DNA REPAIR AND TRANSCRIPTION DEFICIENCY SYNDROMES

DNA HERSTEL EN TRANSCRIPTIE DEFICIENTE SYNDROMEN

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 20 december 1995 om 9.45 uur

door

WILLEM VERMEULEN
geboren te Hardinxveld-Giessendam

PROMOTIECOMMISSIE

Pomotoren:	Prof. Dr. D. Bootsma
	Prof. Dr. J.H.J. Hoeijmakers
Overige leden:	Prof. Dr. F.G. Grosveld
	Prof. Dr. D. Lindhout
	Prof. Dr. A. Westerveld

Printed by Offsetdrukkerij Ridderprint B.V., Ridderkerk

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuid-West Nederland. Het onderzoek is financieel gesteund door het KWF (Koningin Wilhelmina Fonds). Subsidie voor de drukkosten van dit proefschrift werd verleend door Paes Nederland (Olympus). Omslag: ontwerp en druk door Drukkerij van den Berg en Versluijs, Dordrecht.

Life is the shape it is for a purpose.
When you see how things really are,
all the hurt and and the waste falls away.
What's left is the beauty.

(Rosalind Franklin)

Contents

Chapter 1

General introduction

General introduction

The genetic information of all living organisms is stored in DNA, a long macromolecule composed of four different nucleotides. Preservation of the sequence of nucleotides, defining the genetic code, is a prerequisite for a faithful transmission of the genetic information to subsequent generations and for accurate expression of this coded information. Although DNA is a relatively stable molecule, it is vulnerable to changes by metabolic activity or environmental DNA-damaging agents (128). Spontaneous DNA modifications are mainly due to an intrinsic instability of the glycosyl bond between the base and the sugar moiety of a nucleotide or are induced by chemical cellular processes such as oxidation, hydrolytic deamination or alkylation of nucleotides. Furthermore, chemical as well as physical agents in the cellular environment threaten the integrity and stability of DNA. A variety of chemical agents interact with or modify DNA, resulting in inter- and intra-strand crosslinks, single strand- and double strand breaks, oxidized nucleotides, alkylated bases and sugars, bulky adducts on nucleotides, protein-DNA crosslinks and products intercalated into the double-helix. The most prominent physical DNA-damaging agents are γ-rays, X-rays and UV-light.

DNA damage can disturb vital cellular processes, which directly depend on the integrity of DNA, such as transcription and replication. Blockage of the transcription of important housekeeping genes causes malfunctioning of the cell, which may result in cell death.

Replicative DNA synthesis is inhibited by lesions on the DNA. This hindrance can be circumvented by an error-prone replication mechanism. The resulting sequence alterations can influence expression of genes or affect the function of coded gene products. For example, such mutations can activate (proto)oncogenes and/or inactivate tumour suppressor genes, both resulting in a disturbance of either cell-cycle regulation or life-span controlling activities, and provoke uncontrolled cellular growth. Consequently, induction and accumulation of DNA lesions can be considered an early event in the multi-step process of oncogenesis.

Disturbances of normal replication and transcription due to xenobiotic exposure are not only a result of DNA lesions, but also a consequence of the radiation-induced "signal transduction pathway". It has been shown that activities which regulate transcription, replication and cell-cycle progression are also influenced by mutagenic radiation (83). Modifying growth factor receptors by xenobiotics induces a cascade of reactions via the mitogen-activated kinase-pathway, resulting in phosphorylation and dephosphorylation of key transcriptional activators (204). Post-translational modifications affect transcription of responsive genes positively or negatively. Furthermore, DNA damage stabilizes the tumour suppressor gene product, p53 (40). Accumulated p53 induces transcription of genes encoding cell-cycle controlling proteins, resulting in a G1 arrest (40, 206). Postponement of replication allows the cell to repair its DNA, thereby preventing the generation of mutations.

Together with the shortening of telomeres (76) persisting DNA damage can be an

additional factor in the process of cellular aging of somatic tissues.

During evolution several DNA repair pathways have emerged (61) in order to cope with the above mentioned deleterious effects of DNA lesions. Most of the repair mechanisms have been strongly conserved during evolution, with evident parallels between human and yeast and even *Eschericia coli*. In only a few cases have species-specific repair systems evolved. The most relevant repair mechanisms are: 1) direct reversal of damage, 2) base excision repair, 3) nucleotide excision repair, 4) mismatch repair, 5) recombination repair, and 6) post replication repair. For a comprehensive review on DNA repair mechanisms see reference (60).

The first evidence that DNA repair plays an important role in tumour prevention was reported more than 25 years ago, by Cleaver (34). He showed that the cancer-prone hereditary disorder xeroderma pigmentosum (XP), has as its bases on a defective nucleotide excision repair (NER) pathway. This rare, autosomal recessive disorder is characterized by extreme sensitivity of affected individuals to UV-light (resulting in DNA damage), in addition to a predisposition to the development of skin-cancer (35). In addition to this prototype repair disorder, other cancer-prone syndromes based on a defect in one of the repair processes, other than NER, are known. Examples are: Fanconi's anaemia (FA) and Bloom syndrome (BS).

Two other excision repair-deficient hereditary syndromes have been recognized: Cockayne syndrome (CS) (167) and trichothiodystrophy (TTD) (235). However, no predisposition to skin-cancer is present among CS and TTD patients. Individuals suffering from these repair-deficient conditions display a broad spectrum of clinical traits, sometimes with overlapping features of two different syndromes. Most of these disorders are also heterogeneous at the genetic level, and generally multiple genes are involved in a given syndrome (chapter 3).

The aim of the experimental work described in this thesis is to further characterize and dissect the genetic complexity of NER-deficient syndromes. Genetic analysis of patients with complex (and combined) clinical features resulted in the assignment of their inherited defect to a selected group of NER genes (appendices I, II, III and V). Additional studies provide a molecular basis for some of the previously unexplained clinical features of such individuals (appendices IV and V). For this reason, a more detailed introduction to NER, NER-syndromes and NER-factors is presented in chapters 2, 3 and 4. Biochemical studies revealed that another basic cellular process, transcription, is tightly connected to NER. Basic information on this process is also provided in this thesis (chapter 5).

Chapter 2

Nucleotide excision repair

Nucleotide Excision Repair (NER)

The major UV-induced lesions are cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidone (6-4PP) photoproducts (between two adjacent pyrimidines). These lesions, bulky chemical DNA adducts and intra-strand crosslinks are targets for the NER pathway.

NER research has acquired more attention recently, because of the importance of this repair mechanism in relation to the expected increase of environmental UV-light exposure. Our environment is polluted with an increasing amount of chemical agents due to human industrial activities. The accumulation of CFCs in the atmosphere, which are thought to degrade the stratospheric ozone (64), is a significant potential threat. Reduction of the ozone layer results in an increased penetration of the mutagenic UV-light on the earth's surface. A prediction can be made that as a consequence of the elevated UV exposure an increase in the frequency of solar-induced skin cancer will arise (136, 300), since it is well-established that the incidence of cutaneous malignancies can be correlated with the amount of solar exposure.

As with many other DNA repair processes, NER is evolutionary conserved (86, 87). Although the different factors involved in NER have diverged extensively between prokaryotes and eukaryotes (86), the overall basic pathway appears to be similar. The molecular reaction mechanism involves the following steps:

1. Damage recognition factors scan the DNA for lesions. Recognition factors bound to lesions and mediate the assembly of excision factors.
2. Local remodelling or opening of the dense chromatin structure to give access to the NER components.
3. The triggered or activated excision machinery makes a dual incision (excinuclease) on either site (5′ and 3′) at some distance from the lesion.
4. Strand displacement of the excised, damage-containing fragment and turnover of the excinuclease complex.
5. Filling in of the resulting gap by DNA polymerase and auxiliary factors.
6. Rejoining of the newly synthesized DNA to the existing DNA by a ligase.

The exact order of steps 1 and 2 is not known, it is possible that these occur simultaneously. This also holds for steps 4 and 5.

2.1. Nucleotide excision repair in *E. coli*.

Genetic analysis of a panel of UV-sensitive, NER-deficient *E.coli* mutants, gene cloning and biochemical studies on NER-gene products have resulted in a fairly well understood mechanism of this process (70). Two UvrA and one UvrB molecule form a hetero-trimer (Uvr A_2B) (step 1) that scans the DNA-helix for lesions. The A_2B complex hooks onto a lesion, UvrA dissociates and the lesion-bound UvrB bends the DNA and attracts a UvrC molecule (step 1, 2). This BC-complex possesses a dual endonuclease activity (excinuclease), cutting eight bases 5′ (probably catalyzed by UvrC) of the lesion,

Table 1 **Properties *S.cerevisiae* NER genes and mutants**

Gene	UV[5]	Affected NER TCR[1]	GGR[2]	Protein size (aa)[3]	Homologous gene in: human	*S.pombe*	Remarks
RAD1	++	-	-	1100	*ERCC4* ?	*rad16*$^+$	complex with Rad10, 5'-incision [5]
RAD2	++	-	-	1031	*XPG/ERCC5*	*rad13*$^+$	3' incision
RAD3[4]	++	-	-	778	*XPD/ERCC2*	*rad15*$^+$	TFIIH subunit, 5'→3' helicase, vital
RAD4	++	-	-	754	*XPC* ?	?	single-strand DNA binding?
RAD7	+	+	-	565	?	?	SIR3 interaction
RAD10	++	-	-	210	*ERCC1*	*swi10*	complex with Rad1, 5' incision [5]
RAD14	++	-	-	247	*XPA*	?	damage recognition, Zn^{2+}-finger
RAD16	+	+	-	790	?	?	Zn^{2+}-finger, SNF2-like helicase domains
RAD23	+	-?	-?	398	*HHR23A, B*	?	ubiquitin fusion, complex with Rad4?
RAD25[4]	++	-	-	843	*XPB/ERCC3*	*rhp3*$^+$	TFIIH subunit, 3'→5' helicase, vital
RAD26	-	-	+	1085	*CSB/ERCC6*	?	SNF2-like helicase domains
SSL1[4]	-/+	?	?	461	*p44/HHSSL1*	?	TFIIH subunit, 2 Zn^{2+}-fingers
TFB1[4]	-/+	?	?	642	*p62*	?	TFIIH subunit
RFA1	-/+	?	?	622	*RPA1*	?	single-strand DNA binding
RFA2	-/+	?	?	273	*RPA2*	?	single-strand DNA binding
RFA3	-/+	?	?	121	*RPA3*	?	single-strand DNA binding

1) transcription coupled repair. 2) global genome repair. 3) predicted number of amino acids, based on sequence (ORF) of cloned gene.
4) probably also other TFIIH subunits have an additional NER function. 5) additional role in recombination.
A number of other mutants/genes; MMS, RAD24, SNM1, may play a role in NER as well.

and 5 phosphodiester bonds 3' of it (likely performed by UvrB) (step 3). Displacement of the excised strand is performed by the collaborative action of UvrD (or helicase II) and DNA polymerase I (step 4), followed by DNA synthesis, filling the resulting gap (step 5). Steps 4 and 5 are probably performed simultaneously. The reaction is completed by a ligase (step 6).

Lesions can block the transcription process by stalling the elongating RNA polymerase II complex at the injured site (49). It is important for normal functioning of the cell to rapidly eliminate these serious impairments. For this reason, a specialized NER sub-pathway is thought to have arisen in *E. coli*: "transcription coupled repair", or "preferential repair" (150). This pathway is limited to the transcribed strand of active genes. Preferential repair is faster than its counterpart: "genome overall repair" (or repair of non-transcribed DNA). This subdivision of NER was first described for mammalian NER (17). Preferential repair of the transcribed strand in *E. coli* has been shown *in vivo* as well as *in vitro* (217). Selective strand-specific repair is facilitated by the TRCF protein (transcription-repair coupling factor), which is encoded by the *mfd* gene (221). *mfd* mutants appear to be slightly UV-sensitive but have a 5x elevated frequency of UV-induced mutagenesis in comparison to wild-type strains. TRCF functions in dissociating a stalled RNA polymerase from DNA at the site of a lesion and attracting the NER machinery by binding to the UvrA component (219), thereby providing a fast and efficient repair of DNA damage in more critical regions of the genome.

2.2 Eukaryotic NER

In contrast to the situation in *E. coli*, understanding of the eukaryotic NER mechanism is less complete, although insight into this process is rapidly increasing. A number of genes and proteins involved in yeast (*Saccharomyces cerevisiae*) NER have been isolated and characterized, again with the aid of a set of NER-deficient mutants, or by using sequence conservation (190). Recently, some repair genes of the distantly related fission yeast *Schizosaccharomyces pombe* have been cloned (118). Several of these genes show homology with the *S.cerevisiae* genes (146).

Properties of the yeast excision repair proteins are summarized in Table 1. Since in this thesis the emphasis is on genetic and biochemical defects in (human) repair syndromes, the overview of NER presented in the next chapters is limited to that occuring in mammals. Important parallels on structure and function between yeast and man NER are discussed when relevant.

2.3 Mammalian NER mutants.

Identification and characterization of mammalian NER-deficient mutants is important to the study of mammalian DNA repair (as with *E.coli* and *S.cerevisiae*). The mammalian group of NER mutants is divided into two classes:

1- laboratory-induced, rodent, UV-sensitive cell lines

2- naturally existing human syndromes

Table 2 Features of rodent NER-deficient complementation groups.

Complementation group	sensitivity[1]		human equivalent	cloned, correcting gene
	UV	MMC[2]		
1	+ +	+ + +	none	*ERCC1*
2	+ +	+	XP-D	*XPD/ERCC2*
3	+ +	+	XP-B	*XPB/ERCC3*
4	+ +	+ + +	XP-F?	*ERCC4*
5	+(+)	±	XP-G	*XPG/ERCC6*
6	+	+	CS-B	*CSB/ERCC6*
7	+	±	?	?
8	+	+	?	?
9	+	+	?	?
10	+	+	?	?
11	+(+)	+	?	?

1) +, 2-5x wt; + +, 5-10x wt; + + + > 10x wt. 2) mitomycin-C, a crosslinking agent.

2.3.1 Rodent mutants.

UV-sensitive NER-deficient rodent mutant cell lines have been isolated after mutagenic treatment of established rodent cell strains and appropriate selection protocols. Most mutants were generated from chinese hamster ovary cells (CHO) (25, 80, 234). The partial hemi-zygocity, frequently occurring in CHO cultures, has been of great help in isolating mutants with a recessive repair phenotype (human NER syndromes are all recessively inherited). Cell hybridization experiments have resulted in the identification of 11 excision repair deficient complementation groups (Table 2) (25).

A significant difference is observed between rodent and human NER. The overall genome repair of pyrimidine dimers is much less efficient in rodents (10-20% within 24 h) than in man (60-70% within 24 h). However, the toxic effect of a given UV-dose is almost identical in cells derived from both species (271). This apparent discrepancy between these two species (the so-called: human-rodent repair paradox) has not yet been explained, but it should be taken into account whenever experimental data is extrapolated from one species to the other.

Despite these observed differences a considerable similarity between rodent and human NER is apparent (86, 87). Characteristics of the CHO cells, including the immortalized status and suitable transfection properties due to a relatively high capacity to integrate transferred DNA (88), made this group of mutants a valuable tool for the

isolation of mammalian NER genes via transfer of genomic DNA (Table 2).

2.3.2 Human NER-deficiencies

Three rare, autosomal recessive disorders are known to be based on a NER-deficiency: the prototype NER-disease, xeroderma pigmentosum (XP)(34), Cockayne Syndrome (CS)(167), and the photosensitive form of trichothiodystrophy (TTD)(95, 236). In total ten NER-deficient complementation groups are recognized within these three disorders: seven for XP (XP-A to -G), two for CS (CS-A and CS-B) and TTD-A. Clinical and genetical characteristics of these syndromes are described in chapter 3. A significant overlap between the two groups of mammalian NER mutants has become apparent.

Both groups of NER-deficient mutants were of much help for the identification and cloning of the mammalian NER genes.

2.4 Cloning of mammalian NER genes

In order to study the molecular mechanism of mammalian NER, it is necessary to isolate and characterize its components. Cloning of NER genes is a key step for further biochemical analysis. In addition, sequence information is a prerequisite for performing gene targeting experiments which is used for the generation of animal models, mimicking human repair-deficient syndromes.

Using genomic DNA transfection the first human NER gene (*ERCC1*) was cloned a decade ago (293). This gene corrected the NER defect in a Chinese hamster ovary (CHO) cell line from complementation group 1. Subsequent genomic DNA transfections to representatives of the different UV^s CHO groups has resulted in the isolation of a set of human repair genes: *ERCC2/XPD* (289), *ERCC3/XPB* (291), *ERCC5/XPG* (160), and *ERCC6/CSB* (262). The *XPA* gene was cloned by genomic DNA transfer to XP-A cells (254). Using an episomally replicating cDNA library, successful cloning of the *XPC* gene (116) and *CSA* gene (82) were reported. *HHR23B* and its homologue; *HHR23A*, were isolated by virtue of their evolutionary conserved nature. These genes were isolated after database searches, comparing the yeast protein (Rad23) with cloned mammalian sequences of unknown function (EST). Degenerative nucleotide primers, deduced from the micro-sequenced damage-specific DNA-binding (DDB) protein (32) which is altered in XP-E, were used to clone the gene encoding the 127 kDa protein (DDB)(250). Also with this approach, both genes encoding the XPC/HHR23B complex (see 4.4.3) were cloned (140). Recently the *ERCC4* gene was cloned after transfection of a chromosome 16 cosmid library (258), since this gene is localized to this chromosome (129).

2.5 NER-assays.

For monitoring NER, several parameters can be used: cellular survival after UV, UV-induced mutagenesis, chromosomal aberrations, reactivation of UV-irradiated viruses or reporter genes, and removal of specific lesions. These assays, however, monitor the

biological consequence of NER. A direct NER assay is the measurement of the DNA synthesis step of the process, either *in vivo* (at the single cell level, with UDS) or *in vitro* in cell-free systems. To control or manipulate NER at the cellular level, it is necessary to introduce repair factors and inhibitors into the cells. Several methods for introducing components into cells have been used in the past, including transiently permeabilized cells, vesicle-mediated transfer or fusion with enveloped carriers, and microneedle injection with glass capillaries. This last method is the best controlled and has proven to be the most successful tool in studying cellular NER. More recently, a cell-free *in vitro* repair system has been developed. The use of the microneedle injection technique and more importantly the *in vitro* NER assay have both contributed to an increasing knowledge of mammalian NER. Since both assays were important tools for the research presented in this thesis, they will be briefly discussed below.

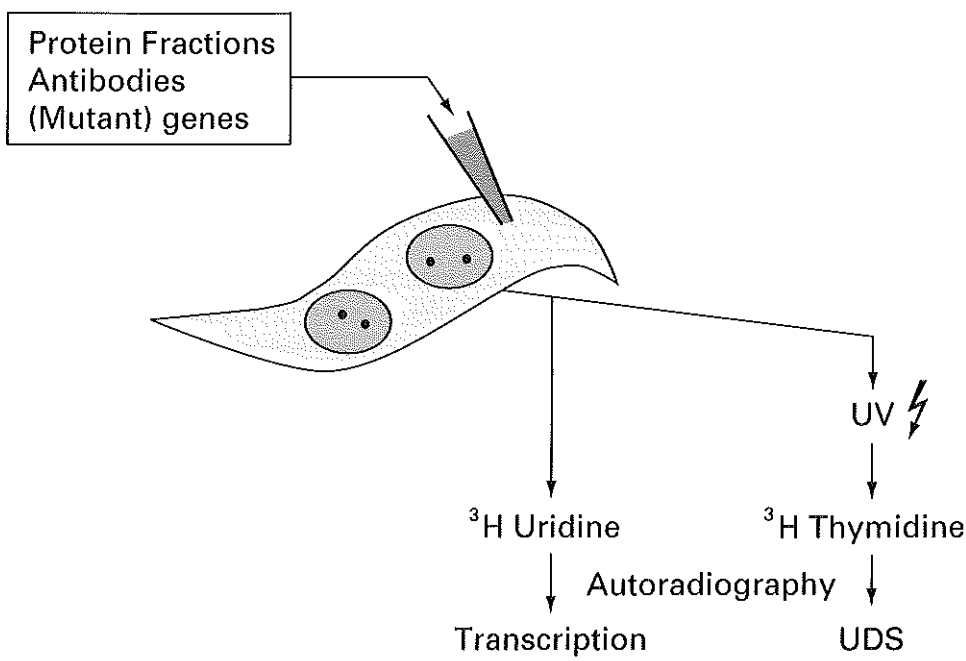


Figure 1. *Scheme of microneedle injection DNA repair assay.*

2.5.1 Microneedle injection in DNA repair

With the microneedle injection technique, the living cell is used as a test tube. The usage of microinjection in NER has been extensively reviewed by Hoeijmakers (85).

With the aid of a glass microcapillary, substances [such as crude cell-extracts, (partly) purified proteins, antibodies, peptides, and nucleic acids (mRNA, DNA)], can be

directly targeted to the cytoplasm or the nuclei (Figure 1), of living repair-proficient or -deficient (XP, CS or TTD) fibroblasts. After microinjection (and a desired incubation time), cells are exposed to UV-light (inducing DNA damage), followed by incubation in culture medium containing tritiated thymidine. During the course of repair replication, labelled nucleotides will be incorporated into DNA and can be visualized after autoradiography (UDS-assay). In a comparable manner, the recovery of RNA synthesis (RRS) after UV can be measured. In this procedure, some time after irradiation (dependent on the UV-dose), cells are labelled with tritiated uridine (added to the culture medium), fixed and exposed for autoradiography. The repair capacity of the injected cells can be quantified by counting the grains above the nuclei (both after UDS and RRS) and are compared to the number of grains above the uninjected surrounding cells.

The advantage of this technique is that it is direct, efficient, not toxic to the cells, and that it consumes only small quantities of compounds. Moreover, NER is examined in its native setting (nuclear chromatin), and neigbouring non-injected cells serve as optimal (negative) controls. However, the method also has limitations: it is time-consuming and laborious, and injection solutions are required to be free of particles and of toxic agents (detergents). Moreover, the NER reaction cannot be controlled or dissected into its several steps, and defined or specific lesions cannot be examined. Despite these limitations, microinjection studies have provided a better understanding of mammalian NER.

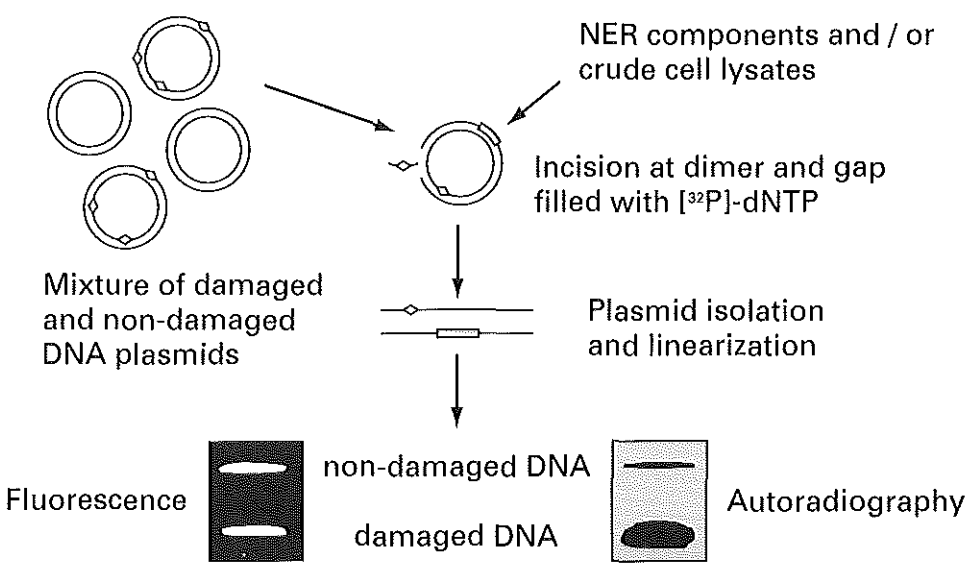


Figure 2. *Scheme of cell-free (in vitro) DNA repair assay.*

2.5.2 Cell-free mammalian NER

To perform controlled biochemical studies on NER and to further dissect the different stages of the reaction, considerable effort has been invested in the development of an *in vitro* mammalian repair assay, similar to the repair system which has been used to elucidate the NER in *E. coli* (70). Almost simultaneously, two labs succeeded in the development of such a cell-free repair system (230, 295). Crude cell-lysates were incubated, in the presence of an energy regenerating system, with different damaged and un-damaged plasmids, together with labelled nucleotides. After recovery and linearization of the plasmid DNAs, incorporation of nucleotides (into the repair patches) was monitored by autoradiography (Figure 2). Radio-active nucleotides are preferentially incorporated into the damaged plasmid in an ATP-dependent manner. ATP consumption is in agreement with previous reports using permeable cells (106). Based on this principle, Hanaoka and coworkers developed an *in vitro* repair assay, using isolated SV40 mini-chromosomes, in which the DNA is in a somewhat more natural configuration (16, 139, 140). These repair systems were capable of performing in vitro complementation after mixing extracts of different XP, and UVs rodent complementation groups (295, 240, 270). Using this cell-free NER system, several repair proteins were purified (140, 177, 200), the repair-patch size was determined (92) and activities required for the repair synthesis were identified (39, 227). Similar cell-free NER assays were established for the fission yeast *Schizosaccharomyces pombe* (231) and the bakers yeast *Saccharomyces cerevisiae* (287). Very recently, a successful *in vitro* reconstitution of the mammalian NER reaction with (partly) purified components has been reported (1, 159).

Chapter 3

Human NER-deficient syndromes

Human NER-deficient Syndromes

3.1 Xeroderma pigmentosum

XP patients are extremely sensitive to solar exposure due to the DNA damaging effect of the UV component of sunlight (35). Individuals predominantly express cutaneous and ocular abnormalities on sun-exposed areas, including acute sun-sensitivity (erythema and bullae), marked hyper- and hypopigmented spots, a dry aged-like and scaling skin, keratosis and telangiectasia. A large group of XP patients exhibit a high risk (more than 1000x elevated) towards the development of cutaneous malignancies (basal cell carcinomas, squamous cell carcinomas and melanomas).

A complex of different symptoms was found to be associated with a subgroup of patients originally designated; De Sanctis-Cacchione syndrome (DSC)(46). DSC describes mainly neurologic and developmental abnormalities of individuals with a freckling (pigmentosum) and parchment (xeroderma) -like skin. It later transpired that all DSC patients belong to XP, and the DSC designation has gone out of use. The (progressive) neurological symptoms in XP are: intellectual deterioration, loss of the ability to talk and increasing spasticity, which are all thought to be due to primary neuronal degeneration (198). Developmental problems are: microcephaly, dwarfism and immature sexual development.

Also at the cellular level (XP-derived cell cultures), an abnormal UV-sensitivity is also observed. DNA repair synthesis after UV-irradiation, as measured by tritiated thymidine incorporation (autoradiographically), is severely reduced in XP (34)(Figure 3). Moreover, a series of mutagenesis studies on these cells revealed that XP cells are hyper-mutable after a UV challenge. The spectrum of UV-induced mutations in XP cells is also different from that in NER-proficient cells (35). The elevated mutation frequency in XP after exposure to DNA damaging agents, and the high risk of skin tumour development, are in agreement with the idea that inappropriate processing of DNA lesions is an early step in oncogenesis. Persisting lesions can induce mutations after replication which may alter the function or expression of genes involved in cellular growth, replication fidelity, cell-cycle control or apoptosis.

A broad clinical heterogeneity is observed among XP individuals. This varies from patients with severe neurological implications and a high number of skin tumours to individuals with mild pigmentary abnormalities without signs of neurological abnormalities. This variation can either be due to differences in genetic background of affected persons or to allelic variation. Cell fusion studies revealed in the early seventies that XP is a multi genic disorder (44). Cells of different patients were fused with the aid of inactivated Sendai-virus, and assayed for repair replication or UDS (unscheduled DNA synthesis) after UV exposure. In some combinations of these somatic XP hybrids complementation of the defective repair replication can be observed (Figure 3). At the present time seven different NER-deficient XP complementation groups (XP-A to XP-G) have been identified (Table 3)(278). This implies that at least seven genes are responsible

for a functional NER and that a defect in one of these results in a XP phenotype.

Approximately 20% of XP patients do not have deficient NER and have been are designated XP-variant (XP-V)(35). The clinical spectrum of this group is also variable, ranging from severe cutaneous alterations (including skin malignancies) to very mildly affected individuals. In general neurological abnormalities have been observed in only a few patients within this group (35). Although cell survival of XP-variants after UV-irradiation is in the near-normal range, UV-induced mutagenesis is enhanced. The spectrum of mutations is also different from that in normal cells (284). The primary defect in XP-V is thought to reside in a process called post replication repair (PRR)(119). In XP-V cells, DNA synthesis is more inhibited, and newly synthesized DNA is of a smaller size than in normal cells after UV-irradiation. The apparent mechanism is that DNA synthesis is blocked at a lesion, and replication had to re-start after the lesion. After a certain delay, the rate of daughter strand synthesis becomes identical to the normal situation. PRR is in fact a mechanism to avoid termination of replicative synthesis after DNA damage. Two PRR pathways are known, an error-free and an error-prone one (114a). Because of the elevated mutation frequency in XP-V, it is likely that the error-free pathway is disturbed in XP-V.

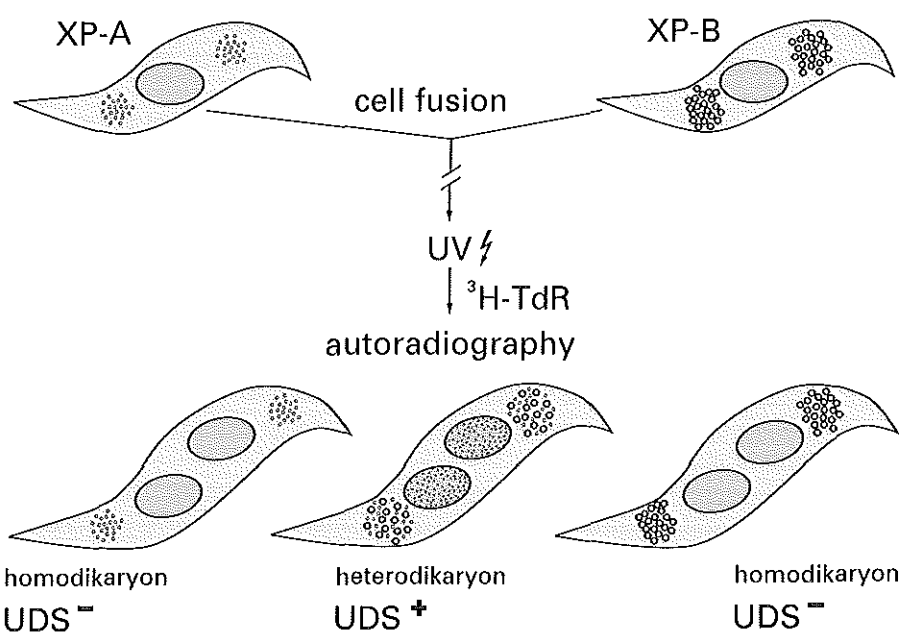


Figure 3. *Complementation analysis by cell-hybridisation. Prior to (Sendai-virus induced) fusion, cells were labelled with micro-spheres of different sizes. The different fusion products can be distinguished by their differential bead content. NER capacity is visualised as auto-radiographic grains above the nuclei, which is reduced or absent by XP cells. In case of complementation, the number of grains above the nuclei in the hybrid cell is identical as above repair-proficient cells.*

3.2 Cockayne syndrome

CS patients display mainly neurologic and developmental abnormalities (121, 167). Individuals have a sun-sensitive skin (erythema after minimal exposure). However, in contrast to XP, there are no pigmentation abnormalities or predisposition to skin cancer. In some cases, thin and dry hair and skin are present. Ocular features are frequently found, these include pigmented retina, cataracts, optic disk atrophy, meiotic pupils and photophobia. Neurological signs are mental retardation (intelligence decline and behaviour problems), loss of gait and speech capacity, hyper-reflexia, occasionally seizures and sensorineuronal hearing loss. These neurological features might be explained by calcification of basal ganglia and perhaps more likely, by dysmyelination of the central- and peripheral nervous system. This neuropathology is of another kind than in XP, where abnormalities are due to primary neuronal degeneration (198). Developmental (as well as neurological) problems have a postnatal onset in almost all cases. These include cachectic dwarfism (in which weight is relatively more affected than height), microcephaly, decreased subcutaneous fat, peculiar (birdlike) facies or "wizened appearance" (preceding chin, beaked nose, protruding ears and deep sunken eyes), and immature sexual development (hypogonadism, undescended small testes).

The NER defect in CS has only recently been established. Cells derived from CS patients are only moderately UV-sensitive and the repair synthesis (or UDS) is in the near normal (NER-proficient) range (3). The repair defect in CS-cells is manifest as a retarded or absent recovery of DNA (120) or RNA synthesis (144) after an UV challenge which normally recoveres within a few hours. The repair defect in CS was shown to be based on an impaired preferential repair of the transcribed strand, called transcription-coupled repair (TCR) (267, 273)(see chapter 4.4). This also explains the observed defect in recovery of RNA synthesis (RRS) after UV. The selective removal of ionizing-radiation-induced lesions from the transcribed-strand also seems impaired in CS (115) suggesting that CS patients have mutations in genes that code for (a) general factor(s) which couple(s) transcription to repair.

Complementation analysis has disclosed three groups in CS (Table 3)(117, 252): A, B and C. Groups A and B harbour the "classical" CS patients. Group C comprises only one patient (XP11BE), who exhibits also the typical XP symptoms (belonging also to XP group B). This combined phenotype, or XP/CS-complex, will be discussed in more detail in the following paragraphs and in appendices I-V.

The group of CS patients is clinically rather heterogeneous. A clinical differentiation among CS individuals can be made on the basis of the age of onset and severity of the disease: the classical type or CS I, and the severe early onset type, CS II (167). Clinical variation is also apparent from the notion that other syndromes like CAMFAK (cataracts, *m*icrocephaly, *f*ailure to thrive, *k*yphoscoliosis) and COFS (*C*erebro-*o*culo-*f*acial *s*yndrome) display a phenotype which resembles many of the traits commonly observed in CS. Both CAMFAK and COFS express the hallmarks of early onset CS with some additional features. However no DNA-repair characteristics nor UV-

Table 3 Properties of XP, CS and PIBIDS complementation groups

Complementation group	UV sens.	residual UDS[1]	Affected NER TCR[2]	Affected NER GGR[3]	relative frequency	skin cancer	neurologic abnormalities	remarks
XP-A	+++	<5%	-	-	high	+	++	
XP-B	++	10-40%	-	-	3 families	+/-	++/+	XP/CS and TTD
XP-C	+	15-30%	+	-	high	+	-	
XP-D	++	15-50%	-	-	intermediate	+/-	++/±	XP,XP/CS and TTD
XP-E	±	≥50%	?	?	rare	+/-	-	
XP-F	+	15-30%	-	-	rare	+/-	-/±	
XP-G	++	<5-25%	-	-	rare	+/-	++/+	XP and XP/CS
TTD-A	+	10%	-	-	1 family	-	+	
CS-A	+	normal range	-	+	intermediate	-	++	
CS-B	+	normal range	-	+	high	-	++	
XP-V[4]	+/±	normal range	+	+	high	+/-	±	

1) Unscheduled DNA Synthesis, expressed as percentage of repair synthesis in normal cells. 2) transcription coupled repair.
3) global genome repair. 4) XP-variant, defect in post replication repair, proficient NER.

sensitivity within these syndromes have been described (167). Recently, Itoh (97) described two siblings without clear CS signs, but with CS-like biochemical DNA-repair properties (UV-sensitivity, normal UDS but a reduced post-UV RRS). Genetic analysis revealed that these two siblings can be assigned to a new NER-deficient complementation group (designated UV^S syndrome), since they complement all known XP and CS groups (96). Two other patients (69) with a CS-type of repair defect also exhibit only XP symptoms. No complementation analysis has as yet been performed with these patients. At the other side of the spectrum, there are individuals with clear CS hallmarks (dwarfism, mental retardation, deafness and peculiar facies) but without a reduced post-UV RRS (121) and photosensitivity.

3.3 Combined XP/CS

A few patients have been described with the rare combination of XP and CS features. The combined XP/CS patients are divided over three groups: XP-B, -D, and -G (21, 277) (Table 3). Patient XP11BE (199), who was for a long time the only known case of XP group B, expressed severe symptoms of both XP and CS including numerous skin tumours, severe mental retardation, and poor physical development. Two siblings (XPCS1BA, and -2BA) with mild features of XP and CS were recently assigned to XP-B (277). Developmental abnormalities are hardly present in these individuals and neurologic symptoms became evident only after the second decade of life (216).

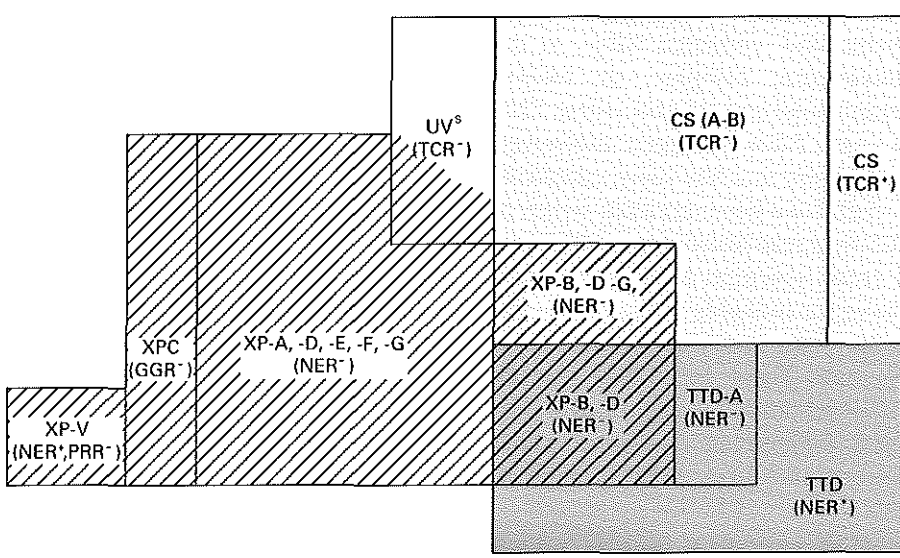


Figure 4. *Clinical and genetic continuum of NER-deficient (and related) syndromes. The type of repair defect is indicated. The hatched group represents individuals expressing XP features, the light-gray box CS patients and the dark-gray group patients with TTD features.*

A remarkable clinical variation is observed between the two XP-B families. The two siblings do not display any cutaneous malignancies despite a for XP relatively advanced age of more than 40 years, whereas patient XP11BE had skin tumours at a very young age (18 years). Nevertheless, the level of UDS, representing the repair defect, is similar in both families (5-10% of NER-proficient cells).

Patient XPCS2 exhibited clear signs of both XP and CS (114, 157) and was assigned to XP group D (278). Cells of this patient have a relatively high residual DNA repair capacity (≈40% UDS). The mean residual UDS level in XP-D is around 20-25% of control. Patient XP8BR, with a clear XP-skin and severe CS features (died at age 2½ years) and a residual UDS of 30-40%, was assigned to XP-D (21). Two very severe early onset CS patients (99) were assigned to XP group G (appendix I). Notably, these patients do not have any XP signs, despite a XP-like repair defect. Previously, it was suggested (197) that the combined XP/CS cases represent a distinct clinical entity. It is, however, tempting to assume that the characteristic features of XP, XP/CS and CS are part of a clinical continuum based on a defective NER (Figure 4), which can be designated as "NER-syndromes". Two reports (69, 97) describing patients with a confusing phenotype support this concept (see 3.2.2)

3.4 Trichothiodystrophy

The most prominent TTD features are sulphur-deficient brittle hair (also dry and sparse) and nails (95). With polarizing light microscopy a typical, "tiger tail" pattern of the hair is visible. Cutaneous signs include photosensitivity, ichthyosis, keratosis, erythema and 'collodion baby'. In many cases the brittle hair is associated with a heterogeneous complex of neuro-ectodermal abnormalities. Neurologic and developmental impairments within TTD are reminiscent to those found in CS (see 3.2.2). TTD cases have not been extensively examined for the origin of the neurologic abnormalities. In a few cases, calcification of the basal ganglia and dysmyelination (30, 187, 260) have been reported, which could be the cause of the clinical features, like in CS. Clinical manifestations and their severity vary extensively between TTD individuals. This broad spectrum of symptoms partly explains the confusing nomenclature in the literature for probably the same disease (95, 147). Different names have been used to describe cases in which some of the above features are present in combination with brittle hair. These are Pollitt syndrome (s.), Tay s., Amish brittle hair s., ONMR (*o*nychotrichodysplasia, *n*eutropenia, *m*ental *r*etardation), Sabinas s., Marinesco-Sjögren s., BIDS (*b*rittle hair, *i*ntellectual impairment, *d*ecreased fertility, *s*hort stature), IBIDS (*i*chthyosis and BIDS), PIBIDS (*p*hotosensitivity and IBIDS), SIBIDS (*o*steosclerosis and IBIDS).

The association of TTD with deficient NER was discovered by Yong (296). Photosensitive TTD patients have a deficient NER (reduced UDS) and complementation studies have revealed that the majority of these TTD patients belong to XP group D (235, 236). However, genetic heterogeneity within NER-deficient TTD is present (Table 3)(235). A single patient (TTD1BR), expressing the TTD features with severe sun-sensitivity,

represents a new NER-deficient complementation group, and was designated TTD-A (237). Very recently, the genetic heterogeneity was extended to a third group by the finding that the repair defect of a single TTD-family is based on a mutated *XPB* gene (279). The genetic overlap of TTD (identical to CS), with different XP groups, strengthens the suggestion above that in addition to XP, XP/CS and CS, UV^5-TTD also belong to the phenotypical extremes of "NER-syndromes" (Figure 4).

3.5 Genetic basis of clinical heterogeneity

Clinical variation could be explained by locus heterogeneity (different genes), allelic heterogeneity (variable mutations of the same gene) and by a contribution from genetic background. To date, several allelic variations of distinct XP loci have been identified, since most of the XP genes have been cloned and mutational analysis has been performed on some patients.

The assignment of two siblings with TTD features to XP-B, extends the already reported clinical heterogeneity within this complementation group (279). This heterogeneity is even more remarkable when the very low incidence of XP-B patients is taken into account (three different phenotypes among three families). In patient XP11BE a splice acceptor mutation of the last intron of *ERCC3/XPB* leads to a 4 bp insertion in the last exon, resulting in a frameshift of the last 40 amino acids (292)(Figure 5). A missense mutation in the same gene at the N-terminus, converting amino acid 99, a phenylalanine into a serine (Figure 5), leads to a similar low cellular repair level, but with a much milder phenotype (277). The mutation of the TTD/XPB family is located at the same N-terminal region of XPB: tyrosine 119 is substituted into a proline (G.Weeda, unpublished observations), resulting in a mild TTD and intermediate NER deficiency. This demonstrates that at least allelic heterogeneity (probably in combination with differences in genetic background) contributes to the different phenotypes within XP-B.

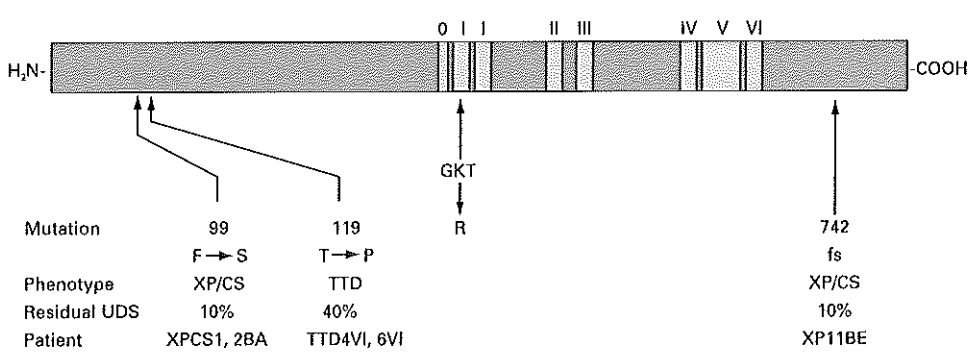


Figure 5. *Schematic representation of the XPB protein. The N-, and C-terminal parts, as well as the conserved helicase domains (0 - VI), are indicated. The three mutations identified in XP-B patients, and the dominant negative mutation are also pointed.*

A similar broad spectrum of symptoms is seen within XP-D (101). The majority of XP-D patients exhibit features of classical XP, most UV-sensitive TTD patients belong to XP-D and two patients show the XP/CS features. However, mutational analysis of XP-D patients does not uncover a clear genotype/phenotype correlation (20, 21, 60), although until now, causative mutations for XP and TTD phenotypes do not overlap.

Twelve different mutations have been identified (174) in the *XPA* gene (253). A high frequency of splice mutations (at the 3' part of the third intron) creating an unstable mRNA has been observed in the Japanese population (homozygous and compound heterozygous), providing evidence for a founder effect of this mutation (208). This mutation gives rise to a homogeneous group of patients with a severe phenotype and a virtually absent NER (208). Some nonsense mutations at the C-terminal part of XPA generate a truncated protein, with residual repair capacity resulting in patients with a less severe phenotype (209, 210). The observed mutations in XP-A are in concordance with the phenotype.

Different mutations of the *XPC* gene also seem to correlate with different cellular sensitivities and severity of symptoms (122). Since only one XP-G patient has been analyzed for mutations in the *XPG* gene (175), no conclusions can be drawn on a correlation between different mutations and the severity of the disease, within this extremely heterogeneous XP-G group (276).

Some of the observed clinical symptoms (for example, photo-sensitivity, pigmentation, premature aging of the skin and cutaneous malignancies) within the above syndromes can be rationalized on the basis of defective NER. However additional manifestations, present mainly in CS and TTD (for example neurodysmyelination, growth retardation, impaired sexual development, cataracts, dental caries, ichthyosis and brittle hair) are difficult to explain by a DNA repair defect. Clues to the nature of these clinical features have been derived from recent observations that some NER-factors play additional roles in fundamental cellular processes like basal transcription (see appendices IV and V and next chapter).

The absence of predisposition to skin cancer in CS and TTD patients is notable, in view of the defective NER, and is as yet unexplained. The absence of cutaneous malignancies does not seem to correspond with the level of NER, since some TTD/XP-D patients with no increased cancer risk have a severe repair defect (20) (comparable to "classical" XP-D patients, which are cancer-prone). The postulated transcription defect in CS and TTD might suppress the initiation and/or progression of the carcinogenic process.

The generation of mouse models mimicking mutations of both classical XP-D and TTD/XP-D in mice, may provide answers to the obscure relationship between NER-defect and susceptibility to malignancy and the contribution of allelic variation and systemic (genetic) influences to carcinogenicity and expression of phenotype. Moreover, mice mimicking the mutations of different patients (of various XP loci), could teach us more about the relevance of specific alleles and the genetic background to phenotypical expression.

Chapter 4

Mammalian NER mechanism

Mammalian NER Mechanism

The available data on the identification, characterization, cloning and biochemical analysis of NER factors can be combined into a model of the mechanism for mammalian NER (Figure 6). The different steps, as presented in this model, together with the experimental evidence for them are discussed in the following paragraphs.

4.1 Damage recognition

The first step in NER is expected to be the binding of a damage recognition factor to the lesion, which enables the association of further repair components. Both XPA and XPE (or DDB) are candidate "recognition-factors", since both proteins have a high affinity for UV-damaged DNA and are involved in NER.

4.1.1 The XPA-correcting protein.

XPA protein was purified from calf thymus and HeLa cells, either using the cell free system (200) or with the microinjection assay (52, 239). XPA has a high affinity for DNA (52, 200), with a preference for UV-damaged DNA (200). The enhanced binding of XPA to damaged DNA is specific for lesions which are targets for NER (4, 102). The strong bias (200-300 times) for UV-photoproducts is mainly due to 6-4PPs, since removal of CPDs (by photolyase) hardly affected the preferential binding (102). The lesion-specific affinity of XPA probably also accounts for the higher rate (5-10 times) of 6-4PP repair in comparison to CPDs (152, 249). Nevertheless, XPA should still be able to recognize CPDs *in vivo*, since XP-A cells lack the ability to repair these lesions. The absence of a detectable preference of XPA for CPD lesions probably reflects the limitations of these *in vitro* studies, since in these assays the configuration of DNA is different from DNA packed into nuclear chromatin.

The deduced amino acid sequence of the cloned *XPA* gene (253), suggested the presence of a zinc-finger motif (4C-type), which might contribute to the DNA-binding properties of this protein. Atomic absorbtion analysis, confirmed the binding of zinc to XPA (4). Mutation studies demonstrates that an intact Zn-finger is indispensable for proper function of the protein (4, 154, 155). The *S.cerevisiae* homolog of *XPA* is *RAD14* (9), having 27% identical and 54% similar residues. Rad14 also binds preferentially to 6-4PPs and chelates zinc (75).

4.1.2 DNA-damage binding protein (DDB).

The other potential damage recognition factor, DDB (DNA damage binding), was first identified in human cell-lysates, by specific retention with UV-damaged oligo-nucleotides in a gel-mobility-shift assay (32). This DDB activity is possibly implicated in XP-E (32), since it is absent in extracts derived from some, but not all, XP-E patients (103,

107). Microinjection of purified DDB transiently corrects the partial NER-defect, only in XP-E cells which lack the DDB-activity (105).

A controversy on the nature of the DDB activity has appeared in the literature. DDB is either purified as a hetero-dimer of 41- and 125 kDa (104, 250), or as a single 125 kDa polypeptide (2, 94). With the aid of amino acid sequence information of the 125 kDa protein (tryptic digests are micro-sequenced), the gene encoding this polypeptide was cloned from a cDNA library (250). The deduced primary structure of this protein does not reveal any consensus sequence for known DNA binding domains. Structural homologs (in *D.discoideum*, and *O.sativa*) were found in a data base search and a DDB-like polypeptide in human and *C.elegans* (250) (van der Spek, personal communication). These gene products probably represent a novel class of DNA-binding proteins.

DDB (or XPE), specifically binds to UV-induced lesions, with a preference for 6-4PPs (10X higher affinity than to CPDs)(104, 192). Despite the very high affinity for damaged DNA, a specific role for DDB in NER is still in debate because of the partial NER defect in XP-E (slightly UV-sensitive, 50-80% UDS, normal in *in vitro* repair). Moreover, XPE is not involved in the core NER reaction *in vitro*, and is thought to act as an accessory factor (1).

4.2 Lesion demarcation (melting of the double helix)

It is likely that somewhere in the NER reaction a DNA-unwinding activity (or helicase) is required, either before and/or after the incision. Two helicase activities have been recognized in *E.coli* NER: $UvrA_2B$, which scans the DNA for lesions in an ATP-dependent reaction and UvrD or Helicase II, which removes the excised damaged stretch of DNA and assists in the turnover of the UvrBC endonucleases.

XPB(ERCC3) (292) and XPD(ERCC2) (290) both contain the seven conserved (consensus) domains of the superfamily of DNA and RNA helicases (67). In addition, both proteins have been shown to possess DNA unwinding activity (202, 241).

4.2.1 XPD/ERCC2 and Rad3 helicase

XPD is highly conserved during evolution, as was revealed after amino acid sequence comparison between human (XPD/ERCC2), Chinese hamster (CXPD), *S.pombe* (rad15/rhp3$^+$) and *S.cerevisiae* (Rad3) (110, 162, 168, 194, 195, 290). Purified Rad3 displays a DNA-dependent ATPase and 5'→3' DNA-helicase activity (243). The enzyme requires a single-strand stretch of DNA to bind to and initiate unwinding (77). Recently, it was shown that the human counterpart of Rad3, XPD, exhibits similar activities (241).

Rad3 helicase- and ATPase-activities are inhibited after UV-irradiation of the substrate. Only lesions in the DNA-strand on which the enzyme translocates are inhibitory. Moreover, the protein seems to be sequestered to the lesion (164). These findings suggest a model in which Rad3 scans the DNA and binds to a lesion (analogous to $UvrA_2B$). However, a specific mutant (K48→R)(242), which is ATPase-, helicase-, and

NER-deficient, still binds to UV-lesions. This makes the damage recognition function of Rad3 after scanning rather unlikely (245). Lysine 48 (K48) is an invariable amino acid in the Walker A-type nucleotide binding box, required for ATP binding and hydrolysis. Furthermore, translocation of most helicases is inhibited by a distortion of the DNA structure.

Different mutations in *RAD3* resulted into a pleiotropic phenotype. Mutants vary from virtually complete NER-deficiency to a moderate UV-sensitivity and to a Rem$^-$ (recombination-mutagenesis) phenotype (156, 233). Deletion mutants are even lethal in haploid yeast cells (84, 169). Mutations in *XPD* also resulted into a heterogeneous phenotype (101). These data together suggest an additional (essential) role for Rad3 (and XPD). The nature of this other function has recently been uncovered by the finding that these proteins are components of the basal transcription initiation complex TFIIH (factor b) (54, 211)(see appendix V and next paragraph).

