Critical Reviews in Biochemistry and Molecular Biology, 36(3):261–290 (2001)

Unraveling DNA Repair in Human: Molecular Mechanisms and Consequences of Repair Defect

*Narendra Tuteja and Renu Tuteja**

International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi–110070, India

* To whom correspondence should be addressed. Phone: 91-11-6197931. Fax: 91-11-6162316 e-mail: renu@icgeb.res.in; renu_tuteja@hotmail.com

ABSTRACT: Cellular genomes are vulnerable to an array of DNA-damaging agents, of both endogenous and environmental origin. Such damage occurs at a frequency too high to be compatible with life. As a result cell death and tissue degeneration, aging and cancer are caused. To avoid this and in order for the genome to be reproduced, these damages must be corrected efficiently by DNA repair mechanisms. Eukaryotic cells have multiple mechanisms for the repair of damaged DNA. These repair systems in humans protect the genome by repairing modified bases, DNA adducts, crosslinks and double-strand breaks. The lesions in DNA are eliminated by mechanisms such as direct reversal, base excision and nucleotide excision. The base excision repair eliminates single damaged-base residues by the action of specialized DNA glycosylases and AP endonucleases. Nucleotide excision repair excises damage within oligomers that are 25 to 32 nucleotides long. This repair utilizes many proteins to remove the major UV-induced photoproducts from DNA, as well as other types of modified nucleotides. Different DNA polymerases and ligases are utilized to complete the separate pathways. The double-strand breaks in DNA are repaired by mechanisms that involve DNA protein kinase and recombination proteins. The defect in one of the repair protein results in three rare recessive syndromes: xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy. This review describes the biochemistry of various repair processes and summarizes the clinical features and molecular mechanisms underlying these disorders.

KEY WORDS: DNA damage, UV photoproducts, excision repair, double-strand break repair, DNA repair disorders.

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I. INTRODUCTION

It is of vital importance for all living systems to ensure proper functioning and propagation of their genetic information. Numerous structural lesions or disorders accumulate in DNA either spontaneously or following genotoxic stress and as a result of this a severe threat to the stability of DNA is posed. The various chemical and physical genotoxic agents damage DNA and thereby induce mutations. These DNA lesions hamper processes like replication and transcription at the cellular level and this result in cell-cycle arrest, genomic instability and cell death. Both exogenous and endogenous agents cause the damage to DNA. Some of the exogenous DNA mutating agents are ultraviolet radiation, ionizing radiation, and alkylating agents. Ultraviolet irradiation and alkylating agents can cause a number of specific base changes, as well as cross-linking bases together. Ionizing radiation is thought to generate the majority of its mutational load by free radical production. On the other hand, the endogenous mutagenesis is the inevitable consequence of a large complex molecule present in a metabolically active environment. Some of the examples are depurination (which occurs because of the reaction of DNA in water), the effect of oxygen and free radicals (causing base damage and DNA strand breaks), and the errors caused by DNA replication (causing base mismatches and deletions).

The DNA double helix seems to have evolved so that mutations, even as small as individual base damage are easily recognized. Such recognition is usually by a change in the physical structure of the DNA double helix. Living cells have developed various strategies to eliminate most of these lesions so as to preserve a life-compatible genetic information and to do this efficiently an intricate network of DNA repair system has been generated. The repair systems protect the genome by repairing modified bases, DNA adducts, and crosslinks and double-strand breaks. Eukaryotic cells have multiple mechanisms for repairing damaged DNA. Furthermore, these pathways are co-

ordinated with other cellular functions, in particular gene transcription and cell cycle.

The three basic mechanisms by which lesions are eliminated from DNA are

1. Direct reversal (DR), carried out by O^6-methylguanine-DNA methyltransferase, which directly reverses some simple alkylation adducts.
2. Base excision repair (BER), which eliminates single damaged-base residues.
3. Nucleotide excision repair (NER), which excises damage within oligomers that are about 25 to 29 nucleotides long. This type of repair removes primarily bulky, helix distorting adducts. However, considerable overlap exists in substrate specificity of repair pathways and certain proteins are used in more than one pathway (Lindahl et al., 1997).