4.2.2 XPB/ERCC3 and Rad25/Ssl2 helicase

Like XPD, XPB is an evolutionarily conserved protein. With the aid of the mammalian sequence (using low-stringency hybridization on a *S.cerevisiae* genomic-library) the *S.cerevisiae* homolog, *RAD25* was isolated and characterized (185). Independently, the same gene, designated *SSL2*, was isolated as a suppressor mutant of an artificial stem-loop structure in the 5' UTR region of *HIS4* (71). This genetic screening was designed to pick up genes involved in translation initiation. The helicase function might be necessary for unwinding the stem-loop in the mRNA.

Some mutant alleles of *ssl2/rad25* give rise to a UV-sensitive phenotype (71, 185). In addition, a C-terminal deletion mutant which mimicks the XP11BE mutation (292) caused a deficiency in CPD removal of both genome overall and transcription-coupled repair (248). Deletion mutants of *RAD3*, *SSL2/RAD25* are not viable in haploid yeast cells (71, 185). Substitution of the highly conserved lysine^{392} (in the Walker A-type ATP binding box) into an arginine, is not vital (185). This suggests that the ATPase activity (and by implication also the helicase activity, which depends on ATP-hydrolysis) is indispensable for viability, which is in contrast to a similar mutation in *RAD3* (242). Notably, micro-injection of human *XPB* cDNA, containing the same mutation induces a dominant negative effect on NER and transcription (appendix V).

A *Drosophila* homolog of *XPB* was cloned (111, 158). A XP11BE-mimicking mutation gives rise to UV-sensitive flies (158). The *Drosophila XPB* is implicated in mutants expressing the pleiotropic *haywire* phenotype (158). One of the peculiar features of *haywire* is male sterility, probably due to a decreased expression of the testis-specific ß2-tubulin, which plays a role in meiotic spindle formation. Spermatogenesis is sensitive to the level of ß2-tubulin expression. This suggests that XPB plays a role in the expression of this testis-specific tubulin, either at the transcriptional or the translational level. These data together suggest that XPB (as XPD) plays an additional role in a (other than DNA-repair) vital cellular processes. This might also explain the extreme rarity and clinical heterogeneity of XPB patients (see chapter 3 and appendix V).

4.2.3 TFIIH involvement in NER

Unexpectedly, XPB was identified as the 89 kDa subunit of the basal transcription initiation factor TFIIH/BTF2 (hereafter designated TFIIH). This multi-subunit protein complex possesses a helicase activity which is possibly derived from XPB (212). Also, the yeast homolog of XPB is associated with transcription factor b (yeast counterpart of TFIIH, recently designated (y)TFIIH)(54, 74, 191). Later, XPD was also found to reside both in human and yeast TFIIH (50, 54, 211). Even a third NER factor, TTDA, is present in TFIIH (appendix V). The heterogeneous and peculiar phenotypes found in XP-B, XP-D and TTD-A, are probably explained by the dual involvement of their respective factors in NER and the basal transcription machinery (appendix V).

TFIIH is a multi-subunit protein complex containing at least 9 different polypeptides (chapter 5) and the entire complex is probably functional in NER and in transcription (appendix V). In both processes it may act as a functional helicase unit. In transcription initiation this helicase unit catalyses promoter clearance or opens the DNA prior to transcriptional start (chapter 5). A similar function can be envisaged for TFIIH in NER: after damage recognition and subsequent complex formation (Figure 6) (analogous to the preinitiation complex), unwinding activity is required to give access to the other NER factors. Or the excision machinery is directed to the "demarcated lesion" after unwinding. Alternatively, or in addition, the helicase function might also be required for further processing or turnover of reaction intermediates. In a stage after the dual incision, the damage-containing oligo nucleotide has to be removed before the gap-filling can start (analogous to the *E.coli* UvrD). A third possibility is that TFIIH acts as a lesion scanning activity, comparable to the $UvrA_2B$ complex: when a lesion is encountered, translocation of the complex stops and bound proteins attract incision activities. The exact place and role of TFIIH in NER will hopefully soon be uncovered with the aid of the recently developed *in vitro* reconstituted NER system (1, 159).

4.3 Excision of the damaged patch

After damage demarcation, either before or after unwinding, the next step in NER is the removal of the damaged nucleotides. Removal can be performed either by endonucleolytic cleavage followed by exonuclease activity, or by an excision nuclease activity (dual incision on either site of the lesion). Repair patch size analysis revealed that during NER a gap of 20-30 bases around the CPD (or 6-4PP) is produced as a reaction intermediate (257). The identification of a released oligo-nucleotide of 27-29 nt., after treatment of damaged plasmids with cell-free extracts (CFE), confirmed the estimated NER patch size and confirmed the dual incision model (92).

4.3.1 The 3' endonuclease (XPG)

Recently, it was shown that the XPG protein is one of the mammalian endonucleases involved in NER (176). XPG cuts structure-specifically, 3' of a single-stranded bubble (unpaired) region, in duplex DNA. A similar structure can be envisaged as a reaction intermediate in the course of NER, after lesion demarcation and/or unwinding (Figure 6). XP-G extracts were not able to make this 3' incision on a substrate containing a single cisplatin adduct at a defined position, whereas this activity was present in extracts from repair-proficient cells (176). The *XPG* gene is identical to the previously cloned human *ERCC5* gene (135, 178, 213, 224), which corrects the rodent complementation group 5 (160).

The primary amino acid sequence encoded by *XPG/ERCC5* revealed that this gene is the mammalian counterpart of *S.cerevisiae RAD2* (137, 172) and *S.pombe Rad13* (26). Mutants in each of these yeast genes give rise to an UV-sensitive phenotype. Moreover, this group of genes (*XPG, RAD2* and *Rad13*) belongs to a new gene-family encoding proteins which are structurally (and possibly also a functionally) related. This gene-family is divided into two sub-groups, first, the *XPG/RAD2/Rad13* group, and second, murine *FEN-1* (79), *S.cerevisiae YKL510* (98) and *S.pombe Rad2* (163). The former group acts in NER, while the latter is thought to be involved in Okazaki-fragment rejoining during lagging-strand DNA synthesis or double-strand break DNA-repair (78). FEN-1 (flap endonuclease-1) only cuts flap structures with 5' termini. Both groups are sequence-independent, unidirectional and structure-specific endonucleases, however each group acts on different substrates. Homology between the two sub-groups is restricted to three conserved domains ("N", "I" and "C" regions), with asyet unidentified functions. A structural distinction between the two sub-groups is made on the bases of a differential spacer size ("S") between the domains N and I, which is extended in the RAD2 group in comparison with the FEN-1 group (79). This S-region probably defines the substrate specificity.

Although *RAD2* mutants are moderately UV-sensitive, epistasis analysis excluded this gene from the NER-deficient epistatic group (163). Although over-expression of *YKL510* does not complement the UV-sensitivity of *RAD2* mutants, a (partial) redundancy for, an as yet unknown, RAD2 function can not be excluded.

The structure-specific cleavage (176), suggested that XPG acts after (partial) unwinding around the lesion (Figure 6).

4.3.2 The 5' endonuclease (complex)

Since it has been shown that during NER a fragment of about 30 nt. is removed, another endonuclease with opposite directionality (incision 5' of the lesion) compared to XPG, can be envisaged. In yeast this activity is probably executed by the NER factors RAD1 and RAD10 (12).

RAD1 and RAD10 (61) form a stable complex both *in vitro* (13) and *in vivo* (6, 11). Previously, it was demonstrated that this complex possesses an endonucleolytic

activity, both on single-stranded and double-stranded DNA (244, 261). Very recently, using Y-shaped substrates, a specific 5' endonuclease activity on duplex DNA at single-strand/double-strand junctions was established for this RAD1-RAD10 complex (12). Incision is performed at the second or third nt. at a branchpoint of duplex-DNA with 3' single-strand extensions (opposite direction as XPG/RAD2).

Mutants in both *RAD1* and *RAD10* have, besides deficient NER, a defect in one of the mitotic recombination pathways (214, 215). Moreover, damage-induced reciprocal recombination is dependent on a functional RAD1 and RAD10 (205). A similar endonucleolytic function (as in NER) displayed by this complex can be envisaged during recombination events.

The mammalian homolog of *RAD10* is *ERCC1* (265). Complementation studies with cell-free lysates revealed that extracts derived from *ERCC1*- deficient cells were not able to correct the repair defect of cells from CHO-groups 4 and 11 and XP-F, respectively (16, 193, 270). Cell-extracts of these groups were still able to complement the NER defect of other groups. Genetic studies, however, clearly demonstrated that the CHO groups 1, 4 and 11 are defined by distinct genetic loci (24, 196). Immuno-depletion and sedimentation experiments (270) together with partial purification of ERCC1 (269) provided evidence that ERCC1, ERCC4, ERCC11 and XPF reside in a complex of tightly associated proteins. The absence (or altered form) of one of the components influences the stability of the other, as was shown in XP-F extracts on immuno-blot analysis, where ERCC1 is strongly reduced (16, 269).

Although not formally proven, it is generally believed that this ERCC1-ERCC4-ERCC11 complex is the functional mammalian counterpart of RAD1-RAD10. Mutants of CHO-groups 1 and 4 are, besides UV, also extremely sensitive to DNA crosslinking agents, whereas group 11 is slightly sensitive to these agents (a phenomenon not observed in XP-F). These lesions are normally targets for the recombination repair process. This indicates that in addition to its function in NER, the ERCC1-ERCC4-ERCC11-XPF complex is probably also involved in a recombination mechanism, analogous to its potential yeast counterpart. Furthermore, the recently cloned *ERCC4* gene (258) shows a significant homology with *RAD1* (Thompson, personal communication). The *ERCC4* gene also induces a partial correction of the XP-F repair defect (Thompson, personal communication), suggesting that *ERCC4* may be involved in XP-F. The total number of components in this complex is now reduced to three proteins, at least two of which have a yeast equivalent. It is, however, not formally excluded that the yeast incision complex contains additional components (6, 13).

Although the candidate endonucleases in NER are probably identified, the order of cleavage action is not yet known. It is even likely that a complete assembly of incision factors is required for the dual incisions which may act simultaneously. In addition to lesion recognition, helix unwinding and endonucleases the *in vitro* assay revealed that additional factors, like RPA and IF7, are required for an efficient excison of damaged DNA. IF7 is as yet poorly characterised and it is not known where it acts in the excision process (1).

4.3.3 Single stranded binding protein (RPA)

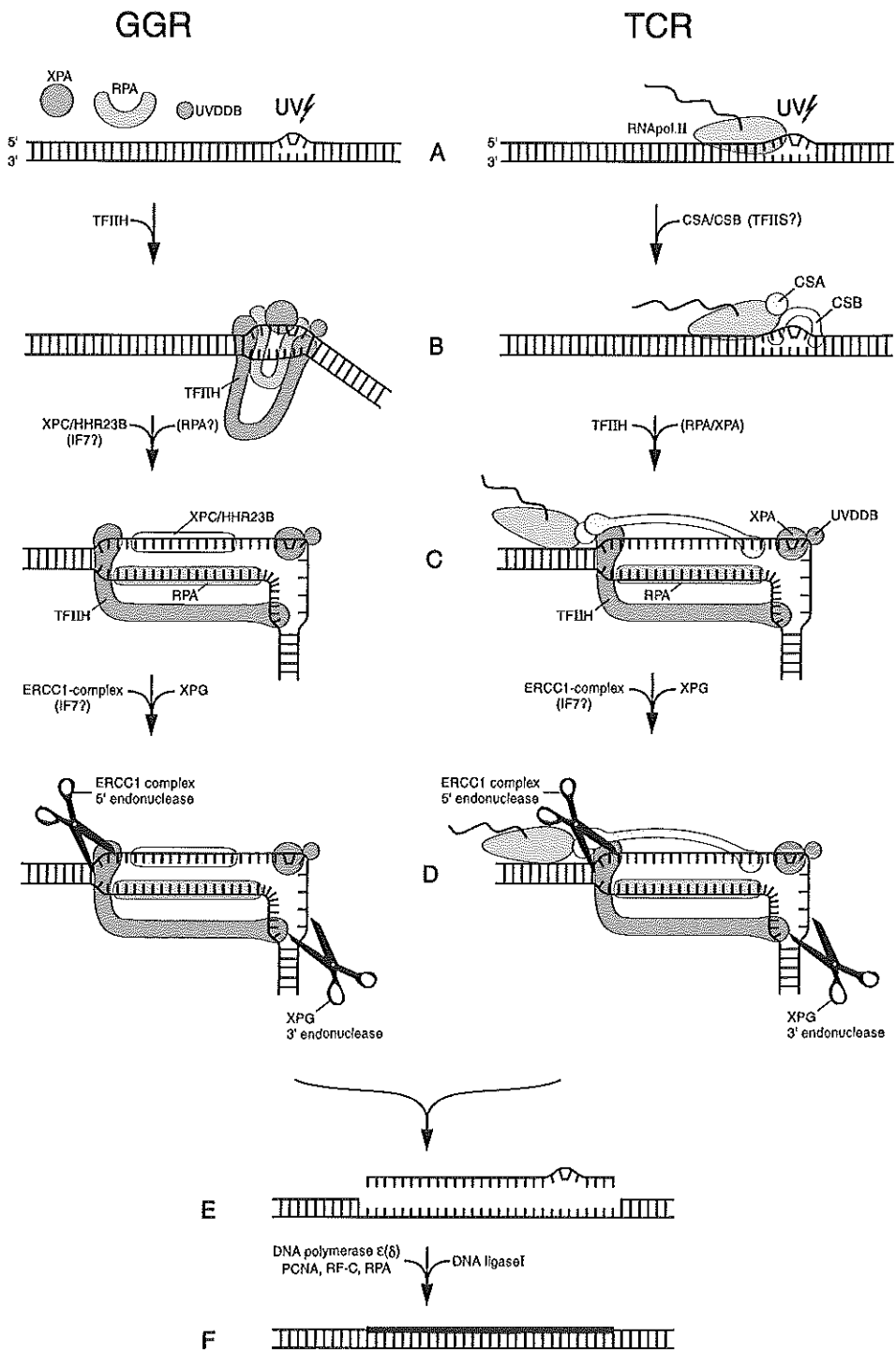
The human single stranded binding protein (HSSB), or RPA (RF-A), plays a role in semi-conservative DNA replication (238). RPA is a tightly associated complex composed of three polypeptides: 70-, 34-, and 11- kDa (resp. RPA1, RPA2 and RPA3) (238). The function of each subunit has not been entirely resolved, although the 70 kDa polypeptide has been identified as the DNA binding subunit and the 34 kDa subunit is a target for phosphorylation during the cell-cycle (48, 51). RPA2 is phosphorylated, only during S- and G2-phase (48), by the combined action of cyclin A-CDC2/CDK2 complexes and DNA-PK (51, 181). Hyper-phosphorylation of RPA2 occurs after genomic stress, including UV-irradiation (27, 130).

In addition to its function in replication (and recombination), RPA also acts in NER. Addition of antibodies directed against either the RPA1 or the RPA2 subunits inhibit repair synthesis, both after microinjection (Vermeulen, unpublished observations) and in cell-free extracts (39). Purified RPA enhances NER in extracts from repair-proficient cells, but not in extracts of XP groups A to G. When, in XP extracts incision is bypassed by the *E.coli* UvrABC, RPA does not stimulate NER, suggesting that RPA acts in a similar stage as UvrABC (38). Further dissection of *in vitro* repair revealed that RPA is an important factor in the incision step of NER, rather than in the expected repair-synthesis (1, 227).

A speculative role for RPA in NER is the stabilisation of the single-stranded nature of DNA around a lesion, generated by the cooperative action of damage recognition proteins and helicases (resp. XPA and TFIIH). One single RPA unit protects a region of $\pm$30 nt. (108) and matches well with the size of an excision repair patch (92), supporting the suggested role of RPA in NER.

A second (or additional) possible function for RPA is that it serves as a bridging factor between the different NER factors. RPA2 was shown to interact with XPA in the yeast two-hybrid system (141). This interaction is confirmed by GST-XPA pull-down, co-immuno-precipitation and XPA affinity column binding (81, 141). RPA-XPA complex formation synergistically enhanced the DNA-binding properties of each activity: either RPA assists damage recognition of XPA, or stabilizes XPA-DNA complex formation. In addition, interactions between RPA1 and XPA, and RPA2 and XPG were shown (81). These interactions suggest a bridging function for RPA in NER

A functional homolog of RPA, 'RF-A', has been identified in *S.cerevisiae*. This activity also contains three subunits (69-, 36-, and 13- kDa), which are encoded by essential genes (resp. *RFA1, RFA2* and *RFA3*)(19). Some mutant alleles of *RFA1* give rise to an UV-sensitive phenotype, suggesting an additional role for RF-A in NER, like its mammalian counterpart (131).


GGR
TCR
XPA
RPA
UVDDB
UV
5'
3'
A
RNApol.II
TFIIH
CSA/CSB (TFIIS?)
CSA
CSB
B
XPC/HHR23B
(IF7?)
(RPA?)
(RPA/XPA)
C
XPA
UVDDB
ERCC1-complex
(IF7?)
XPG
ERCC1 complex
5' endonuclease
D
XPG
3' endonuclease
E
DNA polymerase ε(δ)
PCNA, RF-C, RPA
DNA ligase I
F

opposite page:
Figure 6. *Scheme of mammalian NER mechanism. Model of the two different NER-pathways, genome overall (GGR, see 4.5.3) and transcription coupled repair (TCR, see 4.5.2). Step A and B, lesion recognition (4.1) and stalling of RNA pol II (4.5.2). Step B and C, lesion demarcation and local unwinding (4.2). Dual incision on either site of the lesion, step D (4.3). Repair replication and ligation, respectively step E and F (4.4).*

4.4 Repair replication

The last step in the NER mechanism, gap-filling of the excised patch, is probably the least specific, but this part of the process is used most frequently for assaying NER, both *in vivo* (UDS) and *in vitro*. DNA repair synthesis is performed by common DNA replication factors.

In early studies with permeabilized cells and specific inhibitors, it was shown that the DNA polymerases δ and ϵ act in mammalian NER (106). Studies with cell-free extracts revealed that PCNA, a DNA pol. δ and ϵ accessory factor, is involved in repair synthesis (171, 227). It was suggested for some time that PCNA acts in NER, since it is localised to specific spots in the nucleus after UV-irradiation (repair patches) independently of the cell-cycle (28). With the recently established *in vitro* reconstituted repair reaction, it was shown that efficient repair-synthesis occurred after addition of the mammalian replication factors: PCNA, RF-C, RPA and DNA pol. ϵ (228). DNA pol. δ can substitute for pol. ϵ, but it produces repair patches which are not easy to ligate. RF-C assists PCNA to load on a gapped DNA template (189), a NER-like intermediate. After PCNA assembly on single strand gaps, it forms an anchor site for the polymerase and stimulates the processivity of DNA pol. ϵ (or δ) (189). The yeast homologs of both DNA polymerases δ and ϵ also have been shown to be involved in UV-damage repair (22). Only double mutants of pol. δ and ϵ are UV-sensitive (22), suggesting that each activity can be substituted by the other.

Finally, the NER process is completed by sealing (ligation) of the newly synthesized DNA to the existing DNA. DNA ligase I was predicted to be a likely candidate for this action, since mutations in the corresponding gene can give rise to a UV-sensitive phenotype (14). Addition of purified ligase I to the reconstituted NER reaction induced ligation, although not very efficiently (1). No other ligases have been tested so far. Whether other activities, in addition to a ligase, are necessary for completion of the NER reaction, is under investigation (1).

4.5 Differential NER

Induction of lesions after UV-irradiation is not homogeneous throughout the genome. The type of damage and its relative frequency depend on the location in chromatin and on the transcriptional status of specific sequences (62, 153, 188).

Distribution of CPDs, rather than 6-4PPs, in core nucleosome regions, exhibit a periodicity of 10.3 bases (62). This indicates that shielding by histones is an important parameter in lesion formation, since this periodicity is identical to a single helical turn in duplex DNA.

Not only is the induction of lesions non-uniform, but also a differential rate of repair is present at distinct parts of the genome (15). The repair rate is primarily determined by the transcriptional activity, rather than a non-homogeneous distribution of lesions on nucleosomes (100). However, nucleosome stability influences the rate of repair and of transcription. Moreover, different types of lesions influence the repair rate, e.g. 6-4PPs are repaired much faster than CPDs (152).

4.5.1 Active gene repair

The pioneering work of Hanawalt and Bohr (17) revealed an accelerated NER in actively transcribed regions of the genome, when compared to NER of the genome overall. This preferential repair of CPDs was first discovered in mammalian cells. The amplified *DHFR* gene of CHO cells was about 5 times more rapidly repaired than inactive sites of the genome (17). This transcription-coupled repair (TCR) is evolutionary conserved, from prokaryotes and yeast up to man (149, 150, 256). A notable and as yet unexplained exception is *Drosophila* (42), in which no TCR could be detected in embryonal cell strains. TCR was recently also demonstrated in *in vivo* irradiated epidermal cells of mice (203).

Although transcriptional activity is regulated by intricate interactions between transcription factors and chromatin, it is not likely that preferential NER is driven only by the chromatin structure, since TCR is present in various types of organisms with completely different nucleoprotein structures. Moreover, TCR occurs also in *in vitro* reconstituted NER reactions with purified *E.coli* enzymes, where no nucleoprotein complexes are present (218).

Recently, evidence was provided that the 6-4PPs, which are faster removed than CPDs, also are targets for TCR (268).

4.5.2 Transcription-coupled repair (TCR)

Preferential repair of active genes is mainly caused by selective repair of the transcribed strand (150). This strand bias relies on RNA-polymerase II transcription, but is independent of the level of transcription (15). It is claimed, however, that the non-transcribed strand of active genes is more efficiently repaired than silent or non-coding loci (161, 272). These observations suggest that NER is regulated at three hierarchic levels: the first, accelerated repair of transcribed strand; the second, preferential repair of active genes; and the third, slow repair of transcriptionally silent regions.

The biological significance of a proficient TCR is reflected in the NER-deficient Cockayne syndrome (CS). Cells derived from CS-patients (of both complementation groups), have defective RRS (recovery of RNA synthesis) after UV, but a normal UDS (see

3.2). This phenomenon can be explained by a deficient TCR, which forms the basis of the repair defect in CS (267, 273). Preferential repair of the transcribed-strand suggested that specific factors are required to couple NER to the process of transcription. Two candidates of transcription-repair coupling factors are CSA and CSB, respectively encoded by the recently cloned and sequenced genes *CSA* (82) and *CSB* (263). However, nothing is yet known of their specific function in coupling repair to transcription.

The protein sequence of CSA contains the consensus of five "WD-repeats" (82). This type of motif is present in many proteins, and these proteins are believed to possess a regulatory, rather than a catalytical function (170). Many of these WD-repeat proteins reside in multi-protein complexes. CSA is thought to associate with p44 (a component of TFIIH) and with CSB (82). *CSB* encodes a potential helicase (263), since it contains the seven conserved domains which define a large superfamily of DNA and RNA helicases (67). In addition, CSB shows a striking homology of the entire region spanning the seven helicase domains with proteins belonging to a new sub-family of helicases, the SNF2 sub-family (67, 263). The exact function of these proteins is not yet known, but there is evidence that some of these polypeptides reside in large complexes, which play a role in chromatin modulation. None of the members of this sub-family of helicases have been shown to possess DNA-unwinding activity. The fact that Mot1 (a member of the SNF2 sub-family), removes TFIID from template DNA (5) suggests a protein-DNA dissociating activity. These domains might therefore define an energy-consuming activity, required for either DNA/RNA unwinding or stripping proteins from DNA in a processive manner. Other members of the SNF2 sub-family, are required for efficient transcription of specific target genes or function as a global transcription factor (83a).

A functional prokaryotic homolog of the TCR-factor is the *E.coli* transcription repair coupling factor (TRCF) encoded by the *mfd* gene. TRCF specifically directs NER to stalled (on UV-lesions for example) RNA polymerase (RNAP) complexes (219, 220, 221). No DNA nor RNA-unwinding activities can be measured for TRCF, despite the presence of helicase domains in the protein sequence (219). It has been shown, however, that TRCF itself binds specifically to stalled RNA polymerase complexes and to DNA in an ATP-dependent manner. TRCF dissociates RNAP complexes stalled on UV lesions from DNA and recruits repair factors to the damaged site, via a direct interaction between TRCF and UvrA (220). Helicase motifs in TRCF are likely to be involved in DNA-binding and ATP hydrolysis, which changes the conformation of TRCF with a concomitant stripping off bound proteins (RNAP) from DNA.

A similar function might be expected for CSB with CSA, i.e. directing core mammalian NER-factors to a stalled polymerase ternary complex. However, stalled RNA polymerase II complexes in mammalian transcription elongation may not be simply removed from the template after arresting, as in *E.coli* (49). Templates containing a single CPD efficiently block mammalian RNA polymerase II elongation. It has been postulated (49) that elongation factor SII (TFIIS) together with the CS-factors, plays a role in directing NER to a stalled RNAP complex. SII plays a role in promoting elongation complexes through transcriptional pause sites. The activity catalyses transcript shortening (tracking back of the ternary complex) of nascent RNA on pause sites or when RNAPII is stalled

at a CPD (49). However, SII alone is not able to stimulate bypass of stalled RNAPII over a UV-lesion, and may need the cooperation of CSA with CSB.

The yeast homolog of *CSB*, *RAD26* was recently cloned (266). Disruption mutants of this gene, have a deficient TCR. However, in contrast to mammalian mutants (266), these cells are not sensitive to UV-irradiation, suggesting that UV-resistance in this organism is primarily due to the GGR pathway.

4.5.3. Global genome repair (GGR)

The repair defect in XP-C cells is caused by a deficiency in the repair of the non-transcribed strand of active loci and the overall genome (GGR), while TCR is unaffected (274). These findings fit well with earlier cellular observations. In contrast to the other XP and CS groups, XP-C cells, do not have a defective recovery of RNA synthesis after UV treatment (RRS) (144).

The XP-C correcting factor (XPC), independently purified by two groups (140, 225), consists of two polypeptides of 125 kDa and 58 kDa which are stably associated (140). The size of this complex is in agreement with the hydrodynamic properties of the correcting activity as determined by Shivji and coworkers (225). Micro-sequencing both proteins allowed the cloning of the corresponding genes (140). The gene encoding the 125 kDa protein is a N-terminally-extended version of a previously isolated incomplete *XPC* cDNA (116). Surprisingly, the 58 kDa polypeptide is encoded by one of the two human homolog's of the *S.cerevisiae RAD23* repair gene: *HHR23B*. To date, no known human NER syndromes have been associated with the *HHR23B* gene and a simple explanation might be that the highly homologous *HHR23A* diminishes the effect of loss of HHR23B function. The notion that HHR23B is a ubiquitin(-like) fusion protein suggests that this polypeptide may function as a molecular chaperon for XPC, since ubiquitin moieties can serve as a molecular-chaperon (56). In contrast to XPC, HHR23B is abundantly expressed and only a minority of HHR23B is associated with XPC (van de Spek, ms. in prep.). This suggests a second non-NER function for HHR23B. The single yeast homolog (Rad23) definitely plays a role (directly or indirectly) in both NER pathways (TCR and GGR) (145, 288).

Limited amino acid homology exists between human XPC and yeast Rad4 (116). However, the XPC-complex functions in GGR, whereas Rad4 is indispensable for both NER pathways in yeast (145, 275). In addition to the difference in function of yeast Rad23 and human HHR23B, a further divergence concerning TCR is present between the two species. Yeast *rad7* and *rad16* mutants both behave as functional homologs of the XPC-complex, because they are specifically involved in GGR (8, 275). However, human XPC and HHR23B do not share homology with Rad7 or Rad16 and no mammalian counterparts of RAD7 and RAD16 have been identified to date. The notion that RAD7 interacts with SIR3, a protein involved in transcriptional silencing of chromatin (180), suggests a role for RAD7 in chromatin modification.

At present it is only possible to speculate upon the function of the XPC/HHR23B-complex in NER. Its unique involvement in GGR suggests that this complex uncouples

NER from the tight connection with transcription. With the intrinsic high affinity for ssDNA (140), this complex may enhance unwinding or stabilize partly melted regions around a lesion, and facilitate the action of the excision nucleases. Binding to ssDNA might occur in the opposite strand to where RPA is bound. This DNA-protein complex could form a substrate for the further assembly and action of NER enzymes. In this model, the XPC/HHR23B-ssDNA complex competes with stalled RNAPII in recruiting the excinuclease.

XPC correcting activity is indispensable for *in vitro* repair (1, 140, 225), which indicates that it is involved in the core of the NER reaction. However, NER activity in this assay is independent of transcription and thus exclusively reflects GGR.

4.6 Higher order NER complexes

Although most of the important 'players' in mammalian NER are identified, it is not known yet how these factors assemble on a lesion and how they interact with each other. Recently, several reports appeared in the literature dealing with proposed interactions between NER factors. However, many of these studies are either based on associations in a rather artificial environment (immobilized fusion proteins), or after overexpression in yeast (two-hybrid system). In both systems, transient or low affinity interactions can be selected, which might not reflect the *in vivo* situation.

A central role for the XPA protein is likely in view of the very severe NER-deficient phenotype of XP-A cells (35). Biochemical studies with XPA suggest that this factor serves as a nucleation point in the repair reaction. Interactions of XPA (in addition to that with damaged DNA), have been described with ERCC1 (123, 125, 165, 184), RPA2 (81, 141) and TFIIH (183). Binding of both ERCC1 and RPA to XPA has a synergistic effect on the DNA damage-specific binding affinity of XPA (81, 165). In yeast, TFIIH association with Rad14 (XPA) is reported to occur via Rad23 (73), although in a later report by the same authors, Rad23 was stated to be associated with Rad4 (72). RPA2 is claimed to interact with XPG (81), whereas XPG (Rad2) and XPC (Rad4) might be associated with TFIIH (10, 50). Several of these assumed interactions are dependent on isolation conditions (159) and could not be confirmed by other laboratories (1, 279). Using specific isolation conditions, even a NER super-complex, designated, "repairosome", was purified from yeast cells (247). The majority of the different yeast NER factors are claimed to reside in this repairosome, whereas this isolated complex is not active in a reconstituted NER reaction (247). Also the amount of each of the NER factors residing in this complex is not known.

Chapter 5

Transcription/repair factor TFIIH

Transcription/Repair Factor TFIIH.

Basal transcription initiation factor TFIIH plays an important role in RNA polymerase II-driven transcription. As discussed in chapter 4.2.3, TFIIH possesses a dual function in transcription and NER. Elucidation of the role of TFIIH in basal transcription will also provide clues to its role in NER.

5.1 Basal transcription factors

As for nucleotide excision repair, transcription of protein-encoding genes by RNA polymerase II (RNAPII), is a multi-step process. Basal transcription is likely to start by the sequential loading of transcription factors onto a core promoter region. It is generally accepted that prior to the assembly of an initiation complex, the dense chromatin structure has to be modulated or made accessible (47, 294).

The assembly of the initiation complex, as described below, can be considered as a working model, since the exact order of loading of factors is not rigorously established (29, 37, 299). Transcriptional start site selection on minimal promoters requires binding of the TATA-box binding protein (TBP). TBP is not only involved in RNAPII transcription, but also plays a central role in RNA polymerase I and polymerase III-driven transcription (37, 65). TBP, together with the other basal transcription factors is sufficient to initiate basal, but not activated transcription. For the response to transcriptional activators, TBP has to be incorporated into (holo)TFIID: TBP plus TBP-associated factors (TAFs) (65).

The role of TFIIA (a three subunit factor) in basal transcription is controversial. The requirement of TFIIA depends on the purity of transcription factors in reconstituted systems. While TFIIA is not essential, it promotes loading and stabilizes the association of TFIID on the promoter (23). Negative transcriptional regulators disrupt the TBP-DNA interaction in an ATP-dependent manner. Mot1 (a yeast TAF), is such a negative regulator, possibly involved in stripping TBP from the DNA. Addition of excess TFIIA suppresses this Mot1 inhibitory action (5). TFIIA probably stabilizes TFIID binding and is involved in complex formation in activated transcription in conjunction with the TFIID TAFs (91).

Basal factor TFIIB is thought to act as a bridging factor between the initial complex DNA-TFIIA-TFIID (DA-DNA complex) and RNAPII. TFIIB interaction is important in the accuracy of start site selection (37). Some upstream transcriptional activators interact with TFIIB and are considered to assist in the formation of the initiation complex (223).

TFIIB forms a nucleation point for RNAPII. The delivery of the large RNAPII complex to the DAB-DNA is facilitated by TFIIF which is directly associated with RNAPII (68). Moreover, TFIIF, which consists of two subunits: RAP30 and RAP74, prevents nonselective binding of RNAPII to DNA (68). In addition to the absolute requirement of TFIIF in transcription initiation, TFIIF is also engaged in the elongation stage of transcription (68, 251).

The actual polymerase, RNA polymerase II (RNAPII), is a multi-subunit enzyme. The composition of mammalian RNAPII is not yet completely known. The largest subunit of human RNAPII was recently cloned (151). In contrast, yeast RNAPII is well characterized. It consists of 12 polypeptides (298) varying in size from 240 to 10 kDa (RPB1-12). The different subunits have been cloned and display a high degree of evolutionary conservation among eukaryotes. The homology extends to prokaryotic counterparts of the two largest subunits (resp. ß' and ß). Neither the stoichiometry and architectural composition nor the specific function of each of the subunits are resolved yet. The two largest subunits (220 and 150 kDa) bind respectively to DNA and to ribonucleotides (298).

A feature not found in the prokaryotic RNA polymerase is the presence of a highly conserved hepta-repeat (YSPTSPS) at the C-terminal domain (CTD) of the 220-240 kDa subunit (41). The number of repeats varies from 17 in *Plasmodium falciparum* to 52 in mammals. The CTD is an efficient target for phosphorylation, which occurs as a response to transcriptional activators and is thought to be effective during elongation (41). The CTD kinase activity, displayed by TFIIH, will be discussed in the next paragraph. Recently, it was observed that RNAPII is incorporated into an, even larger, higher order complex: "RNA polymerase II holoenzyme", comprised of approximately 30 different polypeptides (RPBs, TFIIF and SRBs)(109, 112, 113). These associated factors are together referred to as the "mediator". The mediator acts in transmitting the response of transcriptional activators to the basal machinery, by phosphorylating CTD (109). Recently, two of these SRBs (SRB10 and SRB11) were identified as a cyclin/cdk-like complex, specifically kinating CTD in response to activators (127). Besides activated transcription, the mediators also stimulate basal transcription (109, 113).

5.2 Activities of TFIIH

ATP hydrolysis is required to convert the preinitiation complex into a transcription- competent complex (37). However, none of the above mentioned basal factors possesses any catalytic activity, including ATPase. Human TFIIH, previously also called BTF2 (63), its rat homolog factor δ (36, 222) and yeast equivalent factor b, now renamed (y)TFIIH (53), all exhibit ATPase activity and are essential for *in vitro* transcription. Entry of TFIIH into the preinitiation complex is supported by TFIIE (58). TFIIE is a tetramer containing two subunits of 56 kDa and two of 34 kDa protein (37). TFIIE does not only aid in the loading of TFIIH onto the preinitiation complex, but it also stimulates the different TFIIH catalytic activities (132, 179). Until recently, no specific activity could be attributed to TFIIE. However, recent observations indicate that TFIIE might function in melting the template DNA, independent from TFIIH (90).

5.2.1 ATPase and helicase activities

Different activities can be ascribed to the multi subunit TFIIH, composed of at least 8 or 9 different polypeptides (Table 4)(55, 201). The ATP-hydrolysing activity of

TFIIH is DNA-dependent and specific for ATP (36, 179, 202). In addition, TFIIH displays also a bidirectional helicase activity (211, 212) and contains the two helicases, XPB and XPD (50, 211). Recently, ATPase and 3'→5' helicase activities of XPB were actually shown with an *E.coli*-produced recombinant protein (202), later confirmed with XPB produced by the Baculovirus system (134). The yeast XPB homolog (Rad25/Ssl2) also displays helicase activity (74). XPD catalyzes the opposite direction of unwinding, 5'→3' helicase (241).

Table 4. Homology and properties of TFIIH.

Human TFIIH		Yeast TFIIH		protein properties
subunit[1)]	cloned gene	subunit[1)]	cloned gene	
782(p89)	*XPB/ERCC3*	843(p105)	*SSL2/RAD25*	3'→5' helicase
760(p80)	*XPD/ERCC2*	778(p85)	*RAD3*	5'→3' helicase
548(p62)	*p62*	642(p75)	*TFB1*	??
460(p41)	*p52*	495(p55)	*TFB2*	-
395(p44)	*p44*	461(p50)	*SSL1*	2 Zn^{2+}-fingers
346(p38)[2)]	*Mo15/CDK7*	306(p33)[2)]	*KIN28*	CDK-like kinase
303(p34)	*p34*	?		Ssl1-like Zn^{2+}-finger
323(p34)[2)]	*cyclinH*	393(p47)[2)]	*CCL1)*	homology to cyclins
312(p32)[2)]	*MAT1*	312	*TFB3*	ring Zn^{2+}-finger

For references to the subunits and respective genes, see Roy, 1994 and Feaver, 1994 and references therein.
1) subunits are presented as number of amino acids, based on ORF of cloned gene, with size in SDS-PAGE between brackets. 2) subunits of CAK activity, either asociated with TFIIH, or in a free form.

TFIIH helicase is thought to convert the closed DNA with the pre-initiation complex into a more open configuration, allowing RNAPII to enter the elongation phase (212). Using an abortive transcription assay, Tjian and coworkers (66) have provided evidence that TFIIH and TFIIE are not essential for the initiation step, but act in a transition stage between initiation and elongation, referred to as "promoter clearance" (66, 143). The authors argued that TFIIH is not involved in unwinding the template prior to initiation but directly following it, and that TFIIH plays a role in dissociating (clearing) the pre-initiation complex from the promoter. TFIIH and TFIIE (and ATP hydrolysis) are

dispensable for transcription from negatively-supercoiled templates, but are required for transcription from linear templates (186, 259). Superhelicity of the template may provide partially unwound regions, explaining the optional requirement of a helicase (TFIIH). The *in vivo* implications of this are somewhat confusing, because after the process of nucleosome opening (decondensation) prior to transcription, superhelicity can be induced, suggesting that unwinding by TFIIH is not absolutely required *in vivo*. However, genes encoding TFIIH components are essential for viability in yeast (71, 84, 169, 185, 297), consistent with an indispensable role for TFIIH in transcription *in vivo*.

5.2.2. CTD-kinase activity

In addition to ATPase and helicase activities, TFIIH also possesses a phosphorylation activity specific for the C-terminal repetitive domain (CTD) of the largest subunit of RNAPII (132). The function of CTD phosphorylation is not yet known, although it is generally accepted that it plays a role in the elongation stage of transcription (41). Isolation of elongating RNAPII complexes showed that the majority is hyper-phosphorylated RNAPII (RNAPIIo). Initiation of reconstituted transcription using hypo-phosphorylated CTD (RNAPIIa) is as efficient as with RNAPIIo, suggesting that CTD-kinase activity is not required for *in vitro* transcription. However, core-TFIIH fractions, without kinase activity, are inactive in basal transcription (246). Recently, Kim and coworkers reported that when using the holo-RNAPII complex CTD-phosphorylation stimulates not only activated but also basal transcription (109). Moreover, CTD deletion mutants are not viable in several organisms (41), although such a truncated protein is still functional *in vitro*. The conversion of RNAPIIa into RNAPIIo might also play a role in the release of RNAPII from the initiation complex (264).

CTD-kinase also consumes ATP, which is likely to be performed by another (intrinsic) component of TFIIH than the DNA-dependent ATPase (XPB and XPD), since a distinction can be made on the basis of a differential sensitivity to inhibitors (202). Moreover, in a transcription assay, using RNAPII which lacks the CTD, ATP is still required.

The CTD kinase activity of TFIIH can be dissociated from the core TFIIH complex (201, 246). The components (3 both in human and yeast) responsible for CTD kinase have recently been identified (55, 201). Analysis of these subunits revealed that the largest kinase subunit ($\approx$ 40 kDa), is highly homologous to the *Xenopus Mo15* gene (229). Mo15, and its equivalents in different species, were originally identified as the catalytic subunit of the CDK-activating kinase or "CAK" (255).

CAK is an important activator (kinase) of several CDK/cyclin complexes, which are involved in cell-cycle regulation (33, 173). Surprisingly, it turned out that Mo15, based on its amino acid sequence, also belongs to the family of cyclin-dependent kinases (CDK's), and is now designated "CDK7". CAK also contains, besides CDK7, two other subunits, p34 and p32 (255), which were also identified as being associated with TFIIH. The 34 kDa component turned out to belong to the family of cyclins and is termed "cyclin H" (57, 138). The identity of the other subunit (32 kDa) is not yet known.

TFIIH-associated CAK is able to phosphorylate both CTD of RNAPII and CDK/cyclin complexes (201). However, the target sequence for phosphorylation of CTD (YSPTSPS) is rather different than the consensus T-loop region in CDK's (33, 173). An explanation for this apparent discrepancy is that by the assembly of the initiation complex CAK is physically situated in the proximity of CTD and because of that, able to phosphorylate this other (distinct) target.

5.3 Dual involvement of TFIIH.

Highly purified TFIIH specifically corrects the NER defect in XP-B, XP-D and TTD-A, both with microinjection and in the *in vitro* repair assay. With the cell-free NER-assay it was shown that TFIIH directly acts in NER, since in this assay no transcription nor translation can occur, due to the lack of NER-genes in this reaction. Correcting activities of XP-B, XP-D and TTD-A exactly co-elute with transcription- and helicase- activities of TFIIH (appendix IV, V). NER-competent cell lysates and purified TFIIH fractions can be deprived of repair activity after immuno-precipitation with antibodies directed against some components of TFIIH (anti-XPB, -p62, -p44, -p34) (93, 279). These data suggest that the entire transcription initiation factor, TFIIH, is probably involved in NER. The dual functionality of the entire TFIIH complex is also evident from recent studies with yeast TFIIH (54, 74, 247, 286). Moreover, several mutant alleles of TFIIH subunits, *rad3, ssl2, ssl1* and *tfb1* are UV-sensitive, suggesting a role in NER (71, 142, 285, 297). NER-activity and viable function (basal transcription) of Rad25/XPB can be uncoupled as shown by different *ssl2* alleles (71, 185, 248) again indicating that the repair defect is not caused by a deficiency of transcription of repair genes. Anbtibody injections against several TFIIH components inhibits both NER and transcription (appendices IV and V)(201), providing for the first time evidence that TFIIH functions in transcription *in vivo*.

It was postulated by Gulyas and Donahue (71) that Ssl2 acts in translation initiation, probably by a RNA-unwinding activity (of the artificial stem-loop structure), which enables ribosomes to assemble on the mutant *HIS4* RNA. However, in retrospect, with the current knowledge and with transcriptional expression data from the original paper (71), it is very likely that Ssl2 functions in transcription. Suppression of the *his4* mutant phenotype by the *ssl2-1* allele is probably due to a gain-of-function mutation in the *SSL2* gene. The specific mutation in SSL2 may result in a more potent helicase function of TFIIH, which enables this complex to unwind even the altered structure of the leader sequence of the *HIS4* gene. This will result in a higher transcription of the mutant *HIS4*. However, for the the other "translation-component" of TFIIH, Ssl1 it is somewhat more complicated. Although Ssl1 is a core component of TFIIH (54, 93), some *ssl1* alleles do not affect transcription, whereas they influence translation (297)

Chapter 6

Concluding remarks

Concluding Remarks

Previously, the NER-related syndromes xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy were considered distinct hereditary diseases. Genetic analysis of rare NER-deficient individuals with a complicated and heterogenous set of clinical symptoms (appendices I, II, III and V) revealed a large genetic overlap between these repair disorders. Severe cases with clear CS features were genetically linked to the *XPG* gene (appendix I). Mutational analysis of the two severe XP-G/CS patients showed a seriously affected *XPG* (S.Clarkson pers. comm.). Recently, three other cases with either CS symptoms only, or with combined XP/CS features were assigned to the same locus (B. Hamel, submitted; A. Raams, NGJ. Jaspers and W. Vermeulen, unpublished observations). An even more heterogeneous array of clinical symptoms was observed in complementation groups XP-B and XP-D, ranging from XP to XP/CS to TTD (21, 101, 278)(appendices II and V). This genetic overlap and clinical heterogeneity within complementation groups argues in favour of the postulated model (Figure 4) that the previously distinct genetic syndromes are part of a large clinical continuum of "NER-deficent syndromes". The very different features are due to locus and allelic-heterogeneity, in combination with different expression due to genetic background and additional roles of NER factors in other cellular processes (see 4.2.3 and 6.1).

6.1 TFIIH involvement in NER syndromes

The observed link between the XPB, XPD and TTDA subunits with the TFIIH complex may provide a clue for the previously unexplained diverse features in these groups (appendix V). Most of the symptoms displayed by CS and TTD, such as neurodysmyelination, impaired sexual development, retarded growth, dental caries, cataract, and dismorphic facies, had been difficult to rationalize on the basis of a defective NER. The specific correlation between these features and mutations in TFIIH prompted us to postulate that part of these symptoms are a reflection of a subtly disturbed transcription due to a defect in TFIIH. We proposed to designate these syndromes (XP-B, XP-D and TTD-A) as: "transcription/repair syndromes" (18)(appendix V). Extrapolation of this hypothesis led to the suggestion that pure "transcription syndromes" could also exist, without disturbed NER. Non-photosensitive forms of TTD and CS are likely candidates for such a transcriptionally defect. Conversely, classical XP-D patients can be explained as having only a disturbed NER (see Figure 7, appendix V). To verify this hypothesis, these variant forms of CS and TTD can be screened for mutations in one of the TFIIH-encoding genes, a survey which is currently in progress.

An obvious and important question in this model is: why does a mutation in a basal transcription factor only affect transcription of specific genes? The most simple explanation is that TFIIH is not required for transcription of all genes, but only for a subset of genes. The necessity for TFIIH might depend on the topological state of the promoter region, and by inference, the chromatin structure. This would mean that TFIIH

is an auxiliary transcription factor, only necessary for *in vitro* runoff assays and for transcription of genes, e.g. in highly condensed chromatin or in a relative relaxed status. However, the fact that all known components of TFIIH are essential for viability in yeast renders this assumption less likely, although it does not rule out this possibility.

A second, more likely, explanation is that the TFIIH function in the postulated transcription syndromes is only marginally affected. Serious alterations in its function are presumably lethal. A slightly lower transcription, either by diminished initiation or by a less efficient elongation, only affects those cellular processes whose functioning critically depends on the level of transcripts (see appendix V).

Another reason for the selective effect of TFIIH mutations on transcription levels might be a reduced stability of the complex as a consequence of a mutation in one of the components. The effect of an altered stability will mainly be apparent in terminally differentiated cells, where the majority of the genome is transcriptionally silent, and the cells are specialized in high level production of a few proteins. The majority of the salient features in CS and TTD are expressed in highly differentiated tissues of neuro-ectodermal origin.

A fourth explanation is that TFIIH functions as a regulatory element for some transcriptional activators. Mutations which modify the structure of TFIIH could reduce the response of the activator signals on the basal transcription machinery. A new group of factors which serve as intermediates between basal transcription and activators are found to co-purify with RNAPII, and are designated "mediators" (109)(see also chapter 5.1). Two of the mediator components form an active CDK/cyclin pair (127), comparable (but different) to the CDK7/cyclin H pair, present in TFIIH. Both kinases are involved in CTD phosphorylation, and both likely function in concert to potentiate RNAPII by hyper-phosphorylation.

This hypothesis does not explain, however, the "CS" features of some patients of CS-A, CS-B and XP-G. The transcription-repair coupling factors CSA and CSB may play a role in shuttling TFIIH from its transcription mode into the repair mode and/or *visa versa*. A disturbed functioning possibly also affects transcription in addition to TCR. When the 3' incision (near the lesion) by XPG is performed late in the excision reaction, at least after the action of TFIIH, a malfunctioning of this activity may sequester TFIIH in frozen repair intermediates. In both cases, a reduced amount of TFIIH would become available for transcription, resulting in the observed features. In this model, however, CS features are secondary to improperly-processed repair intermediates and thus dependent on the amount of lesions. On the other hand, the very severe CS symptoms in many cases of early onset CS, suggest that these features do not depend on DNA damage. CSA, CSB and XPG may have an additional, as of yet unresolved, (dual) role in transcription, similar to TFIIH.

6.2 DNA repair versus cell-cycle control.

In addition to the induction of DNA repair, exposure to genotoxic agents also affects cell-cycle progression, transcriptional control, and it may provoke apoptosis. These

responses to DNA damage play an important role in preservation of genomic integrity. Cell-cycle progression is regulated by a variety of "cellular checkpoints". Some of these checkpoints are activated by DNA damage and consequently induce a cell-cycle arrest, allowing the cell to process the lesions. Regulation and communication between checkpoints, transcriptional control and repair should be present. Several cell-cycle controlling factors have been found to be transcriptional regulators; some influence the replication machinery.

The intriguing observation of a CDK/cyclin pair in TFIIH makes it tempting to speculate upon a possible cell-cycle controlling function for TFIIH, in addition to its repair and transcription role. If indeed TFIIH is functional in cell-cycle regulation, it is a candidate manager in the genotoxic response. CDK/cyclin complexes are important cell-cycle regulators (173). Activation (by phosphorylation) of these complexes is performed by CAK, or CDK7/cyclin H (33). CAK activity is not differentially expressed and is equally active throughout the phases of the cell-cycle (255). The TFIIH-associated kinase may be a target in the signal transduction pathway(s) (e.g. after genomic stress), which influence both transcription and cell-cycle progression. It would be interesting to identify the kinase which activates CDK7 and see whether it fits in such a model.

A potential target for phosphorylation by CDK/cyclin complexes is RPA, since CDK/cyclin pairs are able to phosphorylate RPA *in vitro* (51). RPA has an essential role in NER and in replication: this makes RPA a likely candidate as a regulator of both replication and repair. Moreover, RPA2 (p34) is differentially phosphorylated throughout the cell-cycle (48) and hyper-phosphorylated after exposure to genotoxic agents (e.g. UV-irradiation)(27, 130). Hyper-phosphorylation inhibits *in vitro* DNA replication (27). During the NER process, TFIIH may unwind the template and provide a good substrate for RPA to bind (see 4.3.3 and 4.6). TFIIH kinase activity (CAK) would then be in close proximity to RPA and may be able to catalyse phosphorylation of the 34 kDa subunit. The altered status of RPA is still capable of acting in NER, but is non-functional in replication. By this process, RPA is forced into repair (in the equilibrium between repair and replication) either by potentiated recruitment to repair patches or a conformational change. Whether hyper-phosphorylated RPA is active in NER has to be investigated. At least the phosphorylation status of RPA2 does not influence XPA binding (124). In contrast to the above proposed model, Pan and coworkers (182) recently reported that the phosphorylation status of RPA neither influences *in vitro* repair nor replication. However, no hyper-phosphorylated RPA was used, and the absence of kinase activities was not shown in their *in vitro* reaction.

Another important regulator of cell-cycle progression in response to genomic stress (at the G1/S checkpoint) is p53 (40, 206). This protein is stabilized by DNA damage and is responsible for the transcriptional activation of some damage-inducible genes (133). p21 (WAF1, CIP1 or SDI1) is one of the p53-activated genes and acts as an inhibitor of G1/S CDK/cyclin complexes. In addition, p21 also binds to PCNA and inhibits *in vitro* replication (59, 280). In contrast, p21 does not affect the function of PCNA in the repair reaction in mammalian extracts (126, 226). In conclusion, p21 inhibits DNA replication after damage, but allows repair to continue.

A third candidate regulator of cell-cycle and DNA repair is GADD45, which is also transcriptionally induced by p53 after DNA damage (89). GADD45 also interacts with PCNA (232), and it is claimed to stimulate NER and to inhibit S-phase entry (232). However, the *in vivo* significance of this postulated regulatory role of GADD45 between NER and replication is in debate (206).

An observed association between TFIIH and p53 protein (281, 283) might even connect the major cellular responses to DNA damage, including apoptosis. Although this association is confirmed *in vivo*, it only modulates the helicase activity of TFIIH, but not its transcriptional potency (283) nor its repair activity (unpublished observations). Recently, it was found that p53-induced apoptosis is delayed in XP-B and XP-D cells (282). The p53 apoptotic pathway is likely to be independent of transcription, so the observed delay of apoptosis in these cells cannot simply be explained by a subtle transcription defect (282). Moreover, p53-independent apoptosis is not altered in XP-B and XP-D. On the other hand, it is difficult to imagine that all different interactions and pleiotropic functions of TFIIH in very vital cellular processes are displayed by a single protein complex. Direct *in vivo* evidence, where all components are expressed at physiological levels, is required to verify the biological relevance of all the postulated TFIIH functions.

6.3 Future perspectives

The recently attained successful *in vitro* reconstitution of NER is probably the most powerful tool for further elucidation of the mammalian NER mechanism. Although a variety of important NER factors are characterized and assigned to a specific step in the process, a complete picture is not yet available. The *in vitro* NER-assay has already recognized previously unknown essential NER factors like RPA and the recently described initiation factor 7 (IF7). This factor has to be further characterized before a specific function can be attributed to this activity. With the aid of this reconstituted NER and by using well-defined artificial substrates, the sequence of events during NER can be unravelled. Unfortunately, not all factors are completely purified yet, nor is the native composition of each factor known, particularly in the cases of the TFIIH and ERCC1-ERCC4 complexes. Purification of these complexes with classical protein purification protocols is laborious and yields only minute quantities. In addition, the native composition of quite intricate protein complexes may be destroyed. Moreover, the ERCC1-containing complex seems to be refractory to further purification (1, 269). A potential solution to this problem might be the use of recombinant tagged genes. When such a fusion gene is stably transfected into a mammalian recipient cells, purification from mass cultures can be performed with the aid of specific affinity (based on the tag) chromatographic columns. The advantage of this system is that it reduces the number of purification steps. Furthermore, purification through elution from affinity columns, is performed by competition with specific ligands instead of high salt or detergent washes, which are deleterious to delicate protein interactions. The relatively mild purification conditions may be helpful for the isolation of intact (native) complexes.

Although the current *in vitro* repair assays have permitted further insight into the mammalian NER mechanism, this system has limitations as well, e.g. the enhancement of NER by transcription cannot be measured in this system. Several attempts have been undertaken to develop a cell-free TCR repair assay without success. It is likely that the chromatin structure contributes to NER. However, the relative contribution of chromatin structure to NER cannot be addressed in present *in vitro* assays.

6.4 Mouse models.

A second important line of research is the generation of DNA repair-deficient mice. Specific inactivation of repair pathways will facilitate the analysis of genotoxic and mutagenic properties of unknown compounds under physiological conditions. Repair-deficient animal models will be valuable for cancer research in general, and in particular for assessing the systematic influences of environmental and endogenous factors on carcinogenesis. Generation of either knock-out or subtly mutated repair deficient mice, by gene replacement is currently in progress in several laboratories. Mice with mutant NER genes, mimicking the situation in existing human repair syndromes, can help to elucidate the correlation between genotype and the consequent phenotype at the level of a whole organism. Interbreeding of a particular repair mutant mice into different mouse-strains will be helpful in determining the contribution of the genetic background on the phenotype. Moreover, genetic crosses of repair-deficient mice with existing tumour suppressor and/or cellular checkpoint mutant mice (p53, Rb, etc) can be performed. This is of potential interest for the research on the relationship between cell-cycle control, DNA repair and carcinogenesis.

The first report of a NER-deficient mouse appeared in 1993 (148). Growth of ERCC1-deficient (-/-) mice is severely retarded, when compared to heterozygote (+/-) and homozygote (+/+) littermates. Unfortunately, ERCC1-deficient mice die within a few weeks after birth. The cause of death is unclear, but severe liver and kidney pathology is observed in addition to iron deposition in the spleen. No other clear abnormalities, except for stunted growth and cellular repair deficiency, are apparent in these mice. The high mortality at a very young age may provide an explanation for the absence of a human syndrome due to ERCC1 deficiency.

Recently, XPA-deficient knock-out mice were independently generated in two laboratories (43, 166). These mice grow normally, and except for defective cellular NER, they do not express any other phenotype. This is in sharp contrast with the human XP-A patients, which display severe neurological abnormalities. This species specific difference in phenotypic consequences of a repair defect is a potential problem in interpreting results obtained from studies on mouse models. The human-rodent repair paradox, might be a serious drawback. However, XPA mice were found to be extremely susceptible to skin cancer after treatment with carcinogenic agents. Thus, despite the above discrepancies with the XP-A patients, these mice serve as a valid model for studies of the relationship between repair-deficiency and carcinogenesis. In addition, homozygous *XPC* knock-out mice are also highly susceptible to UV-induced skin malignancies and display

UV-induced skin pathology (31, 207).

Currently, generation and analysis of mice, either knock-outs for *CSB* (B. van der Horst, pers. comm.) and subtle mutations in *XPB* and *XPD* are in progress (I. Donker, J. de Boer and G. Weeda pers.comm.).

Knock-out mice of two different mismatch repair genes, *MSH2* and *PMS2*, show an enhanced micro-satellite instability and are cancer-prone (7, 45).

References

1. **Aboussekhra, A., M. Biggerstaff, M.K.K. Shivji, J.A. Vilpo, V. Moncollin, V.N. Podust, M. Protic, U. Hubscher, J.-M. Egly and R.D. Wood.** 1995. Mammalian DNA nucleotide excision repair reconstituted with purified components. Cell 80:859-868.
2. **Abramic, M., A.S. Levine and M. Protic.** 1991. Purification of an ultraviolet-inducible, damage-specific DNA-binding protein from primate cells. The Journal of Biological chemistry 266:22493-22500.
3. **Andrews, A.D., S.F. Barrett, F.W. Yoder and J.H. Robbins.** 1978. Cockayne's syndrome fibroblasts have increased sensitivity to ultraviolet light but normal rates of unscheduled DNA synthesis. Journal of Investigative Dermatology 70:237-239.
4. **Asahina, H., I. Kuraoka, M. Shirakawa, E.H. Morita, N. Miura, I. Miyamoto, E. Ohtsuka, Y. Okada and K. Tanaka.** 1994. The XPA protein is a zinc metalloprotein with an ability to recognize various kinds of DNA damage. Mutation Research 315:229-237.
5. **Auble, D.T., K.E. Hansen, C.G.F. Mueller, W.S. Lane, J. Thorner and S. Hahn.** 1994. Mot 1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. Genes and Development 8:1920-1934.
6. **Bailly, V., C.H. Sommers, P. Sung, L. Prakash and S. Prakash.** 1992. Specific complex formation between proteins encoded by the yeast DNA repair and recombination genes *RAD1* and *RAD10*. Proc. Natl. Acad. Sci. USA 89:8273-8277.
7. **Baker, S.M., C.E. Bronner, L. Zhang, A.W. Plug, M. Robatzek, G. Warren, E.A. Elliott, J. Yu, T. Ashley, N. Arnheim, R.A. Flavell and R.M. Liskay.** 1995. Male mice defective in the DNA mismatch repair gene *PMS2* exhibit abnormal chromosome synapsis in meiosis. Cell 82:309-319.
8. **Bang, D.D., R. Verhage, N. Goosen, J. Brouwer and P.v.d. Putte.** 1992. Molecular cloning of *RAD16*, a gene involved in differential repair in *Saccharomyces cerevisiae*. Nucleic Acids Research 20:3925-3931.
9. **Bankmann, M., L. Prakash and S. Prakash.** 1992. Yeast *RAD14* and human xeroderma pigmentosum group A DNA repair genes encode homologous proteins. Nature 355:555-558.
10. **Bardwell, A.J., L. Bardwell, N. Iyer, J.Q. Svejstrup, W.J. Feaver, R.D. Kornberg and E.C. Friedberg.** 1994. Yeast nucleotide excision repair proteins rad2 and rad4 interact with RNA polymerase II basal transcription factor b (TFIIH). Mol. Cell. Biol. 14:3569-3576.
11. **Bardwell, A.J., L. Bardwell, D.K. Johnson and E.C. Friedberg.** 1993. Yeast DNA recombination and repair proteins RAD1 and RAD10 constitute a complex *in vivo* mediated by localized hydrophobic domains. Mol. Microbiol. 8:1177-1188.
12. **Bardwell, A.J., L. Bardwell, A.E. Tomkinson and E.C. Friedberg.** 1994. Specific cleavage of model recombination and repair intermediates by the yeast Rad1-Rad10 DNA endonuclease. Science 265:2082-2085.
13. **Bardwell, L., A.J. Cooper and E.C. Friedberg.** 1992. Stable and specific association between the yeast recombination and DNA repair proteins RAD1 and RAD10 *in vitro*. Molecular and Cellular Biology 12:3041-3049.
14. **Barnes, D.E., A.E. Tomkinson, A.R. Lehmann, A.D.B. Webster and T. Lindahl.** 1992. Mutations in the *DNA ligase I* gene of an individual with immunodeficienies and cellular hypersensitivity to DNA-damaging agents. Cell 69:495-503.
15. **Bedoyan, J., R. Gupta, F. Thoma and M.J. Smerdon.** 1992. Transcription, nucleosome stability, and DNA repair in a yeast minichromosome. The Journal of Biological Chemistry 267:5996-6005.
16. **Biggerstaff, M., D.E. Szymkowski and R.D. Wood.** 1993. Co-correction of ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects *in vitro*. EMBO J. 12:3685-3692.
17. **Bohr, V.A., C.A. Smith, D.S. Okumoto and P.C. Hanawalt.** 1985. DNA repair in an active gene: removal of pyrimidine dimers from the *DHFR* gene of CHO cells is much more efficient than in

the genome overall. Cell 40:359-369.

18. **Bootsma, D. and J.H.J. Hoeijmakers.** 1993. Engagement with transcription. Nature 363:114-115.
19. **Brill, S.J. and B. Stillman.** 1991. Replication factor-A from *S.cerevisiae* is encoded by three essential genes coordinately expressed at S phase. Genes and Development 5:1589-1600.
20. **Broughton, B.C., H. Steingrimsdottir, C.A. Weber and A.R. Lehmann.** 1994. Mutations in the xeroderma pigmentosum group D DNA repair/transcription gene in patients with trichothiodystrophy. Nature Genetics 7:189-194.
21. **Broughton, B.C., A.F. Thompson, S.A. Harcourt, W. Vermeulen, J.H.J. HoeijmakerS, E. Botta, M. Stefanini, M.D. King, C.A. Weber, j. Cole, C.F. Arlett and A.R. Lehmann.** 1995. Molecular and cellular analysis of the DNA repair defect in a patient in xeroderma pigmentosum complementation group D who has the clinical features of xeroderma pigmentosum and cockayne syndrome. American Journal of Human Genetics 56:167-174.
22. **Budd, M.E. and J.L. Campbell.** 1995. DNA polymerases required for repair of UV-induced damage in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 15:2173-2179.
23. **Buratowski, S.** 1994. The basics of basal transcription by RNA polymerase II. Cell 77:1-3.
24. **Busch, D., C. Greiner, K. Lewis, R. Ford, G. Adair and L. Thompson.** 1989. Summary of complementation groups of UV-sensitive CHO mutants isolated by large-scale screening. Mutagenesis 4:349-354.
25. **Busch, D., C. Greiner, K.L. Rosenfeld, R. Ford, J. de Wit, J.H.J. Hoeijmakers and L.H. Thompson.** 1994. Complementation group assignments of moderately UV-sensitive CHO mutants isolated by large-scale screening. Mutagenesis 9:301-306.
26. **Carr, A.M., K.S. Sheldrick, J.M. Murray, R. Al-Harity, F.Z. Watts and A.R. Lehmann.** 1993. Evolutionary conservation of excision repair in *S.pombe*: evidence for a family of sequences related to the *S.cerevisiae*. Nucleic Acids Research 21:1345-1349.
27. **Carty, M.P., M. Zernik-Kobak, S. McGrath and K. Dixon.** 1994. UV light-induced DNA synthesis arrest in HeLa cells is associated with changes in phosphorylation of human single-stranded DNA-binding protein. The EMBO Journal 13:2114-2123.
28. **Celis, J.E. and P. Madsen.** 1986. Increased nuclear cyclin/PCNA antigen staining of non S-phase transformed human amnion cells engaged in nucleotide excision DNA repair. FEBS Lett. 209:277-283.
29. **Chalut, C., V. Moncollin and J.-M. Egly.** 1994. Transcription by RNA polymerase II: a process linked to DNA repair. Bio Essays 16:651-655.
30. **Chen, E., J.E. Cleaver, C.A. Weber, S. Packman, A.J. Barkovich, T.K. Koch, M.L. Williams, M. Golabi and V.H. Price.** 1994. Trichothiodystrophy: clinical spectrum, central nervous system imaging, and biochemical characterization of two siblings. The Journal of Investigative Dermatology 103:154S-158S.
31. **Cheo, D.L., R. Hammer, L. Nieves, K. Wilson and E.C. Friedberg.** 1995. Mouse models of human nucleotide excision repair/transcription deficiency syndromes. Journal of Cellular Biochemistry Suppl.21A:309.
32. **Chu, G. and E. Chang.** 1988. Xeroderma pigmentosum group E cells lack a nuclear factor that binds to damaged DNA. Science 242:564-567.
33. **Clarke, P.R.** 1995. CAK-handed kinase activation. Current Biology 5:40-42.
34. **Cleaver, J.E.** 1968. Defective repair replication in xeroderma pigmentosum. Nature 218:652-656.
35. **Cleaver, J.E. and K.H. Kraemer.** 1994. Xeroderma Pigmentosum and Cockayne syndrome. p. *In* Scriver, C.R., A.L. Beaudet, W.S. Sly, D. Valle (ed.), The metabolic basis of inherited disease. Seventh edition. McGraw-Hill Book Co., New York.
36. **Conaway, R.C. and J.W. Conaway.** 1989. An RNA polymerase II transcription factor has an associated DNA-dependent ATPase (dATPase) activity strongly stimulated by the TATA region of promoters. Proc. Natl. Acad. Sci. USA 86:7356-7360.
37. **Conaway, R.C. and J.W. Conaway.** 1993. General initiation factors for RNA polymerase II. Annu.

Rev. Biochem. 62:161-190.

38. **Coverley, D., M.K. Kenny, D.P. Lane and R.D. Wood.** 1992. A role for the human single-stranded DNA binding protein HSSB/RPA in an early stage of nucleotide excision repair. Nucl. Acids Res. 20:3873-3880.
39. **Coverley, D., M.K. Kenny, M. Munn, W.D. Rupp, D.P. Lane and R.D. Wood.** 1991. Requirement for the replication protein SSB in human DNA excision repair. Nature 349:538-541.
40. **Cox, L.S. and D.P. Lane.** 1995. Tumour suppressors, kinases and clamps: how p53 regulates the cell cylce in response to DNA damage. Bio Essays 17:501-508.
41. **Dahmus, M.E.** 1995. Phosphorylation of C-terminal domain of RNA polymerase II. Biochemica et Biophysica Acta 1261:71-82.
42. **de Cock, J.G.R., E.C. Klink, P.H.M. Lohmann and J.C.J. Eeken.** 1992. Absence of strand-specific repair of cyclobutane pyrimidine dimers in active genes in *Drosophila melanogaster Kc* cells. Mutation Research 274:85-92.
43. **de Vries, A., C.T.M. van Oostrom, F.M.A. Hofhuis, P.M. Dortant, R.J.W. Berg, F.R. de Gruijl, P.W. Wester, C.F. van Kreijl, P.J.A. Capel, H. van Steeg and S.J. Verbeek.** 1995. Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene *XPA*. Nature 377:169-173.
44. **De Weerd-Kastelein, E.A., W. Keijzer and D. Bootsma.** 1972. Genetic heterogeneity of xeroderma pigmentosum demonstrated by somatic cell hybridization. Nature (London) New Biology 238:80-83.
45. **de Wind, N., M. Dekker, A. Berns, M. Radman and H. te Riele.** 1995. Inactivation of the mouse *MSH2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. Cell 82:321-330.
46. **DeSanctis, C. and A. Cacchione.** 1932. I'idiozia xerodermica. Riv.Sper.Freniatr. 56:1932.
47. **Dillon, N. and F. Grosveld.** 1994. Chromatin domains as potential units of eukaryotic gene function. Current Biology 4:260-264.
48. **Din, S., S.J. Brill, M.P. Fairman and B. Stillman.** 1990. Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. Genes and Development 4:968-977.
49. **Donahue, B.A., S. Yin, J.-S. Taylor, D. Reines and P.C. Hanawalt.** 1994. Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. Proc. Natl. Acad. Sci. USA 91:8502-8506.
50. **Drapkin, R., J.T. Reardon, A. Ansari, J.C. Huang, L. Zawel, K. Ahn, A. Sancar and D. Reinberg.** 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. Nature 368:769-772.
51. **Dutta, A. and B. Stillman.** 1992. *cdc2* family kinases phophorylate a human cell DNA replication factor, RPA, and activate DNA replication. The EMBO Journal 11:2189-2199.
52. **Eker, A.P.M., W. Vermeulen, N. Miura, K. Tanaka, N.G.J. Jaspers, J.H.J. Hoeijmakers and D. Bootsma.** 1992. Xeroderma pigmentosum group A correcting protein from calf thymus. Mutation Research 274:211-224.
53. **Feaver, W.J., O. Gileadi and D. Kornberg.** 1991. Purification and characterization of yeast RNA polymerase II transcription factor b. J. Biol. Chem. 266:19000-19005.
54. **Feaver, W.J., J.Q. Svejstrup, L. Bardwell, A.J. Bardwell, S. Buratowski, K.D. Gulyas, T.F. Donahue, E.C. Friedberg and R.D. Kornberg.** 1993. Dual roles of a multiprotein complex from S. cerevisiae in transcription and DNA repair. Cell 75:1379-1387.
55. **Feaver, W.J., J.Q. Svejstrup, N.L. Henry and R.D. Kornberg.** 1994. Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. Cell 79:1103-1109.
56. **Finley, D., B. Bartel and A. Varshavsky.** 1989. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature 338:394-401.
57. **Fisher, R.P. and D.O. Morgan.** 1994. A novelcyclin associayes with Mo15/CDK7 to form the CDK-activating kinase. Cell 78:713-724.

58. **Flores, O., H. Lu and D. Reinberg.** 1992. Factors involved in specific transcription by mammalian RNA polymerase II. J.Biol.Chem. 267:2786-2790.
59. **Flores-Rozas, H., Z. Kelma, F.B. Dean, Z.-Q. Pan, J.W. Harper, S.J. Elledge, M. O'Donnell and J. Hurwitz.** 1994. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA replication polymerase delta holoenzyme. Proc.Natl.Acad.Sci.USA 91:8655-8659.
60. **Frederick, G.D., R.H. Amirkhan, R.A. Schultz and E.C. Friedberg.** 1994. Structural and mutational analysis of the xeroderma pigmentosum group D (*XPD*) gene. Human Molecular Genetics 3:1783-1788.
61. **Friedberg, E.C., G.C. Walker and W. Siede.** 1995. DNA repair and mutagenesis. ASM Press, Washington D.C.
62. **Gale, J.M. and M.J. Smerdon.** 1990. UV induced (6-4) photoproducts are distributed differently than cyclobutane dimers in nucleosomes. Photochem Photobiol 51:411-7.
63. **Gerard, M., L. Fischer, V. Moncollin, J.-M. Chipoulet, P. Chambon and J.-M. Egly.** 1991. Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. J. Biol. Chem. 266:20940-20945.
64. **Gleason, J.F., P. Bhartia, J.R. Herman, R. McPeters, P. Newman, R.S. Stolarski, L. Flynn, G. Labow, D. Larko, C. Seftor, C. Wellemeyer, W.D. Komhyr, A.J. Miller and W. Planet.** 1993. Record low global ozone in 1992. Science 260:523526.
65. **Goodrich, J.A. and R. Tjian.** 1994. TBP-TAF complexes: selectivity factors for eukaryotic transcription. Current Biology 6:403-409.
66. **Goodrich, J.A. and R. Tjian.** 1994. Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. Cell 77:145-156.
67. **Gorbalenya, A.E. and E.V. Koonin.** 1993. Helicases: amino acid sequence comparisons and structure-function relationships. Current Biology 3:419-429.
68. **Greenblatt, J.** 1991. RNA polymerase-associated transcription factors. Trends in Biochem. Sc. 16:408-411.
69. **Greenhaw, G.A., A. Hebert, M.E. Duke-Woodside, I.J. Butler, J.T. Hecht, J.E. Cleaver, G.H. Thomas and W.A. Horton.** 1992. Xeroderma pigmentosum and Cockayne syndrome: overlapping clinical and biochemical phenotypes. American Journal of Human Genetics. 50:677-689.
70. **Grossman, L. and S. Thiagalingam.** 1993. Nucleotide excision repair, a tracking mechanism in search of damage. J. Biol. Chem. 268:16871-16874.
71. **Gulyas, K.D. and T.F. Donahue.** 1992. *SSL2*, a suppressor of a stem-loop mutation in the *HIS4* leader encodes the yeast homologue of human *ERCC3*. Cell 69:1031-1042.
72. **Guzder, S., P. Sung, S. Prakash and L. Prakash.** 1995. Lethality in yeast of trichothiodystrophy (TTD) mutations in the human xeroderma pigmentosum group D gene. The Journal of Biological Chemistry. 270:17660-17663.
73. **Guzder, S.A., V. Bailly, P. Sung, L. Prakash and S. Prakash.** 1995. Yeast DNA repair protein Rad23 promotes complex formation between transcription factor TFIIH and DNA damage recognition factor RAD14. The Journal of Biological Chemistry 270:8385-8388.
74. **Guzder, S.N., P. Sung, V. Bailly, L. Prakash and S. Prakash.** 1994. RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. Nature 369:578-581.
75. **Guzder, S.N., P. Sung, L. Prakash and S. Prakash.** 1993. Yeast DNA-repair gene *RAD14* encodes a zinc metalloprotein with affinity for ultraviolet-damaged DNA. Proc. Natl. Acad. Sci. USA 90:5433-5437.
76. **Harley, C.B., A.B. Futcher and C.W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature 345:458-460.
77. **Harosh, I., L. Naumovski and E.C. Friedberg.** 1989. Purification and characterization of Rad3 ATPase/DNA helicase from Saccharomyces cerevisiae. J Biol Chem 264:20532-9.
78. **Harrington, J.J. and M.R. Lieber.** 1994. The characterization of a mammalian DNA

structure-specific endonuclease. The EMBO Journal 13:1235-1246.

79. **Harrington, J.J. and M.R. Lieber.** 1994. Functional domains within FEN-1 and RAD2 define a family of structure-specific endonucleases: implications for nucleotide excision repair. Gen. & Devel. 8:1344-1355.

80. **Hata, H., M. Numata, H. Tohda, A. Yasui and A. Oikawa.** 1991. Isolation of two chloroethylnitrosourea-sensitive Chinese hamster cell lines. Cancer Res. 51:195-198.

81. **He, Z., L.A. Henricksen, M.S. Wold and C.J. Ingles.** 1995. RPA involvement in the damage-recognition and incision stepa of nucleotide excision repair. Nature 374:566-569.

82. **Henning, K.a., L. Li, N. Iyer, L. McDaniel, M.S. Reagan, R. Legerski, R.A. Schultz, M. Stefanini, A.R. lLehmann, L.V. Mayne and E.C. Friedberg.** 1995. The Cockayne syndroem group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell 82:555-564.

83. **Herrlich, P. and H.J. Rahmsdorf.** 1994. Transcriptional and post-transcriptional responses to DNA-damaging agents. Current Biology 6:425-431.

83a. **Gibbons, R.J., D.J. Picketts, L. Villard and D.R. Higgs.** 1995. Mutations in a putative global transcriptional regulator cause X-linked mental retardation with α-thalassemia (ATR-X syndrome). Cell 80:837-845.

84. **Higgins, D.R., S. Prakash, P. Reynolds, R. Polakowska, S. Weber and L. Prakash.** 1983. Isolation and characterization of the *RAD3* gene of *Saccharomyces cerevisiae* and inviability of *RAD3* deletion mutants. Proc. Natl. Acad. Sci. USA 80:5680-5684.

85. **Hoeijmakers, J.H., A.P. Eker, R.D. Wood and P. Robins.** 1990. Use of in vivo and in vitro assays for the characterization of mammalian excision repair and isolation of repair proteins. Mutat Res 236:223-38.

86. **Hoeijmakers, J.H.J.** 1993. Nucleotide excision repair I: from *E.coli* to yeast. Trends in Genetics 9:173-177.

87. **Hoeijmakers, J.H.J.** 1993. Nucleotide excision repair II: from yeast to mammals. Trends in Genetics 9:211-217.

88. **Hoeijmakers, J.H.J., H. Odijk and A. Westerveld.** 1987. Differences between rodent and human cell lines in the amount of integrated DNA after transfection. Exp. Cell Res. 169:111-9.

89. **Hollander, M.C., I. Alamo, J. Jackman, M.G. Wang, O.W. McBride and J.A.J. Fornace.** 1993. Analysis of the mammlian *GADD45* gene and its response to DNA damage. Journal of Biological Chemistry 268:24385-24393.

90. **Holstege, F.C.P., D. Tantin, M. Carey, P.C. van der Vliet and H.T.M. Timmers.** 1995. The requirement for the basal transcription factor IIE is determined by the helical stability of promoter DNA. The EMBO Journal 14:810-819.

91. **Hori, R. and M. Carey.** 1994. The role of activators in assembly of RNA polymerase II transcription complexes. Current Biology 4:236-244.

92. **Huang, J.C., D.L. Svoboda, J.T. Reardon and A. Sancar.** 1992. Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer. Proc. Natl. Acad. Sci. USA 89:3664-3668.

93. **Humbert, S., A.J. van Vuuren, Y. Lutz, J.H.J. Hoeijmakers, J.-M. Egly and V. Moncollin.** 1994. Characterization of p44/SSL1 and p34 subunits of the BTF2/TFIIH transcription/repair factor. EMBO J 13:2393-2398.

94. **Hwang, B.J. and G. Chu.** 1993. Purification and characterization of a human protein that binds to damaged DNA. Biochem. 32:1657-1666.

95. **Itin, P.H. and M.R. Pittelkow.** 1990. Trichothiodystrophy: review of sulfur-deficient brittle hair syndromes and association with the ectodermal dysplasias. J. of the American Acadamy of Dermatology 22:705-717.

96. **Itoh, T., Y. Fujiwara, T. Ono and M. Yamaizumi.** 1995. UV^s syndrome, a new general category of photosensitive disorder with defective DNA repair, is distinct from xeroderma pigmentosum

variant and rodent complementation group 1. American Journal of Human Genetics. 56:1267-1276.

97. **Itoh, T., T. Ono and M. Yamaizumi.** 1994. A new UV-sensitive syndrome not belonging to any complementation groups of xeroderma pigmentosum or Cockayne syndrome: siblings showing biochemical characteristics of Cockayne syndrome without typical clinical manifestations. Mutation Research 314:233-248.

98. **Jacquier, A., P. Legrain and B. Dujon.** 1992. Sequence of a 10.7 Kb segment of yeast chromosome-XI identifies the APN1 and the BAF1 loci and reveals one transfer-RNA gene and several new open reading frames including homologs to RAD2 and protein kinases. Yeast 8:121-132.

99. **Jaeken, J., H. Klocker, H. Schwaiger, R. Bellmann, M. Hirsh-Kauffmann and M. Schweiger.** 1989. Clinical and biochemical studies in three patients with severe early infantile Cockayne syndrome. Hum. Genet. 83:339-346.

100. **Jensen, K.A. and M.J. Smerdon.** 1990. DNA repair within nucleosome cores of UV-irradiated human cells. Biochemistry 29:4773-82.

101. **Johnson, R.T. and S. Squires.** 1992. The XPD complementation group. Insights into xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy. Mutation Research 273:97-118.

102. **Jones, C.J. and R.D. Wood.** 1993. Preferential binding of the xeroderma pigmentosum group A complementing protein to damaged DNA. Biochem. 32:12096-12104.

103. **Kataoka, H. and Y. Fujiwara.** 1991. UV damage-specific DNA-binding protein in xeroderma pigmentosum complementation group E. Biochemical and Biophysical Research Communications 175:1139-1143.

104. **Keeney, S., G.J. Chang and S. Linn.** 1993. Characterization of human DNA damage binding protein implicated in xeroderma pigmentosum E. The Journal of Biological Chemistry 268:21293-21300.

105. **Keeney, S., A.P.M. Eker, T. Brody, W. Vermeulen, D. Bootsma, J.H.J. Hoeijmakers and S. Linn.** 1994. correction of the DNA repair defect in xeroderma pigmentosum group E by injection of a DNA damage-binding protein. Proc. Natl. Acad. Sci. USA 91:4053-4056.

106. **Keeney, S. and S. Linn.** 1990. A critical review of permeabilized cell systems for studying mammalian DNA repair. Mutation Research 236:239-252.

107. **Keeney, S., H. Wein and S. Linn.** 1992. Biochemical heterogeneity in xeroderma pigmentosum complementation group E. Mutation Research 273:49-56.

108. **Kim, C., R.O. Snyder and M.S. Wold.** 1992. Binding properties of replication protein-A from human and yeast cells. Molecular and Cellular Biology 12:3050-3059.

109. **Kim, Y.-J., S. Bjorklund, Y. Li, M.H. Sayre and R.D. Kornberg.** 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell 77:599-608.

110. **Kirchner, J.M., E.P. Salazar, J.E. Lamerdin, M.A. Montgomery, A.V. Carrano and C.A. Weber.** 1994. Cloning and molecular characterization of the Chinese hamster *ERCC2* nucleotide excision repair gene. Genomics 23:592-599.

111. **Koken, M.H.M., C. Vreeken, S.A.M. Bol, N.C. Cheng, I. Jaspers-Dekker, J.H.J. Hoeijmakers, J.C.J. Eeken, G. Weeda and A. Pastink.** 1992. Cloning and characterization of the *Drosophila* homolog of the xeroderma pigmentosum complemetation-group B correcting gene, *ERCC3*. Nucleic Acids Research 20:5541-5548.

112. **Koleske, A.J. and R.A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. Nature 368:466-469.

113. **Koleske, A.J. and R.A. Young.** 1995. The RNA polymerase II holoenzyme and its implications for gene regulation. Trends Biochem. Sci. 20:113-116.

114. **Lafforet, D. and J.M. Dupuy.** 1978. Photosensibilite et reparation de l'ADN. Possibilite d'une parente nostologique entre xeroderma pigmentosum et syndrome de Cockayne. Arch. franç.

Pediat. (Suppl') 35:65-74.

114a. **Lawrence, C.** 1994. The Rad6 DNA repair pathway in *Saccharomyces Cerevisiae*: What does it do and how does it do it?. Bio Essays 16:253-258.

115. **Leadon, S.A. and P.K. Cooper.** 1993. Preferential repair of ionizing-radiation induced damage in the transcribed strand of an active human gene is defective in Cockayne syndrome. Proc.Natl.Acad.Sci.USA 90:10499-10503.

116. **Legerski, R. and C. Peterson.** 1992. Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. Nature 359:70-73.

117. **Lehmann, A.R.** 1982. Three complementation groups in Cockayne syndrome. Mutation Research 106:347-356.

118. **Lehmann, A.R., A.M. Carr, F.Z. Watts and J.M. Murray.** 1991. DNA repair in the fission yeast, Schizosaccharomyces pombe. Mutat Res 250:205-10.

119. **Lehmann, A.R., S. Kirk-Bell, C.F. Arlett, M.C. Paterson, P.H.M. Lohman, E.A. de Weerd-Kastelein and D. Bootsma.** 1975. Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. Proc. Natl. Acad. Sci. USA 72:219-223.

120. **Lehmann, A.R., S. Kirk-Bell and L. Mayne.** 1979. Abnormal kinetics of DNA synthesis in ultraviolet light irradiated cells from patients with Cockayne syndrome. Cancer Research 3:4237.

121. **Lehmann, A.R., A.F. Thompson, S.A. Harcourt, M. Stefanini and P.G. Norris.** 1993. Cockayne's syndrome: correlation of clinical features with cellular sensitivity of RNA synthesis to UV irradiation. J Med Genet 30:679-682.

122. **Li, L., E.S. Bales, C.A. Peterson and R.J. Legerski.** 1993. Characterization of molecular defects in xeroderma pigmentosum group C. Nature Genetics 5:413-417.

123. **Li, L., S.J. Elledge, C.A. Peterson, E.S. Bales and R.J. Legerski.** 1994. Specific association between the human DNA repair proteins XPA and ERCC1. Proc. Natl. Acad. Sci. USA 91:5012-5016.

124. **Li, L. and R. Legerski.** 1995. Mechanistic implications of protein-protein interactions in nucleotide excision repair. Journal of Cellular Biochemistry Suppl.21A:286.

125. **Li, L., C.A. Peterson, X. Lu and R.J. Legerski.** 1995. Mutations in XPA that prevent association with ERRC1 are defective in nuclear excision repair. Molecular and Cellular Biology 15:1993-1998.

126. **Li, R., S. Waga, G.J. Hannon, D. Beach and B. Stillman.** 1994. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature 371:534-537.

127. **Liao, S.-M., J. Zhang, D.A. Jeffery, A.J. Koleske, C.M. Thompson, D.M. Chao, M. Viljoen, H.J.J. van Vuuren and R.A. Young.** 1995. A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature 374:193-196.

128. **Lindahl, T.** 1993. Instability and decay of the primary structure of DNA. Nature 362:709-715.

129. **Liu, P., J. Siciliano, B. White, R. Legerski, D. Callen, S. Reeders, M.J. Siciliano and L.H. Thompson.** 1993. Regional mapping of human DNA excision repair gene *ERCC4* to chromosome 16p13.13-p13.2. Mutagen. 8:199-205.

130. **Liu, V.F. and D.T. Weaver.** 1993. The ionizing radiation-induced replication protein A phosphorylation response differs between ataxia telangiectasia and normal human cells. Molecular and Cellular Biology 13:7222-7231.

131. **Longhese, M.P., P. Plevani and G. Lucchini.** 1994. Replication factor A is required in vivo for DNA replication, repair, and recombination. Molecular and cellular Biology 14:7884-7890.

132. **Lu, H., L. Zawel, L. Fisher, J.-M. Egly and D. Reinberg.** 1992. Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. Nature 358:641-645.

133. **Lu, X. and D.P. Lane.** 1993. Differential induction of transcriptional active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell 75:765-778.

134. **Ma, L., E.D. Siemssen, M.H.M. Noteborn and A.J. van der Eb.** 1994. The xeroderma pigmentosum group B protein ERCC3 produced in the baculovirus system exhibits DNA helicase activity. Nucleic Acids Research 22:4095-4102.

135. **MacInnes, M.A., J.A. Dickson, R.R. Hernandez, D. Learmont, G.Y. Lin, J.S. Mudgett, M.S. Park, S. Schauer, R.J. Reynolds, G.F. Strniste and J.Y. Yu.** 1993. Human *ERCC5* cDNA-cosmid complementation for excision repair and bipartite amini acid domains conserved with RAD proteins of *S.cerevisiae* and *S.pombe*. Molecular and Cellular Biology 13:6393-6402.

136. **Madronich, S. and F.R. de Gruijl.** 1993. Skin cancer and UV irradiation. Nature 366:23.

137. **Madura, K. and S. Prakash.** 1986. Nucleotide sequence, transcript mapping, and regulation of the *RAD2* gene of *Saccharomyces cerevisiae*. J. Bacteriol. 166:914-923.

138. **Mäkelä, T.P., J.-P. Tassan, E.A. Nigg, S. Frutiger, G. Hughes and R.A. Weinberg.** 1994. A cyclin associated with the CDK-activating kinase MO15. Nature 371:254-257.

139. **Masutani, C., K. Sugasawa, H. Asahina, K. Tanaka and F. Hanaoka.** 1993. Cell-free repair of UV-damaged simian virus 40 chromosomes in human cell extracts. The Journal of Biological Chemistry 268:9105-9109.

140. **Masutani, C., K. Sugasawa, J. Yanagisawa, T. Sonoyama, M. Ui, T. Enomoto, K. Takio, K. Tanaka, P.J. van der Spek, D. Bootsma, J.H.J. Hoeijmakers and F. Hanaoka.** 1994. Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homolog of yeast RAD23. EMBO J. 13:1831-1843.

141. **Matsuda, T., M. Saijo, I. Kuraoka, T. Kobayashi, Y. Nahatssu, A. Nagai, T. Enjoji, C. Masutani, K. Sugasawa, F. Hanaoka, A. Yasui and K. Tanaka.** 1995. DNA repair protein XPA binds to replication protein A (RPA). The Journal of Biological Chemistry 270:4152-4157.

142. **Matsui, P., J. DePaulo and S. Buratowski.** 1995. An interaction between the Tfb1 and Ssl1 subunits of yeast TFIIH correlates with DNA repair activity. Nucleic Acids Research 23:767-772.

143. **Maxon, M.E., J.A. Goodrich and R. Tjian.** 1994. Transcription factor IIE binds preferentially to RNA polymerase IIa and recruits TFIIH: a model for promoter clearance. Genes & Development 8:515-524.

144. **Mayne, L.V. and A.R. Lehmann.** 1982. Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. Cancer Research 42:1473-1478.

145. **McCready, S.** 1994. Repair of 6-4 photoproducts and cylcobutane pyrimidine dimers in *rad* mutants of *Saccharomyces cerevisiae*. Mutation Research 315:261-273.

146. **McCready, S., A.M. Carr and A.R. Lehmann.** 1993. Repair of cyclobutane pyrimidine dimers and 6-4 photoproducts in the fission yeast *Schizosaccharomyces pombe*. Mol. Microbiol. 10:885-890.

147. **McKusick, V.A.** 1992. Mendelian inheritance in man. Catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes. The John university press, Baltimore.

148. **McWhir, J., J. Seldridge, D.J. Harrison, S. Squires and D.W. Melton.** 1993. Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. Nat. Gen. 5:217-223.

149. **Mellon, I., V.A. Bohr, C.A. Smith and P.C. Hanawalt.** 1986. Preferential DNA repair of an active gene in human cells. Proc Natl Acad Sci U S A 83:8878-8882.

150. **Mellon, I. and P.C. Hanawalt.** 1989. Induction of the *Escherichia coli* lactose operon selectively increases repair of its transcribed DNA strand. Nature 342:95-98.

151. **Mita, K., H. Tsuji, M. Morimyo, E. Takahashi, M. Nenoi, S. Ichimura, M. Yamauchi, E. Hongo and A. Hayashi.** 1995. The human gene encoding the largest subunit of RNA POLYMERASE II. Gene 159:285-286.

152. **Mitchell, D.L. and R.S. Nairn.** 1989. The biology of the (6-4) photoproduct. Photochem. Photobiol. 49:805-819.

153. **Mitchell, D.L., T.D. Nguyen and J.E. Cleaver.** 1990. Nonrandom induction of pyrimidine-pyrimidone(6-4) photoproducts in ultraviolet-irradiatedhuman chromatin. J Biol Chem 265:5353-6.

154. **Miura, N., I. Miyamoto, H. Asahina, I. Satokata, K. Tanaka and Y. Okada.** 1991. Identification and characterization of XPAC protein, the gene product of the human *XPAC* (xeroderma

pigmentosum group A complementing) gene. Journal of Biological Chemistry 266:19786-19789.

155. **Miyamoto, I., N. Miura, H. Niwa, J. Miyazaki and K. Tanaka.** 1992. Mutational analysis of the structure and function of the xeroderma pigmentosum group A complementing protein. J. Biol. Chem. 267:12182-12187.

156. **Montelone, B.E. and R.E. Malone.** 1994. Analysis of the *rad3-101* and *rad3-102* mutations of *S.cerevisiae*: implications for structure/function of Rad3 protein. Yeast 10:13-27.

157. **Moshell, A.N., M.B. Ganges, M.A. Lutzner, H.G. Coon, S.F. Barrett, J.-M. Dupuy and J.H. Robbins.** 1983. A new patient with both xeroderma pigmentosum and Cockayne syndrome establishes the new xeroderma pigmentosum complementation group H. p. 209-213. *In* Friedberg, E.C., B.A. Bridges (ed.), Cellular responses to DNA damage. Liss, New York.

158. **Mounkes, L.C., R.S. Jones, B.-C. Liang, W. Gelbart and M.T. Fuller.** 1992. A drosophila model for xeroderma pigmentosum and Cockayne's syndrome: *haywire* encodes the fly homolog of ERCC3, a human excision repair gene. Cell 71:925-937.

159. **Mu, D., C.-H. Park, T. Matsunaga, D.S. Hsu, J.T. Reardon and A. Sancar.** 1995. Reconstitution of human DNA repair excision nuclease in a highly defined system. The Journal of Biological Chemistry 270:2415-2418.

160. **Mudgett, J.S. and M.A. MacInnes.** 1990. Isolation of the functional human excision repair gene *ERCC5* by intercosmid recombination. Genomics 8:623-633.

161. **Mullenders, L.H., H. Vrieling, J. Venema and A.A. van_Zeeland.** 1991. Hierarchies of DNA repair in mammalian cells: biological consequences. Mutat Res 250:223-8.

162. **Murray, J.M., C.L. Doe, P. Schenk, A.M. Carr, A.R. Lehmann and F.Z. Watts.** 1992. Cloning and characterisation of the *S.pombe rad15* gene, a homologue to the *S.cerevisiae RAD3* and human *ERCC2* genes. Nucleic Acids Research 20:2673-2678.

163. **Murray, J.M., M. Tavassoli, R. Al-Harithy, K.S. Sheldrick, A.R. Lehmann, A.M. Carr and F.Z. Watts.** 1994. Structural and functional conservation of the human homolog of the *S.pombe rad2* gene, which is required for chromosome segregation and recovery from DNA damage. Molecular and Cellular Biology 14:4878-4888.

164. **Naegeli, H., L. Bardwell and E.C. Friedberg.** 1992. The DNA helicase and adenosine triphosphatase activities of yeast Rad3 protein are inhibited by DNA damage. A potential mechanism for damage-specific recognition. J Biol Chem 267:392-8.

165. **Nagai, A., M. Saijo, I. Kuraoka, T. Matsuda, N. Kodo, Y. Nakatsu, T. Mimaki, M. Mino, M. Biggerstaff, R.D. Wood, A. Sijbers, J.H.J. Hoeijmakers and K. Tanaka.** 1995. Enhancement of damage-specific DNA binding of XPA by interaction with the ERCC1 DNA repair protein. Biochemical and Biophysical Research Communications 211:960-966.

166. **Nakane, H., S. Takeuchi, S. Yuba, M. Saijo, Y. Nakatsu, T. Ishikawa, S. Hirota, Y. Kitamura, Y. Kato, Y. Tsunoda, H. Miyauchi, T. Horio, T. Tokunaga, T. Matsunaga, O. Nikaido, Y. Nishimune, Y. Okada and K. Tanaka.** 1995. High incedence of ultraviolet-B- or chemical-carcinogen-induced skin tomours in mice lacking the xeroderma pigmentosum group A gene. Nature 377:165-168.

167. **Nance, M.A. and S.A. Berry.** 1992. Cockayne syndrome: Review of 140 cases. American Journal of Medical Genetics 42:68-84.

168. **Naumovski, L., G. Chu, P. Berg and E.C. Friedberg.** 1985. RAD3 gene of Saccharomyces cerevisiae: nucleotide sequence of wild-type and mutant alleles, transcript mapping, and aspects of gene regulation. Mol Cell Biol 5:17-26.

169. **Naumovski, L. and E.C. Friedberg.** 1983. A DNA repair gene required for the incision of damaged DNA is essential for viability in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 80:4818-4821.

170. **Neer, E.J., C.J. Schmidt, R. Nambudripad and T.F. Smith.** 1994. The ancient regulatory-protein family of WD-repeat proteins. Nature 371:297-300.

171. **Nichols, A.F. and A. Sancar.** 1992. Purification of PCNA as a nucleotide excision repair protein. Nucleic Acids Res. 20:2441-2446.

172. **Nicolet, C.M., J.M. Chenevert and E.C. Friedberg.** 1985. The RAD2 gene of Saccharomyces cerevisiae: nucleotide sequence and transcript mapping. Gene 36:225-34.
173. **Nigg, E.A.** 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bio Essays 17:471-480.
174. **Nishigori, C., M. Zghal, T. Yagi, S. Imamura, M.R. Komoun and H. Takebe.** 1994. High prevalence of the point mutation in exon 6 of the xeroderma pigmentosum group A-complementing (XPAC) gene in xeroderma pigmentosum group A patients in Tunisia. American Journal of Human Genetics 53:1001-1006.
175. **Nouspikel, T. and S.G. Clarkson.** 1994. Mutations that disable the DNA repair gene *XPG* in a xeroderma pigmentosum group G patient. Human Molecular Genetics 3:963-967.
176. **O'Donovan, A., A.A. Davies, J.G. Moggs, S.C. West and R.D. Wood.** 1994. XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. Nature 371:432-435.
177. **O'Donovan, A., D. Scherly, S.G. Clarkson and R.D. Wood.** 1994. Isolation of active recombinant XPG protein, a human DNA repair endonuclease. The Journal of Biological Chemistry 269:15965-15968.
178. **O'Donovan, A. and R.D. Wood.** 1993. Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5. Nature 363:185-188.
179. **Ohkuma, Y. and R.G. Roeder.** 1994. Regulation of TFIIH ATPase and kinase activity by TFIIE during active initiation complex formation. Nature 368:160-163.
180. **Paetkau, D.W., J.A. Riese, W.S. MacMorran, R.A. Woods and R.D. Gietz.** 1994. Interaction of the yeast *RAD7* and *SIR3* proteins: implications for DNA repair and chromatin structure. Gen. & Dev. 8:2035-2045.
181. **Pan, Z.-Q., A.A. Amin, E. Gibbs, H. Niu and J. Hurwitz.** 1994. Phosphorylation of the p34 subunit of human single-stranded-DNA-binding in cyclin A-activated G1 extracts is catalyzed by cdk-cyclin A complex and DNA-dependent protein kinase. Proc.Natl.Acad.Sci.USA 91:8343-8347.
182. **Pan, Z.-Q., C.-H. Park, A.A. Amin, J. Hurwitz and A. Sancar.** 1995. Phosphorylated and unphosphorylated forms of human single-stranded DNA-binding protein are equally active in simian virus 40 DNA replication and in nucleotide excision repair. Proc.Natl.Acad.Sci.USA 92:4636-4640.
183. **Park, C.-H., D. Mu, J.T. Reardon and A. Sancar.** 1995. The general transcription-repair factor TFIIH is recruited to the excision repair complex by the XPA protein independent of the TFIIE transcription factor. The Journal of Biological Chemistry 270:4896-4902.
184. **Park, C.-H. and A. Sancar.** 1994. Formation of a ternary complex by human XPA, ERCC1 and ERCC4(XPF) excision repair proteins. Proc. Natl. Acad. Sci. USA 91:5017-5021.
185. **Park, E., S. Guzder, M.H.M. Koken, I. Jaspers-Dekker, G. Weeda, J.H.J. Hoeijmakers, S. Prakash and L. Prakash.** 1992. *RAD25*, a yeast homolog of human xeroderma pigmentosum group B DNA repair gene is essential for viability. Proc. Natl. Acad. Sci. USA 89:11416-11420.
186. **Parvin, J.D. and P.A. Sharp.** 1993. DNA topology and a minimum set of basal factors for transcription by RNA polymerase II. Cell 73:533-540.
187. **Peserico, A., P.A. Battistella and P. Bertoli.** 1992. MRI of a very hereditary ectodermal dysplasia:PIBI(D)S. Neuroradiology 34:316-317.
188. **Pfeifer, G.P., R. Drouin, A.D. Riggs and G.D. Holmquist.** 1992. Binding of transcription factors creates hot spots for UV photoproducts in vivo. Molecular and Cellular Biology 12:1798-1804.
189. **Podust, L.M., V.A. Podust, C. Floth and U. Hubscher.** 1994. Assembly of DNA polymerase delta and epsilon holoenzymes depends on the geometry of the DNA template. Nucleic Acids Research 22:2970-2975.
190. **Prakash, S., P. Sung and L. Prakash.** 1993. DNA repair genes and proteins of *Saccharomyces cerevisiae*. Annu. Rev. Genet. 27:33-70.
191. **Qiu, H., E. Park, L. Prakash and S. Prakash.** 1993. The *Saccharomyces cerevisiae* DNA repair gene *RAD25* is required for transcription by RNA polymerase II. Genes and Development

7:2161-2171.

192. **Reardon, J.T., A.F. Nichols, S. Keeney, C.A. Smith, J.S. Taylor, S. Linn and A. Sancar.** 1993. Comparative analysis of binding of human damaged DNA-binding protein (XPE) and *escherichia-coli* damage recognition protein (UvrA) to the major ultraviolet photoproducts - T[CS]TT[Ts]TT[6-4]T and T[Dewar]T. J. Biol. Chem. 268:21301-21308.

193. **Reardon, J.T., L.H. Thompson and A. Sancar.** 1993. Excision repair in man and the molecular basis of xeroderma pigmentosum. Cold Spring Harbor Symposium on Quantitative Biology 58:605-617.

194. **Reynolds, P., D.R. Higgins, L. Prakash and S. Prakash.** 1985. The nucleotide sequence of the *RAD3* gene of *Saccharomyces cerevisiae*: A potential adenine nucleotide binding amino acid sequence and a nonessential carboxyl terminal domain. Nucleic Acids Research 13:2357-2372.

195. **Reynolds, P.R., S. Biggar, L. Prakash and S. Prakash.** 1992. The *S.pombe rhp3*$^{+}$ gene required for DNA repair and cell viability is functionally interchangeable with the *RAD3* gene *S.cerevisiae*. Nucleic Acids Research 20:2327-2334.

196. **Riboni, R., E. Botta, M. Stefanini, M. Numata and A. Yasui.** 1992. Identification of the eleventh complementation group of UV-sensitive excision repair-defective rodent mutants. Cancer Res. 52:6690-6691.

197. **Robbins, J.H.** 1988. Defective DNA repair in xeroderma pigmentosum and other neurologic diseases. Curr. Opin. Neurol. Neurosurg. 1:1077-1083.

198. **Robbins, J.H., R.A. Brumback, M. Mendiones, S.F. Barrett, J.R. Carl, S. Cho, M.B. Denckla, M.B. Ganges, L.H. Gerber, R.A. Guthrie, J. Meer, A.N. Moshell, R.J. Polinsky, P.D. Ravin, B.C. Sonies and R.E. Tarone.** 1991. Neurological disease in xeroderma pigmentosum. Documentation of a late onset type of the juvenile onset form. Brain 114:1335-1361.

199. **Robbins, J.H., K.H. Kraemer, M.A. Lutzner, B.W. Festoff and H.G. Coon.** 1974. Xeroderma pigmentosum. An inherited disease with sun sensitivity, multiple cutaneous neoplasms and abnormal repair. Ann. Intern. Med. 80:221-248.

200. **Robins, P., C.J. Jones, M. Biggerstaff, T. Lindahl and R.D. Wood.** 1991. Complementation of DNA repair in xeroderma pigmentosum group A cell extracts by a protein with affinity for damaged DNA. EMBO J. 10:3913-3921.

201. **Roy, R., J.P. Adamczewski, T. Seroz, W. Vermeulen, J.-P. Tassan, L. Schaeffer, E.A. Nigg, J.H.J. Hoeijmakers and J.-M. Egly.** 1994. The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell 79:1093-1101.

202. **Roy, R., L. Schaeffer, S. Humbert, W. Vermeulen, G. Weeda and J.-M. Egly.** 1994. The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. Journal of Biological Chemistry 269:9826-9832.

203. **Ruven, H.J.T., R.J.W. Berg, M.J. Seelen, J.A.J.M. Dekkers, P.H.M. Lohman, L.H.F. Mullenders and A.A. van Zeeland.** 1993. Ultrviolet-induced cyclobutane pyrimidine dimers are selectively removed from transcriptionally active genes in the epidermis of the hairless mouse. Cancer Research 53:1642-1645.

204. **Sachsenmaier, C., A. radler-Pohl, R. Zinck, A. Nordheim, P. Herrlich and H.J. Rahmsdorf.** 1994. Involvement of growth factor receptors in the mammalian UVC response. Cell 78:963-972.

205. **Saffran, W.A., R.B. Greenberg, M.S. Thaler and M.M. Jones.** 1994. Single strand and double strand DNA damage-induced reciprocal recombination in yeast. Dependence on nucleotide excision repair and *RAD1* recombination. Nucleic Acids Research 22:2823-2829.

206. **Sanchez, Y. and S.J. Elledge.** 1995. Stopped for repairs. Bio Essays 17:545-548.

207. **Sands, A.T., A. Abuin, A. Sanchez, C.J. Conti and A. Bradley.** 1995. High susceptibility to ultraviolet-induced carcinogenesis in mice lacking *XPC*. Nature 377:162-165.

208. **Satokata, I., K. Tanaka, N. Miura, I. Myamoto, Y. Satoh, S. Kondo and Y. Okada.** 1990. Characterization of a splicing mutation in group A xeroderma pigmentosum. Proc. Natl. Acad. Sci. USA 87:9908-9912.