BER pathway is an essential pathway for DNA maintenance. It works mainly on nonbulky base adducts such as those caused by hydrolysis, oxygen free radicals and simple alkylating agents. The most obvious function of NER in eukaryotes is to remove the major UV-induced photoproducts caused by sunlight from irradiated DNA or it can repair the DNA containing bulky adducts. In particular the NER system repairs virtually everything and requires the action of multiple interacting proteins that locate the damage in DNA, remove it as a short oligonucleotide and synthesize a replacement patch.

UV radiation produces damage to DNA and induces formation of two major UV photoproducts, the cyclobutane pyrimidine dimers and 6-4 photoproducts (Figure 1). The cyclobutane pyrimidine dimers can be formed between any two adjacent pyrimidines. In Figure 1A, a thymine-thymine cyclobutane pyrimidine dimer is shown which is the most frequently occuring UV photoproduct (Protic-Sabljic et al., 1986; Doetsch, 1995). The 6-4 photoproducts are formed by covalent bond between the carbon 6 and carbon 4 of adjacent pyrimidines. These are the most frequently occurring UV photoproducts and occur at 5′-T-C-3′ (Figure 1 B), 5′-C-C-3′ and 5′-T-T-3′, but not at 5′-C-T-3′ sites in DNA (Doetsch, 1995). Both of these photoproducts are toxic and mutagenic lesions, which can be repaired by a number of pathways. These repair pathways include DNA excision repair (comprising the BER and NER pathways), enzymatic photoreactivation, recombination repair and post replications repair (Friedberg et al., 1995).

In animal cells, the major lesions caused by 254-nm UV light, cyclobutane pyrimidine dimers, are removed from the bulk of the genome 5 to 10 times more slowly than the second most abundant lesions, 6–4 photoproducts (Mitchell and Nairn, 1989; Szymkowski et al., 1993b). In UV-irradiated primates a DNA-binding protein specific for 6–4 photoproducts has been detected, suggesting that in mammalian cells different pathways may be used for the repair of these two photoproducts (Pfeifer et al., 1991).

Apart from chemical alterations to the bases in DNA, an important type of damage produced in DNA is the double-strand breaks. These are made under physiological conditions during somatic recombination and transposition. They are also one of the major products of ionizing radiation and of oxidative stress. There are at least two ways of repairing double-strand breaks in eukaryotes. The first, which mainly takes place in yeast, involves homologous recombination with a sister duplex. The second pathway which, predominates in mammals is an end-joining process, which employs the DNA-dependent protein kinase.