209. **Satokata, I., K. Tanaka, N. Miura, M. Narita, T. Mimaki, Y. Satoh, S. Kondo and Y. Okada.** 1992. Three nonsense mutations responsible for group A xeroderma pigmentosum. Mutation Research 273:193-202.
210. **Satokata, I., K. Tanaka, S. Yuba and Y. Okada.** 1992. Identification of splicing mutations of the last nucleotides of exons, a nonsense mutation, and a missense mutation of the *XPAC* gene as causes of group A xeroderma pigmentosum. Mutation research 273:203-212.
211. **Schaeffer, L., V. Moncollin, R. Roy, A. Staub, M. Mezzina, A. Sarasin, G. Weeda, J.H.J. Hoeijmakers and J.M. Egly.** 1994. The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. EMBO J. 13:2388-2392.
212. **Schaeffer, L., R. Roy, S. Humbert, V. Moncollin, W. Vermeulen, J.H.J. Hoeijmakers, P. Chambon and J. Egly.** 1993. DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science 260:58-63.
213. **Scherly, D., T. Nouspikel, J. Corlet, C. Ucla, A. Bairoch and S.G. Clarkson.** 1993. Complementation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast RAD2. Nature 363:182-185.
214. **Schiestl, R.H. and S. Prakash.** 1988. *RAD1,* an excision repair gene of *Saccharomyces cerevisiae,* is also involved in recombination. Molecular and Cellular Biology 8:3619-3626.
215. **Schiestl, R.H. and S. Prakash.** 1990. *RAD10,* an excision repair gene of *Saccharomyces cerevisiae,* is involved in the *RAD1* pathway of mitotic recombination. Molecular and Cellular Biology 10:2485-2491.
216. **Scott, R.J., P. Itin, W.J. Kleijer, K. Kolb, C. Arlett and H. Muller.** 1993. Xeroderma pigmentosum-Cockayne syndrome complex in two, new patients: absence of skin tumors despite severe deficiency of DNA excision repair. J. Am. Acad. Dermatol. 29:883-889.
217. **Selby, C.P. and A. Sancar.** 1990. Transcription preferentially inhibits nucleotide excision repair of the template DNA strand in vitro. J Biol Chem 265:21330-21336.
218. **Selby, C.P. and A. Sancar.** 1991. Gene- and strand-specific repair in vitro: partial purification of a transcription-repair coupling factor. Proc Natl Acad Sci U S A 88:8232-8236.
219. **Selby, C.P. and A. Sancar.** 1994. Mechanisms of transcription-repair coupling and mutation frequency decline. Microbiological Reviews 58:317-329.
220. **Selby, C.P. and A. Sancar.** 1995. Structure and function of transcription-repair coupling factor. The Journal of Biological Chemistry 270:4882-4895.
221. **Selby, C.P., E.M. Witkin and A. Sancar.** 1991. *Escherichia coli mfd* mutant deficient in "mutation frequency decline" lacks strand-specific repair: in vitro complementation with purified coupling factor. Proc Natl Acad Sci U S A 88:11574-11578.
222. **Serizawa, H., R.C. Conaway and J.W. Conaway.** 1993. Multifunctional RNA polymerase II initiation factor δ from rat liver. J. Biol. Chem. 268:17300-17308.
223. **Sheldon, M. and D. Reinberg.** 1995. Tuning-up transcription. Current Biology 5:43-46.
224. **Shiomi, T., Y. Harada, T. Saito, N. Shiomi, Y. Okuno and M. Yamaizumi.** 1994. An ERCC5 gene with homology to yeast RAD2 is involved in group G xeroderma pigmentosum. Mutation Research 314:167-175.
225. **Shivji, M.K.K., A.P.M. Eker and R.D. Wood.** 1994. DNA repair defect in xeroderma pigmentosum group C and complementing factor from HeLa cells. J. of Biological Chemistry 269:22749-22757.
226. **Shivji, M.K.K., S.L. Grey, U.P. Strausfeld, R.D. Wood and J.J. Blow.** 1994. Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision repair. Current Biology 4:1062-1068.
227. **Shivji, M.K.K., M.K. Kenny and R.D. Wood.** 1992. Proliferating cell nuclear antigen is required for DNA excision repair. Cell 69:367-374.
228. **Shivji, M.K.K., V.N. Podust, U. Hubscher and R.D. Wood.** 1995. Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. Biochemistry 34:5011-5017.
229. **Shuttleworth, J., R. Godfrey and A. Colman.** 1990. p40 (Mo15), a cdc-2 related protein kinase

involved in negative regulation of meiotic maturation of *Xenopus* oocytes maturation. The EMBO Journal 9:3233-3240.

230. **Sibghat-Ullah, I. Husain, W. Carlton and A. Sancar.** 1989. Human nucleotide excision repair in vitro: repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract. Nucleic Acids Res 17:4471-84.

231. **Sidik, K., H.B. Lieberman and G.A. Freyer.** 1992. Repair of DNA damaged by UV light and ionizing radiation by cell-free extracts prepared from *Schizosaccharomyces pombe*. Proc.Natl.Acad.Sc.USA 89:12112-12116.

232. **Smith, M.L., I.-T. Chen, Q. Zhan, I. Bae, C.-Y. Chen, T.M. Gilmer, M.B. Kastan, P.M. O'Connor and A.J. Fornace Jr.** 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266:1376-1380.

233. **Song, J.M., B.A. Montelone, W. Siede and E.C. Friedberg.** 1990. Effects of multiple yeast rad3 mutant alleles on UV sensitivity, mutability, and mitotic recombination. J Bacteriol 172:6620-30.

234. **Stefanini, M., A.R. Collins, R. Riboni, M. Klaude, E. Botta, D.L. Mitchell and F. Nuzzo.** 1991. Novel Chinese hamster ultraviolet-sensitive mutants for excision repair form complementation groups 9 and 10. Cancer Res 51:3965-3971.

235. **Stefanini, M., P. Lagomarisini, S. Gilliani, T. Nardo, E. Botta, A. Peserico, W.J. Kleyer, A.R. Lehmann and A. Sarasin.** 1993. Genetic heterogeneity of the excision repair defect associated with trichothiodystrophy. Carcinogenesis 14:1101-1105.

236. **Stefanini, M., P. Lagomarsini, C.F. Arlett, S. Marinoni, C. Borrone, F. Crovato, G. Trevisan, G. Cordone and F. Nuzzo.** 1986. Xeroderma pigmentosum (complementation group D) mutation is present in patients affected by trichothiodystrophy with photosensitivity. Hum. Genet. 74:107-112.

237. **Stefanini, M., W. Vermeulen, G. Weeda, S. Giliani, T. Nardo, M. Mezzina, A. Sarasin, J.L. Harper, C.F. Arlett, J.H.J. Hoeijmakers and A.R. Lehmann.** 1993. A new nucleotide-excision-repair gene associated with the disorder trichothiodystrophy. Am. J. Hum. Genet. 53:817-821.

238. **Stillman, B.** 1989. Initiation of eukaryotic DNA replication *in vitro*. Annu.Rev.Cell.Biol. 5:197-245.

239. **Sugano, T., T. Uchida and M. Yamaizumi.** 1991. Identification of a specific protein factor defective in group A xeroderma pigmentosum cells. J. Biochem. 110:667-664.

240. **Sugasawa, K., C. Masutani and F. Hanaoka.** 1993. Cell-free repair of UV-damaged simian virus 40 chromosomes in human cell extracts. The Journal of Biological Chemistry 268:9098-9104.

241. **Sung, P., V. Bailly, C. Weber, T. L.H., L. Prakash and S. Prakash.** 1993. Human xeroderma pigmentosum group D gene encodes a DNA helicase. Nature 365:852-855.

242. **Sung, P., D. Higgins, L. Prakash and S. Prakash.** 1988. Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. EMBO J. 7:3263-3269.

243. **Sung, P., L. Prakash, S.W. Matson and S. Prakash.** 1987. RAD3 protein of *Saccharomyces cerevisiae* is a DNA helicase. Proc. Natl. Acad. Sci. USA 84:8951-8955.

244. **Sung, P., P. Reynolds, L. Prakash and S. Prakash.** 1993. Purification and characterization of the *Saccharomyces cerevisiae* RAD1/RAD10 endonuclease. J. Biol. Chem. 268:26391-26399.

245. **Sung, P., J.F. Watkins, L. Prakash and S. Prakash.** 1994. Negative superhelicity promotes ATP-dependent binding of yeast Rad3 protein to UV-damaged DNA. The Journal of Biological chemistry 269:8303-8308.

246. **Svejstrup, J.Q., W.J. Feaver, J. LaPointe and R.D. Kornberg.** 1994. RNA polymerase transcription factor IIH holoenzyme from yeast. submitted.

247. **Svejstrup, J.Q., Z. Wang, W.J. Feaver, X. Wu, D.A. Bushnell, T.F. Donahue, E.C. Friedberg and R.D. Kornberg.** 1995. Different forms of TFIIH for transcription and DNA repair: holo-TFIIH and a nucleotide excision repairosome. Cell 80:21-28.

248. **Sweder, K.S. and P.C. Hanawalt.** 1994. The COOH terminus of suppressor of stem loop (Ssl2/Rad25) in yeast is essential for overall genomic excision repair and transcription-coupled

repair. The Journal of Biological Chemistry 269:1852-1857.

249. **Szymkowski, D.E., K. Yarema, J.M. Essigmann, S.L. Lippard and R.D. Wood.** 1992. An intrastrand d(GpG) platinum crosslink in duplex M13 DNA is refractory to repair by human cell extracts. Proc. Natl. Acad. Sci. USA 89:10772-10776.

250. **Takao, M., M. Abramic, M. Moos, V.R. Otrin, J.C. Wootton, M. Mclenigan, A.S. Levine and M. Protic.** 1993. A 127 kDa component of a UV-damaged DNA-binding complex which is defective in some xeroderma pigmentosum group E patients is homologous to a slime mold protein. Nucl. Ac. Res. 21:4111-4118.

251. **Tan, S., T. Aso, R.C. Conaway and J.W. Conaway.** 1994. Roles for both the RAP30 and RAP74 subunits of transcription factor IIF in transcription initiation and elongation by RNA polymerase II. The Journal of Biological Chemistry 269:25684-25691.

252. **Tanaka, K., K. Kawai, Y. Kumahara, M. Ikenaga and Y. Okada.** 1981. Genetic complementation groups in Cockayne syndrome. Somatic Cell Genetics 7:445-455.

253. **Tanaka, K., N. Miura, I. Satokata, I. Miyamoto, M.C. Yoshida, Y. Satoh, S. Kondo, A. Yasui, H. Okayama and Y. Okada.** 1990. Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum and containing a zinc-finger domain. Nature 348:73-76.

254. **Tanaka, K., I. Satokata, Z. Ogita, T. Uchida and Y. Okada.** 1989. Molecular cloning of a mouse DNA repair gene that complements the defect of group A xeroderma pigmentosum. Proc. Natl. Acad. Sci. USA 86:5512-5516.

255. **Tassan, J.P., S.J. Schultz, J. Bartek and E.A. Nigg.** 1994. Cell cycle analysis, subcellular localization, and subunit composition of human CAK (CDK-activating kinase). The Journal of Cell Biology 127:467-478.

256. **Terleth, C., C.A.v. Sluis and P.v.d. Putte.** 1989. Differential repair of UV damage in *Saccharomyces cerevisiae*. Nucleic Acids Research 17:4433-4439.

257. **Th'ng, J.P.H. and I.G. Walker.** 1983. DNA repair patch size measurements with nucleosomal DNA. Carcinogenesis 4:975-978.

258. **Thompson, L.H., K.W. Brookman, C.A. Weber, E.P. Salazar, J.T. Reardon, A. Sancar, Z. Deng and M.J. Siciliano.** 1994. Molecular cloning of the human nucleotide-excision-repair gene *ERCC4*. Proc. Natl. Acad. Sci. USA 91:6855-6859.

259. **Timmers, H.T.M.** 1994. Transcription initiation by RNA polymerase II does not require hydrolysis of the beta-gamma phosphoanhydride bond of ATP. EMBO J. 13:391-399.

260. **Tolmie, J.L., D. de Berker, R. Dawber, C. Galloway, D.W. Gregory, A.R. Lehmann, J. McClure, J.R. Pollitt and J.P.B. Stephenson.** 1994. Syndromes associated with trichothiodystrophy. Clinical Dysmorphology 1:1-14.

261. **Tomkinson, A.E., A.J. Bardwell, N. Tappe, W. Ramos and E.C. Friedberg.** 1994. Purification of Rad1 protein from *Saccharomyces cerevisiae* and further characterization of the Rad1/Rad10 endonuclease complex. Biochemistry 33:5305-5311.

262. **Troelstra, C., H. Odijk, J. De Wit, A. Westerveld, L.H. Thompson, D. Bootsma and J.H.J. Hoeijmakers.** 1990. Molecular cloning of the human DNA excision repair gene *ERCC-6*. Mol Cell Biol 10:5806-5813.

263. **Troelstra, C., A. van Gool, J. de Wit, W. Vermeulen, D. Bootsma and J.H.J. Hoeijmakers.** 1992. *ERCC6*, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell 71:939-953.

264. **Usheva, A., E. Maldonado, A. Goldring, H. Lu, C. Houbavi, D. Reinberg and Y. Aloni.** 1992. Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. Cell 69:871-881.

265. **van Duin, M., J. de Wit, H. Odijk, A. Westerveld, A. Yasui, M.H.M. Koken, J.H.J. Hoeijmakers and D. Bootsma.** 1986. Molecular characterization of the human excision repair gene *ERCC-1*: cDNA cloning and amino acid homology with the yeast DNA repair gene *RAD10*. Cell 44:913-923.

266. **van Gool, A.J., R. Verhage, S.M.A. Swagemakers, P. van de Putte, J. Brouwer, C. Troelstra, D. Bootsma and J.H.J. Hoeijmakers.** 1994. *RAD26*, the functional *S.cerevisiae* homolog of the Cockayne syndrome B gene *ERCC6*. EMBO J. 13:5361-5369.

267. **van Hoffen, A., A.T. Natarajan, L.V. Mayne, A.A. van Zeeland, L.H.F. Mullenders and J. Venema.** 1993. Deficient repair of the transcribed strand of active genes in Cockayne syndrome. Nucleic Acids Research 21:5890-5895.

268. **van Hoffen, A., J. Venema, R. Meschini, A.A. van Zeeland and L.H.F. Mullenders.** 1995. Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and i a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts. The EMBO Journal 14:360-367.

269. **van Vuuren, A.J., E. Appeldoorn, H. Odijk, S. Humbert, V. Moncollin, A.P.M. Eker, N.G.J. Jaspers, J.-M. Egly and J.H.J. Hoeijmakers.** 1995. Partial characterization of the DNA repair protein complex, containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities. Mutation Research 337:25-39.

270. **van Vuuren, A.J., E. Appeldoorn, H. Odijk, A. Yasui, N.G.J. Jaspers, D. Bootsma and J.H.J. Hoeijmakers.** 1993. Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F. EMBO J. 12:3693-3701.

271. **van Zeeland, A.A., C.A. Smith and P.C. Hanawalt.** 1981. Sensitive determination of pyrimidine dimers in the DNA of UV irradiated mammalian cells: introduction of T4 endonuclease V into frozen and thawed cells. Mutation Research 82:173-189.

272. **Venema, J., Z. Bartosova, A.T. Natarajan, A.A. van Zeeland and L.H.F. Mullenders.** 1992. Transcription affects the rate but not the extent of repair of cyclobutane pyrimidine dimers in the human adenosine deaminase gene. Journal of Biological Chemistry 267:8852-8856.

273. **Venema, J., L.H.F. Mullenders, A.T. Natarajan, A.A. Van Zeeland and L.V. Mayne.** 1990. The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. Proc. Natl. Acad. Sci. USA 87:4707-4711.

274. **Venema, J., A. Van Hoffen, A.T. Natarajan, A.A. Van Zeeland and L.H.F. Mullenders.** 1990. The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA. Nucleic Acids Research 18:443-448.

275. **Verhage, R., A. Zeeman, N. de Groot, F. Gleig, D. Bang, P. van der Putte and J. Brouwer.** 1994. The *RAD7* and *RAD16* genes are essential for repair of non-transcribed DNA in *Saccharomyces ceriviae*. Mol. Cell. Biol. in press.

276. **Vermeulen, W., J. Jaeken, N.G.J. Jaspers, D. Bootsma and J.H.J. Hoeijmakers.** 1993. Xeroderma pigmentosum complementation group G associated with Cockayne's syndrome. Am. J. Human Genet. 53:185-192.

277. **Vermeulen, W., R.J. Scott, S. Potger, H.J. Muller, J. Cole, C.F. Arlett, W.J. Kleijer, D. Bootsma, J.H.J. Hoeijmakers and G. Weeda.** 1994. Clinical heterogeneity within xeroderma pigmentosum associated with mutations in the DNA repair and transcription gene ERCC3. Am. J. Human Gen. 54:191-200.

278. **Vermeulen, W., M. Stefanini, S. Giliani, J.H.J. Hoeijmakers and D. Bootsma.** 1991. Xeroderma pigmentosum complementation group H falls into complementation group D. Mutat Res 255:201-208.

279. **Vermeulen, W., A.J. van Vuuren, M. Chipoulet, L. Schaeffer, E. Appeldoorn, G. Weeda, N.G.J. Jaspers, A. Priestley, C.F. Arlett, A.R. Lehmann, M. Stefanini, M. Mezzina, A. Sarasin, D. Bootsma, J.-M. Egly and J.H.J. Hoeijmakers.** 1994. Three unusual repair deficiencies associated with transcription factor BTF2(TFIIH). Evidence for the existence of a transcription syndrome. Cold Spring Harbor 59:in press.

280. **Waga, S., G.J. Hannon, D. Beach and B. Stillman.** 1994. The p21 inhibitor of cylcin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369:574-578.

281. **Wang, X.W., K. Forrester, H. Yeh, M.A. Feitelson, J.-R. Gu and C.C. Harris.** 1994. Hepatitis B

virus X protein inhibits p53 sequence-specific DNA binding, transcritional activity, and association with transcrition factor ERCC3. Proc. Natl. Acad. Sci. USA 91:2230-2234.

282. **Wang, X.W., W. Vermeulen, J.D. Coursen, M. Gibson, S.E. Lupold, K. Forrester, G. Xu, L. Elmore, H. Yeh, J.H.J. Hoeijmakers and C.C. Harris.** 1995. p53-induced apoptosis mediated by the XPB and XPD DNA helicases. submitted

283. **Wang, X.W., H. Yeh, L. Schaeffer, R. Roy, V. Moncollin, J.-M. Egly, Z. Wang, E.C. Friedberg, M.K. Evans, B.G. Taffe, V.A. Bohr, G. Weeda, J.H.J. Hoeijmakers, K. Forrester and C.C. Harris.** 1995. p53 modulation of TFIIH-associated NER activity. Nature Genetics 10:188-195.

284. **Wang, Y.-C., V.M. Maher, D.L. Mitchell and J. McCormick.** 1993. Evidence from mutation spectra that the UV hypermutability og xeroderma pigmentosum variant cells reflects abnormal, error-prone replication on a template conyaining photoproducts. Molecular and Cellular Biology 13:4276-4283.

285. **Wang, Z., S. Buratowski, J.Q. Svejstrup, W.J. Feaver, X. Wu, R.D. Kornberg, T.D. Donahue and E.C. Friedberg.** 1995. The yeast *TFB1* and *SSL1* genes, which encode subunits of transcription factor IIH, are required for nucleotide excision repair and RNA polymerase II transcription. Molecular and Cellular Biology 15:2288-2293.

286. **Wang, Z., J.Q. Svejstrup, W.J. Feaver, X. Wu, R.D. Kornberg and E.C. Friedberg.** 1994. Transcription factor b (TFIIH) is required during NER in yeast. Nature 368:74-76.

287. **Wang, Z., X. Wu and E.C. Friedberg.** 1993. Nucleotide-excision repair in cell-free extracts of the yeast *Saccharomyces cerevisiae*. Proc.Nat.Acad.Sci.USA 90:4907-4911.

288. **Watkins, J.F., P. Sung, L. Prakash and S. Prakash.** 1993. The *Saccharomyces cerevisiae* DNA repair gene *RAD23* encodes a nuclear protein containing a ubiquitin-like domain required for biological function. Mol. Cell. Biol. 13:7757-7765.

289. **Weber, C.A., E.P. Salazar, S.A. Stewart and L.H. Thompson.** 1988. Molecular cloning and biological characterization of a human gene, *ERCC2*, that corrects the nucleotide excision repair defect in CHO UV5 cells. Molecular and Cellular Biology 8:1137-1146.

290. **Weber, C.A., E.P. Salazar, S.A. Stewart and L.H. Thompson.** 1990. *ERCC2*: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast *RAD3*. EMBO Journal 9:1437-1447.

291. **Weeda, G., R.C.A. Van Ham, R. Masurel, A. Westerveld, H. Odijk, J. De Wit, D. Bootsma, A.J. Van Der Eb and J.H.J. Hoeijmakers.** 1990. Molecular cloning and biological characterization of the human excision repair gene *ERCC-3*. Mol Cell Biol 10:2570-2581.

292. **Weeda, G., R.C.A. Van Ham, W. Vermeulen, D. Bootsma, A.J. Van der Eb and J.H.J. Hoeijmakers.** 1990. A presumed DNA helicase encoded by *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. Cell 62:777-791.

293. **Westerveld, A., J.H.J. Hoeijmakers, M. van Duin, J. de Wit, H. Odijk, A. Pastink, R.D. Wood and D. Bootsma.** 1984. Molecular cloning of a human DNA repair gene. Nature 310:425-429.

294. **Wolffe, A.P.** 1994. The transcription of chromatin templates. Current Biology 4:245-254.

295. **Wood, R.D., P. Robins and T. Lindahl.** 1988. Complementation of the xeroderma pigmentosum DNA repair defect in cell-free extracts. Cell 53:97-106.

296. **Yong, S.L., J.E. Cleaver, G.D. Tullis and M.M. Johnston.** 1984. Is trichothiodystrophy part of the xeroderma pigmentosum spectrum? American Journal of Human Genetics 36:82S.

297. **Yoon, H., S.P. Miller, E.K. Pabich and T.F. Donahue.** 1992. SSL1, a suppressor of a HIS4 5'-UTR stem-loop mutation, is essential for translation initiation and effects UV resistance in yeast. Genes and Development 6:2463-2477.

298. **Young, R.A.** 1991. RNA polymerase II. Annu. Rev. Biochem. 60:689-715.

299. **Zawel, L. and D. Reinberg.** 1995. Common themes in assembly and function of eukaryotic transcription complexes. Annual Review of Biochemistry 64:533-561.

300. **Ziegler, A., A.S. Jonason, D.J. Leffell, J.A. Simon, H.W. Sharma, K. J., L. Remington, T. Jacks and D.E. Brash.** 1994. Sunburn and p53 in the onset of skin cancer. Nature 372:773-776.

Summary

The genetic information stored in the nucleotide sequence of the DNA is vulnerable to alterations induced by internal cellular DNA damaging metabolites and by environmental DNA damaging agents. Unremoved DNA lesions interfere with essential processes such as transcription and replication, which may even lead to cell death. Accumulation of DNA injuries results also in enhanced mutagenesis. Mutations can cause cellular malfunctioning. Alterations in cell growth controlling genes are an early step in the process of carcinogenesis. Furthermore, mutations in germ cells can lead to congenital diseases. To diminish these deleterious consequences all living organisms are equipped with a complex network of sophisticated DNA repair processes. Most of the DNA repair processes are highly conserved during evolution. An important repair mechanism for the removal of a wide range of bulky chemical adducts in DNA and UV-light induced DNA lesions is nucleotide excision repair (NER). The consequences of accumulation of DNA damage are briefly discussed in chapter 1, an introduction to the NER-mechanism is presented in chapter 2. A more extensive overview of the current knowledge on mammalian NER is presented in chapter 4.

Several hereditary disorders which have a defect in one of the DNA repair pathways are recognized. The rare, UV-sensitive, cancer-prone disease xeroderma pigmentosum (XP) is generally considered as the prototype repair syndrome and is caused by a defect in NER. XP patients are marked by a dry, aged-like skin and pigmentation abnormalities on sun-exposed areas. In addition, patients have a highly elevated risk for skin cancer development. This disorder is accompanied by a complex and heterogeneous set of clinical features, including neurologic deterioration. Two other, UV-sensitive, genetic syndromes with defective NER have recently been discerned, Cockayne syndrome (CS) and trichothiodystrophy (TTD). However, the most prominent features exhibited by these diseases are of neurologic and developmental origin. These features are difficult to explain on the basis of a defective DNA repair, in contrast to the XP features, UV-sensitive skin and enhanced skin cancer. A small group of patients is recognized which even expresses a combined phenotype of both XP and CS (XP/CS).

In addition to a broad spectrum of clinical traits, these NER-syndromes are also heterogeneous at the genetic level. Genetic complementation analysis with the aid of somatic cell fusions revealed that, at least for XP and CS, multiple genes are responsible. Within the group of XP patients seven genetic complementation groups (XP-A, -G) are identified and two for CS (CSA, CSB). An overview of the clinical symptoms and genetic complexity of the NER-syndromes is provided in chapter 3. The experimental work described in appendix papers I and II (microneedle injection and classical somatic cell-hybridisation), corroborates the phenotypical overlap between XP and CS. Paper II describes the assignment of two new cases (siblings) to the very rare XP complementation group B. These patients express the hallmarks of both XP and CS, they display however, a much less severe phenotype than the original XP-B patient. Analysis of the *XPB/ERCC3* gene from these patients uncovered a mutation in the N-terminal

region of the encoded gene product. In addition, in paper I evidence is provided for a further extension of the genetic overlap between XP and CS, by the assignment of two severe CS patients to XP group G. Within at least three complementation groups (XP-B, -D, and -G) this XP/CS overlap is now confirmed.

Also the repair defect of the brittle hair disease trichothiodystrophy (TTD) appeared not to be restricted to one gene. Previously, UV-sensitive TTD patients were only found within XP group D. Genetic analysis of a single patient, described in appendix paper III, revealed that the repair defect in this particular patient resides in a previously not identified NER gene. The NER defect is complemented by all the known XP groups (including XP-D) and thus this patient (TTD1BR) represents a new NER-deficient complementation group, TTD-A. A further extension of genetic complexity within TTD is shown in appendix paper V, where the mild UV-sensitive TTD-phenotype of two siblings is linked to the *XPB/ERCC3* gene.

The observation that the *XPB* gene product is associated with the basal transcription factor TFIIH, sheds new light on the previously unexplained clinical features observed within this complementation group. In appendix paper IV experimental evidence is provided, with the aid of microneedle injections and an *in vitro* NER system, that the XPB protein in the context of transcription factor TFIIH is functional both in NER and basal transcription *in vitro* and *in vivo*. Moreover, it is even likely that the entire TFIIH complex functions in both processes. Because of the observed connection of NER with basal transcription, a short introduction on the mechanism of basal (RNA polymeraseII-driven) transcription is presented in chapter 5. A better understanding of the exact function of TFIIH in transcription may also be helpful to further unravel the NER mechanism. A functional and significant role for the observed DNA-unwinding activity of TFIIH, can be envisaged for both NER (chapter 4) and transcription initiation (chapter 5).

The work presented in appendix paper V, further establishes the concept of the involvement of the entire TFIIH in NER. Besides XP-B, also the repair defect in XP-D and TTD-A are restored with highly purified TFIIH preparations. Immuno-depletion experiments confirm a physical linkage of XPB, XPD, and TTDA, with the core of TFIIH. The observation that patients with a mutated TFIIH component exhibit a heterogeneous phenotype with features partly unexplained by a DNA repair defect, leads us to propose the 'transcription syndrome' model (appendix V). In this model the neurologic and developmental abnormalities, observed among CS and TTD individuals are explained by a subtly disturbed functioning of TFIIH in transcription of a specific set of genes, whose transcription is critically dependent on optimal TFIIH activity. The validity and implications of this model are discussed in appendix paper V and in chapter 6.

Samenvatting

De integriteit van de genetische informatie, die opgeslagen ligt in de nucleotide volgorde van DNA, staat bloot cellulaire metabolieten en omgevingsfactoren (o.a. UV-licht en talloze chemische verbindingen), die het DNA kunnen aantasten. DNA beschadigingen verstoren essentiële cellulaire processen zoals transcriptie en replicatie. DNA schades kunnen tevens leiden tot een permanente veranderingen in de basevolgorde van een gen; wanneer dit gebeurt in geslachtscellen kunnen aangeboren afwijkingen het gevolg zijn. Mutaties in genen kunnen ook het normale functioneren van de cel verstoren. Veranderingen van de basevolgorde in genen, die de celgroei controleren, zijn een belangrijke factor bij het ontstaan van kanker. Oorzaken en gevolgen van DNA schades zijn kort samengevat in hoofdstuk 1.

Om het hoofd te bieden aan de nadelige gevolgen van DNA veranderingen zijn alle organismen uitgerust met een ingewikkeld en efficiënt stelsel van DNA herstel processen. Één van deze DNA herstelmechanismen is nucleotide excisie reparatie of NER, dat zorgt voor de verwijdering van een groot aantal verschillende DNA lesies (waaronder DNA schades, veroorzaakt door UV-licht). In hoofdstuk 2 wordt kort de werking van NER beschreven. Dit DNA herstel proces is geconserveerd gedurende de evolutie, het mechanisme in zoogdier cellen lijkt sterk op dat van de darmbacterie (*E.coli*). NER is een ingewikkeld proces, waarbij een groot aantal verschillende factoren betrokken zijn. Allereerst moet de schade herkend worden, aan weerszijde wordt een knip gemaakt en de lesie wordt verwijderd, waarna het ontstane gat weer wordt opgevuld. De individuele stappen en werkzame factoren worden besproken in hoofdstuk 4.

Er zijn verschillende erfelijke ziekten bekend, die veroorzaakt worden door een defect in één van de DNA herstel processen. Het meest bekende voorbeeld is xeroderma pigmentosum (XP), een zeldzame autosomaal recessieve aandoening met een overgevoeligheid voor zonlicht en aanleg voor huidkanker, die berust op een gebrekkig NER. XP patiënten hebben een droge, perkamentachtige huid met pigmentatievlekken op plaatsen die blootstaan aan zonlicht. Verder vertonen de patiënten een uitgebreid en variabel scala aan symptomen, waaronder neurologische afwijkingen. Bij twee andere erfelijke aandoeningen is recent ook een NER defect aangetoond: Cockayne's syndroom (CS) en trichothiodystrofie (TTD). Naast een overgevoeligheid voor zonlicht, zijn de meest in het oog springende kenmerken van deze syndromen neurologische- en ontwikkelings-stoornissen. In tegenstelling tot de afwijkingen in de huid, kunnen deze verschijnselen bij CS en TTD moeilijk verklaard worden door een gebrekkig DNA herstel. In hoofdstuk 3 staan de klinische verschijnselen van XP, CS en TTD samengevat.

Ook in genetisch opzicht zijn deze syndromen heterogeen: meerdere genen blijken verantwoordelijk te zijn voor een efficiënt NER (hoofdstuk 3). Wanneer gekweekte huidcellen van verschillende patiënten met elkaar gefuseerd worden, wordt bij bepaalde combinaties het DNA herstel defect opgeheven (gecomplementeerd). Op deze manier zijn bij XP zeven complementatiegroepen (XP-A t/m XP-G), en dus genen,

gevonden en bij CS twee (CS-A en CS-B). Er zijn ook enkele patiënten, die duidelijk de karakteristieken van zowel XP als CS (gecombineerd XP/CS) vertonen. Tot voor kort werden deze patiënten alleen gevonden in XP groep B en D (beide één patiënt). Met behulp van micronaald injecties en celfusies zijn in appendix artikelen I en II meerdere XP/CS patiënten gekarakteriseerd. Twee broers met milde verschijnselen van XP (geen huidtumoren ondanks gevorderde leeftijd) en CS, blijken te behoren tot de uiterst zeldzame XP complementatiegroep B (beschreven in appendix artikel II). Het ziektebeeld van deze personen is veel minder ernstig dan de oorspronkelijke XP groep B patiënt, hetgeen een klinische heterogeniteit binnen die groep aantoont. Analyse van het *XPB(ERCC3)* gen van deze patiënten bracht aan het licht, dat bij het gecodeerde eiwit slecht één aminozuur veranderd is. De overlap tussen XP en CS is verder uitgebreid met een derde XP-groep, doordat twee ernstige CS patiënten bij XP groep G blijken te behoren, hetgeen beschreven staat in appendix artikel I. Het samen voorkomen van XP- en CS-verschijnselen in tenminste drie groepen doet vermoeden, dat XP en CS verschillende manifestaties zijn van een deficiënt NER.

Ook het DNA herstel defect van trichothiodystrofie (TTD) is niet tot één gen beperkt. Tot voor kort werden alle UV-gevoelige TTD patiënten gevonden in XP groep D. In appendix artikel III, wordt echter de genetische analyse beschreven van een UV-gevoelige TTD patiënt, waarvan het herstel defect wordt gecomplementeerd door alle bekende XP groepen (ook XP-D). Deze patiënt vertegenwoordigt dan ook een nieuwe complementatie groep (en een niet eerder geïdentificeerd gen): TTD-A. Een nog verdere uitbreiding van de genetische complexiteit binnen TTD wordt behandeld in appendix artikel V, waarin wordt aangetoond dat het UV-gevoelige fenotype van twee andere TTD patiënten berust op een defect *XPB* gen.

Het XPB eiwit blijkt een onderdeel te zijn van een complexe eiwitfactor (TFIIH), die een rol speelt bij de initiatie, van door RNA polymerase II gestuurde, transcriptie. Met micronaald injecties en een *in vitro* NER-systeem, wordt in appendix artikel IV bewijs geleverd, dat het XPB eiwit in de structuur van het TFIIH complex een dubbele rol vervult, zowel in transcriptie als in NER. Met behulp van sterk gezuiverde TFIIH fracties en genoemde methoden in appendix V aangetoond, dat naast XPB, ook XPD en TTDA deel uitmaken van het TFIIH eiwitcomplex. Met hulp van specifieke antilichamen tegen andere (dan XPB, XPD of TTDA) TFIIH componenten, wordt aangetoond dat mogelijk het gehele TFIIH complex (9 verschillende eiwitten) een rol speelt in NER. TFIIH heeft een helicase (ontwinden van de dubbele helix structuur van DNA) activiteit. Dit is waarschijnlijk ook de gemeenschappelijke functie van TFIIH in transcriptie en NER. In hoofdstuk V wordt kort het mechanisme beschreven van de initiatie van basale (door RNA polymerase II gestuurde) transcriptie. Kennis over dit proces en de rol van TFIIH hierin is indirect ook van belang voor inzicht in NER, gezien de parallellen tussen de beide mechanismen.

Patiënten met een gemuteerde TFIIH component (XPB, XPD of TTDA), vertonen symptomen, die moeilijk te rijmen zijn met een NER defect. Mogelijk zijn deze verschijnselen te verklaren vanuit een verstoorde transcriptie functie van TFIIH. We stellen dan ook voor, dat deze aandoeningen aangeduid kunnen worden als een nieuwe

klasse van erfelijke afwijkingen,"DNA herstel/transcriptie syndromen" (appendix V). Waarschijnlijk bestaan er ook afzonderlijke "transcriptie syndromen", waar alleen de basale transcriptie functie is aangetast en het DNA herstel mechanisme normaal functioneert, zoals bijvoorbeeld bij TTD patiënten die geen verstoord NER hebben (appendix V en hoofdstuk 6).

Nawoord

Talloze personen hebben een bijdrage geleverd aan de totstandkoming van dit boekje. Het doet me dan ook deugd dat ik nu in de gelegenheid ben om enkele mensen persoonlijk te bedanken. Ik realiseer me wel dat er altijd personen vergeten worden. Bij voorbaat wil ik me dan ook excuseren voor deze eventuele omissie.

Allereerst een woord van waardering voor het thuisfront. Hanny, dankzij jouw liefdevolle loyaliteit en steun kon ik me volledig storten op de afronding van dit werk. Door jouw inzet draaide ons kleine gezinnetje gewoon door. Maar bovenal ben je voor mij véél meer dan een relativerende steun en toeverlaat. Frank en Paul, wellicht onbewust, maar jullie levenslust is voor mij een blijvende inspiratie bron. Ook hebben jullie mij helpen realiseren dat er belangrijkere zaken in het leven zijn dan wetenschap. Pa en Ma, het is een voorrecht om bij jullie opgegroeid te zijn, bedankt voor de zorg, steun en stimulans. Ma Odijk, Jacques, Jules en Anja, jullie vele oppas uren, waren voor ons van onschatbare waarde.

Mijn beide promotoren, Prof. D. Bootsma en Prof. J. Hoeijmakers ben ik zeer erkentelijk voor het in mij gestelde vertrouwen en de geboden mogelijkheden. Beste Dick en Jan, dankzij jullie gezamenlijke inspanning, stimulans en durf kon ik mij ontwikkelen tot een beginnend wetenschapper. Het enthousiasme en de energie van jou Jan zijn verbluffend en stimulerend en vormen met de rust en visie van Bootsma een goede complementatie. De leden van de promotie commissie, Prof. F. Grosveld, Prof. D. Lindhout en Prof. A. Westerveld bedank ik voor het kritisch lezen en de waardevolle suggesties.

Jules, bedankt voor de hulp bij de lay-out, de geleverde litho's en het ontwerp en de verzorging van de omslag. Dear Michael (Hé meneer) thanks for your excellent english corrections. Jan(Jos) en Koos zorgden ervoor dat ik, als computer analfabeet, toch dit manuscript kon bewerken. Koos, tevens bedankt voor al je andere hulp en de vele levensbeschouwelijke en wetenschappelijke filosofische verhandelingen.

Onder leiding van Jan Hoeijmakers en Bram de Jonge ben ik begonnen aan de microinjectie, de celkweek geheimen werden me bijgebracht door Wilma, Andries en Jan (dW). Jan, al heel lang zitten we bij elkaar op de kamer en dat heeft vele prettige gesprekken opgeleverd, ik hoop dat we daar nog lang mee door kunnen gaan. André lichtte enkele tipjes van de eiwit zuivering-sluier voor me op. Christine, Marcel(K) en Geert hebben verwoede pogingen gedaan mij wat moleculaire wijsheid bij te brengen. Nog steeds jammer dat je weg bent, Christine. Ik durf het bijna niet te zeggen, maar Marcel(K) waar blijft ie van jou nou? Geert, ook bedankt voor de leiding van de voorheen "ERCC3-groep". In dit verband wil ik ook Libin, Carrol en Prof. L. van der Eb bedanken voor de samenwerking op het ERCC3 front. Zeker Hanneke wil ik ook met name noemen, juist in de laatste periode is onze samenwerking intensief, plezierig en zinvol geweest. Gelukkig dat mensen zoals jij samenwerken als iets vanzelfsprekends ervaren. Alle andere reparerende labgenoten uit heden en verleden: Jan(H), Jan(dW), Joke (Z), Koos, Andries, Wilma, Marcel(vD), Marcel(K), Jacolien, Carla, Akira, André, Mirek,

Christine, Ian, Iris, Marianne, Geert, Peter (succes met het schrijven), Joke, Hanneke, Alain (begin maar vast), Ingrid, Jan (vK), Anneke, Esther & Anja (jawel, jullie zijn genoemd), Sigrid, Jan-Huib, Wouter, Henk, Bert, Jan (dB), Jeroen, Bas, Cécile, Suzanne, Michael, Roland, Kaoru, en alle studenten en stagiaires, wil ik bedanken voor de prettige werksfeer, (natte) proppen, dropjes, cake, koffie, bier en het vrij houden van m'n bench.

Irma, Ard, Hans, Jos, Coen en Michel, hebben als studenten veel bijgedragen aan het experimentele werk wat hier gepresenteerd is.

I am very indebted to Jean-Marc Egly and to the people from his group, for providing the precious TFIIH samples, antibodies, genes and usefull suggestions. I enjoyed our fruitfull collaboration and I hope we can continue this in the future. I am pleased that you are willing to take part in the promotion committee. Dear Miria (Stefanini), it is a pleasure for me that you are also in this committee. The complementation between the Pavia and Rotterdam groups on genetic screening of repair syndromes worked very well. The collaboration with the labs of Alan Lehmann and Alain Sarasin were also of invaluable importance for the experimental work described in this thesis. I appreciate the cooperation with the lab of Gilbert de Murcia and his kind hospitality during my stay there. Also the combined work on helicases with the group of Narendra Tuteja in the lab of Prof. A. Falaschi, and my stay in Trieste helped me a lot. I enjoyed the stays of our foreign guests who came to use the micro-injection facilities in our lab: Gilbert, Marie-Josèphe, Miguel, Narendra, Scott, Mauro, Xin, Doris and others.

De infrastructuur op de afdeling zijn van groot belang voor het gladjes verlopen van het "labgebeuren". Rita, de immer vrolijke rots in de branding, jouw medeleven en relativerende opmerkingen zal ik niet snel vergeten. Marieke, bedankt voor de koekjes. Jopie, Elly, Joke en Rob zorgen voor schoon glaswerk en de belangrijkste vloeistof van de hele afdeling: koffie. Piet, bedankt voor het meedenken en vervaardigen van de huidige microinjectie opstelling. Rein, Melle en Mieke, gelukkig ben ik niet zo duur als mijn bestellingen. Mirko, Ruud en Tom (en voorheen Joop en Tar) door jullie vaardigheid werden mijn autoradiografische preparaten nog enigszins toonbaar.

Ook wil ik het Koningin Wilhelmina Fonds (KWF) bedanken voor de financiering van dit onderzoek en van het vervolg onderzoek.

Verder wil ik al mijn vrienden, familieleden en alle andere labgenoten bedanken voor de interesse in mij en m'n werk.

Curriculum vitae

Willem Vermeulen Geboren te Hardinxveld-Giessendam	13 juli, 1960
MAVO-4 eindexamen aan: "De Calvijnschool" te H'veld-G'dam	juni, 1977
Voorbereidend jaar HBO, aan: het "Van 't Hoff Instituut" te Rotterdam	juli, 1978
HTS-Chemie, specialisatie Biochemie, aan: het "Van 't Hoff Instituut" te Rotterdam Afstudeer stage op de afdeling Celbiologie en Genetica, Erasmus Universiteit Rotterdam (o.l.v. Dr. J.H.J. Hoeijmakers).	november, 1982
Vervangende dienst	maart, 1983
Aanstelling als Analist bij het instituut Genetica van de Erasmus Universiteit Rotterdam	maart, 1983 - december, 1994
Aanstelling als onderzoeks medewerker bij het instituut Genetica van de Erasmus Universiteit Rotterdam	december, 1994- heden

Publications

1. **A.J.R. de Jonge, W. Vermeulen, B. Klein and J.H.J. Hoeijmakers.** Microinjection of human cell extracts corrects xeroderma pigmentosum defect. EMBO J. 2: 637-641 (1983).
2. **J.H.J. Hoeijmakers, J.C.M. Zwetsloot, W. Vermeulen, A.J.R. de Jonge, C. Backendorf, B. Klein and D. Bootsma.** Phenotypic correction of xeroderma pigmentosum cells by microinjection of crude extracts and purified proteins. In: 'Cellular Responses to DNA Damage' (E.C. Friedberg and B.A. Bridges, eds.). Alan R. Liss, Inc. New York.: 173-181 (1983).
3. **J.C.M. Zwetsloot, W. Vermeulen, J.H.J. Hoeijmakers, A. Yasui, A.P.M. Eker and D. Bootsma.** Microinjected photoreactivating enzymes from *Anacystis* and *Saccharomyces* monomerize dimers in chromatin of human cells. Mutat.Res. 146: 71-77 (1985).
4. **A.J.R. de Jonge, W. Vermeulen, W. Keijzer, J.H.J. Hoeijmakers and D. Bootsma.** Microinjection of *Micrococcus luteus* UV-endonuclease restores UV-induced unscheduled DNA synthesis in cells of 9 xeroderma pigmentosum complementation groups. Mutat.Res. 150: 99-105 (1985).
5. **J.H.J. Hoeijmakers, A. Westerveld, M. van Duin, A. Yasui, W. Vermeulen, H. Odijk, J. de Wit and D. Bootsma.** Partial characterization of genes and gene products involved in excision repair of human cells. First Research Conference on Familial Cancer, Karger, Basel.: 231-233 (1985).
6. **J.C.M. Zwetsloot, J.H.J. Hoeijmakers, W. Vermeulen, A.P.M. Eker and D. Bootsma.** Unscheduled DNA synthesis in xeroderma pigmentosum cells after microinjection of yeast photoreactivating enzyme. Mutat.Res. 165: 109-115 (1985).
7. **J.H.J. Hoeijmakers, A. Westerveld, M. van Duin, W. Vermeulen, H. Odijk, J. de Wit and D. Bootsma.** Identification of genes and proteins involved in excision repair of human cells. In: Biochem. and Molec. Epidemiology of Cancer. Alan R. Liss Inc.: 337-390 (1986).
8. **J.C.M. Zwetsloot, A.P. Barbeiro, W. Vermeulen, J.H.J. Hoeijmakers and C. Backendorf.** Microinjection of *Escherichia coli* UvrA, B, C and D proteins into fibroblasts of xeroderma pigmentosum complementation groups A and C does not result in restoration of UV-induced DNA synthesis. Mutat.Res. 166: 89-98 (1986).
9. **W. Vermeulen, P. Osseweijer, A.J.R. de Jonge and J.H.J. Hoeijmakers.** Transient correction of excision repair defects in fibroblasts of 9 xeroderma pigmentosum complementation groups by microinjection of crude human cell extract. Mutat.Res. 165: 199-206 (1986).
10. **L.Roza, W. Vermeulen, G.P. van der Schans and P.H.M. Lohman.** The induction and repair of cyclobutane thymidine dimers in human skin. In: Human exposure to ultraviolet radiation: Risks and regulations (W.F.Passhier and B.F.M. Bosnjakovic, eds.). Elsevier Science Publishers B.V.: 27-32 (1987).
11. **M. van Duin, G. Vredeveldt, L.V. Mayne, H. Odijk, W. Vermeulen, G. Weeda, B. Klein, J.H.J. Hoeijmakers, D. Bootsma and A. Westerveld.** The cloned human DNA excision repair gene *ERCC-1* fails to correct xeroderma pigmentosum complementation groups A through I. Mutat.Res. 217: 83-92 (1989).
12. **N.G.J. Jaspers, L. Roza, W. Vermeulen, A. Eker, R.D.F.M. Taalman, J.H.J. Hoeijmakers and D. Bootsma.** *In vitro* correction of cells from patients with mutagen hypersensitivity. In: "DNA Damage and Repair" (A. Castellani, ed.) Plenum Press New York and London.: 73-82 (1989).
13. **L. Roza, W. Vermeulen, J.B.A. Bergen Henegouwen, A.P.M. Eker, N.G.J.Jaspers, P.H.M. Lohman and J.H.J. Hoeijmakers.** Effects of microinjected photoreactivating enzyme on thymine dimer removal and DNA repair synthesis in normal and xeroderma pigmentosum fibroblasts. Cancer Research 50: 1905-1910 (1990).
14. **G. Weeda, R.C.A. van Ham, W. Vermeulen, D. Bootsma, A.J. van der Eb and J.H.J. Hoeijmakers.** A presumed DNA helicase, encoded by the excision repair gene *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. Cell 62: 777-791 (1990).
15. **W. Vermeulen, M. Stefanini, S. Giliani, J.H.J. Hoeijmakers and D. Bootsma.** Xeroderma

pigmentosum complementation group H falls into complementation group D. Mutat.Res. 255: 201-208 (1991).

16. **A.P.M. Eker, W. Vermeulen, N. Miura, K. Tanaka, N.G.J. Jaspers, J.H.J. Hoeijmakers and D. Bootsma.** Xeroderma pigmentosum group A correcting protein from Calf Thymus. Mutat.Res. 274: 211-224 (1992).
17. **C. Troelstra, A. van Gool, J. de Wit, W. Vermeulen, D. Bootsma and J.H.J. Hoeijmakers.** *ERCC6*, a member of a subfamily of putative helicases is involved in Cockayne's syndrome and preferential repair of active genes. Cell 71: 939-953 (1992).
18. **W. Vermeulen, J. Jaeken, N.G.J. Jaspers, D. Bootsma and J.H.J. Hoeijmakers.** Xeroderma pigmentosum complementation group G associated with Cockayne's syndrome. Am. J. Hum. Genet. 53: 185-193 (1993).
19. **M. Stefanini, W. Vermeulen, G. Weeda, S. Giliani, T. Nardo, M. Mezzina, A. Sarasin, J.I. Harper, J.H.J. Hoeijmakers and A.R. Lehmann.** A new nucleotide excision repair gene associated with the genetic disorder trichothiodystrophy. Am. J. Hum. Genet. 53: 817-821 (1993).
20. **M. Molinete, W. Vermeulen, A. Bürkle, J. Ménissier-de Murcia, J.H. Küpper, J.H.J. Hoeijmakers and G. de Murcia.** Overproduction of the poly(ADP-ribose) polymerase DNA binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. EMBO J. 12: 2109-2117 (1993).
21. **L. Schaeffer, R. Roy, S. Humbert, V. Moncollin, W. Vermeulen, J.H.J. Hoeijmakers, P. Chambon and J-M. Egly.** DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science 260: 58-63 (1993).
22. **M. Mezzina, E. Eveno, O. Chevallier-Lagente, A. Benoit, M. Carreau, W. Vermeulen, J.H.J. Hoeijmakers, M. Stefanini, A.R. Lehmann, C. A. Weber, and A. Sarasin.** Correction by the *ERCC2* gene of UV sensitivity and repair deficiency phenotype in a subset of trichothiodystrophy cells. Carcinogenesis 15: 1493-1498 (1994)
23. **W. Vermeulen, R.J. Scott, S. Potger, H.J. Müller, J. Cole, C.F. Arlett, W.J. Kleijer, D. Bootsma, J.H.J. Hoeijmakers and G. Weeda.** Clinical heterogeneity within xeroderma pigmentosum associated with mutations in the DNA repair and transcription gene *ERCC3*. Am. J. Hum. Genet. 54: 191-200 (1994).
24. **A.J. van Vuuren, W. Vermeulen, L. Ma, G. Weeda, E. Appeldoorn, N.G.J. Jaspers, A. J. van der Eb, D. Bootsma, J.H.J. Hoeijmakers, and S. Humbert, L. Schaeffer, and J-M. Egly.** Correction of xeroderma pigmentosum repair defect by basal transcription factor BTF2 (TFIIH). EMBO J. 13: 1645-1653 (1994).
25. **R. Roy, L. Schaeffer, S. Humbert, W. Vermeulen, G. Weeda, and J-M Egly.** The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. J. of Biol. Chem. 269: 9826-9832 (1994).
26. **S. Keeney, A.P.M. Eker, T. Brody, W. Vermeulen, D. Bootsma, J.H.J. Hoeijmakers, and S. Linn.** Correction of the DNA repair defect in xeroderma pigmentosum group E by injection of a DNA damage binding protein. Proc. Natl. Acad. Sci. USA 91: 4053-4056 (1994).
27. **R. Roy, J.P Adamczewski, T.Seroz, W. Vermeulen, J-P, Tassan, L. Schaeffer, E.A. Nigg, J.H.J. Hoeijmakers, and J-M Egly.** The Mo15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell, 79: 1093-1101 (1994).
28. **W. Vermeulen, A.J. van Vuuren, M. Chipoulet, L. Schaeffer, E. Appeldoorn, G. Weeda, N.G.J.Jaspers, A. Priestley, C.F. Arlett, A.R. Lehmann, M. Stefanini, M. Mezzina, A. Sarasin, D. Bootsma, J-M Egly, and J.H.J. Hoeijmakers.** Three unusual repair deficiencies associated with transcription factor BTF2(TFIIH). Evidence for the existence of a transcription syndrome. Cold Spring Harb. Symp. Quant. Biol. 59: 317-329 (1994).
29. **W. Vermeulen, A.J. van Vuuren, G. Weeda, D. Bootsma, J-M Egly, and J.H.J Hoeijmakers.** Clinical aspects and functional analysis of transcription/repair unit TFIIH. J. of Cellular Biochemistry, supplement 21A: 308 (1995).

30. **B.C. Broughton, A.F. Thompson, S.A. Harcourt, W. Vermeulen, J.H.J. Hoeijmakers, E. Botta, M. Stefanini, M.D. King, C.A Weber, J. Cole, C.F. Arlett, and A.R. Lehmann.** Molecular and cellular analysis of the DNA repair defect in a patient with xeroderma pigmentosum complementation group D who has the clinical features of xeroderma pigmentosum and Cockayne syndrome. Am. J. Hum. Genet. 56: 167-174 (1995).

31. **D. Bootsma, G. Weeda, W. Vermeulen, A.J van Vuuren, C. Troelstra, P.J. van der Spek, and J.H.J. Hoeijmakers.** Nucleotide excision repair syndromes: molecular basis and clinical symptoms. Phil. Trans. R. Soc. Lond. 347: 75-81 (1995).

32. **M. Carreau, E.Evino, X. Quilliet, O. Chevalier-Lagente, A. Benoit, B.Tanganelli, M. Stefanini, W. Vermeulen, J.H.J. Hoeijmakers, A.Sarasin, and M. Mezzina.** Development of a new easy complementation assay for DNA repair deficient human syndromes using cloned repair genes. Carcinogenesis 16: 1003-1009 (1995).