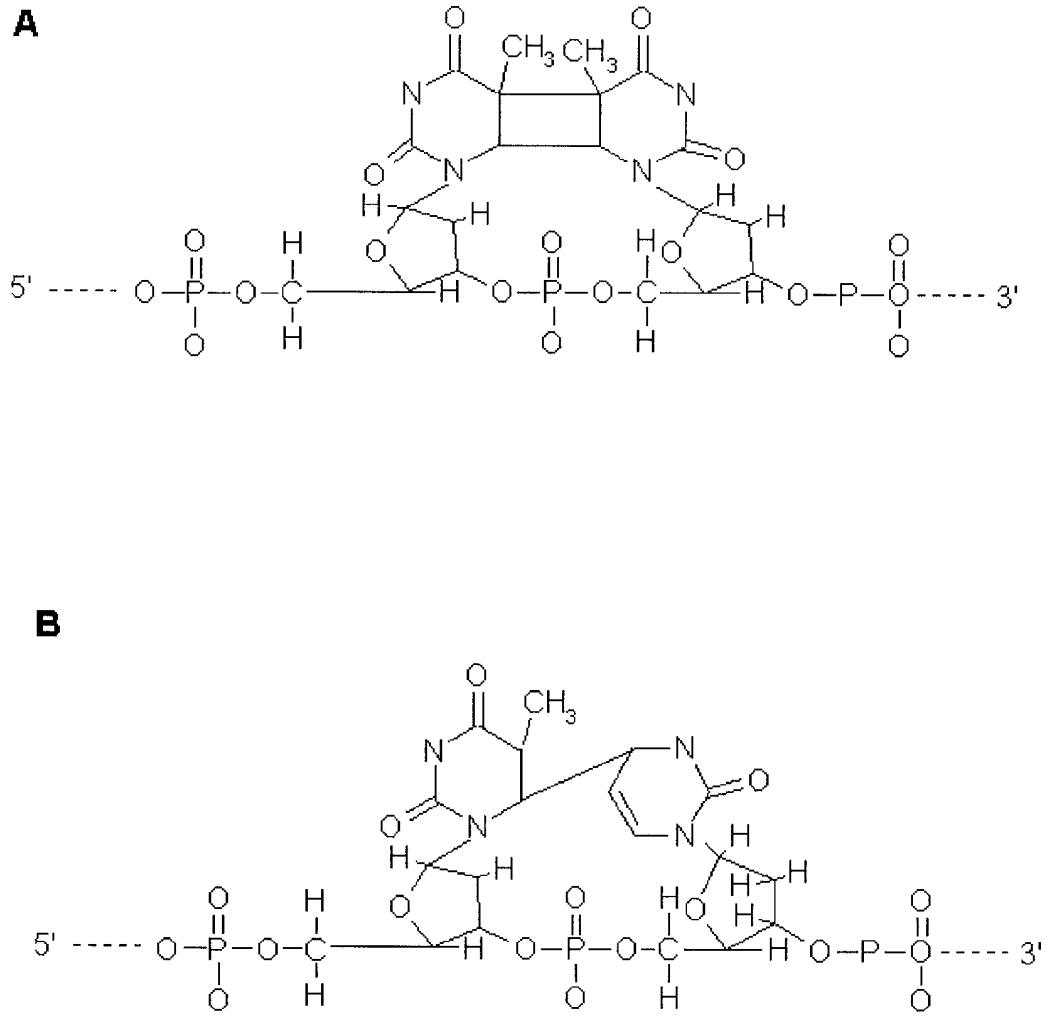

FIGURE 1. UV-light-induced DNA photoproducts. (A) Cyclobutane pyrimidine dimers (T-T). (B) 6-4 Photoproducts (5′-T-C-3′).

II. DIRECT REVERSAL

DR is the simplest mechanism, which involves a single enzyme reaction for removal of certain types of DNA damage. Alkyltransferases simply extract the alkyl group from alkylated bases that is transferred to an internal cysteine residue, and thus inactivating themselves (Teo et al., 1984). The best example for DR is the correction of the miscoding alkylation lesions O^6–methylguanine, which are generated endogenously in small amounts by reactive cellular catabolites. DR is carried out by a specific enzyme called methylguanine-methyltransferase (MGMT), which removes the methyl group from the guanine residue of DNA, and transfers it to one of its own cysteine residues in a rapid and error free repair process (Moore et al., 1998). O^6–methylguanine can pair with both C and T and thereby causes transition mutations, which are sometime corrected by, mismatch repair mechanism (O'Driscoll et al., 1998; Lindahl and Wood, 1999). Photolyases, on the other hand, revert UV-induced dimers in a light dependent reaction called photoreactivation (Sancar, 1990; Yasui and Eker 1998; Todo, 1999).

III. BASE EXCISION REPAIR

BER works mainly on DNA damages that can arise spontaneously in a cell from hydrolytic events such as deamination or base loss, fragmented bases resulting from ionizing radiation and oxidative damage or methylation of ring nitrogens by endogenous agents. BER pathway is the most important cellular protection mechanism responding to oxidative DNA damage, whether it occurs from reactive oxygen species formed during normal metabolism or from exposure to exogenous agents. A model for BER pathway is shown in Figure 2. There are two key events in base excision repair. First is the hydrolysis of the N-glycosyl bond linking a modified (or damaged) base to the deoxyribose-phosphate chain, which excises the base residue in the free form and creates an AP (apurinic/apyrimidinic) site on sugar-phosphate backbone of DNA. The class of enzymes called glycosylases carries out this release. Different DNA glycosylases remove different kinds of damage, and the specificity of repair pathway is determined by the type of glycosylase involved (Seeberg et al., 1995). In the second event of the reaction the resulting abasic sugar is cleaved by an AP endonuclease at 5′ to the AP site. There are a number of well-characterized glycosylases but only one major endonuclease has been reported in humans so far (Wood, 1996). This type of repair has a limited substrate range since the DNA glycosylases that initiate the repair process are in intimate contact with the lesion during catalysis. Completion of base excision repair requires the removal of the 5′ terminal deoxyribose-phosphate residue generated by the endonuclease, which is catalyzed by the phosphodiesterase activity of DNA polymerase β. The resulting one-nucleotide gap is filled by DNA polymerase β and sealed by either DNA ligase I or DNA ligase III with its accessory protein XRCC1 (Figure 2) (Lehmann, 1998). One of the several available alternate pathways can often repair a given lesion because complete loss of base excision repair would be incompatible with life.

A double knockout mutation of polymerase β in mice causes embryonic lethality (Gu et al., 1994). This finding suggests that either the single-patch mode of BER is essential for maintaining normal viability or that polymerase β has an additional essential role in mammalian cells such as in chromosomal DNA replication.

IV. NUCLEOTIDE EXCISION REPAIR

The main function of the ubiquitous nucleotide excision repair pathway is the removal of UV-induced lesions from DNA. It is the process whereby DNA damage is removed as part of an oligonucleotide fragment, followed by replacement with new DNA using the intact strand as template. This type of repair requires the action of multiple interacting proteins that locate the damage in DNA, remove it as a short oligonucleotide and synthesize a replacement patch. This process occurs in stepwise fashion, beginning with recognition of the DNA lesion, followed by enzymatic incisions in the damaged strand on both sides of the lesion, removal of the damaged single-stranded segment, repair synthesis to fill in the resultant gapped DNA duplex and ligation of the repair patch to the existing DNA strand. Six core factors, comprising 15 to 18 polypeptides, are required for dual incision of damage and another dozen or so polypeptides are needed for the repair synthesis step. The nucleotide excision repair machinery has very broad substrate specificity, being able to recognize a wide variety of

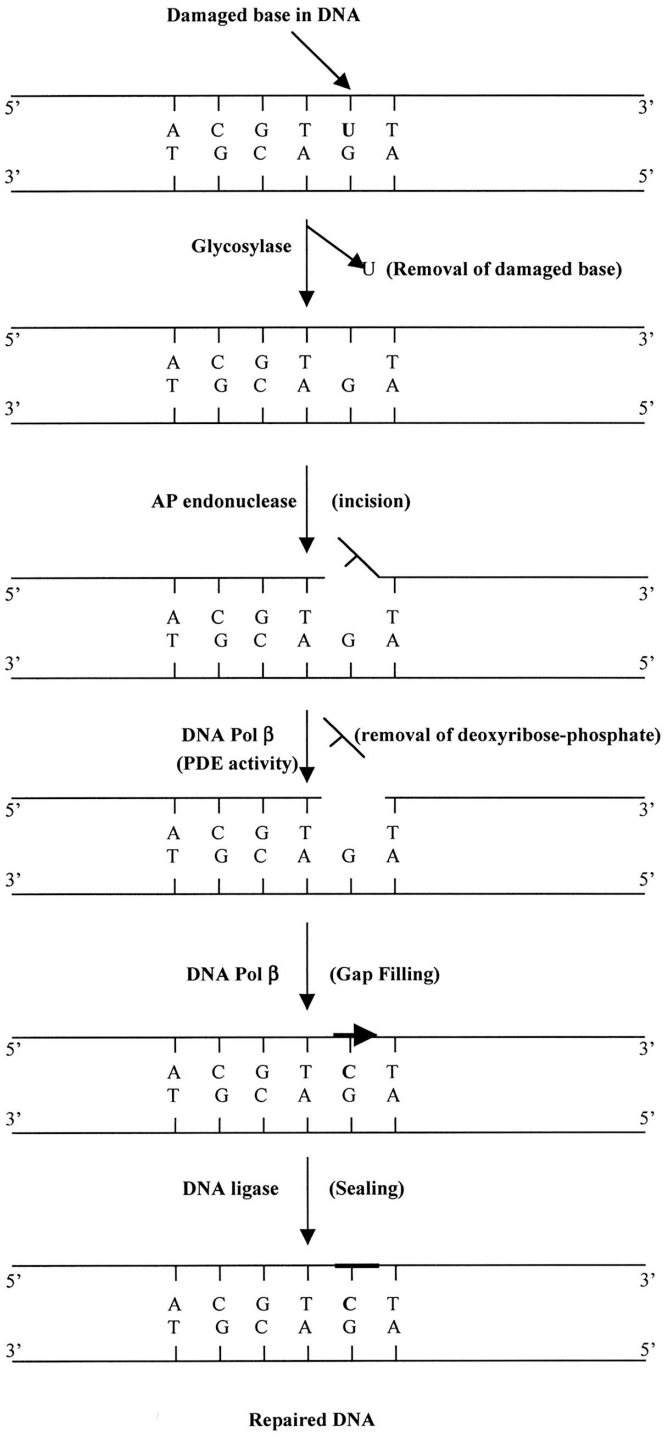

FIGURE 2. A model for base excision repair pathway in mammalian cells. AP endonuclease = apurinic/apyrimidinic endonuclease. PDE = phosphodiesterase.

chemical alterations to DNA that result in large local distortions of the DNA structure.

A. Steps and Proteins Involved in Nucleotide Excision Repair

The molecular mechanism of NER pathway in human is shown in Figure 3. This process involves the products of at least 30 genes. A defect in any of these genes leads to defective NER and results in a series of genetic disorders, which are discussed later.

Most of the mammalian NER genes have been isolated by transfection of genomic DNA into UV-sensitive rodent repair mutants followed by selection of UV-resistant transformants and retrieval of the correcting sequence (Hoeijmakers, 1993). The human genes correcting rodent repair defects are called ERCC genes, for excision repair cross-complementing rodent repair deficiency genes. The number refers to the rodent group that has been corrected.