33. **X.W. Wang, W. Vermeulen, J.D. Coursen, M. Gibson, S.E. Lupold, K. Forrester, G. Xu, L. Elmore, H. Yeh, J.H.J. Hoeijmakers, and C.C. Harris.**
p53-induced apoptosis mediated by the XPB and XPD DNA helicases. *submitted.*

34. **W. Vermeulen, J.H.J. Hoeijmakers, and D. Bootsma.** Maintaining nature's perfection. Helix, *in the press.*

35. **J.R. Wang, V. Moncollin, W. Vermeulen, A.J. van Vuuren, J.H.J. Hoeijmakers, and J-M. Egly.** A 3' to 5' XPB helicase defect in repair/transcription factor TFIIH of xeroderma pigmentosum group B affects both DNA repair and transcription. *submitted*

Abbreviations

CAK	CDK activating kinase
CDK	cyclin dependent kinase
cDNA	copy DNA
CFC	chloro-fluoro-carbonhydrate
CG	complementation group
CHO	Chinese Hamster ovary
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
CTD	C-terminal domain of RNA polymerase II largest subunit (hepta-peptide repeat)
(UV)DDB	(UV-light) DNA damage binding protein
DNA	deoxyribonucleic acid
DNA pol.δ (ϵ)	DNA polymerase δ (of ϵ)
dsDNA	double-stranded DNA
E.coli	*Escherichia coli*
ERCC (ERCC)	excision repair cross complementing gene (protein)
GGR	global genome repair
HHR	human homolog of *S.cerevisiae* Rad
IF7	initiation factor 7
kDa	kilodalton
NER	nucleotide excision repair
nt.	nucleotide
PCNA	proliferating cell nuclear antigen
6-4PP	6-4 pyrimidine-pyrimidone dimer
PRR	post replication repair
RAD (Rad)	*S.cerevisiae* repair gene (protein)
RPA (HSSB)	replication factor A (human ss binding protein)
RF-C	replication factor C
(m)RNA	(messenger) ribonucleic acid
RNAP (II)	RNA polymerase (II)
RRS	recovery of RNA synthesis
S.cerevisiae	*Saccharomyces cerevisiae*
S.pombe	*Saccharomyces pombe*
ssDNA	single-stranded DNA
TCR	transcription coupled repair
TFIIH (BTF2)	transcription initiation factor IIH
TRCF	transcription repair coupling factor
TTD	trichothiodystrophy
UDS	unscheduled DNA synthesis
UV	ultraviolet light
UV^s	ultraviolet light sensitive
Uvr (Uvr)	*E.coli* NER gene (protein)
XP	xeroderma pigmentosum
XP-A (-G)	XP complementation group A (G)

Appendix paper I

American J. of Human Genetics,
Vol. 53: 185-192, 1993

Xeroderma Pigmentosum Complementation Group G Associated with Cockayne Syndrome

W. Vermeulen,* J. Jaeken,† N. G. J. Jaspers,* D. Bootsma,* and J. H. J. Hoeijmakers*

*MGC Department of Cell Biology and Genetics, Erasmus University, Rotterdam; and †Division of Metabolism and Nutrition, University Hospital Gasthuisberg, Leuven, Belgium

Summary

Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are two rare inherited disorders with a clinical and cellular hypersensitivity to the UV component of the sunlight spectrum. Although the two traits are generally considered as clinically and genetically distinct entities, on the biochemical level a defect in the nucleotide excision-repair (NER) pathway is involved in both. Classical CS patients are primarily deficient in the preferential repair of DNA damage in actively transcribed genes, whereas in most XP patients the genetic defect affects both "preferential" and "overall" NER modalities. Here we report a genetic study of two unrelated, severely affected patients with the clinical characteristics of CS but with a biochemical defect typical of XP. By complementation analysis, using somatic cell fusion and nuclear microinjection of cloned repair genes, we assign these two patients to XP complementation group G, which previously was not associated with CS. This observation extends the earlier identification of two patients with a rare combined XP/CS phenotype within XP complementation groups B and D, respectively. It indicates that some mutations in at least three of the seven genes known to be involved in XP also can result in a picture of partial or even full-blown CS. We conclude that the syndromes XP and CS are biochemically closely related and may be part of a broader clinical disease spectrum. We suggest, as a possible molecular mechanism underlying this relation, that the XPGC repair gene has an additional vital function, as shown for some other NER genes.

Introduction

Accumulation of DNA damage, caused by chemical or physical agents, can hamper transcription as well as replication and can induce mutations. To prevent these deleterious consequences, all living organisms are equipped with a network of DNA repair systems (for an extensive review, see Friedberg 1985). One of the best-understood and universal DNA repair mechanisms is the nucleotide excision-repair (NER) pathway. This is a multistep process that recognizes and removes lesions from the DNA by a dual incision around the lesion in the damaged strand and excision of some flanking nucleotides (Huang et al. 1992), followed by repair synthesis (also referred to as "unscheduled DNA synthesis" [UDS]), using the nondamaged strand as template, and ligation. In this way the system eliminates a wide variety of structurally unrelated lesions. Detailed studies on the repair of unique genes have led to the differentiation of NER into two subpathways: preferential repair of the transcribed strand of active genes to a slower and less efficient "overall genome" repair (Bohr 1991; Hanawalt 1991).

The importance of the NER system is reflected by the existence of two rare, human, hereditary diseases caused by a defective excision repair: xeroderma pigmentosum (XP) (Cleaver 1968) and Cockayne syndrome (CS) (Venema et al. 1990*a*). Cells from most excision-defective XP patients are impaired in both NER subpathways, and some are impaired in the "overall" pathway alone (Venema et al. 1990*b*). In CS patients, however, the repair deficiency seems to be restricted to the preferential subpathway (Venema et al. 1990*a*). Since nontranscribed sequences represent the bulk of the genome, measurement of repair synthesis (i.e., UDS) in whole cells primarily reflects the rate of the "overall"

Received January 31, 1993; revision received March 17, 1993.

Address for correspondence and reprints: Dirk Bootsma, Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

0002-9297/93/5301-0022$02.00

NER modality. As a consequence, CS cells display normal rates of UV-induced UDS, whereas XP cells have UDS levels reduced to a varying extent. Since UV-induced DNA damage acts as a block to transcription, the rate of RNA synthesis is depressed following UV exposure, and subsequent recovery is dependent on preferential NER. CS patients' cells thus fail to recover RNA synthesis rates, as do those XP cells with defective preferential NER.

Clinically, the two autosomal recessive disorders show clear differences as well as similarities. They share an extreme sun sensitivity of the skin. XP patients exhibit, in addition, other pronounced cutaneous abnormalities: atrophic skin with hyper- and hypopigmented spots in sun-exposed areas, in most cases combined with skin malignancies at an early age (Cleaver and Kraemer 1989). The disease is frequently associated with progressive neuronal degeneration. In CS patients, cutaneous abnormalities are less pronounced and are often restricted to sun sensitivity, whereas neurological disease is obligate and of a different type than that in XP (Nance and Berry 1992). Neurological dysfunctioning is predominantly due to demyelination in the cases of CS, while the neurological abnormalities of XP are believed to be based on primary neuronal degeneration (Robbins et al. 1991). In addition, retinal degeneration (salt-and-pepper pigmentation) together with retinal artery narrowing are characteristically associated with CS. Often intracranial calcifications are observed in CS patients but not in XP. Although different in origin, some neurological features, such as microcephaly, sensorineural deafness, and psychomotor retardation are shared by the two syndromes. A striking difference between XP and CS is the absence of cancer predisposition in CS patients, compared with a 2000-fold increased risk of developing cutaneous malignancies in XP individuals (Cleaver and Kraemer 1989). Within both DNA repair syndromes the clinical manifestations are rather heterogeneous. At the genetic level this heterogeneity is partly reflected by the existence of seven distinct genetic complementation groups in XP (XP-A to XP-G) (Vermeulen et al. 1991) and at least two complementation groups within classical CS (CS-A and CS-B) (Tanaka et al. 1981; Lehmann 1982). But even within some groups the clinical picture may vary considerably.

In only two cases a combination of clinical hallmarks of XP and CS has been reported, and these have been assigned to XP groups B (Robbins et al. 1974) and D, respectively (Lafforet and Dupuy 1978; Johnson et al. 1989; Vermeulen et al. 1991). Robbins (1988) has proposed to consider this XP/CS combination a distinct clinical entity, called "XP-CS complex." On the other hand, the exceptional conjunction could also fall within the extreme clinical heterogeneity encountered in some of the XP complementation groups, which could imply that the distinction between XP and CS is smaller than originally thought.

Here we report a genetic study of two unrelated patients combining very severe CS symptoms with a strongly reduced level of UDS typical of XP cells. Both are assigned to the rare XP complementation group G, which previously was not associated with CS. This finding demonstrates that the overlap between XP and CS is more extensive than believed thus far and that it involves at least three of the seven excision-deficient XP complementation groups.

Material and Methods

Cell Strains, Culture Conditions, and Cell Hybridization

All primary fibroblast cultures (table 1) were grown on Ham's F10 medium (Gibco) supplemented with 11% FCS (Biological Industries), 100 IU penicillin/ml, and 100 μg streptomycin/ml. Prior to Sendai virus–mediated cell hybridization, the cytoplasm of each fusion partner was labeled by culturing for 2–3 d in the presence of latex beads of different sizes (Jaspers and Bootsma 1982; Vermeulen et al. 1991); the cells were trypsinized, mixed in a 1:1 ratio, and extensively washed with PBS to remove free beads. Fused cells were seeded on coverslips and were cultured under standard conditions for 2 d prior to assays of UV-induced UDS or RNA synthesis recovery. Homopolykaryons, to be used for the microinjection experiments, were generated in the same way, except that the addition of latex beads was omitted and only one cell strain was used for the fusion.

Microneedle Injection

Nuclear microinjection of cDNA was performed according to a method described elsewhere (van Duin et al. 1989). In short, *XPBC/ERCC3* cDNA was cloned into the mammalian expression vector pSVL, yielding the plasmid pSV3H (Weeda et al. 1990) dissolved in PBS at a concentration of 100 μg/ml and, with the aid of a glass microneedle, injected into one of the nuclei of XP homopolykaryons. Coverslips with injected and uninjected cells were cultivated for 24 h to permit expression of the introduced cDNA before they were assayed for their repair capacity.

UV-induced UDS

The UDS assay (Vermeulen et al. 1986) was performed 2 d after fusion and 1 d after microinjection.

Table 1

Repair Activity of Cell Strains Used

Cell Strain	UDS (% of normal)	Source
CS-TK	80–100	Jaeken et al. 1989
XPCS1LV	5	Jaeken et al. 1989
XPCS2LV	5	Jaeken et al. 1989
CS3BE (CS-A)	80–100	Lehmann 1982
CS1BE (CS-B)	80–100	Lehmann 1982
XP25RO (XP-A)	2	
XPCS1BA (XP-B)	8	W. Vermeulen et al., unpublished data
XP2BI (XP-G)	5	Keijzer et al. 1979
C5RO (normal control)	100	

Cells were washed with PBS, irradiated with 15 J/m^2 UV-C light (Philips TUV lamp), incubated for 2 h in 10 μCi [methyl-^{3}H]-thymidine (50 Ci/mmol; Amersham)/ml containing culture medium, were washed with PBS, and were fixed and dipped in a photo-sensitive emulsion (Ilford K2). Repair capacity was quantitated by grain counting after autoradiography.

RNA Synthesis Recovery

RNA synthesis recovery after UV irradiation (Lehmann 1982; Mayne and Lehmann 1982) was determined 2 d after fusion. Cells were UV irradiated (10 J/m^2), cultivated for 16 h to recover, then incubated for 1 h in medium containing 10% dialysed FCS and 10 μCi [5,6-^{3}H]-uridine (50 Ci/mmol; Amersham)/ml, and finally were processed for autoradiography as described above. The relative rate of RNA synthesis was expressed as the number of autoradiographic grains over the UV-exposed nuclei (G_{UV}) divided by the number of grains over the nuclei of nonirradiated cells on parallel slides (G_0) (RNA synthesis; G_{UV}/G_0).

Results

Summary of Clinical Symptoms of Patients

Patients NF and BT have been described in detail elsewhere (Jaeken et al. 1989). These unrelated patients are designated here as "XPCS1LV" and "XPCS2LV," respectively. The most important clinical symptoms are briefly recapitulated here.

XPCS1LV.—Psychomotor retardation and microcephaly of this girl were noted at the age of 9 mo. She attained a maximal level of development of 8–10 mo. Her skin was very sensitive to sunlight, and she had several small pigmented spots on face, trunk, and limbs. Nerve conduction velocity was normal. Salt-and-pepper retinal pigmentation and retinal artery narrowing were noted. She died from bronchopneumonia at age 6.5 years.

XPCS2LV.—This boy did not reach a level of psychomotor development above 2 mo and exhibited extreme microcephaly. Dysplastic ears and large extremities were apparent at age 6 wk. His sun-sensitive skin exhibited pigmented spots on trunk and limbs. Nerve conduction velocity was decreased, and visual and auditory-evoked potentials were absent. Salt-and-pepper retinal pigmentation but no intracranial calcification were observed. He died from a pulmonary infection at age 20 mo.

At the cellular level, both patients showed pronounced UV sensitivity and a reduced rate of overall thymidine-dimer removal, as measured in a radioimmunoassay.

Determination of UV-induced UDS

To further characterize the repair phenotype of these patients, the level of UV-induced UDS was determined and compared with the levels of UDS found in repair-competent and reference XP and CS cell strains. Table 1 shows that XPCS1LV and XPCS2LV cells exhibit a strongly reduced UDS (≤5% of repair-proficient cells; C5RO). This level is in the same range as is usually found for representatives of XP complementation groups A, B, and G and is significantly lower than the residual UV-induced UDS of XP groups C, D, E, and F (≥15% of repair-proficient cells), whereas the UDS in CS cells is in the wild-type range (CS1BE, CS3BE, CS-TK, and C5RO; table 1).

Microinjection with the Cloned XPBC/ERCC3 cDNA

The combination of clinical symptoms and the biochemical features of XPCS1LV and XPCS2LV resem-

Table 2

Microinjection of the *XPBC/ERCC3* cDNA

Injected Cells	DNA[a]	UV Irradiation	UDS (% of normal)
XPCS1BA	pSV3H	+	80–100
XPCS1BA	...	+	8
XPCS1BA	pSV3H	...	0
XPCS1LV	pSV3H	+	5
XPCS1LV	...	+	5
XPCS2LV	pSV3H	+	5
XPCS2LV	...	+	5

[a] Vector pSV3H contains the human *XPBC/ERCC3* gene cloned into the mammalian expression vector pSVL.

ble most closely those of the exceptional XP group B: a low residual UDS and combined XP/CS clinical symptoms. To investigate whether the XP-like CS patients indeed belong to this complementation group, nuclei were microinjected with cDNA from the recently cloned human excision-repair gene *XPBC/ERCC3*, which specifically corrects the repair defect of XP-B (Weeda et al. 1990). As shown in table 2, the introduction of this gene failed to enhance UV-UDS, in both cell strains, whereas, in a parallel experiment, fibroblasts of XP-B (XPCS1BA; W. Vermeulen, unpublished data) were corrected, by the *XPBC/ERCC3* cDNA, to the level seen in normal cells. The microinjection results indicate that patients XPCS1LV and XPCS2LV are not members of XP group B.

Complementation with XP-A and XP-G

In light of the low level of UDS, only two other candidate XP complementation groups exist: A and G. Therefore XPCS1LV and XPCS2LV fibroblasts were hybridized to representative cell strains of these groups. Both XP/CS cell strains were fully complemented by XP-A cells (XP25RO; table 3), as is evident from the approximately normal UDS in the heterokaryons. An XP-G representative (XP2BI; table 3) caused no such complementation. Consistent with this observation, XPCS1LV and XPCS2LV failed to complement each other. From these results we conclude that the two CS patients belong to the rare XP complementation group G.

Complementation with CS Cell Strains

To investigate the relationship between the two XP/CS patients and classical CS, additional complementation studies were performed with known CS patients' cells. In view of the normal UDS levels in CS cells, we utilized the feature of RNA synthesis recovery in these experiments, which is suitable for complementation analysis of CS (Lehmann 1982).

A significant degree of recovery occurred after fusion of XPCS1LV and XPCS2LV cells with CS3BE (CS-A), CS1BE (CS-B), and XPCS1BA (XP-B) (table 4). Consistent with the absence of UDS complementation observed between XPCS1LV and XPCS2LV cells, no complementation also was found between these lines with RNA synthesis recovery as a repair parameter. As regularly observed in correction of RNA synthesis recovery (Lehmann 1982), the RNA synthesis rates in complementing heterokaryons did not reach the level observed in normal control cells (i.e., C5RO). This phenomenon can be explained by several possible mechanisms (discussed by Troelstra et al. 1992), but which of these possibilities is correct has remained unresolved so far. The significant correction exerted by all CS strains tested, however, clearly excludes assignment of XPCS1LV and XPCS2LV to any of the known classical CS groups.

Discussion

Several clinical hallmarks exhibited by the two severely affected patients analyzed here categorize them as CS, as opposed to classical XP. These include the retinal abnormalities, the decreased physical and mental development, and the conduction abnormalities in some peripheral nerves. Furthermore, lateral ventricles were found to be slightly or moderately enlarged, although intracranial calcifications were not noted. It is possible that this trait, characterized as "not consistently observed in CS individuals" (Nance and Berry 1992, p. 78), would have become apparent at a more advanced age.

On the other hand, there are also some features reminiscent of XP. Small pigmented spots were observed on trunk, limbs, and (in XPCS1LV) face. It cannot be excluded that these cutaneous symptoms typical for XP would have become more prominent when the patients would have been older. Also, the absence of skin cancer may be due to their young age. In this respect it is worth noting that the occurrence of skin tumors is relatively rare in XP group G: in only one patient (of the seven) was a basal cell epithelioma reported, and this occurred at a rather late age (32 years).

Although the CS symptoms are more prominent than the XP features, the repair defect of both patients is typical of XP: UV-induced UDS is severely reduced (in

Table 3

XP Complementation-Group Analysis

Fused Cells[a]	Type of Binucleate Cell	Grains/Nucleus (Mean ± SEM)	UDS (% of normal response)	Complementation?
XPCS1LV × XP25RO(A)	XPCS1LV	2 ± 0	3	
	XP25RO	2 ± 0	3	
	Heterodikaryons	40 ± 2	63	Yes
XPCS1LV × XP2BI(G)	XPCS1LV	2 ± 0	3	
	XP2BI	2 ± 0	3	
	Heterodikaryons	2 ± 0	3	No
XPCS2LV × XP25RO(A)	XPCS2LV	3 ± 0	3	
	XP25RO	2 ± 0	2	
	Heterodikaryons	60 ± 3	68	Yes
XPCS2LV × XP2BI(G)	XPCS2LV	2 ± 0	2	
	XP2BI	2 ± 0	2	
	Heterodikaryons	3 ± 0	3	No
XPCS1LV × XPCS2LV	XPCS1LV	2 ± 0	2	
	XPCS2LV	2 ± 0	2	
	Heterodikaryons	3 ± 0	3	No
XP25RO(A) × XP2BI(G)	XP25RO	2 ± 0	2	
	XP2BI	2 ± 0	2	
	Heterodikaryons	64 ± 3	73	Yes

[a] Letters in parentheses are XP complementation group.

contrast to classical CS). The failure to recover RNA synthesis rates after UV exposure is a characteristic of CS and most XP cells. This property permitted us to perform complementation analysis using both parameters. Our analysis indicates an unequivocal assignment of the NER defect to XP group G and excludes assignment to XP group A and the known classical CS complementation groups. The inability of the microinjected

Table 4

CS Complementation-Group Analysis

Fused Cells[a]	Type of Binucleate Cell	Mean ± SEM RNA Synthesis, 16 h Post UVA: G_{UV} (10 J/m²) (grains/nucleus)	Mean ± SEM RNA Synthesis, 16 h Post UVA: G_0 (0 J/m²) (grains/nucleus)	G_{UV}/G_0	Complementation?
XPCS1LV × CS3BE(A)	XPCS1LV	7 ± 1	64 ± 2	.11	
	CS3BE	13 ± 1	68 ± 3	.19	
	Heterodikaryon	39 ± 2	65 ± 2	.60	Yes
XPCS1LV × CS1BE(B)	XPCS1LV	8 ± 1	80 ± 3	.10	
	CS1BE	15 ± 1	75 ± 3	.20	
	Heterodikaryon	42 ± 2	81 ± 3	.52	Yes
XPCS1LV × XPCS1BA[b]	XPCS1LV	11 ± 1	76 ± 2	.14	
	XPCS1BA	12 ± 1	76 ± 2	.16	
	Heterodikaryon	57 ± 2	77 ± 2	.74	Yes
XPCS1LV × XPCS2LV	XPCS1LV	5 ± 1	78 ± 2	.06	
	XPCS2LV	5 ± 1	72 ± 2	.07	
	Heterodikaryon	4 ± 1	72 ± 2	.06	No
Normal cells (CSRO)	Not fused	86 ± 3	84 ± 2	1.04	

[a] Letters in parentheses are CS complementation group.
[b] New patient of XP complementation group B (W. Vermeulen, unpublished data).

Table 5

Clinical and Biochemical Features of XP-G Patients

XP-G Patient	Reference[a]	Age at Diagnosis	UV[b] Sensitivity	UDS (% of normal)	Acute Sun Sensitivity?	Pigmentation Symptoms[c]	Skin Cancer?	Retarded Growth?	Neurological Abnormalities?	Micro-cephaly?	Ocular Abnormalities?
XP2BI	1,2	16 years	8 ×	≤5	Yes	++	No	Yes	Yes	Yes	No
XP3BR	3	6 years	8 ×	≤5	Yes	++	No	Yes	Yes	Yes	Unknown
XP31KO ...	4	37 years	5 ×	25	Yes	+	Yes[d]	No	No	No	No
XP124LO ..	5	14 years	?	15	Yes	+	No	No	No	No	No
XP125LO ..	5	12 years	?	15	Yes	+	No	No	No	No	No
XPCS1LV ..	6	9 mo	9 ×	≤5	Yes	±	No	Yes	Yes	Yes	Yes
XPCS2LV ..	6	6 wk	9 ×	≤5	Yes	±	No	Yes	Yes	Yes	Yes

[a] 1 = Cheesbrough and Kinmont (1978); 2 = Keijzer et al. (1979); 3 = Arlett et al. (1980); 4 = Ichihashi et al. (1985); 5 = Norris et al. (1987); and 6 = Jacken et al. (1989).

[b] Cellular sensitivity to UV-C irradiation of XP-G patients, compared with normal donor cells.

[c] ++ = Severe XP pigmentation; + = clear XP pigmentation but less severe; and + = mild and limited pigmentation.

[d] Basal cell epithelioma at age 37 years.

XPBC/ERCC3 gene to correct the UDS, in agreement with normal RNA synthesis recovery after hybridization to XP-B cells, ruled out XP complementation group B. The assignment of both strains to the same complementation group is consistent, since they were unable to complement each other's defect with regard to both UDS and recovery of UV-blocked transcription.

The assignment of XPCS1LV and XPCS2LV to XP group G further extends the clinical heterogeneity within this group, which is summarized in table 5. Until now the G group comprised five patients, none of whom has been reported to be associated with CS. Within XP group G the clinical symptoms vary from mild cutaneous and no neurological abnormalities (XP31KO, XP124LO, and XP125LO) to severe dermatological and neurological impairment (XP2BI and XP3BR). For most clinical manifestations, there is a reasonable correlation between the severity and the magnitude of the NER defect. The exception are the cutaneous abnormalities. The patient exhibiting the highest residual UDS (XP31KO; 25%) is the only XP-G patient who developed skin tumor, which appeared at a relatively late age.

Symptoms characteristic of CS have been reported so far in two XP cases. One is patient XP11BE, the only representative of XP group B (identical to CS group C), and the other is patient XPCS2 from XP group D. The latter group also shows extensive clinical heterogeneity (reviewed by Johnson and Squires 1992). Thus, XP groups B, D, and G resemble each other with regard to concurrence of XP and CS symptoms and (for D and G) the pronounced heterogeneity. The genes responsible for XP groups B and D, *XPBC/ERCC3* (Weeda et al. 1990) and *XPDC/ERCC2* (Fletjer et al. 1992; also see citation of Weber, in Lehmann et al. 1992), have recently been isolated and show striking parallels. Both are postulated to encode helicases, based on the basis of the presence, in the predicted amino acid sequence, of seven consecutive motifs that are associated with a superfamily of DNA- and RNA-unwinding enzymes. Furthermore, both human genes have strongly conserved yeast cognates with a distinct "as yet uncharacterized" vital function, in addition to their role in NER. It is likely that the human repair genes also participate in a process essential for viability. The rarity of XP-B and the striking heterogeneity of clinical symptoms of XP-D are certainly consistent with the hypothesis that the affected genes are vital and tolerate only a limited set of mutations disturbing the repair—but not the vital—function. Likewise, a possible explanation for the severity, heterogeneity, and rarity of XP-G could be an additional essential function for the *XPGC* gene, in analogy with XP-B and XP-D.

The findings reported here identify a third XP complementation group in which patients with CS presentations occur. In the case of XP-B and XP-D, the XP/CS patients clearly show a combination of the typical skin manifestations of XP and the characteristic neurological and other features of CS. The distinctive clinical features of the XP/CS patients in XP groups B and D have prompted Robbins et al. (1988) to define a distinct clinical entity, the "XP-CS complex." However, for the two XP-G individuals described here, the clinical traits of CS prevail, whereas those characteristic of XP are only marginally expressed. This demonstrates that the

heterogeneity, at least within group G, can range from exclusively XP to virtually only CS, and it argues against a narrowly defined "XP-CS complex" in this complementation group.

Furthermore, this observation may have important implications for a rigid distinction between XP and CS. In this respect it is relevant to note that recently three siblings were described with clinical symptoms associated with XP but with a repair defect typical of CS (Greenhaw et al. 1992). In this case, however, no (CS) complementation group assignment was performed. A clue to the resolution of the problem of heterogeneity may come from the molecular analysis of the genetic defect in clinically different patients of the same complementation group. Unfortunately, at present this is not possible for XP-G, as the *XPGC* gene has not yet been cloned. A relevant answer probably will come soon from mutation determination in the *XPDC/ERCC2* gene of various XP-D patients. Analysis of additional XP/CS patients will reveal whether specific mutations correlate with a specific phenotype and will help to link the clinical features with the molecular defects. Thus, for one XP/CS patient (XP11BE; XP group B), a single gene defect has been demonstrated (Weeda et al. 1990) to be responsible for the entire complex clinical phenotype.

Acknowledgments

We thank Irma van der Velde and Ard Joossen for expert assistance in the complementation experiments. Dr. Geert Weeda is acknowledged for providing the *XPBC/ERCC3* cDNA, and Mrs. Rita J. Boucke is acknowledged for typing the manuscript. The work reported here was supported by Dutch Cancer Society grant IKR88-2 and by Commission of European Communities project BJ6-141-NL.

References

Arlett CF, Harcout SA, Lehmann AR, Stevens S, Ferguson-Smith WA, Morley WN (1980) Studies of a new case of xeroderma pigmentosum (XP3BR) from complementation group G with cellular sensitivity to ionizing radiation. Carcinogenesis 1:745–751

Bohr VA (1991) Gene specific DNA repair. Carcinogenesis 12:1983–1992

Cheesbrough MJ, Kinmont PDS (1978) Xeroderma pigmentosum, a unique variant with neurologic involvement. Br J Dermatol 99, Suppl 16:61

Cleaver JE (1968) Defective repair replication of DNA in xeroderma pigmentosum. Nature 218:652–656

Cleaver JE, Kraemer KH (1989) Xeroderma pigmentosum. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) Metabolic basis of inherited disease. McGraw-Hill, New York, pp 2949–2971

Fletjer WL, McDaniel LD, Johns D, Friedberg EC, Schultz RA (1992) Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: involvement of the human ERCC2 DNA repair gene. Proc Natl Acad Sci USA 89:261–265

Friedberg EC (1985) DNA repair. WH Freeman, New York

Greenhaw GA, Herbert A, Duke-Woodside ME, Butler IJ, Hecht JT, Cleaver JE, Thomas GH, et al (1992) Xeroderma pigmentosum and Cockayne syndrome: overlapping clinical and biochemical phenotypes. Am J Hum Genet 50: 677–689

Hanawalt PC (1991) Heterogeneity of DNA repair at the gene level. Mutat Res 247:203–211

Huang J, Svoboda DL, Reardon JT, Sancar A (1992) Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the photodimer. Proc Natl Acad Sci USA 89:3664–3668

Ichihashi M, Fujiwara Y, Uehara Y, Matsumoto A (1985) A mild form of xeroderma pigmentosum assigned to complementation group G and its repair heterogeneity. J Invest Dermatol 85:284–287

Jacken J, Klocker H, Schweiger H, Bellmann R, Hirsch-Kauffmann M, Schweiger M (1989) Clinical and biochemical studies in three patients with severe early infantile Cockayne syndrome. Hum Genet 83:339–346

Jaspers NGJ, Bootsma D (1982) Genetic heterogeneity in ataxia telangiectasia as studied by cell fusion. Proc Natl Acad Sci USA 79:2641–2644

Johnson RT, Elliott GC, Squires S, Joysey VC (1989) Lack of complementation between xeroderma pigmentosum groups D and H. Hum Genet 81:203–210

Johnson RT, Squires S (1992) The XPD group: insights into xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy. Mutat Res 273:97–118

Keijzer W, Jaspers NGJ, Abrahams PJ, Taylor AMR, Arlett CF, Zelle B, Takebe H, et al (1979) A seventh complementation group in excision-deficient xeroderma pigmentosum. Mutat Res 62:183–190

Lafforet D, Dupuy JM (1978) Photosensibilité et réparation de l'ADN: possibilité d'une parente nosologique entre xeroderma pigmentosum et syndrome de Cockayne. Arch Fr Pediatr 35:65–74

Lehmann AR (1982) Three complementation groups in Cockayne syndrome. Mutat Res 106:347–356

Lehmann AR, Hoeijmakers JHJ, Van Zeeland AA, Backendorf CMP, Bridges BA, Collins A, Fuchs RPD, et al. (1992) Workshop on DNA repair. Mutat Res 273:1–28

Mayne LV, Lehmann AR (1982) Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from

individuals with Cockayne's syndrome and xeroderma pigmentosum. Cancer Res 42:1473–1478

Nance MA, Berry BA (1992) Cockayne syndrome review of 140 cases. Am J Med Genet 42:68–84

Norris PG, Hawk JLM, Avery JA, Giannelli F (1987) Xeroderma pigmentosum complementation group G—report of two cases. Br J Dermatol 116:861–866

Robbins JH (1988) Xeroderma pigmentosum, defective DNA repair causes skin cancer and degeneration. JAMA 260:384–388

Robbins JH, Branback RA, Mendiones M, Barrett SF, Carl JR, Cho S, Derckla MB, et al (1991) Neurological disease xeroderma pigmentosum. Brain 114:1335–1361

Robbins JH, Kraemer KH, Lutzner MA, Festoff BW, Coon HG (1974) Xeroderma pigmentosum: an inherited disease with sun sensitivity, multiple cutaneous neoplasms and abnormal DNA repair. Ann Intern Med 80:221–248

Tanaka K, Kawai K, Kumahara Y, Ikenaga M, Okada Y (1981) Genetic complementation groups in Cockayne's syndrome. Somat Cell Genet 7:445–455

Troelstra C, Van Gool A, De Wit J, Vermeulen W, Bootsma D, Hoeijmakers JHJ (1992) ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell 70:939–953

van Duin M, Vredeveldt G, Mayne LV, Odijk JHM, Vermeulen W, Klein B, Weeda G, et al (1989) The cloned human excision repair gene *ERCC-1* fails to correct xeroderma pigmentosum complementation group A through I. Mutat Res 217:83–92

Venema J, Mullenders LHF, Natarajan AT, van Zeeland AA, Mayne LV (1990*a*) The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. Proc Natl Acad Sci USA 87:4707–4711

Venema J, van Hoffen A, Natarajan AT, van Zeeland AA, Mullenders LHF (1990*b*) The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA. Nucleic Acids Res 18:443–448

Vermeulen W, Osseweijer P, de Jonge AJR, Hoeijmakers JHJ (1986) Transient correction of excision-repair defects in fibroblasts of 9 xeroderma pigmentosum complementation groups by microinjection of crude human cell extracts. Mutat Res 165:199–206

Vermeulen W, Stefanini M, Giliani S, Hoeijmakers JHJ, Bootsma D (1991) Xeroderma pigmentosum complementation group H falls into complementation group D. Mutat Res 255:201–208

Weeda G, van Ham RCA, Vermeulen W, Bootsma D, van der Eb AJ, Hoeijmakers JHJ (1990) A presumed DNA helicase, encoded by the excision repair gene *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. Cell 62:777–791

Appendix paper II

American J. of Human Genetics,
Vol. 54: 191-200, 1994

Clinical Heterogeneity within Xeroderma Pigmentosum Associated with Mutations in the DNA Repair and Transcription Gene *ERCC3*

W. Vermeulen,* R. J. Scott,‡ S. Rodgers,‡ H. J. Müller,‡ J. Cole,§ C. F. Arlett,§ W. J. Kleijer,† D. Bootsma,* J. H. J. Hoeijmakers,* and G. Weeda*

*MGC-Department of Cell Biology and Genetics and †Department of Clinical Genetics, Erasmus University, Rotterdam; ‡Human Genetics Group, Department of Research, University Hospital, Basel; and §MRC Cell Mutation Unit, University of Sussex, Brighton

Summary

The human DNA excision repair gene *ERCC3* specifically corrects the nucleotide excision repair (NER) defect of xeroderma pigmentosum (XP) complementation group B. In addition to its function in NER, the ERCC3 DNA helicase was recently identified as one of the components of the human BTF2/TFIIH transcription factor complex, which is required for initiation of transcription of class II genes. To date, a single patient (XP11BE) has been assigned to this XP group B (XP-B), with the remarkable conjunction of two autosomal recessive DNA repair deficiency disorders: XP and Cockayne syndrome (CS). The intriguing involvement of the ERCC3 protein in the vital process of transcription may provide an explanation for the rarity, severity, and wide spectrum of clinical features in this complementation group. Here we report the identification of two new XP-B patients: XPCS1BA and XPCS2BA (siblings), by microneedle injection of the cloned *ERCC3* repair gene as well as by cell hybridization. Molecular analysis of the *ERCC3* gene in both patients revealed a single base substitution causing a missense mutation in a region that is completely conserved in yeast, *Drosophila*, mouse, and human *ERCC3*. As in patient XP11BE, the expression of only one allele (paternal) is detected. The mutation causes a virtually complete inactivation of the NER function of the protein. Despite this severe NER defect, both patients display a late onset of neurologic impairment, mild cutaneous symptoms, and a striking absence of skin tumors even at an age of >40 years. Analysis of the frequency of *hprt*$^-$ mutant T-lymphocytes in blood samples suggests a relatively low in vivo mutation frequency in these patients. Factors in addition to NER deficiency may be required for the development of cutaneous tumors.

Introduction

Rare human hereditary syndromes with a predisposition toward the development of cancer—such as Bloom syndrome, Fanconi anemia, ataxia telangiectasia, and xeroderma pigmentosum (XP)—are believed to be based on a defective DNA repair mechanism or on an impaired cellular response to damage inflicted to DNA. XP is the prototype of these disorders, as it is the most intensively studied at the genetic, biochemical, and molecular level. Patients suffering from this autosomal recessive disease are extremely sensitive to the UV spectrum of solar irradiation (Cleaver and Kraemer 1989). This hallmark of the disease is reflected by an atrophic skin with (*a*) hyper- and hypopigmented spots on sun-exposed areas and (*b*) a >1,000-fold increased risk of developing skin cancer. Some patients also exhibit early and severe neurological degeneration. A striking heterogeneity is observed among XP individuals, varying from patients with only mild skin lesions to those with severe developmental disorders such as dwarfism, microcephaly, and neurologic (ataxia and mental retardation) abnormalities (Robbins et al. 1991). This heterogeneity is only partly explained by the involvement of multiple distinct genes as revealed by the identification of seven XP complementation groups

Received August 5, 1993; revision received September 30, 1993.

Address for correspondence and reprints: Dr. W. Vermeulen, Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000DR, Rotterdam, The Netherlands.

0002-9297/94/5402-0004$02.00

(XP-A through XP-G) (Vermeulen et al. 1991). The primary defect in most XP patients resides in a disturbed nucleotide excision repair (NER) pathway (Cleaver 1968) (for a recent review on NER, see Hoeijmakers 1993). A subpopulation of XP patients, designated "XP-variants," appears to have a defect in the poorly defined postreplication repair system (Lehmann et al. 1975). NER consists of two subpathways: (1) preferential repair of the transcribed strand of transcriptionally active genes and (2) the slower and less efficient overall genome repair (Bohr 1991; Hanawalt 1991). Information available to date indicates that both NER subpathways are impaired in the various forms of XP, with the notable exception of XP group C, in which only the overall genome repair system appears to be affected (Venema et al. 1990*b*). Defective NER can be monitored biochemically by measuring the UV-induced DNA repair synthesis referred to as "unscheduled DNA synthesis" (UDS). Absence or reduced levels of UDS are found in XP groups A–G. Recently, it was demonstrated that another human genetic syndrome associated with UV sensitivity, Cockayne syndrome (CS), is also based on a defect in NER. Here only the preferential repair of active genes is disturbed, whereas the overall repair (responsible for the bulk of UDS) is normal (Venema et al. 1990*a*). Consequently, UDS levels in CS fall within the normal range. In addition to a UV-sensitive skin (without pigmentation abnormalities), neurological dysfunction due to demyelination of neurons and calcification of basal ganglia is obligate for the diagnosis of CS. General developmental impairment is often also seen (Nance and Berry 1992). Although CS is based on a partially deficient NER, no significant increased frequency of skin cancer is observed. Clinical and genetic heterogeneity is apparent in CS (Lehmann 1982). A class of extremely rare individuals with a conjunction of features of both syndromes ("XP-CS" complex) has been recognized. Three XP complementation groups are implicated (XP-B, XP-D, and XP-G). The genes responsible for the defect in XP-B, XP-D, and XP-G have recently been cloned; they are, respectively *ERCC3* (*XPBC*), *ERCC2* (*XPDC*), and *ERCC5* (*XPGC*) (Weeda et al. 1990; Flejter et al. 1992; Lehmann et al. 1992; O'Donovan and Wood 1993; Scherly et al. 1993). The *ERCC3* gene product was identified as one of the components of the human BTF2/TFIIH transcription factor, which is required for initiation of transcription of class II genes (Schaeffer et al. 1993).

Until now XP-B has consisted of a single patient (XP11BE), who displayed severe features of XP as well as CS and who developed numerous skin tumors at an early age. Mutational analysis of the *ERCC3* gene of this patient revealed that only one allele was detectably expressed and that it contained a splice-acceptor mutation in the last exon, causing a C-terminal frameshift at the protein level (Weeda et al. 1990). The few remaining fibroblasts of this patient grow poorly. Recently we received fibroblasts from two brothers, designated "XPCS1BA" and "XPCS2BA," who have combined XP and CS symptoms (Scott et al., in press). In the present paper we report both the assignment of these two patients to XP-B and the identification, at the molecular level, of the mutation in *ERCC3* in these patients.

Material and Methods

Cell Strains, Culture Conditions, and Cell Hybridization

Human fibroblast cultures C5RO (control), XP25RO (XP-A), XP11BE (XP-B), XPCS2 (XP-D), XPCS1LV (XP-G), XPCS1BA, and XPCS2BA (assigned to XP-B; present paper) were grown in Ham's F10 medium supplemented with 11% FCS and antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml). Lymphoblastoid cells were grown in RPMI medium supplemented with 10% FCS and antibiotics.

Three days prior to cell hybridization, each fusion partner was labeled with latex beads of different sizes by adding a suspension of beads to normal culture medium (Vermeulen et al. 1991). After trypsinization, 0.5 $\times$ 10^6 cells of each were mixed in a 1:1 ratio, washed extensively to remove free beads, and fused with ß-propiolactone-inactivated Sendai virus. Fused cells were seeded onto coverslips, incubated for 2 d under normal culture conditions, and assayed for UV-induced UDS, which was measured by autoradiography as described below. Homopolykaryons, to be used for the microinjection experiments, were generated as described above, using only one cell strain and omitting the addition of latex beads.

Microneedle Injection

Plasmid cDNA (100 μg/ml) in PBS was injected into one of the nuclei of XP homopolykaryons by using a glass microcapillary, as described elsewhere (Van Duin et al. 1989). For each experiment at least 50 homopolykaryons were injected. Coverslips with injected cells were cultured for 24 h to allow expression of the injected cDNA before they were assayed for their repair capacity by means of UV-induced UDS.

UV-induced UDS

The UDS assay (Vermeulen et al. 1986) was performed 2 d after fusion and 1 d after injection. In brief,

cells grown on coverslips were washed with PBS, irradiated with 15 J/m^2 UV-C (Philips TUV lamp), immediately incubated for 2 h in culture medium containing 10 μCi (*methyl*-^{3}H) thymidine (50 Ci/mmol; Amersham)/ml, washed with PBS, and fixed. Coverslips with radioactively labeled cells were mounted onto slides and dipped in a photosensitive emulsion (Ilford K2). After exposure (2–7 d) slides were developed and stained. Repair capacity was quantitated by counting autoradiographic grains above the nuclei of ≥50 cells. UDS levels are expressed as the percentage of UDS of normal cells on an identically treated coverslip culture in the same experiment.

In Vivo Mutant Frequency at the hprt *Locus*

Mutant frequency estimations were undertaken by published procedures (Cole et al. 1988, 1992). In brief, for each determination, a mass culture of mononuclear cells (≈10^7 cells) was incubated overnight in RPMI 1640 medium supplemented with 10% human AB serum, before being cloned in 96-well microtiter trays in the presence of mitogen (phytohemagglutinin), lethally irradiated lymphoblastoid feeder cells, and the T-cell growth factor interleukin-2 (IL-2). For *hprt* mutant selection, the cells were plated at high density (1 × 10^4/well) in the presence of the purine analogue 6-thioguanine (6TG), in which nonmutants are unable to grow. To determine cloning efficiency in the absence of selection, a low cell density (3 cells/well) was used. The plates were scored for negative (no colony) wells after 15–20 d incubation, and the mutant frequency was calculated from the zero term of the Poisson distribution. Under the conditions used in our experiments, mononuclear cell cloning efficiencies of ≈50% in the absence of selection are routinely obtained; thus a high percentage of the T-lymphocytes in the sample are capable of colony formation in vitro. Therefore the mutant frequency should reflect the in vivo situation.

RNA Isolation, DNA Amplification, and Mutation Detection

RNA was isolated by the LiCl/urea method (Auffray and Rougeon 1980). The RNA was used for preparing cDNA with *ERCC3*-specific primers (as described by Weeda et al. 1990). Amplification was performed by 32 cycles of, consecutively, 2 min denaturing at 95°C, 2 min annealing (the temperature was dependent on the primers used), and 3 min extension at 70°C (Saiki et al. 1985). Amplified DNA was spotted onto Hybond filters (Amersham) and was UV-cross-linked and hybridized to wild-type and mutant ^{32}P-labeled primers. The amplified DNA was purified and digested with the appropriate restriction enzyme and subsequently was cloned into a M13mp18 vector for sequence analysis by the dideoxy-chain termination method (Sanger et al. 1977) using T7 DNA polymerase (Pharmacia). Oligonucleotide primers for cDNA, DNA amplification, and DNA sequencing were synthesized in an Applied Biosystem DNA synthesizer. The primers for mutation detection in cells of XP patients XPCS1BA and XPCS2BA were p68, 5′-CCCAAGACTTCTTGGTGG-3′; p69, 5′-CCCAAGACTCCTTGGTGG-3′.

cDNA Constructs

The wild-type *ERCC3* cDNA expression plasmid (pSVH3) and the mutant containing the XP11BE mutation (pSVH3M), used for microinjection into XP-B fibroblasts, have been described elsewhere (Weeda et al. 1990). The *ERCC3* gene harboring the mutation present in the two brothers was constructed as follows: the 5′ part of PSVH3 was replaced by a 0.7-kb *Eco*RI/*Sac*I DNA fragment that was synthesized by means of PCR-amplified *ERCC3* cDNA derived from mRNA isolated from cells of patient XPCS1BA, yielding pSVH3M2.

Results

UV-induced UDS

A detailed description of the clinical features of XP patients XPCS1BA and XPCS2BA (two brothers of unrelated parents) has been presented elsewhere (Scott et al., in press). The clinical pattern is most consistent with the "XP-CS complex" phenotype seen in a very limited number of patients with NER deficiency. The features of these patients, together with those of four other XP-CS complex patients, are summarized in table 1. The repair capacity of the fibroblasts from the two individuals (XPCS1BA and XPCS2BA), compared with the level found in normal repair-competent fibroblasts, is severely reduced, in contrast to classical CS, which exhibit normal levels of UDS. This is consistent with the high sensitivity of their fibroblasts to the cytotoxic effects of UV-C, as demonstrated by a colony-survival assay (Scott et al., in press) and our own unpublished observations. The level of residual UDS is in the same range as is usually found in XP-A, -B, and -G (i.e., <10% of normal) and is well below the 30%–50% residual UDS displayed by XPCS2 (XP-D). These findings make XP-B and -G likely candidates in the relatively mildly affected individuals.

Microneedle Injection of ERCC3 *cDNA*

Microneedle injection of the *ERCC3* cDNA specifically corrects the NER defect in XP-B cells, but not in

Table 1

Features of XP-CS-Complex Patients

Feature	CS[a]	XP[b]	XPCS2[c]	XPCS1/2LV[d]	XP11BE[e]	XPCS1/2BA[f]
Cutaneous:						
Photodermatitis	+	++	++	++	++	++
Pigmentation abnormalities	−	++	++	−	++	+
Skin cancer	−	>1,000 times	+(2 years)	−	+(18 years)	−(>40 years)
Neurologic:						
Primary defect	Demyelination	Degeneration	Demyelination	Unknown	Demyelination	Demyelination
Ganglia calcification	+	−	Unknown	+	+	−
Psychomotor retardation	+	−(+)[g]	+	+	+	+/−[h]
Hearing impairment	+	−(+)[g]	+	+	+	+/−[h]
Reflexes	Hyper	Hypo	Hyper	Unknown	Unknown	Hyper
Retinal pigmentation	+	−	+	+	+	+
Developmental:						
Dysmorphic dwarfism	+	−(+)[g]	+	+	+	−[i]
Immature sexual development	+	−(+)[g]	+	+	+	+
Microcephaly	+	−(+)[g]	+	+	+	−
Biochemical/cellular:						
UDS (% of control)	100	1–50	30–50	<5	5–10	5–10
UV sensitivity (in situ)[j]	Moderate	Severe/moderate	Unknown	Severe	Severe	Severe

NOTE.—Status of features is as follows: + = presence of symptom; ++ = severe phenotype; and − = absence of feature.

[a] Nance and Berry (1992).

[b] Robbins et al. (1991).

[c] XP-D (Vermeulen et al. 1991).

[d] XP-G (Vermeulen et al. 1993).

[e] XP-B (Robbins et al. 1974).

[f] XP-B (present study).

[g] Absent in classical XP; present in XP with neuropathology.

[h] Present, but relatively late onset.

[i] Weight and height not as low as in CS, but at the 3d percentile.

[j] Colony survival after UV irradiation, compared with control cells.

XP cells of other complementation groups (Weeda et al. 1990). To examine whether the *ERCC3* cDNA was able to correct the repair defect in cells of patients XPCS1BA and XPCS2BA, the cDNA in a mammalian expression vector was introduced into the nuclei of homopolykaryons of both patients. The results, shown in figure 1 and quantified in table 2, demonstrate that injected XPCS1BA and XPCS2BA fibroblasts were able to achieve normal UDS levels. The nuclear labeling was UV dependent, and no correction was obtained using an inactive *ERCC3* gene containing the XP11BE mutation. These data clearly demonstrate that the repair defect is corrected by the *ERCC3* gene and that both patients belong to XP-B.

Complementation by Cell Hybridization

The complementation assignment via the microneedle injection was verified by using the classical cell hybridization technique. When XPCS1BA cells were fused to XP-A fibroblasts (fig. 2*A* and table 3), clear complementation was observed up to the normal level of UDS, found in NER-proficient cells. In contrast, no complementation occurred after fusion with either XP-B cells (fig. 2*B* and table 3) or cells from the sib XPCS2BA. These data confirm the assignment of both patients to XP-B, by microneedle injection.

Determination of the ERCC3 *Mutation*

Analysis by Southern and northern blot hybridization made it clear that, in the cells of both patients, the *ERCC3* gene is not detectably rearranged and is normally expressed; the RNA has the expected size of 2.8 kb (data not shown). This suggests that the presence of a gross alteration in the gene is rather unlikely. Sequence analysis on PCR-amplified mRNA of patient XPCS1BA revealed a single base substitution (T→C transversion; see fig. 3) in the 5′ part of the cDNA, resulting, at the protein level, in a phenylalanine (F)–to–

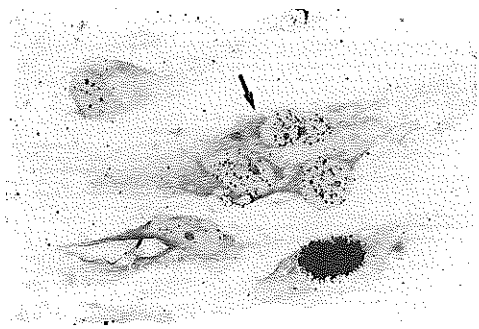


Figure 1 Effect of microinjection of *ERCC3* cDNA on UV-induced UDS in XPCS1BA fibroblasts. Shown is a micrograph of a XPCS1BA homopolykaryon (*arrow*) microinjected with wild-type *ERCC3* cDNA in one of the nuclei and subjected to the UDS procedure. The injected cell has a considerably larger number of grains above its nuclei than do the noninjected, surrounding mononuclear cells. In fact, the number of nuclear grains above the injected cell reaches that of repair-proficient fibroblasts assayed in parallel. The cell containing the heavily labeled nucleus was in S-phase during ^{3}H-TdR incubation.

serine (S) amino acid substitution at position 99. No other changes were observed in the remainder of the *ERCC3* cDNA. To determine whether this was the inherited mutation, the relevant part of the *ERCC3* cDNA of the two patients and their parents and of HeLa cDNA was amplified and subjected to dot blot analysis. The filters were hybridized with ^{32}P-labeled wild-type-specific (p68) and mutant-specific (p69) oligonucleotide probes. HeLa control DNA (fig. 4) hybridized only to the wild-type *ERCC3* oligonucleotide probe, whereas the cDNA of both patients hybridized only with the mutant-specific probe. PCR-amplified cDNA from the father, however, exhibited clear hybridization with both probes. This indicates that the father is heterozygous for the mutation. Since only the paternal allele of *ERCC3* can be detected, the maternal allele of the patient must contain another small mutation causing complete either absence or a greatly reduced level of *ERCC3* transcript.

A cDNA construct carrying the XPCS1BA mutation (PSVH3M2) was microinjected into XPCS2BA fibroblasts. As demonstrated in table 2, no significant increase of UDS is observed with this mutant cDNA. These experiments indicate that the XPCS1BA mutation completely inactivates the ERCC3 repair function, as was previously also shown for the XP11BE mutation (table 2).

In Vivo Mutant Frequency at the hprt$^-$ *Locus*

The frequency of 6-thioguanine-resistant T-lymphocytes in blood samples from the two patients was compared with similar information from our database on normal control subjects and other excision-defective patients, over the relevant age range. We have pub-

Table 2

***ERCC3* cDNA Microinjections into XPCS1BA and 2BA Fibroblasts**

Cell Strain	cDNA Injected	UV Irradiation (15 J/m^2)	UDS (mean ± SEM)	
			Grains per Nucleus	% of Control Value
XPCS1BA	pSV3H[a]	+	120 ± 8	80 ± 6
XPCS1BA	...[b]	+	10 ± 2	6 ± 1
XPCS2BA	pSV3H[a]	+	138 ± 10	93 ± 7
XPCS2BA	...[b]	+	12 ± 2	7 ± 1
XPCS2BA	pSV3H[a]	−	2 ± 1	1 ± 1
XPCS2BA	pSV3HM[c]	+	13 ± 2	7 ± 1
XPCS2BA	pSV3HM2[d]	+	10 ± 2	6 ± 1
CSRO	...	+	150 ± 11	100 ± 8

[a] *ERCC3* cDNA (wild-type).
[b] Uninjected polykaryon.
[c] *ERCC3* cDNA containing the XP11BE mutation.
[d] *ERCC3* cDNA containing the XPCS1BA mutation.

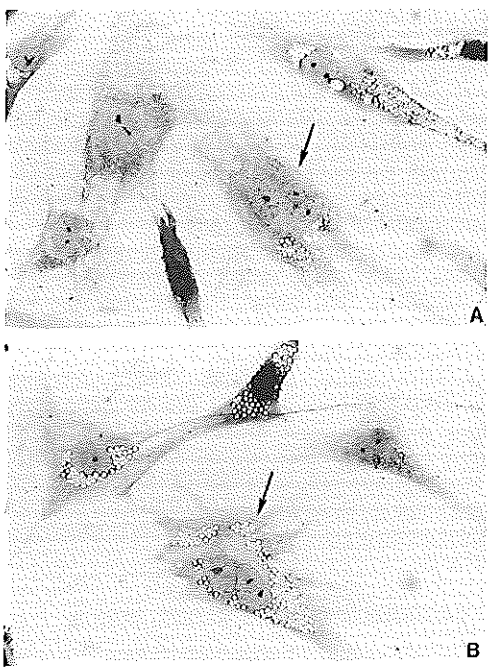


Figure 2 Cell hybridization of XPCS1BA with XP-A and XP-B fibroblasts. *A*, Monokaryons (expressing no detectable UDS): XP25RO, containing small (0.8 μm) latex beads, and XPCS1BA, containing large (2.0 μm) latex beads. The heterodikaryon (*arrow*) generated by Sendai virus–stimulated fusion of XP25RO(A) and XPCS1BA fibroblasts, identified by the presence of two types of beads, reaches a UDS level of NER-competent fibroblasts, which are visualized as autoradiographic grains above the nuclei of the hybrid cell. *B*, Fusion between XP11BE (0.8-μm beads) and XPCS1BA (2.0-μm beads). No increase in UV-induced UDS is observed above the nuclei of the heterodikaryon (*arrow*), compared with the monokaryons.

lished (Cole et al. 1992) additional results on older XP patients, consisting of three XP variants and an unusual XP-C excision-defective patient 65 years of age.