Although most of the important players in mammalian NER are identified but only very recently have we begun to gain some insight into the mechanism by which proteins recognize chemical and structural modifications of canonical DNA, resulting from damage (Pearl and Savva, 1995; Lowndes and Murguia, 2000). Recently a "bipartite" or two-step model for recognition has been proposed (Lindahl and Wood, 1999). In the first step, a distortion is recognized and in the second, the damaged strand and chemical alterations are located.

It has been established that binding of the XP-C is the initial, damage-recognizing step in NER, which recruits the entire repair protein apparatus to the damage (Sugasawa et al., 1998). XP-E also has a role in damage recognition of cyclobutane pyrimidine lesions because it has a high affinity for UV-damaged DNA (Keeney et al., 1993). XP-A protein may be important for verification of the damage and for proper organization of the repair apparatus around the lesion since it has a high affinity for DNA, with a preference for UV-damaged DNA (Tanaka et al., 1989; Robins et al., 1991; Eker et al., 1992; Jones and Wood, 1993). The protein has been cloned and studied in detail (Tanaka et al., 1990). XP-A protein is a core factor in the NER complex, showing key interactions with RPA, ERCC1 and TFIIH (Figure 3). Apart from its ability to bind to damaged DNA via a zinc-finger domain, different parts of it interact with other NER proteins. One domain can bind to the single-strand-binding protein RPA, and this association increases the binding affinity of XP-A for damaged DNA (He et al., 1995). Another part of the XP-A protein can bind to the basal transcription factor TFIIH, which was shown to have a role in NER as well as in basal transcription (Park et al., 1995; Lehmann, 1998). Several DNA-unwinding enzymes termed helicases participate in lesion recognition and in removal of the damaged segment. XP-B (ERCC3) and XP-D (ERCC2) both contain the seven conserved domains of the superfamily of DNA and RNA helicases (Weber et al., 1990; Weeda et al., 1990; Gorbalenya and Koonin, 1993). In addition both proteins have been shown to possess DNA unwinding activity, but of opposite polarity (Sung et al., 1993; Roy et al., 1994). XP-B and XP-D and other proteins like p62, p44, p34, and p52 (Figure 3) are the subunits of TFIIH (Schaeffer et al., 1993; Schaeffer et al., 1994; Lehmann, 1998). The function of these helicases is to open up the structure around the damaged site to enable the structure-specific nucleases to incise the DNA and full opening of the DNA helix around the lesion is dependent on the presence of ATP (Evans et al., 1997). Mice with inactivating mutations in the TFIIH subunits XP-B and XP-D are invi-