As shown in figure 5, the excision-defective XP patients as a group have an elevated mutant frequency compared with normal, nonsmoking subjects. Statistical analysis of these data has confirmed the conclusion of Cole et al. (1992) that the increase in *hprt* mutants in XPs is highly significant. However, the mutant frequencies of both XPCS1BA and XPCS2BA are at the top of the range for normal donors. When there is matching for age, a formal statistical analysis that tests for the inclusion/exclusion of the two patients' mutant frequency in the normal or XP populations shows that they are not significantly different from normal donors, nor can they be discriminated from the population of Xps (we are indebted to Dr. M. H. L. Green of the MRC Cell Mutation Unit for undertaking this analysis).

Table 3

Complementation Analysis by Somatic Cell Hybridization

Fused Cells (complement group) and Type of Dikaryon	UDS[a] (mean ± SEM)	
	Grain per Nucleus	% of Control Value
XP25RO (A) × XPCS1BA:		
XPCS1BA	3 ± 1	7 ± 1
XP25RO	1 ± 1	2 ± 1
Heterodikaryon	37 ± 2	90 ± 5
XP11BE (B) × XPCS1BA:		
XPCS1BA	3 ± 1	7 ± 1
XP11BE	3 ± 1	7 ± 1
Heterodikaryon	3 ± 1	7 ± 1
XPCS1BA × XPCS2BA:		
XPCS1BA	3 ± 1	7 ± 1
XPCS2BA	3 ± 1	7 ± 1
Heterodikaryon	3 ± 1	7 ± 1
C5RO (control)	41 ± 3	100 ± 7

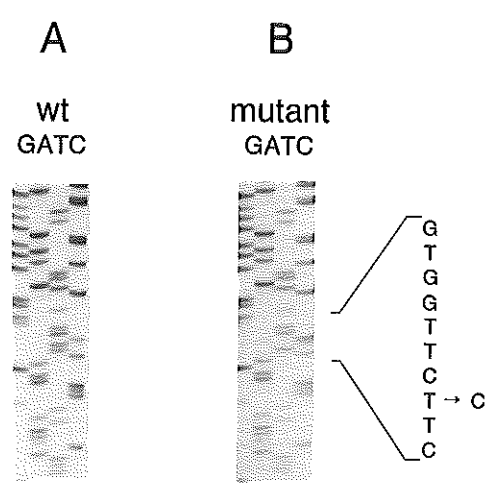


Figure 3 Nucleotide sequence analysis of the XPCS1BA mutation. Nucleotide sequence of a part of the *ERCC3* cDNA of a wild-type allele (wt), from HeLa (*A*) and from patient XPCS1BA (mutant) (*B*).

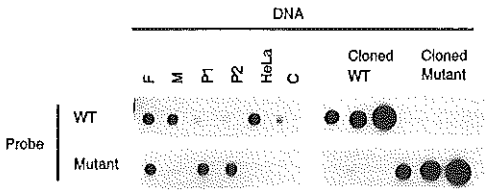


Figure 4 Dot blot analysis of the *ERCC3* in the XPCSBA family. Amplified DNA from HeLa, XPCS1BA (P1), XPCS2BA (P2), XPCSH5BA (F [father]), and XPCSH4BA (M [mother]) (in lane C, no cDNA was added) was spotted onto a nylon filter and hybridized with a labeled wild-type (Cloned WT) probe and with a mutant oligonucleotide (Cloned Mutant) probe (see experimental procedures). As a control for the specificity of hybridization of the mutant and wild-type *ERCC3*-specific oligonucleotide, plasmid DNA from clones PSVH3 (i.e., wild-type) and PSVH3M2 (i.e., XPCS1BA and 2BA mutant) was spotted in serial dilutions.

Two other XP patients, XP125LO and XP7NE, also clearly fall within the normal range. We have shown elsewhere (Cole et al. 1988) that smoking can increase the frequency of mutants recovered from normal donors. Among the population of XP patients discussed here, only one, XP125LO from XP-G (who is indicated by a distinctive symbol in fig. 5), had any history of smoking. We note that he only commenced this habit during the period in which we began our study of this mutant frequency.

Discussion

Cultured fibroblasts of two brothers with relatively mild clinical symptoms of XP and CS and with a virtually complete deficiency of NER were assigned to the extremely rare XP-B. The assignment was performed in two ways: by microinjection of the cloned *ERCC3* repair gene and by the classical somatic cell hybridization technique. Microinjection of NER genes has several advantages compared with the cell-fusion method: (1) It is a direct assay that scores for a positive result (i.e., induction of UDS). Assignment by cell fusion, on the other hand, is based on the absence of complementation (i.e., no correction of UDS). (2) Multiple copies of the gene can be injected into a nucleus, circumventing problems due to low levels of expression. (3) Only a limited number of cells are required, which may be important when only poorly growing cells are available. (4) Microinjection of a cloned NER gene excludes the possibility of intragenic complementation, which may complicate cell hybridization results. (5) Finally, microneedle injection permits assessment of NER genes for which no corresponding human complementation group has been identified thus far. This holds for genes for which only rodent mutants are known, such as the *ERCC1* gene (Van Duin et al. 1989), and for which complementation analysis by cell hybridization is complicated by the occurrence of poor interspecies cell fusion and instability of the resulting hybrids (Thompson et al. 1985). In addition, candidate repair genes for which no mammalian mutants are known can be tested using direct nuclear injection. This may be relevant for mammalian genes that have been isolated on the basis of sequence homology with cloned DNA repair genes of lower species, e.g., *Saccharomyces cerevisiae* or *S. pombe* (Koken et al. 1991). These considerations make the microinjection technique a useful additional method for complementation analysis, particularly in view of the fact that the number of cloned mammalian NER genes is rapidly increasing. At present, at least seven NER genes have been isolated: *ERCC1* (Westerveld et al. 1984); five genes involved in XP—namely, *XPAC* (Tanaka et al. 1989), *ERCC3* (*XPBC*) (Weeda et al. 1990), *XPCC* (Legerski and Peterson 1992), *ERCC2* (*XPDC*) (Weber cited in Lehmann et al. 1992; Flejter et al. 1992), and *ERCC5* (*XPGC*) (O'Donovan and Wood 1993; Scherly et al. 1993)—and, finally, one gene, *ERCC6* (*CSBC*), mutated in CS complementation group B (Troelstra et al. 1992).

The results presented here define the causative mutation in the *ERCC3* gene, responsible for the phenotype

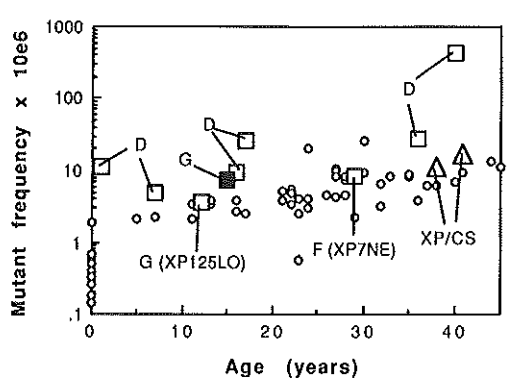


Figure 5 *hprt* mutant frequency in circulating T-lymphocytes in normal, nonsmoking subjects and in XP patients. Frequency of *hprt* mutants is set against the age of the donors, who are normal, nonsmoking donors (○), an XP nonsmoker (□), an XP smoker (■), and XPCS1BA and XPCS2BA (△).

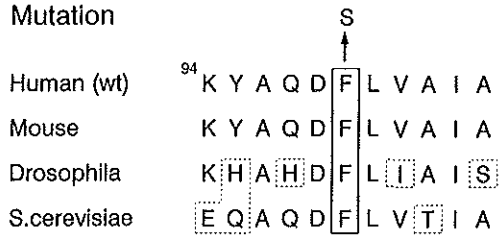


Figure 6 Amino acid sequence conservation of the mutated region in *ERCC3*. XPCS1BA and 2BA mutations in a conserved region of the ERCC3 protein are shown. There is homology between the amino acid sequences of *ERCC3*, around position 99 of human, mouse, *Drosophila*, and *Saccharomyces cerevisiae*. Homologous residues are boxed with a dashed line, and the conserved phenylalanine is boxed with a solid line. The amino acid substitution into a serine is indicated by an arrow.

of the XPCS1BA and XPCS2BA patients. A single-base-pair substitution resulting in an amino acid change of a phenylalanine to a serine at position 99 is found in the only allele that is detectably expressed in these patients. This phenylalanine resides in a region to which, as yet, no specific functional domain has been assigned. However, as shown in figure 6, this residue has not changed in the long evolutionary distance that separates yeast from man, and it is part of a protein segment that is very strongly conserved during eukaryotic evolution. This indicates that this portion of the protein has an important function that may not tolerate much change.

A comparison of the original XP-B case (XP11BE) with the two patients described here reveals a remarkable clinical heterogeneity. DNA repair studies with fibroblasts indicate the same low level (<10% of the normal level) of UDS, the absence of cyclobutane pyrimidine dimer removal, and a low UV-C survival in colony-forming assays, in all three patients (Scott et al., in press). However, despite a common severe deficiency in NER as measured in fibroblasts, the two newly identified XP-B cases are much less severely affected (Scott et al., in press) than is the original patient (Robbins et al. 1974). The original XP-B patient presented numerous cutaneous malignancies before she was 18 years old, while the two new patients were free of skin cancer after they were 40 years old, despite the fact that apparently they took few precautions to protect themselves from sunlight (Scott et al., in press).

The absence of skin cancers in these patients suggests that processes in addition to defects in NER may contribute to carcinogenesis. NER is considered to be error free in human cells (Bridges and Brown 1992), and thus any increase in skin cancer may reflect the efficiency of, or the overloading of, error-prone repair processes such as daughter-strand repair. Differences in immunosurveillance are also believed to be involved in the process of tumorigenesis. However, although there have been a number of reports of immune dysfunction in XP patients (Cole et al. 1992), including deficiencies of natural killer-cell activity in various XP, CS, and TTD patients (Lehmann and Bridges 1990; Mariani et al. 1992), no consistent pattern providing an adequate explanation for the discrepancies in tumor predisposition has emerged from these studies.

Since there is ample evidence indicating that mutation is an important early initiation step in carcinogenesis, it was of interest to see whether the new XP patients have a significantly increased frequency of *hprt* mutations in circulating T-lymphocytes. We have previously shown that XP patients with residual repair synthesis measured in fibroblasts that ranges from 15% to approximately that in wild-type cells have, as a group, a highly significantly increased frequency of this mutation in T-cells (Cole et al. 1992). Since there is no evidence that cells from XP donors have an elevated *spontaneous* mutant frequency, whereas they are clearly hypermutable by UV, it was postulated that the raised in vivo frequency is also induced, possibly by exposure of the lymphocytes to sunlight as they pass through the skin (see Cole et al. 1992, and references therein). Figure 5 shows that the *hprt* mutant frequency in the two new XP/CS cases is clearly not greatly elevated compared with that in normal subjects, despite both the almost total deficiency in NER and the clear hypersensitivity of their lymphocytes to the lethal effects of both UV-C and UV-B (J. Cole and C. F. Arlett, unpublished data). All XP patients whom we had studied previously had, with two exceptions (XP125LO [XP-G] and XP7NE [XP-F]) been shown to have an elevated in vivo *hprt* mutant frequency in T-cells (Cole et al. 1992). XP7NE has no cellular hypersensitivity to the lethal effects of UV-C, in either T-cells or fibroblasts (Arlett et al. 1992). In addition, we have recently shown that in T-lymphocytes there is no hypersensitivity to UV-B, for either XP7NE or XP125LO (J. Cole and C. F. Arlett, unpublished data). These individuals are free of skin cancer. It is plausible that their normal mutant frequency reflects the absence of target-cell hypersensitivity to wavelength of UV irradiation relevant to the in vivo situation. However, T-lymphocytes from the XP/CS patients described here *are* hypersensitive to both UV-C and UV-B (data not shown); thus their normal

mutant frequency and lack of skin cancer set them apart from the other XP patients with elevated mutant frequencies. Possible explanations for the absence of skin cancer in the two new XP/CS patients include (1) an increased efficiency of alternative repair pathways (such as recombination repair), which may compensate for the reduced NER; (2) modified immune surveillance; or (3) the nature of the mutation in the *ERCC3* gene. It is worth noting that the *ERCC3* gene has additional functions in the cell, beyond its role in excision repair. The work of Gulyas and Donahue (1992), Mounkes et al. (1992), and Park et al. (1992) indicates an additional essential function of the *ERCC3* gene in yeast and *Drosophila*. Schaeffer et al. (1993) identified the *ERCC3* gene product as one of the components of the human transcription factor BTF2/TFIIH required for a late step in the initiation of transcription of class II genes. These findings shed new light onto the clinical features of XP and XP/CS, which were difficult to reconcile solely on the basis of deficient NER (Bootsma and Hoeijmakers 1993). Partial dysfunction of *ERCC3* may have a subtle effect on gene expression, resulting in retarded growth, immature sexual development, and demyelination of neurons. A part of the symptomatology of these patients and, probably, of CS in general might be due to a control function, of the involved genes, in transcription. The dual involvement of *ERCC3* could also provide an explanation for the rarity and pronounced clinical heterogeneity of XP-B, perhaps including the predisposition to cancer.

Acknowledgments

We thank Mr. Ard Joosse for his assistance in the cell hybridization, Mrs. Ingrid Donker and Marianne Hoogeveen for technical assistance in mutational analysis, and Dr. M. Green for help with the statistical analysis of the mutant frequency data. Dr. N. G. J. Jaspers, Dr. L. Ma, and Prof. A. J. van der Eb are acknowledged for their helpful discussions. We are grateful to Tom de Vries Lentsch, Mirko Kuit, and Ruud Koppenol for photographic material. This work was supported by Dutch Cancer Society grants EUR92-2 and 90-20, by EC grants EEG-F13P-CT92-0007 and EV5V-CT91-0034, and by Swiss National Fund grant 32-30007.90.

References

Arlett CF, Hartcourt SA, Cole J, Green MHL, Anstey AV (1992) A comparison of the response of unstimulated and stimulated T-lymphocytes and fibroblasts from normal, xeroderma pigmentosum and trichothiodystrophy donors to the lethal action of UV-C. Mutat Res 273:127–135

Auffray JE, Rougeon F (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor DNA. Eur J Biochem 107:303–314

Bohr VA (1991) Gene specific DNA repair. Carcinogenesis 12:1983–1992

Bootsma D, Hoeijmakers JHJ (1993) DNA repair, engagement with transcription. Nature 363:114–115

Bridges BA, Brown GM (1992) Mutagenic DNA repair in *Escherichia coli XXI*: a stable SOS-inducing signal persisting after excision repair of ultraviolet damage. Mutat Res 270:135–144

Cleaver JE (1968) Defective repair replication in xeroderma pigmentosum. Nature 218:652–656

Cleaver JE, Kraemer KH (1989) Xeroderma pigmentosum. In: Scriver CR, Beaudet Al, Sly Ws, Valle D (eds) The metabolic basis of inherited disease. McGraw-Hill, New York, pp 2949–2971

Cole J, Arlett CF, Norris PG, Stephens G, Waugh APW, Beare DM, Green MHL (1992) Elevated *hprt* mutant frequency in circulating T-lymphocytes of xeroderma pigmentosum patients. Mutat Res 273:171–178

Cole J, Green MHL, James SE, Henderson L, Cole H (1988) Human population monitoring: a further assessment of factors influencing measurements of thioguanine-resistant mutant frequency in circulating T-lymphocytes. Mutat Res 204:493–507

Flejter WL, McDaniel LD, Johns D, Friedberg EC, Schultz RA (1992) Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: involvement of the human *ERCC2* DNA repair gene. Proc Natl Acad Sci USA 89:261–265

Gulyas KD, Donahue TF (1992) *SSL2*, a suppressor of a stem-loop mutation in the *HIS4* leader encodes the yeast homologue of human *ERCC3*. Cell 69:1031–1042

Hanawalt PC (1991) Heterogeneity of DNA repair at the gene level. Mutat Res 247:203–211

Hoeijmakers JHJ (1993) Nucleotide excision repair II: from yeast to mammals. Trends Genet 9:211–217

Koken MH, Reynolds P, Jaspers-Dekker I, Prakash L, Prakash S, Bootsma D, Hoeijmakers JHJ (1991) Structural and functional conservation of two human homologs of the yeast DNA repair gene *RAD6*. Proc Natl Acad Sci USA 88:8865–8869

Legerski R, Peterson C (1992) Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. Nature 359:70–73

Lehmann AR (1982) Three complementation groups in Cockayne syndrome. Mutat Res 106:347–356

Lehmann AR, Bridges BA (1990) Sunlight-induced cancer: some new aspects and implications of the xeroderma pigmentosum model. Br J Dermatol 122, Suppl 35:115–119

Lehmann AR, Hoeijmakers JHJ, van Zeeland AA, Backendorf CMP, Bridges BA, Collins A, Fuchs RPD, et al (1992) Workshop on DNA repair. Mutat Res 273:1–28

Lehmann AR, Kirk-Bell S, Arlett CF, Paterson MC, Lohman

PHM, de Weerd-Kastelein EA, Bootsma D (1975) Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. Proc Natl Acad Sci USA 72:219–223

Mariani E, Facchini A, Honorati MC, Lalli E, Berardesca E, Ghetti P, Marinoni S, et al (1992) Immune defects in families and patients with xeroderma pigmentosum and trichothiodystrophy. Clin Exp Immunol 88:376–382

Morison WL, Bucana C, Hashem N, Kripke ML, Cleaver JE, German JL (1985) Impaired immune function in patients with xeroderma pigmentosum. Cancer Res 45:3929–3931

Mounkes LC, Jones RS, Liang BC, Gelbart W, Fuller MT (1992) A *Drosophila* model for xeroderma pigmentosum and Cockayne's syndrome: *haywire* encodes the fly homolog of *ERCC3*, a human excision repair gene. Cell 71:925–937

Nance MA, Berry SA (1992) Cockayne syndrome: review of 140 cases. Am J Med Genet 42:68–84

O'Donovan A, Wood RD (1993) Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5. Nature 363:185–188

Park E, Guzder S, Koken MHM, Jaspers-Dekker I, Weeda G, Hoeijmakers JHJ, Prakash S, et al (1992) *RAD25*, a yeast homolog of human xeroderma pigmentosum group B DNA repair gene is essential for viability. Proc Natl Acad Sci USA 89:11416–11420

Robbins JH, Brumback RA, Mendiones M, Barrett SF, Carl JR, Cho S, Denckla MB, et al (1991) Neurological disease in xeroderma pigmentosum: documentation of a late onset type of the juvenile onset form. Brain 114:1335–1361

Robbins JH, Kraemer KH, Lutzner MA, Festoff BW, Coon HG (1974) Xeroderma pigmentosum: an inherited disease with sun sensitivity, multiple cutaneous neoplasms and abnormal repair. Ann Intern Med 80:221–248

Saiki R, Scharf S, Faloona F, Mullis K, Horn G, Ehrlich HA, Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1353

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schaeffer L, Roy R, Humbert S, Moncollin V, Vermeulen W, Hoeijmakers JHJ, Chambon P, et al (1993) The basic transcription factor BTF2/TFIIH contains a DNA helicase involved in both transcription and DNA repair. Science 260:58–63

Scherly D, Nouspikel T, Corlet J, Ucla C, Bairoch A, Clarkson SG (1993) Complementation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast *RAD2*. Nature 363:182–185

Scott RJ, Itin P, Kleijer WJ, Kolb K, Arlett C, Muller H. Xeroderma pigmentosum-Cockayne syndrome complex in two, new patients: absence of skin tumors despite severe deficiency of DNA excision repair. J Am Acad Dermatol (in press)

Tanaka K, Satokata I, Ogita Z, Uchida T, Okada Y (1989) Molecular cloning of a mouse DNA repair gene that complements the defect of group A xeroderma pigmentosum. Proc Natl Acad Sci USA 86:5512–5516

Thompson LH, Mooney CL, Brookman K (1985) Genetic complementation between UV-sensitive CHO mutants and xeroderma pigmentosum fibroblasts. Mutat Res 150:423–429

Troelstra C, van Gool A, de Wit J, Vermeulen W, Bootsma D, Hoeijmakers JHJ (1992) *ERCC6*, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell 71:939–953

Van Duin M, Vredeveldt G, Mayne LV, Odijk H, Vermeulen W, Klein B, Weeda G, et al (1989) The cloned human DNA excision repair gene *ERCC-1* fails to correct xeroderma pigmentosum complementation groups A through I. Mutat Res 217:83–92

Venema J, Mullenders LHF, Natarajan AT, van Zeeland AA, Mayne LV (1990*a*) The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. Proc Natl Acad Sci USA 87:4707–4711

Venema J, van Hoffen A, Natarajan AT, van Zeeland AA, Mullenders LHF (1990*b*) The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA. Nucleic Acids Res 18:443–448

Vermeulen W, Jacken J, Jaspers NGJ, Bootsma D, Hoeijmakers JHJ (1993) Xeroderma pigmentosum complementation group G associated with Cockayne syndrome. Am J Hum Genet 53:185–192

Vermeulen W, Osseweijer P, de Jonge AJ, Hoeijmakers JHJ (1986) Transient correction of excision repair defects in fibroblasts of 9 xeroderma pigmentosum complementation groups by microinjection of crude human cell extracts. Mutat Res 165:199–206

Vermeulen W, Stefanini M, Giliani S, Hoeijmakers JHJ, Bootsma D (1991) Xeroderma pigmentosum complementation group H falls into complementation group D. Mutat Res 255:201–208

Weeda G, van Ham RCA, Vermeulen W, Bootsma D, van der Eb AJ, Hoeijmakers JHJ (1990) A presumed DNA helicase encoded by *ERCC3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. Cell 62:777–791

Westerveld A, Hoeijmakers JHJ, van Duin M, de Wit J, Odijk H, Pastink A, Wood RD, et al (1984) Molecular cloning of a human DNA repair gene. Nature 310:425–429

Appendix paper III

American J. of Human Genetics,
Vol. 53: 817-821, 1993

A New Nucleotide-Excision-Repair Gene Associated with the Disorder Trichothiodystrophy

M. Stefanini,* W. Vermeulen,† G. Weeda,† S. Giliani,* T. Nardo,* M. Mezzina,‡ A. Sarasin,‡ J. I. Harper,§ C. F. Arlett,‖ J. H. J. Hoeijmakers,† and A. R. Lehmann‖

*Consiglio Nazionale delle Richerche, Instituto di Genetica Biochemica Evoluzionistica, Pavia, Italy; †Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University, Rotterdam; ‡Institut de Recherches Scientifiques sur le Cancer, Villejuif Cedex, France; §Department of Paediatric Dermatology, The Hospitals for Sick Children, London; and ‖MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton, United Kingdom

Summary

The sun-sensitive, cancer-prone genetic disorder xeroderma pigmentosum (XP) is associated in most cases with a defect in the ability to carry out excision repair of UV damage. Seven genetically distinct complementation groups (i.e., A–G) have been identified. A large proportion of patients with the unrelated disorder trichothiodystrophy (TTD), which is characterized by hair-shaft abnormalities, as well as by physical and mental retardation, are also deficient in excision repair of UV damage. In most of these cases the repair deficiency is in the same complementation group as is XP group D. We report here on cells from a patient, TTD1BR, in which the repair defect complements all known XP groups (including XP-D). Furthermore, microinjection of various cloned human repair genes fails to correct the repair defect in this cell strain. The defect in TTD1BR cells is therefore in a new gene involved in excision repair in human cells. The finding of a second DNA repair gene that is associated with the clinical features of TTD argues strongly for an involvement of repair proteins in hair-shaft development.

Introduction

Nucleotide excision repair of UV-induced damage in DNA is a complex process involving 6 genes in *Escherichia coli*, 10 or more genes in *Saccharomyces cerevisiae*, and at least 11 genes in mammals (Hoeijmakers and Bootsma 1990; Lehmann et al. 1992; Riboni et al. 1992). In mammals the different genes are defined by two sets of complementation groups—namely, (*a*) the UV-sensitive rodent mutants (11 groups) and (*b*) patients with XP (7 groups) and Cockayne syndrome (CS) (2 groups). The recent cloning of some of these human DNA repair genes has demonstrated significant overlap in the two sets of complementation groups. The *ERCC3* gene, cloned by its ability to correct the UV sensitivity of certain rodent mutants, has been shown to correct the defect in a patient with clinical symptoms of both XP and CS from XP complementation group B (Weeda et al. 1990). Similarly, the *ERCC2* gene corrects the UV sensitivities of several XP-D cell strains (Flejter et al. 1992; C. A. Weber, cited in Lehmann et al. 1992, pp. 6–7), and *ERCC6* has recently been shown to correct the deficiency in CS patients from CS complementation group B (Troelstra et al. 1992).

Trichothiodystrophy (TTD) is a rare genetic disorder whose clinical symptoms are quite different from those of XP and CS. Sulfur-deficient brittle hair is associated with mental and physical retardation, ichthyosis, an unusual facies, and, in many but not all patients, sun sensitivity. Unlike in XP, there are no reports of skin cancer associated with this disorder. Nevertheless, cells from photosensitive patients with TTD are, like XP cells, deficient in excision repair of UV damage. The extent of this deficiency is very heterogeneous between cells from different patients (Stefanini et al. 1986, 1992; Lehmann et al. 1988; Broughton et al. 1990). Cell fusion experiments have shown that, in all but three TTD cell strains examined so far, the repair deficiency is in

Received February 12, 1993; revision received May 18, 1993.

Address for correspondence and reprints: Dr. A. R. Lehmann, MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton BN1 9RR, England.

0002-9297/93/5304-0004$02.00

the same complementation group as is the defect in XP group D (Stefanini et al. 1986, 1992, 1993; Lehmann et al. 1988). In the three exceptional cases (two from related patients), complementation was observed with XP-D cells. In the present paper we show that one of these, TTD1BR, from a 16-year-old boy with a severe deficiency in excision repair, was able to complement the excision-repair defect in all XP complementation groups, and we show that complementation was not intragenic. This cell strain is therefore a representative of a new excision-repair complementation group.

Clinical Description

The patient (described 11 years ago in Jorizzo et al. 1982) had typical symptoms of TTD (characteristic hair-shaft abnormalities with reduced sulfur content, collodion baby, short stature, ichthyosis, bilateral congenital cataracts, and asthmatic attacks). Material for the current study was taken in 1988. A recent examination at age 20 years showed that he had had recurrent infective exacerbations of his asthma and that he remains severely growth retarded (height and weight below the 3d centile) and of limited intelligence but not severely mentally retarded (IQ 70–80). His ichthyosiform erythroderma continues. He has developed limited joint contractures of the hands that are due to the severe ichthyosiform involvement of the palms, and he has limited mobility. Despite all his problems, he retains a friendly personality with pleasingly good humor.

He has been sensitive to sunlight since early childhood, but, apart from sun sensitivity, his clinical features are quite distinct from those associated with XP patients. There is no significant freckling or other pigmentary changes. There are no telangiectases or actinic keratoses, nor have there been any skin tumors. There is no conjunctivitis or keratitis in the eyes, nor is there any sign of mental deterioration.

His immune function has been reported by Norris et al. (1990), who found that his $CD3^+$ cells were at the lower end of the normal range, with a $CD4^+/CD8^+$ ratio in the normal range. His lymphocytes showed a reduced response to phytohemogglutinin. Natural killer cell activity was in the normal range.

Methods

The procedures used in these studies have all been described in earlier work. Complementation studies were carried out as described elsewhere (Vermeulen et al. 1991; Stefanini et al. 1992). Microinjection experiments using cloned DNA repair genes were as described by Van Duin et al. (1989). An *ERCC2* cDNA product was synthesized by using reverse transcriptase (RT)-PCR and then was used to screen a human cDNA library. A full-length *ERCC2* cDNA clone was identified, and the *ERCC2* cDNA subsequently was inserted into the mammalian expression vector pSLM (Koken et al. 1992) for use in the microinjection experiments.

Table 1

Response of TTD1BR Fibroblasts to UV Irradiation

	Normal	TTD1BR	TTD2GL
Cell survival (D_{37}-Jm^{-2}) ...	6.6	1.6	1.1
UDS (% of normal at 10 Jm^{-2})	100	15	8
RNA synthesis (% of normal at 15 Jm^{-2})	100	12	9
Cyclobutane dimer excision (% removed in 24 h)	55	2	15
6-4 Photoproduct excision (% removed in 3 h)	75	18	18

NOTE.—Data from Broughton et al. (1990).

Results

UV Response of Fibroblasts

Table 1 summarizes our previously published results on the response to UV irradiation of TTD1BR cells, which are compared with a normal cell strain and with TTD2GL, a TTD cell strain with a severe DNA repair defect in the XP-D complementation group (see Lehmann et al. 1988, in which this cell strain was designated "P2"). Both cell strains have very pronounced defects in excision repair. The defect in TTD2GL is slightly more severe, TTD2GL being one of the most sensitive cell strains of the many TTD strains that we have examined (Stefanini et al. 1986, 1992, 1993). (The measurement of cyclobutane dimer excision is subject to large experimental errors, and the difference between TTD1BR and TTD2GL in table 1 is not significant.)

Complementation

The severe deficiency in unscheduled DNA synthesis (UDS) following UV irradiation of TTD1BR (table 1) enabled us to carry out complementation studies by fusing these cells with other TTD cell strains and with XP cells from complementation group D, followed by measurement of UDS in binucleate heterokaryons. Results of these experiments are shown in figure 1 (TTD8PV, XP17PV, and XP3NE). The level of UDS in the nuclei of heterokaryons was, in all cases, consider-

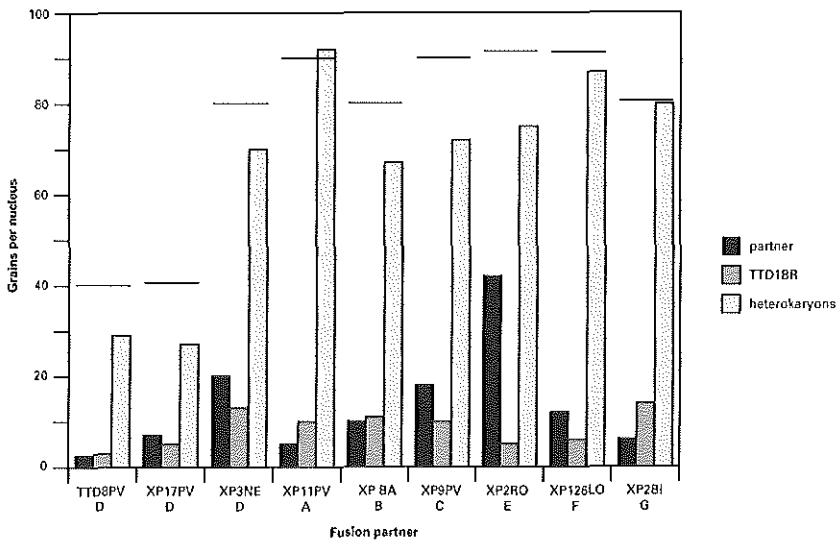


Figure 1 Complementation of TTD1BR. TTD1BR cells were fused with the indicated cell strains, following labeling of each fusion partner with different-sized latex beads. The fused cells were UV irradiated (10–20 Jm^{-2}) and UDS measured by autoradiography following ^{3}H-thymidine incorporation. The horizontal lines show the grains per nucleus in normal cells in the same experiment.

ably higher than that in homokaryons of either fusion partner in the same culture, and it approached that in normal controls. Thus TTD1BR cells clearly complement the repair defects in TTD8PV, XP3NE, and XP17PV, which have all previously been shown, by complementation analysis, to fall into the XP-D complementation group. This confirms our preliminary result reported elsewhere (Stefanini et al., 1993).

These observations suggest that the defect in TTD1BR is not in the XP-D gene, but they do not exclude the possibility of intragenic complementation. In order to evaluate this possibility, microneedle injection experiments were performed with the XP-D gene. It has recently been shown that the previously cloned *ERCC2* gene (Weber et al. 1990) is in fact the XP-D gene (Flejter et al. 1992; C. A. Weber, cited in Lehmann et al. 1992, pp. 6–7). As anticipated, microinjection of *ERCC2* cDNA, cloned into a mammalian expression vector, into various XP and TTD representatives of group D restored UV-induced UDS to normal levels (fig. 2*A*). In contrast, no increase in UDS was found in injected TTD1BR cells (fig. 2*B*), ruling out the possibility of intragenic complementation. This finding was further substantiated by direct sequencing of *ERCC2* cDNA after amplification by PCR. No sequence alterations were found in TTD1BR, whereas in other TTD patients various mutations were found in the *ERCC2* gene (A. R. Lehmann and B. C. Broughton, unpublished observations).

We next fused TTD1BR cells with XP cells from the other six XP complementation groups (i.e., A–C and E–G). Complementation was observed in all cases (fig. 1). To examine intragenic complementation for other XP and CS groups and to assess whether TTD1BR might be the human equivalent of some excision-deficient rodent complementation groups, three cloned human excision-repair genes that we have available—*ERCC1*, *ERCC3* (XPBC), and *ERCC6* (CSBC)—were microinjected into TTD1BR cells. In no case was UDS restored. These results show that TTD1BR is a representative of a new excision-repair complementation group.

Discussion

We have shown that the severe defect in excision repair of UV damage in TTD1BR cells is complemented

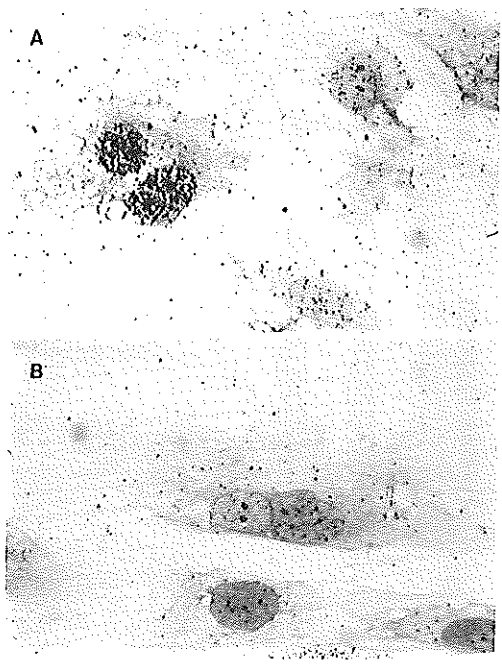


Figure 2 Microinjection of *ERCC2* into TTD1BR cells. The *ERCC2* cDNA inserted into a mammalian expression vector was introduced into homopolykaryons of XP1BR (a representative cell line of XP-D) (*A*) and TTD1BR (*B*) by microneedle injection into one of the nuclei. To permit expression of the injected DNA, cells were incubated for 24 h after injection. Subsequently, the fibroblasts were UV irradiated (15 J/m^2), incubated in the presence of ^{3}H-thymidine for 2 h to label repair patches, and were fixed and processed for autoradiography. The nuclei of the injected XP1BR polykaryon (the binuclear cell) (*A*) shows a complete correction of UDS (apparent from the high number of autoradiographic grains above both nuclei), in contrast to the neighboring, noninjected monokaryons. No induction of UDS is visible in the injected polykaryon of TTD1BR (*B*).

by all known XP complementation groups and is not corrected by several cloned human DNA repair genes. TTD1BR is therefore a representative of a new excision-repair complementation group not yet found in the population of XP patients, in contrast to previously reported TTD cell strains deficient in excision repair, which fall into the XP-D complementation group (Stefanini et al. 1986, 1992; Lehmann et al. 1988). TTD1BR therefore represents the second group in which defective DNA repair is associated with hair-shaft abnormalities, ichthyosis, and the other features of TTD (while showing none of the features of XP). This argues strongly for a causal relationship, rather than the chance association of independent genes involved in DNA repair and hair-shaft development. The involvement of DNA repair genes in apparently unrelated processes is not unprecedented. Three recent papers have shown that the *ERCC3* excision-repair gene is also involved in transcription/translation. Gulyas and Donahue (1992) identified yeast-suppressor mutants that could overcome a block to translation of the *his4-316* mRNA, caused by a strong, artificial stem-loop structure in the 5′ untranslated region. One of these suppressor genes, designated "*SSL2*," turned out to be the yeast homologue of the human *ERCC3* repair gene. Intriguing observations have also been made with the *Drosophila* homologue of this gene (Mounkes et al. 1992). A mutant designated "*haywire*" was characterized by male sterility, UV sensitivity, and defects in the central nervous system, and it displayed abnormalities in microtubule-based processes of spermatogenesis. These properties appeared to result from a mutation in the *Drosophila* homologue of *ERCC3*. Both the yeast and *Drosophila ERCC3* counterparts (and probably also the human gene) were shown to have an undefined function essential for cell viability, a function distinct from their role in excision repair. These observations suggest that the *ERCC3* gene product has two different functions (one involved with excision repair and the other involved with control of transcription/translation), which are not obviously related. This has been confirmed very recently by Schaeffer et al. (1993), who demonstrated that the 89-kD subunit of the transcription factor TFIIH (BTF2) corresponds to the *ERCC3* gene product. It is thus plausible to suggest that both the *ERCC2* gene product (which has several features in common with the ERCC3 protein) and the new gene defective in TTD1BR cells may also be involved in two apparently unrelated processes—namely, excision repair and another process involved in hair-shaft development. The clinical phenotype of the patients (e.g., XP or TTD) may depend on the way in which the causative mutation affects one or the other or both of the hypothesized functions. Further experiments should provide evidence supporting or disproving these speculative ideas on the relationship of excision-repair defects to hair-shaft abnormalities.

Acknowledgments

We are indebted to patient TTD1BR for provision of blood and skin for this study and to Mr. A. Joosse for help in

the cell hybridization experiments. This work was supported in part by EC Stimulation Programme grant SCI-232 and by the Associazione Italiana per la Ricerca sul Cancro. W.V. was supported by Dutch Cancer Society grants IKR88-2 and EUR 92-118.

References

Broughton BC, Lehmann AR, Harcourt SA, Arlett CF, Sarasin A, Kleijer WJ, Beemer FA, et al (1990) Relationship between pyrimidine dimers, 6-4 photoproducts, repair synthesis and cell survival: studies using cells from patients with trichothiodystrophy. Mutat Res 235:33–40

Flejter WL, McDaniel LD, Johns D, Friedberg EC, Schultz RA (1992) Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: involvement of the human *ERCC2* DNA repair gene. Proc Natl Acad Sci USA 89:261–265

Gulyas KD, Donahue TF (1992) *SSL2*, a suppressor of a stem-loop mutation in the *HIS4* leader encodes the yeast homolog of human *ERCC-3*. Cell 69:1031–1042

Hoeijmakers JHJ, Bootsma D (1990) Molecular genetics of eukaryotic DNA excision repair. Cancer Cells 2:311–320

Jorizzo JL, Atherton DJ, Crounse RG, Wells RS (1982) Ichthyosis, brittle hair, impaired intelligence, decreased fertility and short stature (IBIDS syndrome). Br J Dermatol 106:705–710

Koken MHM, Vreeken C, Bol SAM, Cheng NC, Jaspars-Dekker I, Hoeijmakers JHJ, Eeken JCJ, et al (1992) Cloning and characterization of the Drosophila homolog of the xeroderma-pigmentosum complementation-group-B correcting gene, *ERCC3*. Nucleic Acids Res 20:5541–5548

Lehmann AR, Arlett CF, Broughton BC, Harcourt SA, Steingrimsdottir H, Stefanini M, Taylor AMR, et al (1988) Trichothiodystrophy, a human DNA repair disorder with heterogeneity in the cellular response to ultraviolet light. Cancer Res 48:6090–6096

Lehmann AR, Hoeijmakers JHJ, Van Zeeland AA, Backendorf CMP, Bridges BA, Collins A, Fuchs RPD, et al (1992) Workshop on DNA repair. Mutat Res 273:1–28

Mounkes LC, Jones RS, Liang B-C, Gelbart W, Fuller MT (1992) A *Drosophila* model for xeroderma pigmentosum and Cockayne's Syndrome: *haywire* encodes the fly homolog of *ERCC3*, a human excision repair gene. Cell 71:925–937

Norris PG, Limb GA, Hamblin AS, Lehmann AR, Arlett CF, Cole J, Waugh APW, et al (1990) Immune function, mutant frequency and cancer risk in the DNA repair defective genodermatoses xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy. J Invest Dermatol 94:94–100

Riboni R, Botta E, Stefanini M, Numata M, Yasui A (1992) Identification of the eleventh complementation group of UV-sensitive excision repair-defective rodent mutants. Cancer Res 52:6690–6691

Schaeffer L, Roy R, Humbert S, Moncollin V, Vermeulen W, Hoeijmakers JHJ, Chambon P, et al (1993) DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science 260:58–63

Stefanini M, Giliani S, Nardo T, Marinoni S, Nazzaro R, Rizzo R, Trevisan G (1992) DNA repair investigations in nine Italian patients affected by trichothiodystrophy. Mutat Res 273:119–125

Stefanini M, Lagomarsini P, Arlett CF, Marinoni S, Borrone C, Crovato F, Trevisan G, et al (1986) Xeroderma pigmentosum (complementation group D) mutation is present in patients affected by trichothiodystrophy with photosensitivity. Hum Genet 74:107–112

Stefanini M, Lagomarsini P, Giliani S, Nardo T, Botta E, Peserico A, Kleijer WJ, et al (1993). Genetic heterogeneity of the excision repair defect associated with trichothiodystrophy. Carcinogenesis 14:1101–1105.

Troelstra C, Van Gool A, De Wit J, Vermeulen W, Bootsma D, Hoeijmakers JHJ (1992) *ERCC6*, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell 71:1–15

Van Duin M, Vredeveldt G, Mayne LV, Odijk H, Vermeulen W, Klein B, Weeda G, et al (1989) The cloned human DNA excision repair gene ERCC-1 fails to correct xeroderma pigmentosum complementation groups A through I. Mutat Res 217:83–92

Vermeulen W, Stefanini M, Giliani S, Hoeijmakers JHJ, Bootsma D (1991) Xeroderma pigmentosum complementation group H falls into complementation group D. Mutat Res 255:201–208

Weber CA, Salazar EP, Stewart SA, Thompson LH (1990) *ERCC-2*: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast RAD3. EMBO J 9:1437–1448

Weeda G, Van Ham RCA, Vermeulen W, Bootsma D, Van Der Eb AJ, Hoeijmakers JHJ (1990) A presumed DNA helicase encoded by *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. Cell 62:777–791

Appendix paper IV

The EMBO J.,
Vol. 13: 1645-1653, 1994

Correction of xeroderma pigmentosum repair defect by basal transcription factor BTF2 (TFIIH)

A.J.van Vuuren[1], W.Vermeulen[1], L.Ma[2], G.Weeda, E.Appeldoorn, N.G.J.Jaspers, A.J.van der Eb[2], D.Bootsma, J.H.J.Hoeijmakers[3] and S.Humbert[4], L.Schaeffer[4] and J.-M.Egly[4]

Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University, PO Box 1738, 3000 DR, Rotterdam, [2]Laboratory for Molecular Carcinogenesis, Medical Genetics Centre, Sylvius Laboratory, PO Box 9503, 2300 RA, Leiden, The Netherlands and [4]UPR 6520 (CNRS), Unité 184 (INSERM), Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

[3]Corresponding author
[1]The first and second authors have contributed equally to this work

Communicated by D.Bootsma

ERCC3 was initially identified as a gene correcting the nucleotide excision repair (NER) defect of xeroderma pigmentosum complementation group B (XP-B). The recent finding that its gene product is identical to the p89 subunit of basal transcription factor BTF2(TFIIH), opened the possibility that it is not directly involved in NER but that it regulates the transcription of one or more NER genes. Using an *in vivo* microinjection repair assay and an *in vitro* NER system based on cell-free extracts we demonstrate that ERCC3 in BTF2 is directly implicated in excision repair. Antibody depletion experiments support the idea that the p62 BTF2 subunit and perhaps the entire transcription factor function in NER. Microinjection experiments suggest that exogenous ERCC3 can exchange with ERCC3 subunits in the complex. Expression of a dominant negative K436 → R ERCC3 mutant, expected to have lost all helicase activity, completely abrogates NER and transcription and concomitantly induces a dramatic chromatin collapse. These findings establish the role of ERCC3 and probably the entire BTF2 complex in transcription *in vivo* which was hitherto only demonstrated *in vitro*. The results strongly suggest that transcription itself is a critical component for maintenance of chromatin structure. The remarkable dual role of ERCC3 in NER and transcription provides a clue in understanding the complex clinical features of some inherited repair syndromes
Key words: BTF2/chromatin structure/ERCC3/nucleotide excision repair/repair syndromes

Introduction

An intricate network of DNA repair systems protects the genetic information from continuous deterioration due to the damaging effects of environmental genotoxic agents and inherent chemical instability of DNA. Thus these systems prevent mutagenesis leading to inborn defects, cell death and neoplasia, and may counteract the process of ageing.

Nucleotide excision repair (NER) is one of the major, cellular repair pathways. It removes a wide range of structurally unrelated lesions (such as UV-induced pyrimidine dimers and chemical adducts) in a complex multi-step reaction. The mechanistic details of this process in eukaryotes are—in contrast to *Escherichia coli*—poorly understood, but a general picture is emerging. After recognition of the DNA injury by a process not yet resolved, the damaged strand is incised on either side of the lesion, 27–29 nucleotides apart (Huang *et al.*, 1992). Excision of the patch, which appears to require one or more single strand binding proteins, such as HSSB (RP-A) (Coverley *et al.*, 1992) is followed by gap-filling, mediated by DNA polymerase δ and/or ε in a reaction dependent on PCNA (Nichols and Sancar, 1992; Shivji *et al.*, 1992). Finally, ligation is required to seal the newly synthesized repair patch to the pre-existing DNA (for recent reviews see Grossman and Thiagalingam, 1993; Hoeijmakers, 1993a,b; Sancar and Tang, 1993). In fact, in most, if not all, organisms at least two NER sub-pathways exist. Special factors allow for the rapid and efficient removal of lesions in the transcribed strand that interfere with ongoing transcription (transcription-coupled repair). The other sub-pathway deals with the slower repair of the rest of the genome (genome overall repair; Hanawalt and Mellon, 1993).

The dramatic consequences of impaired NER are illustrated by three distinct, inherited diseases characterized by sun (UV) hypersensitivity, elevated genetic instability and a striking clinical and genetic heterogeneity. These are the prototype repair disorder xeroderma pigmentosum (seven complementation groups: XP-A–XP-G), Cockayne's syndrome (two groups: CS-A and CS-B) and PIBIDS (at least two groups, one of which is equivalent to XP-D) (Stefanini *et al.*, 1986, 1993; for a review see Hoeijmakers, 1993b). XP is marked by severe cutaneous abnormalities, including a strong predisposition to skin cancer, and frequently progressive neurological degeneration (reviewed in Cleaver and Kraemer, 1989). CS exhibits poor general development and neurodysmyelination. No increased frequency of skin cancer is noted in this disorder (Lehmann, 1987; Nance and Berry, 1992). The repair defect in CS is limited to the sub-pathway of transcription-coupled repair (Venema *et al.*, 1990). Patients with PIBIDS manifest most of the CS symptoms and, curiously, the hallmark of another disease called trichothiodystrophy: ichthyosis and brittle hair and nails (the latter may be due to a reduced synthesis of a cysteine-rich matrix protein) (Peserico *et al.*, 1992; Bootsma and Hoeijmakers, 1993). In exceptional cases individuals display a combination of XP and CS. These have been assigned to XP complementation groups B, D and G (Vermeulen *et al.*, 1991, 1993, 1994).

Recently, the gene responsible for one of these, the *XP-B* gene: *ERCC3* (Weeda *et al.*, 1990), was unexpectedly found to be identical to the p89 subunit of basal transcription factor BTF2 (TFIIH) (Schaeffer *et al.*, 1993). Human TFIIH, its

rat counterpart factor δ and its yeast equivalent factor b are one of the seven or so components required for proper transcription initiation of RNA polymerase II *in vitro* from a number of model promoters (Conaway and Conaway, 1989; Feaver *et al.*, 1991; Gerard *et al.*, 1991; Flores *et al.*, 1992). The formation of an elongation-competent initiation complex involves a highly ordered cascade of reactions (reviewed in Sawadogo and Sentenac, 1990; Roeder, 1991; Gill and Tijan, 1992; Drapkin *et al.*, 1993), initiated by the binding of factor TFIID to the TATA box and completed by binding of TFIIJ. The multi-subunit BTF2 consists of a minimum of five proteins: p89, p62, p43, p41 and p35. The role of this factor is at least 2-fold. First, the human BTF2/TFIIH as well as the rat δ factor are associated with a protein kinase activity that specifically phosphorylates the C-terminal domain of the large subunit of RNA polymerase II (Lu *et al.*, 1992; Serizawa *et al.*, 1993b). Second, it exhibits a DNA helicase activity (Schaeffer *et al.*, 1993) that is functionally required for ATP-driven local denaturation of the transcriptional start site. The formation of an open configuration precedes the catalysis of the first phosphodiester bond of the transcript. Experimental evidence renders it likely that the ERCC3 gene product participates in the essential unwinding reaction (Schaeffer *et al.*, 1993). This is consistent with the identification of seven so-called 'helicase motifs' in the ERCC3 amino acid sequence (Weeda *et al.*, 1990).

The discovery of the ERCC3–transcription connection provided an adequate explanation for several of the mysterious observations with respect to this gene, such as its unsolved essential function in yeast (Gulyas and Donahue, 1992; Park *et al.*, 1992) and *Drosophila* (Mounkes *et al.*, 1992) and a poorly defined involvement in expression of certain genes. In addition to a direct participation of ERCC3 in both transcription and repair, this finding opened a second possibility to explain the ERCC3–NER connection. When the expression of one or more essential NER genes critically depends on the functioning of *ERCC3*, mutations in this gene might indirectly result in a NER defect (Gulyas and Donahue, 1992; Schaeffer *et al.*, 1993). The aim of this study was to further define the role of *ERCC3* in transcription and repair *in vivo* and *in vitro*. The work presented here has implications for understanding the complex clinical picture of XP-B and other forms of NER syndromes and provides evidence for the involvement of an additional BTF2 component in NER.

Results

NER function of BTF2 measured by microinjection

The presence of XP-B specific NER correcting activity in protein fractions obtained during the purification of transcription factor BTF2 was assessed by microinjection into living XP-B fibroblasts in culture. The effect of the injected proteins on the repair capacity of the cells is measured by UV-induced unscheduled DNA synthesis (UDS), visualized by *in situ* autoradiography and quantified by counting silver grains above nuclei (De Jonge *et al.*, 1983). Figure 1A shows the purification scheme of BTF2 (Gerard *et al.*, 1991) together with the results of the microinjection in XP-B and as a control in XP-G. It is apparent that BTF2-containing fractions induce correction in XP-B. The multinuclear XP-B cell shown in Figure 1B has been injected with purified BTF2/TFIIH (hydroxyapatite fraction; Gerard

A Purification scheme of BTF2(TFIIH)

BTF2 purification step		Correction of NER-defect in XP-B	XP-G
HeLa WCE (Manley)		+	+
Heparin ultrogel	(0.22-0.4M KCl)	NT	NT
DEAE-spherodex	(0.07-0.17M KCl)	NT	NT
Sulphopropyl-5PW	(0.38M KCl, peak)	+	-
Phenyl-5PW	(0.2M $(NH_4)_2SO_4$, peak)	+	-
Hydroxyapatite	(0.37 M K-Pi, peak)	+	-
glycerol gradient		+	-

B

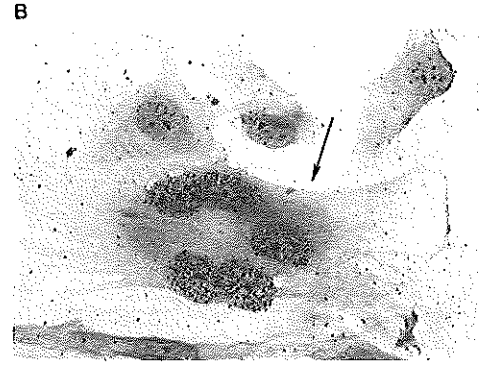


Fig. 1. Assessment of XP-B correcting activity by BTF2 using microneedle injection. (A) Copurification of XP-B repair correction and transcription stimulation of BTF2. Peak fractions of transcription stimulatory activity of each BTF2 purification step (for details on the BTF2 purification see Gerard *et al.*, 1991) were injected into the cytoplasm of XP-B or XP-G homopolykaryons, followed by UV irradiation, incubation in the presence of [^{3}H]TdR, fixation and processing for autoradiography (see Materials and methods for details). The number of silver grains above nuclei is a reflection of the repair capacity of the cell. Fractions were scored positive when the injected cells showed a level of UDS that was more than five times above the background of non-injected neighbouring fibroblasts. WCE: whole cell extract. The samples of the first two purification steps were very diluted compared with the WCE. (B) Micrograph of XP-B fibroblasts (XPCS1BA), microinjected with the highly purified fraction 12 of the HAP chromatography column (see Figure 2), showing induction of the UV dependent UDS in the injected multi-nucleated cell (arrow) compared with the typical XP-B residual UDS level as shown by the surrounding uninjected mononuclear cells.

et al., 1991) and shows a level of UDS in the range of normal cells. In contrast, the non-injected neighbouring mononuclear cells exhibit the very low repair activity characteristic of XP-B. However, the correcting activity for XP-G fibroblasts, which is present in the whole cell extract (WCE), does not copurify with BTF2 (Figure 1A). To follow more closely the relationship between BTF2 and XP-B correction the fractionation profiles of the last two purification steps, the hydroxyapatite (HAP) chromatography and the glycerol gradient sedimentation were screened in a quantitative fashion. Figure 2 shows that there is a direct correlation between the repair complementing activity measured as UDS as well as *in vitro* and the transcription activation determined in an *in vitro* run-off assay using the adenovirus major late promoter with RNA polymerase II and all basal transcription factors except BTF2 (Fischer *et al.*, 1992). In

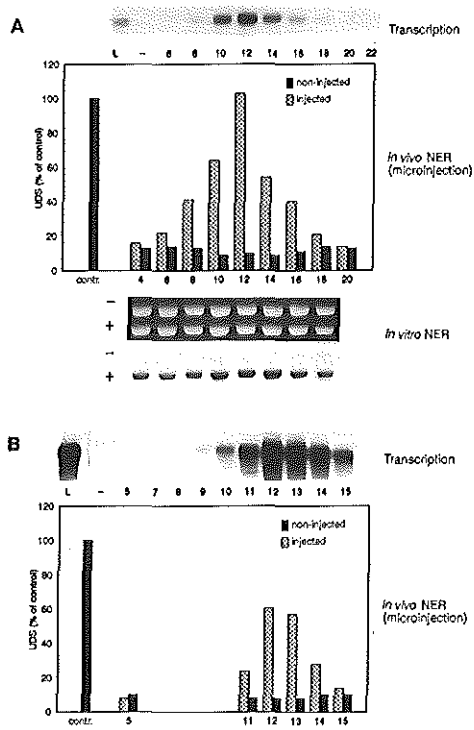


Fig. 2. Cochromatography and cosedimentation of BTF2 transcription stimulating and XP-B/ERCC3 repair-correcting activities. The transcriptionally active fractions that were eluted from the phenyl-5PW column were successively applied on a HAP column and a 15–35% glycerol gradient. All fractions were tested for transcription stimulation *in vitro* using the adenovirus major late promoter. Repair activity was determined *in vivo* by microneedle injection into living XP-B fibroblasts and *in vitro* using a cell-free NER assay. (A) HAP purification step. The upper part shows the autoradiogram of the transcription assay, the middle part presents the results of grain counting (average number of grains per nucleus, 50 nuclei counted) of XP-B cells microinjected with each fraction and assayed for UV-induced UDS. The lower two panels represent the *in vitro* NER activities of the same fractions in 27-1, a rodent NER deficient cell strain of complementation group 3. They show the stained gel to demonstrate equal loading and the autoradiogram to visualize repair synthesis. The AAF-damaged plasmid is indicated with '+', the undamaged internal control with '–'. Fractions 4 and 12 of the HAP profile had undergone an extra cycle of freezing and thawing prior to this test. (B) Glycerol gradient sedimentation. Transcription (upper part) and XP-B *in vivo* repair activity (lower part) of the glycerol fractions were assayed as above.

previous work it was shown that the transcription stimulation of BTF2 parallels its helicase activity (Schaeffer *et al.*, 1993). Thus, all BTF2 activities coincide with the XP-B specific NER correction over six purification steps. We conclude that microinjected BTF2 complex itself and not a minor contaminant causes restoration of the repair defect in living XP-B cells.

NER function of BTF2 in an in vitro repair assay

The results of the microinjection experiments do not exclude the possibility that ERCC3 is only indirectly involved in

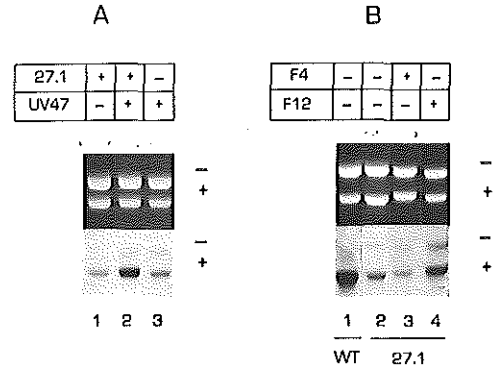


Fig. 3. BTF2 corrects NER defect of rodent complementation group 3 *in vitro*. (A) *In vitro* complementation of NER defect in CHO mutants of rodent complementation groups 3 (mutant 27-1) and 4 (mutant UV47). Reactions contained a damaged plasmid (pBKS, 3.0 kb) and a non-damaged internal control plasmid (pHM14, 3.7 kb), 250 ng each. Protein concentration in all reactions was 200 μg. In lane 2 100 μg of protein of each mutant extract were mixed. After incubation in the presence of [^{32}P]dATP to permit repair, DNA was isolated, linearized by restriction endonuclease treatment and size-fractionated by agarose gel electrophoresis. (B) *In vitro* correction of NER defect of rodent complementation group 3 (mutant 27-1) by purified BTF2 (fractions 4 and 12 of the HAP chromatography shown in Figure 2). Lanes 1 and 2 contained 200 μg protein, lanes 3 and 4 100 μg of extract was used. The amount of protein contributed by the BTF2 HAP fractions is negligible.

NER. It is feasible that introduced BTF2 corrects expression of one or more critical NER genes whose transcription is abolished by the *ERCC2* mutation in XP-B. Therefore, we also measured correction of the XP-B defect in an *in vitro* NER system (Wood *et al.*, 1988) where transcription and translation cannot occur. Figure 3B shows the outcome of administration of highly purified BTF2 (fraction 12 of the HAP chromatography) to cell-free extracts of CHO mutant 27-1. This mutant is a member of rodent complementation group 3, the equivalent of XP-B (Weeda *et al.*, 1990). In the presence of purified BTF2, repair synthesis in the damaged plasmid reaches a level similar to that achieved by mixing a complementing extract (Figure 3A). No such correction is observed with fraction 4 of the HAP eluate, which does not contain BTF2 activity. In further testing all fractions eluted from this column in the *in vitro* NER assay we find that the repair and transcription profiles are again superimposable, as they were using the microinjection assay (Figure 2A, lower panel). These experiments demonstrate that ERCC3 has a direct involvement in both transcription and NER.

Configuration of ERCC3 in the NER reaction

To determine whether the ERCC1 correcting activity in a whole cell extract resides in a BTF2(-like) configuration antibody depletion experiments were conducted. A repair proficient HeLa cell extract was incubated with a monoclonal antibody against the p62 component of BTF2 (Mab3C9), immobilized on protein A–sepharose beads. After removal of the beads by centrifugation the remainder of the extract was tested for repair capacity *in vitro*. Figure 4 shows that removal of p62 causes a clear antibody dependent reduction in repair activity (lane 3, compared with lane 1)

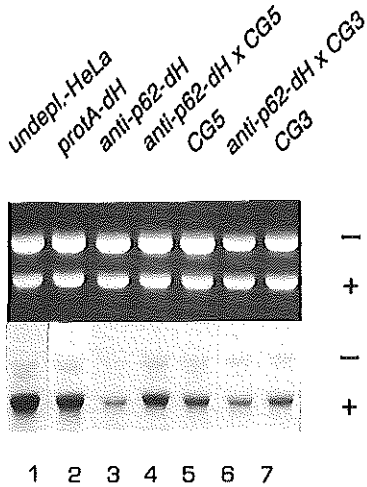


Fig. 4. Monoclonal antibodies against p62 deplete a repair-competent HeLa extract from repair and ERCC3 activity. HeLa cell-free extract (150 μg protein) was incubated with Mab3C9 against the p62 BTF2 subunit immobilized on protein A–sepharose CL-4B beads. After centrifugation to remove the bound proteins the supernatant was tested for its repair and complementing activities using the *in vitro* NER assay. Lane 1, HeLa extract (undepleted 100 μg); lane 2, HeLa extract treated with protein A beads alone (80 μg); lane 3, HeLa extract depleted with Mab3C9 (80 μg); lane 4, Mab3C9-treated HeLa extract (40 μg) mixed with extract of CHO mutant UV135 (complementation group 5, 100 μg); lane 5, UV135 extract alone (200 μg); lane 6, Mab3C9-treated HeLa extract (40 μg) mixed with extract of CHO mutant 27-1 (complementation group 3, 100 μg); lane 7, 27-1 extract alone (200 μg).

suggesting that p62 is also involved in NER. To see whether ERCC3 activity is still present the treated extract was mixed with an extract of CHO group 3. It is apparent from Figure 4 that the $(p62)^{depleted}$-HeLa extract had also lost the ability to complement the group 3 (lane 6) but not the group 5 defect (lane 4), which is equivalent to XP-G (O'Donovan and Wood, 1993; Scherly *et al.*, 1993). These findings indicate that ERCC3 correcting activity in a repair-competent HeLa whole cell extract is associated with p62 and that the ERCC5/XP-G factor is not tightly bound to the p62–ERCC3 complex.

Effect of free ERCC3 protein

The purified BTF2 complex is quite stable, at least *in vitro*, as the proteins involved remain associated even in 1 M KCl (Schaeffer *et al.*, 1993). To find out whether the ERCC3 protein can only function in NER when offered in the form of the BTF2 complex we tested the ability of ERCC3 protein overproduced in *E.coli* to complement the XP-B defect. Figure 5 shows a glutathione–agarose bound fraction containing the GST–ERCC3 fusion protein after SDS–PAGE (lane 1) and the partially purified ERCC3 gene product released from the GST fusion protein after cleavage by thrombin (lane 3). The results summarized in Table I (lower part) demonstrate that the recombinant GST–ERCC3 fusion protein as well as free ERCC3 induce significant correction soon (within 4 h) after microinjection into XP-B

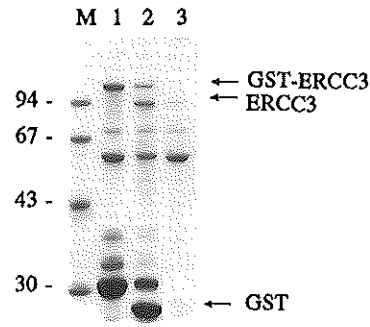


Fig. 5. SDS–PAGE analysis of recombinant-produced ERCC3 fusion protein. Recombinant-produced fusion protein of GST–ERCC3 (using vector pGEX2T) was partly purified on glutathione–agarose beads and eluted with 5 mM reduced glutathione (lane 1). Lane 2, eluted proteins, after digestion with thrombin; lane 3, proteins released after thrombin cleavage from immobilized fusion proteins on GSH beads. Arrows indicate the recombinant ERCC3 proteins. Other bands present in the Coomassie stained gel are probably derived from C-terminal degradation products of GST–ERCC3 and contaminating *E.coli* proteins.

fibroblasts. The fact that ERCC3 in the context of the BTF2 complex as well as free ERCC3 exerts correction must mean that the ERCC3 subunit is exchangeable. The notion that the correction by BTF2 is more rapid (within 2 h) and reaches wild-type level is consistent with the idea that this complex rather than the free protein is involved in the NER reaction. Apparently, the N-terminal GST extension does not seriously interfere with ERCC3 functioning in the BTF2 complex.

Consequence of interference with ERCC3 functioning for NER and transcription

The involvement of ERCC3 in transcription is derived from results using an *in vitro* transcription assay and a limited set of promoters (Gerard *et al.*, 1991). It is important to verify whether the protein performs such a function *in vivo* as well. Therefore, we assessed the effect of antibody injection on RNA synthesis (determined by incorporation of [^{3}H]uridine) and on NER (as determined by the level of UV-induced [^{3}H]TdR incorporation) into normal fibroblasts. As a control, cells on the same slide were injected with preimmune serum of the same rabbit and with antibodies against the ERCC1 protein, a NER component not residing in the BTF2 complex. Previously, we presented the specificity of the antibodies (Schaeffer *et al.*, 1993; van Vuuren *et al.*, 1993). The results are summarized in Table I (upper part). The ERCC3 preimmune serum has no significant effect on the number of grains derived from transcription or NER. The ERCC1 control serum induces a total inhibition of excision repair, demonstrating that this protein is indispensable for NER. However, no reduction of general transcription is observed, demonstrating that transcription does not drop as a non-specific consequence of the inhibition of repair. The ERCC3 serum causes a significant but incomplete repression of transcription as well as repair synthesis. The partial effect can be explained in several ways. The antibodies cannot reach or block the activity of all ERCC3 molecules, e.g. because the antigenic sites are not accessible. Alternatively,

Table I. Microinjection of ERCC3 protein, antibodies and DNA constructs in human cells

Injected substances[a]	Hours after injection	% inhibition of NER[b]	% inhibition of RNA synthesis[b]	NER activity in XP-B (UDS in % of normal)
Anti-ERCC1 (476)	24	97	0	–
Anti-ERCC3 (1151)	24	43	48	–
Preimmune 1151	24	2	5	–
K346 → R *ERCC3*	6	0	0	–
K346 → R *ERCC3*	22	74	70	–
K346 → R *ERCC3*	30	93	78	–
K346 → R *ERCC3*	48	98	95	–
w.t. *ERCC3*	30	10	5	–
Rec. GST–ERCC3[c]	4–6	–	–	39
Rec. ERCC3[c]	4–6	–	–	43
No injection	–	–	–	11

[a]Polyclonal rabbit serum (476, anti-ERCC1; 1151, anti-ERCC3; preimmune 1151), *ERCC3* cDNA in the mammalian expression vector pSVL (wild-type and K346 → R mutant of *ERCC3*); GSH-purified recombinant fusion protein of GST–ERCC3 and thrombin-cut fusion protein of GST–ERCC3 on GSH column (see Figure 5).
[b]Compared with uninjected cells present on the same slide.
[c]Estimated protein concentration 0.05–0.1 μg/ml.

the protein is only involved in part of the transcription and NER reactions in the cell.

As an alternative manner of interfering with ERCC3 functioning *in vivo* a DNA construct was designed carrying a mutation that is expected to have only a subtle effect on the tertiary structure of the protein but a drastic effect on its activity. For this the conserved lysine 346 in the nucleotide binding box (Weeda *et al.*, 1990) was selected and replaced by arginine, thereby preserving the positive charge. It is known from other ATPases and helicases with GKT-type nucleotide binding domains that substitution of the invariant lysine reduces or completely abolishes ATP hydrolysis but does not necessarily affect ATP binding as such (Azzaria *et al.*, 1989; Reinstein *et al.*, 1990; Tijan *et al.*, 1990). In the yeast RAD3 repair helicase it has been demonstrated that a similar K → R replacement does not interfere with ATP binding but blocked the hydrolysis step and helicase activity (Sung *et al.*, 1988). Transfection of the mutant *ERCC3* cDNA into the UV-sensitive, repair-deficient rodent group 3 cell line 27-1 demonstrated that the K346 → R protein was unable to restore the repair defect (L.Ma, A.Westbroek, A.G.Jochemsen, G.Weeda, D.Bootsma, J.H.J.Hoeijmakers and A.J. van der Eb, manuscript submitted). An analogous mutation in the yeast homologue of ERCC3, RAD25, showed that the essential function of the protein was also inactivated (Park *et al.*, 1992). To see whether this mutation exerts a dominant effect the K346 → R *ERCC3* cDNA was microinjected into repair-competent fibroblasts. As a control the wild-type cDNA in the same vector was injected into cells on the same slide. Although at different time points some heterogeneity was seen in the magnitude of the effects, presumably due to differences in the level of expression, a sharp drop in UDS was registered within 22 h which was total by 48 h (see Figure 6D–F and for quantitative results Table I, middle part). Concomitantly, transcription fell down to undetectable levels (Figure 6A–C, Table I). This was closely followed by a dramatic change in nuclear morphology. At first (22 h) the nucleoli increased in size and became less densely stained. At later times the entire Giemsa-stainable chromatin material clumped in a small area, leaving the remainder of the nucleus empty. In contrast, the cytoplasm stayed remarkably normal in morphology (see Figure 6). None of these effects were observed in the cells injected with the wild-type cDNA construct which even exerts correction of the repair defect when injected into XP-B fibroblasts (Weeda *et al.*, 1990). We conclude that the K346 → R substitution confers a dominant-negative effect on both transcription and NER and induces a dramatic chromatin collapse.

Discussion

The findings reported here demonstrate that the ERCC3 gene product functions directly in two quite different aspects of nucleic acid metabolism: basal transcription and nucleotide excision repair. This confirms and extends earlier observations by Egly and coworkers who identified the ERCC3 protein as one of the components of the BTF2/TFIIH complex required for proper transcription initiation of RNA polymerase II *in vitro* (Schaeffer *et al.*, 1993). Thus these results rule out the theoretical possibility raised by these and other remarkable observations with respect to the *ERCC3* gene in the yeast and *Drosophila* systems, namely that the ERCC3–NER connection could be indirect, i.e. ERCC3 might control the expression of one or more (real) NER genes without being involved itself in this process (Gulyas and Donahue, 1992; Mounkes *et al.*, 1992; Schaeffer *et al.*, 1993). In addition, this work establishes that the ERCC3 protein participates in the transcription machinery *in vivo*; the evidence for this was hitherto only based on an *in vitro* transcription assay. Finally, this paper reveals the dramatic consequences of interfering with proper ERCC3 functioning *in vivo* via overexpression of dominant-negative ERCC3 mutant protein or by antibody injections. Our findings have direct implications for the functioning of ERCC3 at the molecular level and for the interpretation of some of the clinical manifestations of the associated human syndrome.

Involvement of BTF2 in NER

In what configuration does ERCC3 function in NER? The fact that purified BTF2 is able to correct the XP-B/rodent

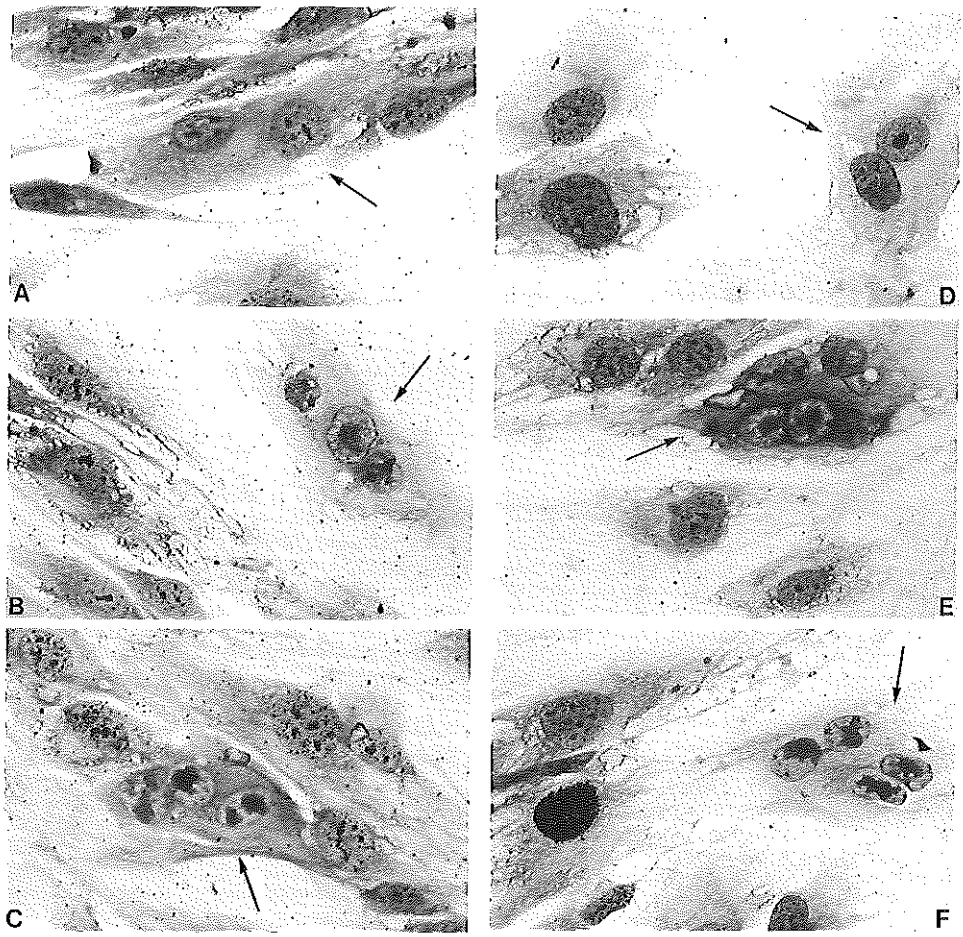


Fig. 6. Effect of K346 → R ERCC3 mutant on transcription and NER. Micrographs A, B and C show the time dependent effect on RNA synthesis, assayed by pulse labelling with [^{3}H]uridine, in control (wild-type) fibroblasts injected with the *ERCC3* cDNA encoding the K346 → R mutated protein. Micrographs D, E and F demonstrate the effect on NER, as revealed by UV-induced UDS. A and D were assayed 22 h, B and E 30 h and C and F 48 h after microinjection. Injected polykaryons (arrows) demonstrate a complete inhibition of RNA synthesis (no incorporation of [^{3}H]uridine) as well as NER (absence of UDS in injected polykaryons) and a dramatic chromatin collapse.

group 3 repair defect *in vivo* and *in vitro* suggests, but does not prove, that the complex as a whole participates in the NER process. It is, however, not excluded that the ERCC3 subunit dissociates from the complex and either functions alone or in a different complex in the NER reaction mechanism. An argument in favour of the involvement of BTF2 in NER is our finding that monoclonal antibodies against p62 are able to deprive a HeLa WCE of its NER capability and all detectable ERCC3 activity. This suggests that at least the p62 subunit of BTF2/TFIIH is tightly associated with the majority of the ERCC3 molecules that are required for *in vitro* NER. A second inference from this observation is that the p62 protein may be yet another NER factor. Whether the same holds for the entire BTF2 complex has to be established. The dual involvement of ERCC3 extends the emerging notion that the eukaryotic NER system recruits many factors from other systems that operate in the nucleus. From parallels with yeast it is likely that the recently discovered ERCC1, ERCC4, ERCC11 and XPFC complex functions simultaneously in NER and in a mitotic recombination pathway (Schiestl and Prakash, 1990; Bailly *et al.*, 1992; Bardwell *et al.*, 1992; Biggerstaff *et al.*, 1993; van Vuuren *et al.*, 1993). Using the *in vitro* NER assay Wood and coworkers have shown that the replication factors PCNA and HSSB (RPA) also participate in the post-incision stages of the NER reaction (Coverley *et al.*, 1992; Shivji *et al.*, 1992) in addition to DNA polymerase δ or ε and ligase I. The dual usage of these proteins for different processes

may be for economical reasons. Alternatively, it may be a reflection of the tight links of excision repair with other processes in the nucleus.

Possible role of ERCC3 in NER

What can be the role of ERCC3 and BTF2 in transcription initiation and NER? From the foregoing it is most logical to search for a common functional step for ERCC3 and perhaps the entire BTF2/TFIIH complex in both processes. It has been demonstrated that the human BTF2/TFIIH complex as well as the rat counterpart exhibit two activities: a protein kinase that is able to phosphorylate the C-terminal domain of the large subunit of RNA polymerase II (Lu *et al.*, 1992; Serizawa *et al.*, 1993a,b) and a helicase activity (Schaeffer *et al.*, 1993). The latter is likely to be associated with the p89 ERCC3 subunit. In this light at least two possibilities (or a combination) can be envisaged for a common catalytic function of ERCC3 in transcription and NER. (i) Induction of a locally melted DNA conformation for RNA polymerase to be loaded onto the template. In the context of NER a similar function can be imagined for putting a scanning complex onto the DNA or the incision complex at the site of a lesion. (ii) Unwinding of the helix as part of the translocation of the transcription or NER scanning complex along the chromatin, as suggested for the $UvrA_2B$ complex in *E.coli* NER (Grossman and Thiagalingam, 1993). In both cases the ERCC3 protein or the BTF2 factor has to interact with components of the transcription as well as the NER machinery. Mutations in the ERCC3 protein found in XP-B patients (such as alteration of the C-terminus: Weeda *et al.*, 1990) primarily inactivate NER. It is tempting to speculate that these regions are specifically involved in the interaction with other NER components.

Interference with ERCC3 functioning

Microinjection of the K346 → R *ERCC3* mutant construct induces a strong reduction or even a complete blockage of NER and general transcription. A similar effect—although quantitatively not as pronounced—was seen with anti-ERCC3 antibodies. Selective inhibition of NER is not the reason for the dramatic decline of transcription, because antibodies against the ERCC1 protein also cause a total blockage of NER but have no measurable impact on transcription. The inhibition of NER and transcription by K346 → R ERCC3 occurs with similar kinetics, indicating that both processes are disturbed in an analogous fashion. Furthermore, it is observed relatively shortly after microinjection when taking into account that the injected gene has to be transcribed, the mRNA translated and the protein incorporated in a sufficient number of BTF2 complexes to exert the observed effect. For many genes expression is first registered 16–24 h after injection. Also the correction of the XP-B repair defect by injection of the purified ERCC3 protein is a quite rapid phenomenon: significant increase in UDS is seen within 4 h after injection. This suggests that exogenous ERCC3 is able to exchange with endogenous ERCC3 in BTF2 complexes in a relatively fast manner. One possibility is that the BTF2 complex disassembles and reassembles at regular times as part of its reaction cycle and in this way incorporates new exogenous ERCC3 protein. For the dominant effect of mutant ERCC3 the following scenario seems most plausible. The conservative K346 → R mutation is not expected to perturb dramatically the tertiary conformation and fitting of the protein into the BTF2 complex. After incorporation into BTF2 and assembly of the preinitiation complex the reaction proceeds up to the stage in which the ATPase activity of ERCC3 is required. At this point the system is paralysed, frustrating the normal progression of both transcription and NER. When a critical threshold of poisoned transcription units is reached this process culminates in a complete and irreversible inhibition of transcription. Ironically, this catastrophic event will also shut down the expression of the injected *ERCC3* mutant construct itself.

BTF2 has been demonstrated to be involved in transcription by RNA polymerase II (Gerard *et al.*, 1991). It is not known whether it is implicated in RNA polymerase I and III transcription as well. The effects of the dominant-negative *ERCC3* mutant indicates that total RNA synthesis becomes impaired. This does not necessarily mean that ERCC3 participates directly in all three modes of transcription. It is possible that the inhibition of e.g. RNA polymerase I is an indirect result of the blockage of RNA polymerase II.

The time-resolved consequences of selective inhibition of transcription by RNA polymerase II have hitherto escaped detection because severe mutants in this process are obviously not viable. In our transient microinjection system the most dramatic and earliest morphological effects concern the nucleus where the nucleolus seems to swell first and eventually all chromatin appears to become clumped in one or a few regions, leaving the remainder of the nucleus empty. These alterations initiate already at a stage when inhibition of transcription is still not complete suggesting that the chromatin structure critically depends on ongoing transcription and functioning ERCC3. Probably all cellular processes which require proteins with a high turnover and a short mRNA half-life will be affected first. Morphologically, the affected cells show features resembling an early stage of apoptosis. However, further studies are warranted to establish a relationship with apoptosis.

Consequences for the clinical features of XP-B

The very rare XP-B complementation group exhibits a number of clinical characteristics that are atypical for a NER defect and difficult to rationalize on the basis of a DNA repair problem. This includes many of the features these patients share with Cockayne's syndrome, such as neurodysmyelination, immature sexual development, absence of subcutaneous fat and a general growth deficiency (Cleaver and Kraemer, 1989; Scott *et al.*, 1993; Vermeulen *et al.*, 1994). In view of the additional function of ERCC3 it is tempting to assign at least some of these features to a subtle impairment of transcription. In mouse models evidence has been collected that the production of the myelin sheet is strongly determined by the amount of mRNA for myelin basic protein (Popko *et al.*, 1987). Thus the neurodysmyelination may be related to a reduced expression of this protein. Viable mutations in the *Drosophila* homologue of *ERCC3* designated '*haywire*' display sterility which is likely to be caused by reduced expression of β-tubulin, required for spindle formation in meiosis (Mounkes *et al.*, 1992). Perhaps the immature sexual development in XP-B could be due to the same problem. Recent findings in the field of basic transcription support the notion that the requirement of transcription factors may vary from promoter to promoter, depending on the sequence around the initiation site, the topological state of the DNA and perhaps other factors such

as the local chromatin structure (see Stanway, 1993 and references therein). This may explain the above features and the poor general development characteristic of this form of XP. In fact two other XP complementation groups, XP-D and XP-G, also display the remarkable clinical features of XP-B (Vermeulen *et al.*, 1993). As demonstrated here the XP-G factor is not present in the BTF2 complex. If it is involved in transcription it is either present in another complex or it is only loosely associated with components of BTF2 and not essential for transcription. The latter idea is in agreement with the notion that the yeast homologue of XP-G, RAD2 (Scherly *et al.*, 1993) is also not an essential gene (Madura and Prakash, 1986). As noted before, XP-D and the corresponding gene *ERCC2* have many parallels with XP-B and *ERCC3*. This has prompted the idea that the ERCC3 and ERCC2 proteins may interact and have a similar function (Weeda *et al.*, 1990). Clinical heterogeneity in XP-D is even more pronounced than in XP-B and includes the peculiar brittle hair symptoms of trichothiodystrophy. We have recently obtained evidence that the functional overlap between repair and transcription also includes ERCC2. This is consistent with the idea that some of the clinical features of these rare pleiotropic disorders are due to basal transcription problems.

Materials and methods

General procedures

Purification of nucleic acids, restriction enzyme digestion, gel electrophoresis of nucleic acids and proteins, transformation of *E.coli*, etc. were performed according to standard procedures (Sambrook *et al.*, 1989).

Purification of BTF2/TFIIH

The purification of BTF2 and all other general transcription factors required for the transcription assay using the adenovirus 2 major late promoter as template was performed starting from HeLa cells as described earlier (Gerard *et al.*, 1991).

Microneedle injection and assay for RNA synthesis and UV-induced unscheduled DNA synthesis

Microneedle injection of XP-B fibroblasts as well as control cells (C5RO) was performed as described (Vermeulen *et al.*, 1994). Briefly, at least 3 days prior to microinjection cells were fused with the aid of inactivated Sendai virus, seeded onto coverslips and cultured in Ham's F-10 medium, supplemented with 12% fetal calf serum and antibiotics. After injection of at least 50 homopolykaryons cells were incubated for the desired time in normal culture medium before being assayed. NER activity was determined after UV-C light irradiation with 15 J/m^2, incubation for 2 h in [^{3}H]thymidine (10 μCi/ml; s.a.: 50 Ci/mmol)-containing culture medium, fixation and exposure to autoradiography. Grains above the nuclei (>100) were counted and represent a quantitative measure for NER activity. RNA synthesis was determined also by counting autoradiographic grains above the nuclei of injected cells, after labelling with [^{3}H]uridine (10 μCi/ml; s.a.: 50 Ci/mmol) during a pulse labelling period of 1 h in normal culture medium. Protein preparations (including antisera) were injected into the cytoplasm, cDNAs were microinjected into one of the nuclei of polykaryons.

In vitro DNA repair assay

Plasmids pBKS (3.0 kb) and pHM14 (3.7 kb) were isolated from *E.coli* and extensively purified as closed circular DNA (Biggerstaff *et al.*, 1991). pBKS was treated with 0.1 mM *N*-acetoxy-2-acetylaminofluorene (AAF) (a kind gift of R.Baan, TNO, Rijswijk), inducing mainly *N*-(guanine-8-yl)–AAF adducts. AAF-modified plasmids were collected by repeated di-ethyl-ether extractions and ethanol precipitation (Landegent *et al.*, 1984) and repurified on a neutral sucrose gradient. pHM14 was mock-treated in parallel. There are 15–20 AAF–guanine adducts per damaged plasmid.

Repair-proficient cell lines: HeLa, Chinese hamster ovary (CHO) cell strain CHO9 and CHO NER mutants: 27-1 (complementation group 3), UV47 (CG 4) and UV135 (CG 5) were cultured in a 1:1 mixture of Ham's F-10 and DMEM medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. Cells were harvested and extracts were prepared from 2–5 ml of packed cell pellet by the method of Manley as modified by Wood (Manley *et al.*, 1983; Wood *et al.*, 1988). Extracts were dialysed in 25 mM HEPES–KOH, pH 7.8, 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT and 17% (v/v) glycerol and stored at −80°C.

The reaction mixture (50 μl) contained 250 ng of both damaged and non-damaged plasmid DNA, 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 20 μM each of dCTP, dGTP and TTP, 8 μM dATP, 74 kBq of (α-^{32}P]dATP, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.45% glycerol, 18 μg bovine serum albumin and 200 μg of cell-free extract. The reaction was incubated for 3 h at 30°C, afterwards the plasmid DNAs were isolated, linearized and separated on an agarose gel electrophoresis. Data were analysed via autoradiography and quantified by scintillation counting of excised DNA bands.

For microinjection and antibody depletion experiments, the following antisera were used: (i) a rabbit polyclonal anti-ERCC1 antiserum raised against a ubiquitin–ERCC1 fusion protein and characterized as previously described (van Vuuren *et al.*, 1993); (ii) a polyclonal antiserum raised against a GST–ERCC3 fusion protein containing an internal part (amino acids 82–480) of ERCC3; (iii) a monoclonal antibody (Mab3C9) against the 62 kDa polypeptide, a component of BTF2, used in depletion experiments was published earlier (Fischer *et al.*, 1992).

To deplete 150 μg of repair-proficient HeLa extract, anti-p62 antibodies (3 μl of ascites fluid) were immobilized on protein A–Sepharose CL-4B beads; after incubation with HeLa extract and centrifugation the supernatant was used as a depleted Hela extract and tested for repair activity *in vitro* as detailed above (van Vuuren *et al.*, 1993).

Overproduction and purification of recombinant ERCC3 protein

ECCR3 cDNA cloned in pGEX2T was transferred to *E.coli* strain BL21 and gene expression was induced during 3 h by IPTG. Cells were homogenized in PBS (containing 2 mM PMSF and 15% glycerol) by sonication and extracts were cleared by centrifugation. Fusion protein was purified by passing the cell homogenate through a glutathione–agarose containing column, proteins were eluted with 5 mM reduced glutathione (in 50 mM Tris, pH 8.0). Alternatively recombinant ERCC3 was cleaved from the GST part by incubating the immobilized fusion protein (on the glutathione–agarose beads) with thrombin (1–2 ng/μg protein) for 45 min at 20°C.

Site-directed mutagenesis

Mutations in the *ERCC3* cDNA sequence were made using the oligonucleotide directed, uracil–DNA method (Kunkel *et al.*, 1987). An internal fragment of *ERCC3* was inserted in a M13 vector, and after mutation induction used to replace the wild-type fragment in the parental plasmid pE3-WT. The desired mutation was verified by sequence analysis.

Acknowledgements

We are grateful to J.van Kampen and A.Bosch for help in some of the experiments, J.L.Weickert for cell culture, Y.Lutz for antibody production and M.Chipoulet for technical assistance. M.Kuit, R.Koppenol and T.de Vries Lentsch are acknowledged for photography. This work was supported in part by grants from the Netherlands Foundation for Chemical Research (SON), the Dutch Cancer Society (EUR 92-118), J.A.Cohen Inter-university Research Institute for Radiopathology and Radiation Protection (IRS), the INSERM, the CNRS, the Ministère de la Recherche et de l'Enseignement Supérieur and the Association pour la Recherche sur le Cancer.

References

Azzaria,M., Schurr,E. and Gros,P. (1989) *Mol. Cell. Biol.*, **9**, 5289–5297.
Bailly,V., Sommers,C.H., Sung,P., Prakash,L. and Prakash,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8273–8277.
Bardwell,L., Cooper,A.J. and Friedberg,E.C. (1992) *Mol. Cell. Biol.*, **12**, 3041–3049.
Biggerstaff,M., Robins,P., Coverley,D. and Wood,R.D. (1991) *Mutat. Res.*, **254**, 217–224.
Biggerstaff,M., Szymkowski,D.E. and Wood,R.D. (1993) *EMBO J.*, **12**, 3685–3692.
Bootsma,D. and Hoeijmakers,J.H.J. (1993) *Nature*, **363**, 114–115.
Cleaver,J.E. and Kraemer,K.H. (1989) *Xeroderma pigmentosum. The Metabolic Basis for Inherited Disease*, Vol. II. McGraw-Hill Book Co., New York, pp.2949–2971.

Conaway,R.C. and Conaway,J.W. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 7356–7360.
Coverley,D., Kenny,M.K., Lane,D.P. and Wood,R.D. (1992) *Nucleic Acids Res.*, **20**, 3873–3880.
De Jonge,A.J.R., Vermeulen,W., Klein,B. and Hoeijmakers,J.H.J. (1983) *EMBO J.*, **2**, 637–641.
Drapkin,R., Merino,A. and Reinberg,D. (1993) *Curr. Biol.*, **5**, 469–476.
Feaver,W.J., Gileadi,O. and Kornberg,D. (1991) *J. Biol. Chem.*, **266**, 19000–19005.
Fischer,L., Gerard,M., Chalut,C., Lutz,Y., Humbert,S., Kanno,M., Chambon,P. and Egly,J.-M. (1992) *Science*, **257**, 1392–1395.
Flores,O., Lu,H. and Reinberg,D. (1992) *J. Biol. Chem.*, **267**, 2786–2790.
Gerard,M., Fischer,L., Moncollin,V., Chipoulet,J.-M., Chambon,P. and Egly,J.-M. (1991) *J. Biol. Chem.*, **266**, 20940–20945.
Gill,G. and Tjian,R. (1992) *Curr. Opin. Gen. Dev.*, **2**, 236–242.
Grossman,L. and Thiagalingam,S. (1993) *J. Biol. Chem.*, **268**, 16871–16874.
Gulyas,K.D. and Donahue,T.F. (1992) *Cell*, **69**, 1031–1042.
Hanawalt,P. and Mellon,I. (1993) *Curr. Biol.*, **3**, 67–69.
Hoeijmakers,J.H.J. (1993a) *Trends Genet.*, **9**, 173–177.
Hoeijmakers,J.H.J. (1993b) *Trends Genet.*, **9**, 211–217.
Huang,J.C., Svoboda,D.L., Reardon,J.T. and Sancar,A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3664–3668.
Kunkel,T.A., Robetts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Landegent,J.E., Jansen in de Wal,N., Baan,R.A., Hoeijmakers,J.H.J. and Van der Ploeg,M. (1984) *Exp. Cell Res.*, **153**, 61–72.
Lehmann,A.R. (1987) *Cancer Rev.*, **7**, 82–103.
Lu,H., Zawel,L., Fisher,L., Egly,J.-M. and Reinberg,D. (1992) *Nature*, **358**, 641–645.
Madura,K. and Prakash,S. (1986) *J. Bacteriol.*, **166**, 914–923.
Manley,J.L., Fire,A., Samuels,M. and Sharp,P.A. (1983) *Methods Enzymol.*, **101**, 568–582.
Mounkes,L.C., Jones,R.S., Liang,B.-C., Gelbart,W. and Fuller,M.T. (1992) *Cell*, **71**, 925–937.
Nance,M.A. and Berry,S.A. (1992) *Am. J. Med. Genet.*, **42**, 68–84.
Nichols,A.F. and Sancar,A. (1992) *Nucleic Acids Res.*, **20**, 2441–2446.
O'Donovan,A. and Wood,R.D. (1993) *Nature*, **363**, 185–188.
Park,E., Guzder,S., Koken,M.H.M., Jaspers-Dekker,I., Weeda,G., Hoeijmakers,J.H.J., Prakash,S. and Prakash,L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11416–11420.
Peserico,A., Battistella,P.A. and Bertoli,P. (1992) *Neuroradiology*, **34**, 316–317.
Popko,B., Puckett,C., Lai,E., Shine,H.D., Readhead,C., Takahashi,N., Hunt,S.W.,III, Sidman,R.L. and Hood,L. (1987) *Cell*, **48**, 713–721.
Reinstein,J., Brune,M. and Wittinghofer,A. (1990) *Biochemistry*, **29**, 7451–7459.
Roeder,R.G. (1991) *Trends Biochem. Sci.*, **16**, 402–408.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sancar,A. and Tang,M.-S. (1993) *Photochem. Photobiol.*, **57**, 905–921.
Sawadogo,M. and Sentenac,A. (1990) *Annu. Rev. Biochem.*, **59**, 711–754.
Schaeffer,L., Roy,R., Humbert,S., Moncollin,V., Vermeulen,W., Hoeijmakers,J.H.J., Chambon,P. and Egly,J.-M. (1993) *Science*, **260**, 58–63.
Scherly,D., Nouspikel,T., Corlet,J., Ucla,C., Bairoch,A. and Clarkson,S.G. (1993) *Nature*, **363**, 182–185.
Schiestl,R.H. and Prakash,S. (1990) *Mol. Cell. Biol.*, **10**, 2485–2491.
Scott,R.J., Itin,P., Kleijer,W.J., Kolb,K., Arlett,C. and Müller,H.J. (1993) *J. Am. Acad. Dermatol.*, **29**, 883–889.
Serizawa,H., Conaway,J.W. and Conaway,R.C. (1993a) *Nature*, **363**, 371–374.
Serizawa,H., Conaway,R.C. and Conaway,J.W. (1993b) *J. Biol. Chem.*, **268**, 17300–17308.
Shivji,M.K.K., Kenny,M.K. and Wood,R.D. (1992) *Cell*, **69**, 367–374.
Stanway,C.A. (1993) *Bioessays*, **15**, 559–560.
Stefanini,M., Lagomarsini,P., Arlett,C.F., Marinoni,S., Borrone,C., Crovato,F., Trevisan,G., Cordone,G. and Nuzzo,F. (1986) *Hum. Genet.*, **74**, 107–112.
Stefanini,M. *et al.* (1993) *Am. J. Hum. Genet.*, **53**, 817–821.
Sung,P., Higgins,D., Prakash,L. and Prakash,S. (1988) *EMBO J.*, **7**, 3263–3269.
Tijan,G., Yan,H., Jiang,R.-T., Kishi,F., Nakazawa,A. and Tsai,M.-D. (1990) *Biochemistry*, **29**, 4296–4304.
van Vuuren,A.J., Appeldoorn,E., Odijk,H., Yasui,A., Jaspers,N.G.J., Bootsma,D. and Hoeijmakers,J.H.J. (1993) *EMBO J.*, **12**, 3693–3701.
Venema,J., Mullenders,L.H.F., Natarajan,A.T., Van Zeeland,A.A. and Mayne,L.V. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4707–4711.
Vermeulen,W., Stefanini,M., Giliani,S., Hoeijmakers,J.H.J. and Bootsma,D. (1991) *Mutat. Res.*, **255**, 201–208.
Vermeulen,W., Jaeken,J., Jaspers,N.G.J., Bootsma,D. and Hoeijmakers, J.H.J. (1993) *Am. J. Hum. Genet.*, **53**, 185–192.
Vermeulen,W., Scott,R.J., Potger,S., Müller,H.J., Cole,J., Arlett,C.F., Kleijer,W.J., Bootsma,D., Hoeijmakers,J.H.J. and Weeda,G. (1994) *Am. J. Hum. Genet.*, **54**, 191–200.
Weeda,G., Van Ham,R.C.A., Vermeulen,W., Bootsma,D., Van der Eb,A.J. and Hoeijmakers,J.H.J. (1990) *Cell*, **62**, 777–791.
Wood,R.D., Robins,P. and Lindahl,T. (1988) *Cell*, **53**, 97–106.

Received on November 18, 1993; revised on December 23, 1993

Appendix paper V

Cold Spring Harb. Symp. Quant. Biol.,
Vol. 59: 317-329, 1994

Three Unusual Repair Deficiencies Associated with Transcription Factor BTF2(TFIIH): Evidence for the Existence of a Transcription Syndrome

W. VERMEULEN,*†† A.J. VAN VUUREN,*†† M. CHIPOULET,† L. SCHAEFFER,† E. APPELDOORN,* G. WEEDA,* N.G.J. JASPERS,* A. PRIESTLEY,‡ C.F. ARLETT,‡ A.R. LEHMANN,‡ M. STEFANINI,§ M. MEZZINA,** A. SARASIN,** D. BOOTSMA,* J.-M. EGLY,† AND J.H.J. HOEIJMAKERS*

*Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University, 3000DR, Rotterdam, The Netherlands; †UPR 6520 (CNRS), Unité 184 (INSERM), Faculté de Médicine, 67085 Strasbourg Cedex, France; ‡MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton BN1 9RR, United Kingdom; §Consiglio Nazionale Della Richerche, Istituto di Genetica Biochemica ed Evoluionistica, 27100 Pavia, Italy; **UPR 42 (CNRS), IFC 1101, 94801 Villejuif Cedex, France*

To counteract the deleterious effects of DNA damage, a sophisticated network of DNA repair systems has evolved, which is essential for genetic stability and prevention of carcinogenesis. Nucleotide excision repair (NER), one of the main repair pathways, can remove a wide range of lesions from the DNA by a complex multistep reaction (for a recent review, see Hoeijmakers 1993). Two subpathways are recognized in NER: a rapid "transcription-coupled" repair and the less efficient global genome repair (Bohr 1991; Hanawalt and Mellon 1993). The consequences of inborn errors in NER are highlighted by the prototype repair syndrome, xeroderma pigmentosum (XP), an autosomal recessive condition displaying sun (UV) sensitivity, pigmentation abnormalities, predisposition to skin cancer, and often progressive neurodegeneration (Cleaver and Kraemer 1994). Two other excision repair disorders have been recognized, Cockayne's syndrome (CS) and trichothiodystrophy (TTD), which present different clinical features. These are, besides sun sensitivity, neurodysmyelination, impaired physical and sexual development, dental caries (in CS and TTD), ichthyosis and sulfur-deficient brittle hair and nails (in TTD)(Lehmann 1987; Nance and Berry 1992), which are difficult to rationalize on the basis of defective NER only. The NER syndromes are genetically heterogeneous and comprise at least 10 different complementation groups: 7 in XP (XP-A to XP-G), 5 in CS (CS-A, CS-B, XP-B, XP-D, and XP-G) and 2 in TTD (TTD-A and XP-D) (Hoeijmakers 1993; Stefanini et al. 1993b). Thus, considerable overlap and clinical heterogeneity are associated with a selected subset of complementation groups, of which XP-D is the most extreme, harboring patients with XP only, or combined XP and CS, or TTD (Johnson and Squires 1992).

Recently, it was discovered that the DNA repair helicase encoded by the *ERCC3* gene (Roy et al. 1994), which is mutated in XP-B (Weeda et al. 1990), is identical to the p89 subunit of the transcription factor BTF2/TFIIH (Schaeffer et al. 1993). ERCC3 in the context of TFIIH is directly involved in NER and transcription in vitro as well as in vivo (van Vuuren et al. 1994). Furthermore, another repair helicase, XPD/ERCC2, was recognized to be associated with TFIIH (Schaeffer et al. 1994), and it was shown that a partially purified TFIIH fraction is able to correct the NER deficiency of XP-D in vitro (Drapkin et al. 1994). These results reveal a link between two distinct DNA-metabolizing processes: repair and transcription.

Initiation of transcription is believed to require the formation of an elongation-competent protein complex in a highly ordered cascade of reactions. A preinitiation (DAB) complex is formed by the binding of the multisubunit TFIID factor to the TATA box element of core promoters, stabilized by TFIIA and followed by the association of TFIIB. The DAB intermediate stimulates the entry of RNA polymerase II mediated by TFIIF. Initiation is completed by the ordered association of TFIIE, TFIIH/BTF2, and TFIIJ (Roeder 1991; Gill and Tjian 1992; Drapkin et al. 1993). TFIIH/BTF2 is thought to be involved in the conversion of a closed to an open initiation complex by local melting of the transcriptional start site and phosphorylation of the carboxy-terminal repeat of the large subunit of RNA polymerase II (Lu et al. 1992; Schaeffer et al. 1993; Serizawa et al. 1993), either at the preinitiation stage, or at a step between initiation and elongation, referred to as promoter clearance (Goodrich and Tjian 1994).

Here we report that defects in the XPB/ERCC3 subunit define a new TTD complementation group extending the clinical heterogeneity associated with ERCC3. Furthermore, we demonstrate that probably the entire TFIIH complex has a dual role in transcription and repair, as it harbors now at least three NER proteins, all associated with the TTD and CS symptoms. The dual function of TFIIH, and the link between mutations in this complex and the pleiotropic features of TTD and CS, strongly support the idea that some of

††These authors have contributed equally to this work.

the clinical manifestations arise from defects in the transcription function of TFIIH. This provides for the first time a molecular explanation for the seemingly unrelated symptoms of these disorders and introduces a novel clinical entity, a heterogenous "transcription syndrome" complex, that may include many more inherited conditions.

METHODS

Purification of BTF2/TFIIH. The purification of BTF2 starting from HeLa whole-cell extract and involving sequential chromatography on Heparin-Ultrogel, DEAE-Spherodex, SP-5PW sulfopropyl, and—after ammonium sulfate precipitation—Phenyl-5PW Sepharose and hydroxyapatite, was carried out essentially as described earlier (Gerard et al. 1991). In some fractions an additional purification step using heparin chromatography was inserted in the standard protocol before the Phenyl-5PW column. The in vitro assay for following the transcriptional stimulation activity of BTF2 on an AD2MLP promoter containing template involving purified RNA polymerase II and all transcription factors except BTF2/TFIIH has been described in detail previously (Gerard et al. 1991).

Cell lines and extracts. The human cell lines used for microinjection and for complementation analysis were XP25RO and XP11PV (both XP-A), XPCS1BA and XPCS2BA (XP-B), XP21RO (XP-C), XP1BR, XP3NE, and TTD8PV (all XP-D), XP2RO (XP-E), XP126LO (XP-F), XP2BI (XP-G), and TTD1BR (TTD-A) and TTD6VI (Stefanini et al. 1993b; Vermeulen et al. 1986, 1994). The primary fibroblasts were cultured in Ham's F10 medium supplemented with antibiotics and 10–15% fetal calf serum.

For preparing cell-free extracts utilized for the in vitro repair assay, the following cell lines were used: a SV40-transformed line belonging to TTD-A (TTD1BRSV), human repair-proficient HeLa cells, mutant Chinese hamster cells 43-3B, UV5, 27.1, UV41, and UV135 assigned to complementation groups 1, 2, 3, 4, and 5, respectively (Busch et al. 1989). The cells were grown in a 1:1 mixture of F10 and DMEM medium; antibiotics and 10% fetal calf serum were added. After harvesting and washing with phosphate-buffered saline (PBS), cell-free extracts were prepared as described previously (Manley et al. 1983; Wood et al. 1988), dialyzed against a buffer containing 25 mM HEPES/KOH (pH 7.8), 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and 17% (v/v) glycerol, and stored at −80°C.

In vitro DNA repair assay. Plasmid pBluescript KS^+ (3.0 kb) was damaged by treatment with 0.1 mM *N*-acetoxy-2-acetylaminofluorene (AAF) (a kind gift of R. Baan, TNO, Rijswijk). As a nondamaged control, plasmid pHM14 (3.7 kb) was used. Circular closed forms of both plasmids were isolated and extensively purified as described previously (Biggerstaff et al. 1991; van Vuuren et al. 1993). The reaction mixture contained 250 ng each of damaged and nondamaged control plasmids, 45 mM HEPES/KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.4 mM EDTA, 0.9 mM DTT, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.5% glycerol, 18 μg bovine serum albumin, 20 μM each of dCTP, dGTP, and TTP, 8 μM dATP, 74 kBq of [α-^{32}P]dATP and 200 μg of cell-free extract for the rodent extracts and 100 μg for HeLa. After 3 hours incubation at 30°C, plasmid DNAs were purified from the reaction mixture, linearized by restriction, and separated by electrophoresis on an 0.8% agarose gel. Results were quantified using an LKB Densitometer and a B&L Phospho-Imager.

Anti-p62 antibody depletion of a repair-proficient extract. Protein A-Sepharose beads in PBS were incubated with monoclonal antibodies against the p62 subunit of BTF2 (Mab3C9; Fischer et al. 1992) or against p89 for 1 hour at 0°C. The beads were washed with dialysis buffer and added to a repair-competent HeLa extract or a (partially) purified heparin or hydroxyapatite BTF2 fraction for 1 hour at 0°C (van Vuuren et al. 1993). The bound proteins were removed by centrifugation and the supernatant was tested in the in vitro repair assay or for microneedle injection. Western blot analysis following SDS-PAGE was carried out according to standard protocols (Sambrook et al. 1989).

Microinjection. Microinjection of XP homopolykaryons was performed as described earlier (Vermeulen et al. 1994). Repair activity was determined after UV irradiation (15 J/m^2), [^{3}H]thymidine incubation (10 μCi/ml; s.a.: 50 Ci/mMole), fixation, and autoradiography. Grains above the nuclei represent a quantitative measure for NER activity. RNA synthesis was detected after labeling with [^{3}H]uridine (10 μCi/ml; s.a.: 50 Ci/mMole). cDNAs were injected into one of the nuclei of the polykaryons; protein fractions and antibodies were injected into the cytoplasm of the cell. Antisera used for microinjection: polyclonal rabbit anti-ERCC1 and anti-ERCC3 (van Vuuren et al. 1994) and polyclonal rabbit anti-ERCC2 (Schaeffer et al. 1994).

Complementation analysis by cell hybridization. Fibroblasts of each fusion partner were labeled with latex beads (0.8 μm or 2.0 μm) 3 days prior to fusion by adding a suspension of beads to the culture medium. Cell fusion was performed with the aid of inactivated Sendai virus or, using polyethylene glycol, cells were seeded onto coverslips and assayed for UV-induced unscheduled DNA synthesis (UDS) as described in detail elsewhere (Stefanini et al. 1993a; Vermeulen et al. 1993).