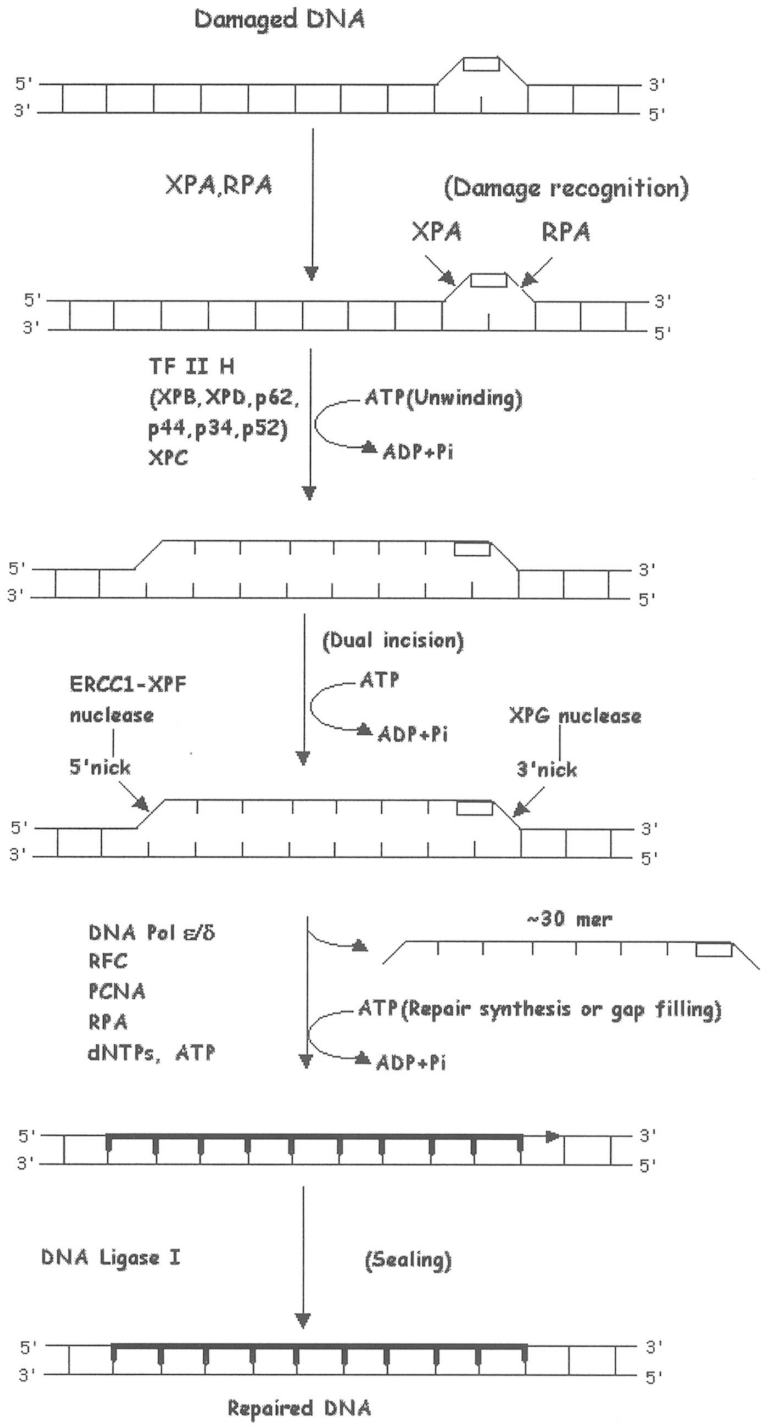

FIGURE 3. A model for nucleotide excision repair in mammalian cells. The figure shows from top to bottom damage recognition, unwinding of a region around the lesion, dual incision, repair synthesis, and ligation of the newly synthesized patch.

able which suggests an essential role of these components in basal transcription (de Boer et al., 1998b).