Other procedures. Isolation of DNA, subcloning, and in vitro transcription and translation were done using established procedures (Sambrook et al. 1989).

RESULTS

Identification of Further Genetic Heterogeneity within TTD

In an effort to assess the genetic heterogeneity within the class of repair-deficient TTD patients, we have conducted a systematic complementation study of a large number of photosensitive TTD families by cell fusion and microneedle injection of cloned repair genes. This resulted recently in the identification of a second complementation group among NER-deficient TTD patients (designated TTD-A) (Stefanini et al. 1993b). Figure 1 shows the results of an exhaustive complementation analysis of fibroblasts from one of two siblings (TTD6VI and TTD4VI) with relatively mild clinical features of TTD and moderately impaired NER characteristics (detailed clinical description to be presented elsewhere). Full complementation of the repair defect was seen when fibroblasts of patient TTD6VI were fused with representants of the known TTD complementation groups: XP group D (3 cell lines tested) and with TTD-A fibroblasts (Fig. 1) (Stefanini et al. 1993a,b). This demonstrates that this family defines a new TTD complementation group. To see whether this group is genetically identical to one of the other XP groups not previously associated with TTD, further cell-hybridization experiments were carried out. No restoration of the deficient UV-induced UDS was found when TTD6VI cells were fused with an XP-B representant (XPCS1BA), whereas normal complementation was observed with the other XP groups (Fig. 1). This indicates that the repair defect in this family resides in the *XPB/ERCC3* gene. Although TTD6VI cells appeared exceptionally sensitive to nuclear microneedle injection of DNA, still a few cells could be found corrected by *ERCC3* cDNA, in agreement with the assignment by cell fusion. Defects in the TFIIH subunit ERCC3 thus extend to the disorder TTD, increasing the already observed clinical heterogeneity among patients carrying mutations in *ERCC3* (Vermeulen et al. 1994).

Systematic Screening of TFIIH for NER Proteins

To examine whether besides XPB/ERCC3 (p89) additional NER factors are hidden in the TFIIH/BTF2 complex, we have systematically screened the existing human and rodent NER mutants for complementation by BTF2. The final fractions of TFIIH purification show at least five tightly associated proteins, including p89 (XPB/ERCC3), p62, p44, p41, and p34 (Gerard et al. 1991; Schaeffer et al. 1993). A purified BTF2-fraction (hydroxyapatite chromatography, gel pattern, and properties; see Gerard et al. 1991) was inserted by

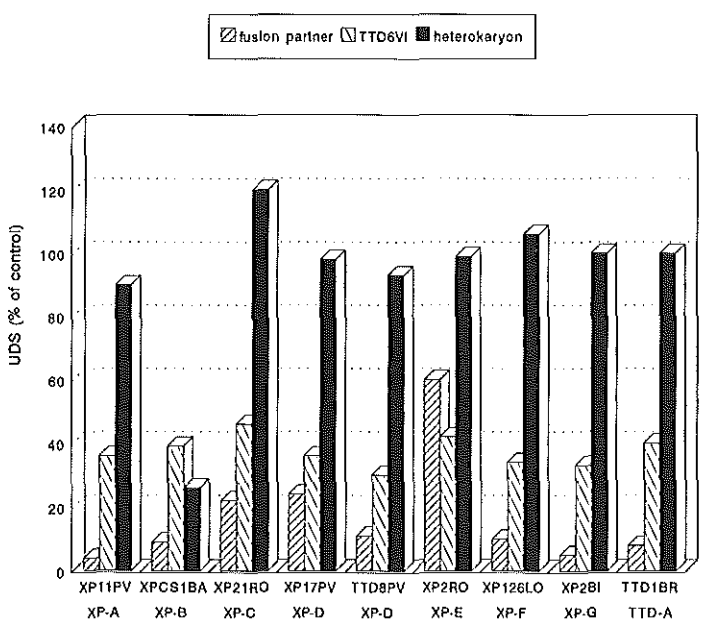


Figure 1. Complementation analysis of TTD6VI cells. Fibroblasts of TTD6VI (labeled with 0.8-μm beads) were fused with representatives of different XP and TTD complementation groups (labeled with 2.0-μm beads). After performing the UV-induced UDS assay, the average number of autoradiographic grains above the nuclei was determined. DNA repair synthesis was expressed as the percentage of control UDS (vertical axis) observed in normal fibroblasts assayed in parallel. The relative standard errors of the mean are in all cases less than 5%. The different types of binuclear cells (homodikaryons of TTD6VI and of the different fusion partners, and heterodikaryons) are recognized by their different bead content and indicated as such in the figure.

microneedle injection into fibroblasts of all known excision-deficient XP, XP/CS, and TTD complementation groups. The injected cultures were exposed to UV and incubated with [^{3}H]thymidine to permit visualization of the repair synthesis step of NER by in situ autoradiography (unscheduled DNA synthesis, UDS). The results, summarized in Table 1, show that 3 out of 8 NER-deficient human complementation groups are corrected by the purified BTF2 transcription factor. Figure 2 presents micrographs of BTF2-injected multinucleated fibroblasts of XP-D, TTD-A, and the new XP-B patient with TTD symptoms. The repair activities in injected cells reach the levels of normal fibroblasts assayed in parallel, indicating full correction of the excision defect (see also Table 2).

The restoration of NER could also be explained by correction of expression of one or more critical repair genes whose transcription is impaired by mutations in BTF2 subunits. Therefore, purified BTF2 was checked for its repair capacity in an in vitro repair assay (Wood et al. 1988; van Vuuren et al. 1993), where neither transcription nor translation can occur, because ribonucleotides and amino acids are lacking. The results in Figure 3 and Table 1 show that the BTF2 fraction not only corrects the repair defect in rodent group 3 (the equivalent of XP-B), as reported earlier (van Vuuren et al. 1994), but also the rodent group equivalent to XP-D (CHO group 2, defective in ERCC2) (Flejter et al. 1992) and an extract from a SV40-transformed TTD1BR cell line (TTD-A). In contrast, no significant NER restoration is observed in extracts of UV-sensitive mutants from groups 1, 4, and 5 (defective in ERCC1, ERCC4, and XPG/ERCC5, respectively). These findings extend the specificity of the repair-correcting activity of TFIIH and confirm the in vivo results obtained by microinjection. Furthermore, they demonstrate that besides XPB/ERCC3, XPD/ERCC2 and TTD-A must also have a direct involvement in NER. The in vitro and in vivo correction of *ERCC2* mutants confirmed the in vitro data of Drapkin et al. (1994), who used a partially purified TFIIH preparation. However, the TFIIH/BTF2 purified fractions used in this study do not contain a XPC-correcting activity, in contrast to the observations made by these authors.

Table 1. Involvement of BTF2 in Different Human and Rodent NER-deficient Complementation Groups

NER-deficient complement. groups		Correction of NER defect by BTF2
human mutants		microneedle injection
XP25RO	XP-A	–
XPCS1BA	XP-B	+
XP21RO	XP-C	–
XP1BR	XP-D	+
XP2RO	XP-E	–
XP126LO	XP-F	–
XP2BI	XP-G	–
TTD1BR	TTD-A	+
		in vitro repair assay
TTD1BRSV	TTD-A	+
rodent mutants		
43.3B	group 1	–
UV 5	group 2	+
27.1	group 3	+
UV 41	group 4	–
UV 135	group 5	–

Relationship of XPD/ERCC2 and TTD-A with the Core of TFIIH

To further strengthen the link between NER and the TFIIH complex, the full elution profile of hydroxyapatite chromatography, the final purification step of BTF2 (Gerard et al. 1991), was quantitatively screened for NER activity by microinjection as well as by in vitro complementation. The XP-D and TTD-A correcting activities coelute with the activities of XP-B correction and transcription initiation (Fig. 4). Identical results were obtained after cosedimentation in glycerol gradients (van Vuuren et al. 1994; W. Vermeulen et al., unpubl.).

Independent evidence for a physical association of the XPD/ERCC2, XPB/ERCC3, and TTDA proteins with each other and with other components in the TFIIH complex can be obtained from antibody depletion experiments. A crude repair-proficient HeLa extract was incubated with a monoclonal antibody against the p62 TFIIH subunit (Fischer et al. 1992), immobilized on protein A-Sepharose beads, and after centrifugation to remove bound proteins, the extract was tested for remaining repair capacity. Western blot analysis verified that the amount of p62 was strongly reduced (Fig. 5). In the in vitro correction assay, the p62^{depleted} HeLa extract had lost most of its repair activity when compared with the repair level of mutant extracts alone and a mock-treated extract (see footnote to Table 2). To examine whether other NER factors were co-depleted, the treated extract was tested for its complementing capacity using both the in vitro assay and the microneedle injection. Previously, we have demonstrated that XPB/ERCC3, but not XPG/ERCC5, is simultaneously removed with p62 (van Vuuren et al. 1994). Table 2 shows that most XPD/ERCC2 and TTDA activities are also removed, whereas XPA, ERCC1, ERCC4, and XPG/ERCC5 are not significantly eliminated (Table 2 and unpublished research). This is confirmed by the Western blot analysis revealing depletion of XPB/ERCC3(p89) at the protein level (Fig. 5, lanes 5,6). The same co-depletion patterns were observed with a monoclonal antibody against p89 (XPB/ERCC3) (Table 2). Furthermore, depletion experiments using monoclonal antibodies against two other components of TFIIH, p44 (the human homolog of yeast SSL1) and p34, revealed tight association with repair (Humbert et al. 1994; and results not shown). As with crude HeLa extracts, antibody depletion of purified BTF2 (hydroxyapatite and heparin purification fractions) using p62 monoclonal antibodies resulted in simultaneous removal of the XPB/ERCC3, XPD/ERCC2, and TTDA factors (Table 2). The depletion

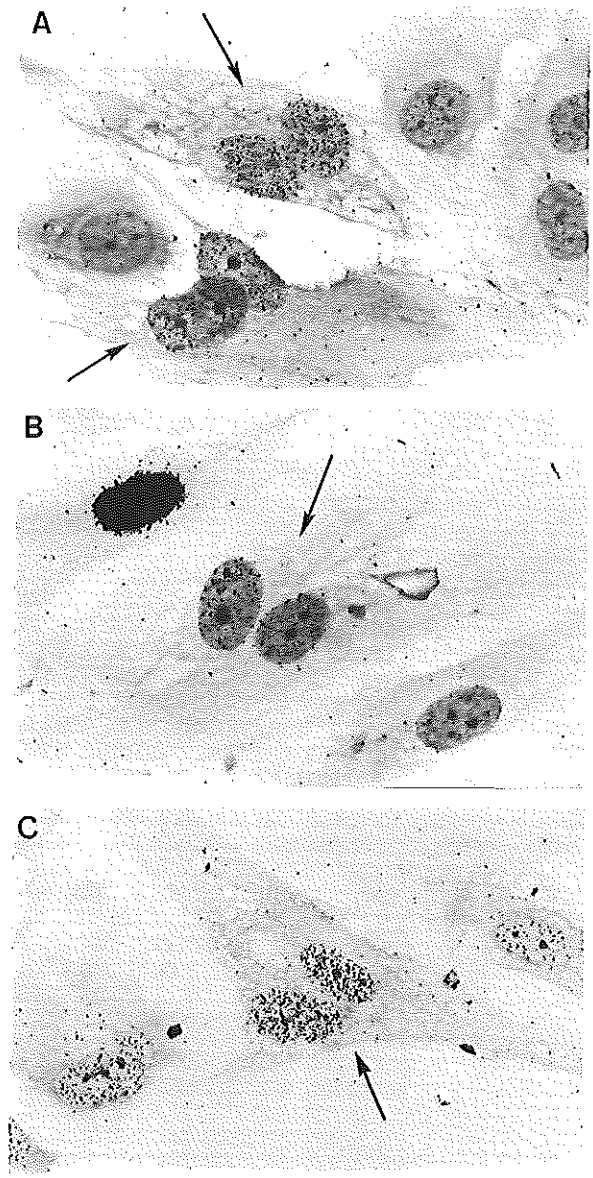


Figure 2. Microneedle injection of BTF2 in XP-D (*A*), TTD-A (*B*), and XP-B (*C*) fibroblasts. Micrographs showing the effect of purified BTF2 (HAP-fraction 12, containing the highest transcriptional activity; Schaeffer et al. 1993) on NER-activity of injected XP-D (cell line XP1BR), TTD-A (TTD1BR), and XP-B (TTD6VI) fibroblasts, respectively. The injected cells (binuclear fibroblasts obtained by cell fusion prior to injection) are indicated by arrows. As apparent from the number of silver grains above their nuclei, they exhibit a high (wild-type) level of UV-induced UDS when compared to the noninjected surrounding cells, which express residual UDS levels typical for these complementation groups. The heavily labeled fibroblast in panel *B* is a cell performing S-phase replicative DNA synthesis at the moment of the UDS assay.

and correction experiments provide strong evidence that three repair factors are physically associated with three different TFIIH subunits and, thus, likely constitute an integral part of transcription factor BTF2.

Table 2 also shows that small amounts of correcting activities persist in the anti-p62-treated extract. This is largely due to incomplete removal of p62 (as shown by the Western blot analysis in Fig. 5). However, it is not fully excluded that a small fraction of the ERCC2, ERCC3, and TTDA molecules exist dissociated from (the p62 part of) TFIIH, or reside in forms of TFIIH lacking p62. The same holds for the anti-p89 experiments. A somewhat looser association between ERCC2 and the p62-ERCC3 core of TFIIH is also derived from independent dissociation studies by Schaeffer and coworkers (1994). Further indications for exchange of

Table 2. Effect of Immunodepletion of BTF2 Components on NER Activity

Repair assay	Immunodepleted material	Tested cells/extracts (compl. group)		NER activity (% of control) protA depl.	anti p62 depl.	anti p89 depl.	residual[a]
Microneedle injection	crude HeLa lysate	XP25RO	(XP-A)	99	100	94	2
		XPCS2BA	(XP-B)	103	39	33	13
		XP1BR	(XP-D)	98	41	39	21
		TTD1BR	(TTD-A)	102	29	23	8
Microneedle injection	BTF2 (heparin 5PW, fraction 12)	XPCS2BA	(XP-B)	92	37	35	12
		TTD6VI	(XP-B)	100	42	44	44
		XP1BR	(XP-D)	94	37	38	23
		TTD1BR	(TTD-A)	100	35	35	8
In vitro repair	crude HeLa lysate[b]	27.1	(group 3)	100	35	n.d.[c]	34
		UV-5	(group 2)	100	32	n.d.	24
		TTD1BR	(TTD-A)	100	32	n.d.	27

[a] Residual NER activity: amount of repair exhibited by mutated cells/extracts.
[b] p62-depleted crude extract exhibits a residual incorporation of 35% compared to a protA-treated control extract, which is close to incorporation by most mutant extracts.
[c] n.d.: Not determined.

various TFIIH subunits can be deduced from the in vitro complementation between ERCC2- and TTD-A-deficient extracts shown in Figure 3A (lanes 1–3), each of these extracts being defective in a different component of the same complex. In line with this, extracts defective in XPD/ERCC2 and XPB/ERCC3 also exhibit complementation in vitro (van Vuuren et al. 1993), and microinjection of free (recombinant) ERCC3 protein is able to induce a partial but clear correction of the repair defect of XP-B cells in vivo (van Vuuren et al. 1994). We conclude that a complex containing at least XPD/ERCC2, XPB/ERCC3, TTDA, and p62, and possibly also p44 (SSL1) and p34, is implicated in NER and that subunits of this complex can exchange in vivo and to some extent also in vitro. It is plausible that this complex represents the entire multisubunit TFIIH transcription factor.

Is TTDA Identical to Any of the Cloned TFIIH Subunits?

A prediction of the findings reported above is that one of the components of BTF2 is responsible for the repair defect in TTD-A for which no repair gene has yet been isolated. Therefore, cDNAs encoding the cloned TFIIH subunits p62, p44, and p34 (Humbert et al. 1994), and *XPD/ERCC2*(p80) and *XPB/ERCC3*(p89) were inserted into a mammalian expression vector and injected into the nucleus of TTD-A fibroblasts. Prior to injection, the cDNA-containing

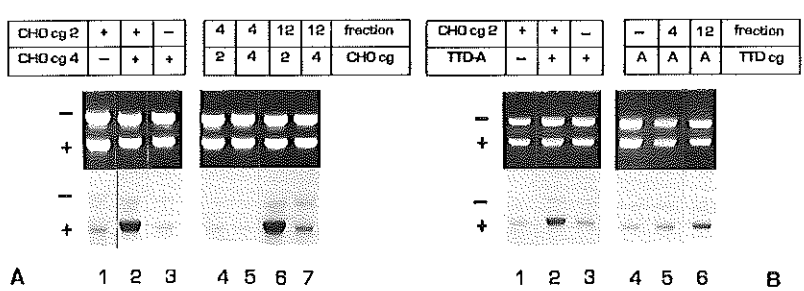


Figure 3. In vitro correction of NER defect by purified BTF2. BTF2 HAP-fraction 12 was added to an extract of rodent complementation group 2 (mutant UV5, equivalent to XP-D)(*A*), and to a human TTD-A lysate (TTD1BRSV)(*B*), and tested for its capacity to restore the NER defect using an in vitro cell-free repair assay (Wood et al. 1988). The level of correction of NER activity using purified BTF2 is of the same order (lanes *6,A* and *B*) as the level of complementation reached when the extract of group 2 is mixed with a group 4 extract (lane *2A*) or with the TTD-A extract (lane *2B*). No significant correction is observed when the purified BTF2-fraction is added to rodent complementation group 4 (mutant UV47) extract (lane *7A*). The upper panel shows the ethidium-bromide-stained DNA gel, the lower panel the autoradiogram of the dried gel. The presence of [^{32}P]dATP indicates repair synthesis. The positions of AAF-damaged and non-damaged DNA substrates, 250 ng each, are indicated by (+) and (–), respectively. Lanes *1–3* contain in total 200 μg of cell-free extract (in complementations, 100 μg of each extract was used); the other lanes contain 100 μg. The protein contribution by the BTF2 fraction is negligible.

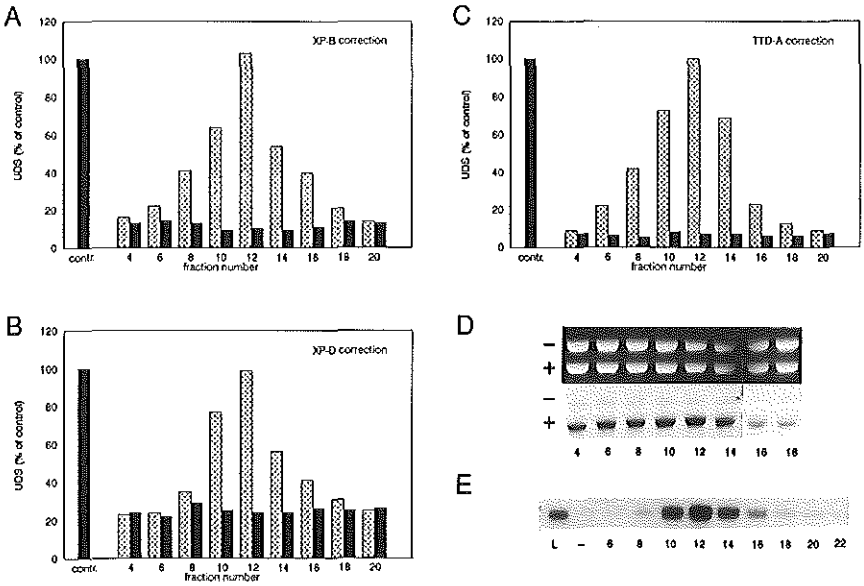


Figure 4. Correlation between transcription activation and correction of the NER defect in XP-D/ERCC2 and TTD-A mutant cells using different fractions from the HAP chromatography column. BTF2-derived in vivo NER-correcting activity was quantitatively determined, after microinjection, by counting the grains above the nuclei (UV-induced UDS) of injected cells and was expressed as the percentage of the UDS-level of repair-proficient control cells (C5RO) assayed in parallel (left black bar) (Vermeulen et al. 1986). The hatched bars indicate the average UDS level (determined by counting grains above 50 nuclei) of multikaryons injected with BTF2. The black bars represent UDS in noninjected neighboring cells. The in vivo NER activity profiles for, respectively, XP-B (XPCS1BA) (*A*), XP-D (XP1BR) (*B*), and TTD-A (TTD1BR) (*C*) are compared to the in vitro repair capacity for rodent complementation group 2 (*ERCC2* mutant UV5) extracts (*D*) and to the transcription activity, as determined in a BTF2-dependent in vitro transcription runoff assay using the adenovirus late promoter (*E*). Note that the UV5 (group 2) extract in panel *D* (like the XP-D cells in panel *B*) has a considerable residual repair. The lower activity seen in the higher salt fractions (lanes *16–18*) is attributed to an inhibition of repair incorporation by increased salt (Wood et al. 1988).

vectors were checked for their ability to specify proteins of the predicted size. None of these genes (*p62, p44, p34*), nor any of the cloned NER genes (*ERCC1, XPD/ERCC2, XPB/ERCC3, CSB/ERCC6, XPA, XPC,* and the genes *HHR23A* and *B*; Masutani et al. 1994), were able to exert correction of the repair defect, whereas microinjected *ERCC2* and *ERCC3* were able to restore UDS to XP-D and XP-B cells, respectively, in the same experiments. It appears that the TTDA factor is yet another non-cloned component of TFIIH.

Evidence for Direct Involvement of XPD/ERCC2 in Transcription In Vivo

The data described thus far tightly link the repair protein XPD/ERCC2 with transcription factor TFIIH/BTF2; however, they do not demonstrate that the protein is directly involved in transcription. Recently, Schaeffer and coworkers (1994) have shown that a p80 protein associated with TFIIH is identical to XPD/ERCC2 and that this protein stimulates the TFIIH-dependent in vitro transcription reaction. In addition, Drapkin and coworkers (1994) have shown that an antibody directed against ERCC2 is able to inhibit the in vitro transcription reaction. To verify whether the in vitro results can be extrapolated to the in vivo situation, we have conducted antibody microinjection experiments into living normal cells. As shown in Figure 6A and quantitatively in Table 3, introduction of antibodies against XPD/ERCC2 causes a strong inhibition of UV-induced UDS, consistent with the direct role of the protein in NER (Figs. 2 and 3) and similar to the effect observed using antibodies against another repair protein (ERCC1) that is not detectably associated with purified BTF2 (Table 3). In addition, a strong inhibition of general transcription (as measured by a 1-hour [^{3}H]uridine labeling) is found (Fig. 6B), which is absent in the injections with ERCC1 antiserum (Table 3). Similar, but less pronounced, effects on transcription and UDS are exerted by a less powerful serum against the XPB/ERCC3 component of TFIIH (Table 3) (see also van Vuuren et al. 1994). These results indicate that XPD/ERCC2, like XPB/ERCC3, is involved in transcription in vivo.

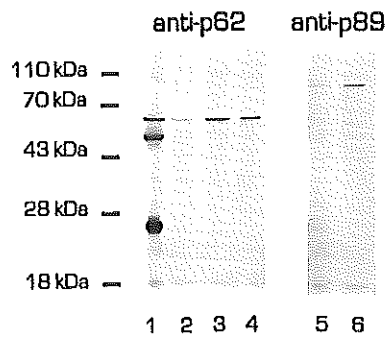


Figure 5. Western blot analysis of p62 and p89 depletion of a repair-proficient HeLa extract. Monoclonal antibody (MAb3C9) against the p62 subunit of BTF2(TFIIH) coupled to protein-A-Sepharose beads was incubated with a repair-proficient extract prepared from HeLa cells. To verify the removal of p62 and simultaneous removal of other components of BTF2, the remaining cell-free extract and the bound fraction were analyzed by immunoblotting using monoclonal antibodies against p62 itself (lanes *1–4*) and against the p89 (XPB/ERCC3) subunit of BTF2 (lanes *5* and *6*). Lane *1* contains the protein fraction bound to the anti-p62 beads, released by SDS (the strong bands at 55 kD and 25 kD represent the heavy and light chains of the antibodies released from the beads). (Lanes *2* and *5*) HeLa whole-cell extract treated with the anti-p62 beads. (Lanes *3* and *6*) HeLa whole-cell extract treated with protein-A beads alone (control). (Lane *4*) Untreated HeLa whole-cell extract. In lanes *2–6*, equal amounts of sample were loaded.

DISCUSSION

The findings reported here have implications in several directions. At the molecular level, the identification of at least three repair factors in TFIIH endows this complex with a dual functionality and extends the functional overlap between two quite different processes, basal transcription and NER. At the clinical level, the identification of a link between TTD and XP/CS group B extends the association between TFIIH subunits and TTD and supports the notion that CS, TTD, and some forms of XP represent different manifestations of one large clinical continuum. The connection between TFIIH and TTD provides new clues to understand the basis of the complex clinical features displayed by these disorders and, potentially, a number of other, strikingly related syndromes.

Mechanistic Implications for Transcription and Repair

A minimum of three NER factors is found to be associated with transcription factor TFIIH. In addition to XPB/ERCC3 (Schaeffer et al. 1993), we demonstrate that XPD/ERCC2 and TTDA reside in this complex. We provide evidence for the involvement of the XPD/ERCC2 helicase in transcription in vivo. Circumstantial data support the idea that the transcription proteins p62, p44, and p34 are also implicated in NER (Humbert et al. 1994; van Vuuren et al. 1994; this paper). As previously anticipated (Bootsma and Hoeijmakers 1993), the human homolog of SSL1, a yeast repair factor which was linked to translation previously (Yoon et al. 1992), was recently identified as the p44 subunit of TFIIH (Humbert et al. 1994). Table 4 summarizes the current evidence for the involvement of different human BTF2/TFIIH and yeast factor b subunits in repair. It is possible that the TTDA protein is identical to the (non-cloned) p41 BTF2 subunit. Together, these data suggest that most, if not all, components of the transcription complex participate in excision repair, converting this complex into a functional unit participating in at least two processes.

It is still difficult to estimate how many polypeptides constitute TFIIH. Because of the absence of XPD/ERCC2 as a major protein in the most pure BTF2 preparation (Schaeffer et al. 1994; Drapkin et al. 1994), it is likely that the five predominant bands present in the final stages of BTF2 purification (Gerard et al. 1991) are derived from the most tightly associated protein fraction constituting the core of the complex. Protein profiles of the rat homolog (factor δ) suggest the presence of at least eight polypeptides (Conaway and Conaway 1989). Thus, BTF2 could well be considerably larger in vivo and form a "supercomplex" of which the more loosely bound factors have a tendency to (partly) dissociate during purification. This could also explain the absence of a XPC-correcting activity in our TFIIH purifications, whereas others found association of XPC with TFIIH, but not in the final purification fraction (Drapkin et al. 1994). Alternatively, it is possible that the XPC-correcting activity represents a spurious copurification with TFIIH. Association of XPC with TFIIH is somewhat unexpected, since XPC is thought to be selectively involved in the global genome (transcription-independent) NER pathway (Venema et al. 1990).

BTF2 possesses a bidirectional unwinding activity involving XPB/ERCC3 and XPD/ERCC2 (Schaeffer et al. 1994), which may promote transition from a closed to an open initiation complex. It is likely that TFIIH can be utilized as an independent unit in the context of transcription and NER; e.g., for loading and/or translocation of the preinitiation complex or a NER scanning/incision complex or for the repair synthesis step. The TFIIH mutants XP-B, XP-D, and TTD-A display defects in both the "transcription-coupled" and the "global genome" NER subpathways, so the complex is likely to play a role in the core of the NER reaction mechanism (Sweder and Hanawalt 1993).

Clinical Heterogeneity and Pleiotropy Associated with Mutations in TFIIH

The spectrum of diseases linked with TFIIH is heterogeneous and pleiotropic, including seemingly unrelated symptoms such as photosensitivity, brittle hair and nails, neurodysmyelination, impaired sexual

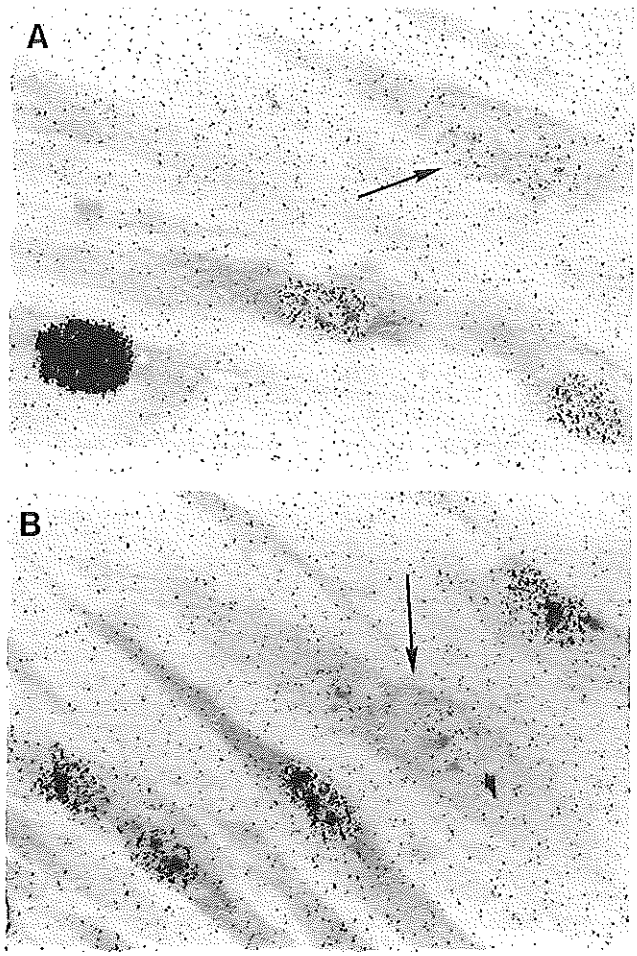


Figure 6. Effect of injection of antibodies against XPD/ERCC2 on transcription and repair of normal cells. Micrograph *A* demonstrates the effect of injection of ERCC2 antibodies on UV-induced UDS of normal fibroblasts, assayed by a 2-hr incubation in [^{3}H]thymidine immediately following UV-irradiation. Micrograph *B* shows the effect of injection of XPD/ERCC2 antiserum on RNA synthesis of control (wild-type) fibroblasts assayed by a 1-hr pulse-labeling with [^{3}H]uridine. The UDS and RNA synthesis assays were performed 20 hr after injection of the antibodies. The strong reduction of autoradiographic grains above the nuclei of injected dikaryons (*arrows*) compared to noninjected cells indicates a virtually complete inhibition of RNA and repair synthesis. The heavily labeled nucleus in *A* is from a cell in S phase at the time of incubation. Note that these preparations have a higher background labeling than the micrographs shown in Fig. 1.

development, ichthyosis, and dental caries. Both the rare XP group B and the more common group D present pronounced clinical heterogeneity: classical XP (only in group D), atypical combination of XP and CS, and TTD (Johnson and Squires 1992; Vermeulen et al. 1994; this paper). The occurrence of patients displaying TTD symptoms within XP-B extends the parallels between XP-B and XP-D and their respective gene products noted before (Weeda et al. 1990). Clinical variability in TTD is even observed within families and also apparent from the close association with at least seven disorders (shown in Fig. 7) appearing in the On-line Mendelian Inheritance in Man (OMIM) database (McKusick 1992). The occurrence of TTD in three NER-deficient complementation groups argues against a chance association between genetic loci separately involved in NER and in brittle hair. Consistent with this notion, mutations in the *XPD/ERCC2* gene have been detected recently in TTD(XP-D) patients (Broughton et al. 1994).

Table 3. Effect of Antibody Injection on Repair and Transcription

Injected antiserum	% inhibition of NER[a]	% inhibition of transcription[b]
Rabbit anti ERCC1[c]	97	0
Rabbit anti ERCC3[c]	43	48
Rabbit anti ERCC2	87	85
Preimmune rabbit serum	2	7

[a] Compared to UDS level observed in uninjected cells on the same slide.

[b] Compared to transcription level (assayed by 1-hr pulse labeling with [^{3}H]uridine) observed in uninjected cells on the same slide.

[c] van Vuuren et al. (1994).

How can we rationalize the pleiotropy and clinical heterogeneity in the above-mentioned conditions? The symptoms associated with a sole NER defect are displayed by the most common XP groups, A (totally deficient in NER) and C (defective in the "genome overall" repair subpathway). Patients in these groups present a relatively uniform clinical picture involving photosensitivity, pigmentation abnormalities, predisposition to skin cancer and, in the case of XP-A, accelerated neurodegeneration (which is not associated with neurodysmyelination), but no CS and TTD symptoms. The gene products affected in these groups are not vital and therefore do not appear to be essential for basal transcription.

Obviously, the hallmarks of a pure NER deficiency do not include the salient features of CS and TTD. It is tempting to link these with the additional transcription-related function. Indeed, it would be highly unlikely when all mutations in the three subunits of this bifunctional complex would only affect the repair function and leave the inherent transcriptional role entirely intact. This interpretation is supported by the *haywire* phenotype of the *Drosophila ERCC3* mutant, involving UV sensitivity, central nervous system abnormalities, and impaired sexual development, as found in XP-B (Mounkes et al. 1992). Spermatogenesis in *Drosophila* is very sensitive to the level of β_2-tubulin (Kemphues et al. 1982). Mutations in the *Drosophila ERCC3* gene seem to affect β-tubulin expression, causing male sterility (Mounkes et al. 1992). In mammals, β-tubulin mRNA is selectively regulated by a unique cotranslational degradation mechanism (Theodorakis and Cleveland 1992). It is possible that this renders β-tubulin expression particularly sensitive to the level of transcription and thereby to subtle mutations in BTF2, resulting in the immature sexual development found in TTD and CS. Similarly, reduced transcription of genes encoding ultrahigh sulfur proteins of the hairshaft may account for the observed reduced cysteine content in the brittle hair of TTD patients (Itin and Pittelkow 1990). Low expression of the myelin basic protein, whose transcription is known to be rate-limiting in the mouse (Readhead et al. 1987), may cause the characteristic neurodysmyelination of CS and TTD (Peserico et al. 1992; Sasaki et al. 1992). A comparable explanation is proposed for the poor enamelation of teeth in CS and TTD (Nance and Berry 1992; McCuaig et al. 1993). The skin abnormalities typical of TTD often involve ichthyosis. Various classes of ichthyoses show abnormalities in the production of filaggrin (Fleckman and Dale 1993). Thus, mutations in TFIIH which subtly disturb its transcription function may affect a specific subset of genes whose functioning critically depends on the level or fine-tuning of transcription. Recent studies indicate that the requirement for basal transcription factors may vary from promoter to promoter depending on the sequence around the initiation site, the topological state of the DNA, and the local chromatin structure (Parvin and Sharp 1993; Stanway 1993; Timmers 1994). These mechanisms can easily explain the pronounced clinical heterogeneity even within families.

On close inspection (Bootsma and Hoeijmakers 1993), many parallels can be found between CS and TTD. To a varying degree, CS patients exhibit features prominent in TTD, such as thin, dry hair and scaly skin (Nance and Berry 1992). TTD has recently been recognized to include neurodysmyelination (Peserico et al. 1992), bird-like facies, dental caries, and cataract (McCuaig et al. 1993), hallmarks normally associated with CS. This suggests that CS and TTD are manifestations of a broad clinical continuum, consistent with the notion that mutations in different subunits of the same (TFIIH) complex give rise to a similar set of

Table 4. The NER connection of BTF2/TFIIH

NER proteins associated with BTF2	NER proteins with yeast factor b	Polypeptides identified in SDS-PAGE
XPB/ERCC3	**RAD25/SSL2**[b]	**p89**
XPD/ERCC2	**RAD3**[c]	**p80**
TTD-A	?	?
p62[a]	TFB1[d]	p62
p44[a]	SSL1[d]	p44
?	?	p41[e]
?	?	p34

Factors for which the involvement in NER is unequivocally demonstrated are in boldface.

[a] NER function based on inference from (presumed) NER involvement of yeast homologs.

[b] References: Park (1992); Feaver (1993).

[c] Reference: Feaver (1993).

[d] Some alleles are UV sensitive, suggesting that they are deficient in NER.

[e] The relationship between p41 and TTD-A is not yet known.

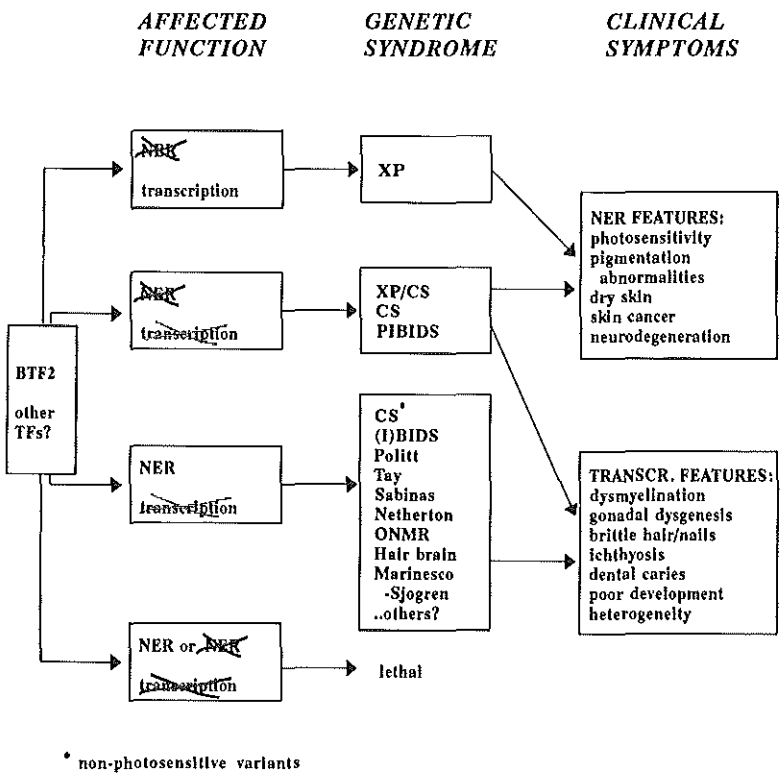


Figure 7. Model for involvement of defects in transcription and repair in human disease (see text for explanation).

phenotypic features. In this proposition, defects in the classical CS genes *CSA* and *ERCC6/CSB* (Troelstra et al. 1992), as well as *XPG/ERCC5* (Bootsma and Hoeijmakers 1993; Vermeulen et al. 1993), are expected to somehow affect basal transcription as well.

Model for Involvement of Transcription and Repair in CS and TTD: Deduction of the Existence of Transcription Syndromes

A tentative model proposed for the etiology of the defects in the conglomerate of CS, TTD, and related disorders is shown in Figure 7. In this model, mutations in BTF2 factors inactivating only the NER function result in a XP phenotype as observed in the classic XP patients of XP-D. If, in addition, the transcription function is subtly affected, the photosensitive forms of combined XP/CS and TTD are found. Theoretically, mutations causing a (still viable) transcription problem without NER impairment are predicted. The notion that the new TTD XP-B members have only a mild repair defect (see Table 2) but nevertheless display TTD features not exhibited by the more repair-deficient original XP-B cases fits perfectly into this reasoning. Indeed, a significant proportion of TTD patients, as well as clinically characteristic CS patients, is not noticeably photosensitive and has normal NER (Lehmann 1987; Nance and Berry 1992; Lehmann et al. 1993). These findings extend the implications to non-repair-defective disorders. Therefore, and in view of the pronounced heterogeneity inherent to the model, we propose that the Sjögren-Larsson (270200), RUD (308200), ICE (146720), OTD (257960), IFAP (308205), CAM(F)AK (214550), Rothmund-Thompson (268400), and KID (242150) syndromes (for references, see Baden 1991; McKusick 1992) also fall within this category. Interestingly, some of these diseases show occurrence of skin cancer.

In conclusion, our findings provide evidence for the presence of a wide class of disorders that we propose to designate collectively as "transcription syndromes." A prediction from our model is that these patients carry mutations in transcription factors that do not affect the NER process. This proposition is testable. The explanation put forward here for this class of disorders would introduce a novel concept into human genetics. It can be envisaged that similar phenomena are associated with subtle defects in translation, implying the potential existence of "translation syndromes" (as suggested

earlier on completely different grounds; Fisher et al. 1990).

ACKNOWLEDGMENTS

We gratefully acknowledge S. Humbert and V. Moncollin for help in BTF2 purification, Elena Botta for help with the complementation analysis, C. Backendorf for helpful information on cornified envelopes, A. van Oudenaren for help with the densitometer scanning, and J.W. van Klaveren for help with the computer. M. Kuit is acknowledged for photography. This work was supported in part by grants from the Netherlands Foundation for Chemical Research (SON), the Dutch Cancer Society (EUR 90-20, 92-118), The Dutch Scientific Organization, Medical Scientific Research (900-501-113 and 093), the INSERM, the C.N.R.S., the Ministère de la Recherche et de l'Enseignement Supérieur, the Association pour la Recherche sur le Cancer, and the Associazione Italiana per la Ricerca sul Cancro.

REFERENCES

Baden, H. 1991. Keratinizing disorders. In *Genetic disorders of the skin* (ed. J.C. Alpen), p.170. Mosby-Year Book, St. Louis, Missouri.

Biggerstaff, M., P. Robins, D. Coverley, and R.D. Wood. 1991. Effect of exogenous DNA fragments on human cell extract-mediated DNA repair synthesis. *Mutat. Res.* **254:** 217.

Bohr, V.A. 1991. Gene specific DNA repair. *Carcinogenesis* **12:** 1983.

Bootsma, D. and J.H.J. Hoeijmakers. 1993. Engagement with transcription. *Nature* **363:** 114.

Broughton, B.C., H. Steingrimsdottin, C.A. Weber, and A.R. Lehmann. 1994. Mutations in the xeroderma pigmentosum group D DNA repair/transcription gene in patients with trichothiodystrophy. *Nat. Genet.* **7:** 189.

Busch, D., C. Greiner, K. Lewis, R. Ford, G. Adair, and L. Thompson. 1989. Summary of complementation groups of UV-sensitive *CHO* mutants isolated by large-scale screening. *Mutagenesis* **4:** 349.

Cleaver, J.E. and K.H. Kraemer. 1994. Xeroderma pigmentosum and Cockayne syndrome. In *the metabolic basis of inherited disease*, 7th edition (ed. C.R. Scriver et al.). McGraw-Hill, New York.

Conaway, R.C. and J.W. Conaway. 1989. An RNA polymerase II transcription factor has an associated DNA-dependent ATPase (dATPase) activity strongly stimulated by the TATA region of promoters. *Proc. Natl. Acad. Sci.* **86:** 7356.

Drapkin, R., A. Merino, and D. Reinberg. 1993. Regulation of RNA polymerase II transcription. *Curr. Biol.* **5:** 469.

Drapkin, R., J.T. Reardon, A. Ansari, J.C. Huang, L. Zawel, K. Ahn, A. Sancar, and D. Reinberg. 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature* **368:** 769.

Fischer, L., M. Gerard, C. Chalut, Y. Lutz, S. Humbert, M. Kanno, P. Chambon, and J.-M. Egly. 1992. Cloning of the 62-kilodalton component of basic transcription factor BTF2. *Science* **257:** 1392.

Fisher, E.M.C., P. Beer-Romero, L.G. Brown, A. Ridley, J.A. McNeil, J.B. Lawrence, H.F. Willard, F.R. Bieber, and D.C. Page. 1990. Homologous ribosomal protein genes on the human X and Y chromosomes: Escape from X inactivation and possible implications for Turner syndrome. *Cell* **63:** 1205.

Fleckman, P. and B.A. Dale. 1993. *Structural protein expression in the ichthyosis* (ed. B.A. Bernard and B. Shroot). Karger, Basel, Switzerland.

Flejter, W.L., L.D. McDaniel, D. Johns, E.C. Friedberg, and R.A. Schultz. 1992. Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: Involvement of the human *ERCC2* DNA repair gene. *Proc. Natl. Acad. Sci.* **89:** 261.

Gerard, M., L. Fischer, V. Moncollin, J.-M. Chipoulet, P. Chambon, and J.-M. Egly. 1991. Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. *J. Biol. Chem.* **266:** 20940.

Gill, G. and R. Tjian. 1992. Eukaryotic coactivators associated with the TATA box binding protein. *Curr. Opin. Genet. Dev.* **2:** 236.

Goodrich, J.A. and R. Tjian. 1994. Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell* **77:** 145.

Hanawalt, P. and I. Mellon. 1993. Stranded in an active gene. *Curr. Biol.* **3:** 67.

Hoeijmakers, J.H.J. 1993. Nucleotide excision repair. II. From yeast to mammals. *Trends Genet.* **9:** 211.

Humbert, S., A.J. van Vuuren, Y. Lutz, J.H.J. Hoeijmakers, J.-M. Egly, and V. Moncollin. 1994. Characterization of p44/SSL1 and p34 subunits of the BTF2/TFIIH transcription/repair factor. *EMBO J.* **13:** 2393.

Itin, P.H. and M.R. Pittelkow. 1990. Trichothiodystrophy: Review of sulfur-deficient brittle hair syndromes and association with the ectodermal dysplasias. *J. Am. Acad. Dermatol.* **22:** 705.

Johnson, R.T. and S. Squires. 1992. The XPD complementation group. Insights into xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy. *Mutat. Res.* **273:** 97.

Kemphues, K.J., T.C. Kaufman, R.A. Raff, and E.C. Raff. 1982. The testis-specific beta-tubulin subunit in *Drosophila melanogaster* has multiple functions in spermatogenesis. *Cell* **31:** 655.

Lehmann, A.R. 1987. Cockayne's syndrome and trichothiodystrophy: Defective repair without cancer. *Cancer Rev.* **7:** 82.

Lehmann, A.R., A.F. Thompson, S.A. Harcourt, M. Stefanini, and P.G. Norris. 1993. Cockayne's syndrome: Correlation of clinical features with cellular sensitivity of RNA synthesis to UV irradiation. *J. Med. Genet.* **30:** 679.

Lu, H., L. Zawel, L. Fisher, J.-M. Egly, and D. Reinberg. 1992. Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. *Nature* **358:** 641.

Manley, J.L., A. Fire, M. Samuels, and P.A. Sharp. 1983. In vitro transcription: Whole cell extract. *Methods Enzymol.* **101:** 568.

Masutani, C., K. Sugasawa, J. Yanagisawa, T. Sonoyama, M. Ui, T. Enomoto, K. Takio, K. Tanaka, P.J. van der Spek, D. Bootsma, J.H.J. Hoeijmakers, and F. Hanaoka. 1994. Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homolog of yeast RAD23. *EMBO J.* **13:** 1831.

McCuaig, C., D. Marcoux, J.E. Rasmussen, M.M. Werner, and N.E. Genter. 1993. Trichothiodystrophy associated with photosensitivity, gonadal failure, and striking osteosclerosis. *J. Am. Acad. Dermatol.* **28:** 820.

McKusick, V.A. 1992. Catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes. In *Mendelian inheritance in man*. Johns Hopkins University Press, Baltimore, Maryland.

Mounkes, L.C., R.S. Jones, B-C. Liang, W. Gelbart, and M.T. Fuller. 1992. A *Drosophila* model for xeroderma pigmentosum and Cockayne's syndrome: *haywire* encodes the fly homolog of *ERCC3*, a human excision repair gene. *Cell* **71:** 925.

Nance, M.A. and S.A. Berry. 1992. Cockayne syndrome: Review of 140 cases. *Am. J. Med. Genet.* **42:** 68.

Parvin, J.D. and P.A. Sharp. 1993. DNA topology and a minimum set of basal factors for transcription by RNA polymerase II. *Cell* **73:** 533.

Peserico, A., P.A. Battistella, and P. Bertoli. 1992. MRI of a very hereditary ectodermal dysplasia: PIBI(D)S. *Neuroradiology* **34:** 316.

Readhead, C., B. Popko, N. Takahashi, H.D. Shine, R.A. Saavedra, R.L. Sidman, and L. Hood. 1987. Expression of a myelin basic protein gene in transgenic shiverer mice: Correction of the dysmyelinating phenotype. *Cell* **48:** 703.

Roeder, R.G. 1991. The complexities of eukaryotic transcription initiation: Regulation of preinitiation complex assembly. *Trends Biochem. Sci.* **16:** 402.

Roy, R., L. Schaeffer, S. Humbert, W. Vermeulen, G. Weeda, and J.-M. Egly. 1994. The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. *J. Biol. Chem.* **269:** 9826.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sasaki, K., N. Tachi, M. Shinoda, N. Satoh, R. Minami, and A. Ohnishi. 1992. Demyelinating peripheral neuropathy in Cockayne syndrome: A histopathologic and morphometric study. *Brain Dev.* **14:** 114.

Schaeffer, L., R. Roy, S. Humbert, V. Moncollin, W. Vermeulen, J.H.J. Hoeijmakers, P. Chambon, and J.-M. Egly. 1993. DNA repair helicase: A component of BTF2 (TFIIH) basic transcription factor. *Science* **260:** 58.

Schaeffer, L., V. Moncollin, R. Roy, A. Staub, M. Mezzina, A. Sarasin, G. Weeda, J.H.J. Hoeijmakers, and J.M. Egly. 1994. The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. *EMBO J.* **13:** 2388.

Serizawa, H., J.W. Conaway, and R.C. Conaway. 1993. Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. *Nature* **363:** 371.

Stanway, C.A. 1993. Simplicity amidst complexity in transcriptional initiation. *BioEssays* **15:** 559.

Stefanini, M., P. Lagomarisini, S. Gilliani, T. Nardo, E. Botta, A. Peserico, W.J. Kleyer, A.R. Lehmann, and A. Sarasin. 1993a. Genetic heterogeneity of the excision repair defect associated with trichothiodystrophy. *Carcinogenesis* **14:** 1101.

Stefanini, M., W. Vermeulen, G. Weeda, S. Giliani, T. Nardo, M. Mezzina, A. Sarasin, J.I. Harper, C.F. Arlett, J.H.J. Hoeijmakers, and A.R. Lehmann. 1993b. A new nucleotide-excision-repair gene associated with the disorder trichothiodystrophy. *Am. J. Hum. Genet.* **53:** 817.

Sweder, K.S. and P.C. Hanawalt. 1993. Transcription coupled DNA repair. *Science* **262:** 439.

Theodorakis, N.G. and D.W. Cleveland. 1992. Physical evidence for cotranslational regulation of beta-tubulin mRNA degradation. *Mol. Cell. Biol.* **12:** 791.

Timmers, H.T.M. 1994. Transcription initiation by RNA polymerase II does not require hydrolysis of the beta-gamma phosphoanhydride bond of ATP. *EMBO J.* **13:** 391.

Troelstra, C., A. van Gool, J. de Wit, W. Vermeulen, D. Bootsma, and J.H.J. Hoeijmakers. 1992. *ERCC6*, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. *Cell* **71:** 939.

van Vuuren, A.J., E. Appeldoorn, H. Odijk, A. Yasui, N.G.J. Jaspers, D. Bootsma, and J.H.J. Hoeijmakers. 1993. Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F. *EMBO J.* **12:** 3693.

van Vuuren, A.J., W. Vermeulen, L. Ma, G. Weeda, E. Appeldoorn, N.G.J. Jaspers, A.J. van der Eb, D. Bootsma, J.H.J. Hoeijmakers, S. Humbert, L. Schaeffer, and J.-M. Egly. 1994. Correction of xeroderma pigmentosum repair defect by basal transcription factor BTF2(TFIIH). *EMBO J.* **13:** 1645.

Venema, J., A. Van Hoffen, A.T. Natarajan, A.A. Van Zeeland, and L.H.F. Mullenders. 1990. The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA. *Nucleic Acids Res.* **18:** 443.

Vermeulen, W., P. Osseweijer, A.J. De Jonge, and J.H.J. Hoeijmakers. 1986. Transient correction of excision repair defects in fibroblasts of 9 xeroderma pigmentosum complementation groups by microinjection of crude human cell extracts. *Mutat. Res.* **165:** 199.

Vermeulen, W., J. Jaeken, N.G.J. Jaspers, D. Bootsma, and J.H.J. Hoeijmakers. 1993. Xeroderma pigmentosum complementation group G associated with Cockayne's syndrome. *Am. J. Hum. Genet.* **53:** 185.

Vermeulen, W., R.J. Scott, S. Potger, H.J. Muller, J. Cole, C.F. Arlett, W.J. Kleijer, D. Bootsma, J.H.J. Hoeijmakers, and G. Weeda. 1994. Clinical heterogeneity within xeroderma pigmentosum associated with mutations in the DNA repair and transcription gene *ERCC3*. *Am. J. Hum. Genet.* **54:** 191.

Weeda, G., R.C.A. Van Ham, W. Vermeulen, D. Bootsma, A.J. Van der Eb, and J.H.J. Hoeijmakers. 1990. A presumed DNA helicase encoded by *ERCC-3* is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. *Cell* **62:** 777.

Wood, R.D., P. Robins, and T. Lindahl. 1988. Complementation of the xeroderma pigmentosum DNA repair defect in cell-free extracts. *Cell* **53:** 97.

Yoon, H., S.P. Miller, E.K. Pabich, and T.F. Donahue. 1992. SSL1, a suppressor of a HIS4 5'-UTR stem-loop mutation, is essential for translation initiation and effects UV resistance in yeast. *Genes Dev.* **6:** 2463.