An oligonucleotide of about 30 nucleotides is excised after local unwinding and demarcation of the lesion. The incision pattern and size of the excised fragment are highly variable. Two incisions (5′ nick and 3′ nick) are introduced into the damaged DNA strand, one on each side of a DNA lesion (Figure 3). A multisubunit ATP-dependent nuclease known as excision nuclease or excinuclease makes the dual incisions, one on either side of the lesion and excises the oligonucleotide carrying the damaged portion. For psoralen monoadducts and cyclobutane pyrimidine dimers, one incision is made 5 to 6 phosphodiester bonds 3′ to the lesion, and another incision 22 to 24 phosphodiester bonds away from the lesion on the 5′ side, resulting in excision fragments 27 to 32 nucleotides long (Svoboda et al., 1993). The dual incision is absolutely dependent on ATP hydrolysis (Svoboda et al., 1993). Two different nucleases are used to create the dual incisions. ERCC1-XPF and XP-G are both structure-specific nucleases with different specificity's (O'Donovan et al., 1994a; O'Donovan et al., 1994b). Both can cleave Y-type structures at the junction of the single and double-stranded regions. XP-G cuts structure specifically, 3′ of a single-stranded bubble region in duplex DNA (O'Donovan et al., 1994a). ERCC1/XP-F cuts on the strand that leads off from the junction in the 5′ to 3′ direction (Scherly et al., 1993; Bardwell et al., 1994; Sijbers et al., 1996).

The specificity of ERCC1/XP-F seems to be coordinated by RPA, which binds with defined polarity to the undamaged strand. Its 3′-oriented side stimulates ERCC1/XP-F whereas the 5′-oriented side inhibits endonuclease activity of ERCC1/XP-F, in the undamaged strand (de Laat et al., 1998). The specificities of these two nucleases make them ideally suited to cleave the opened up damaged site on either side of the damage. The result is the removal of a piece of DNA about 25 to 30 nucleotides in length (Figure 3). The size of the repair patch formed during nucleotide excision repair is about 25 to 30 nucleotides long as determined by *in vivo* (Th'ng et al., 1986; Cleaver et al., 1991) and *in vitro* studies (Hansson et al., 1989; Huang et al., 1992; Shivji et al., 1992; Szymkowski et al., 1993a). Disruption of the ERCC1 subunit of the ERCC1-XP-F nuclease has severe consequences. Animals with this disorder are abnormally small, die before weaning, and show chromosome and tissue abnormalities in the liver and other organs (McWhir et al., 1993; Weeda et al., 1997a). Knockouts of XP-G in mice and humans are also very severe. Such mice die early because of failure to properly develop the intestine (Harada et al., 1999).

The human single-stranded DNA binding protein replication protein A (RPA) plays important role in NER. A speculative role for RPA in this type of repair is the stabilization of the single-stranded nature of DNA around a lesion, generated by the cooperative action of damage recognition proteins and helicases. One single RPA unit protects a region of nearly 30 nucleotides and matches well with the size of an excision repair patch, supporting the suggested role of RPA in NER (Kim et al., 1992; Shivji et al., 1995). XP-G and RPA interact directly, but RPA alone is not sufficient to endow strand specificity on XP-G. The strong interaction between TFIIH and XP-G suggest that TFIIH is required for proper positioning of XP-G (Iyer et al., 1996).

The last step in the NER mechanism, gap-filling of the excised patch, is least specific and this DNA repair synthesis is performed by common DNA replication factors. An *in vitro* reconstituted repair reaction showed that efficient repair synthesis requires the mammalian replication factors

RPA, replication factor C (RF-C), proliferating cell nuclear antigen (PCNA), and DNA polymerase δ and ε (Hubscher and Thommes, 1992; Shivji et al., 1995). It was proposed that repair synthesis requires polymerase δ and polymerase α (Dresler and Frattini, 1986; Nishida et al., 1988; Keeney and Linn, 1990; Syvaoja et al., 1990; Hunting et al., 1991; Coverley et al., 1992; Popanda and Thielmann, 1992; Aboussekhra et al., 1995; Lehmann, 1995; Lehmann, 1998). This role was further strengthened by the finding that PCNA is required for NER by mammalian cell extracts (Nicholas and Sancar, 1992; Shivji et al., 1992) as only polymerase δ and polymerase α require PCNA (Kelman, 1997). It has been shown that PCNA functions in NER synthesis and in filling of short single-stranded gaps by assisting in the initiation of DNA synthesis (Podust et al., 1994; Shivji et al., 1995). In repair synthesis PCNA may serve as an anchoring clamp at the 3′-OH terminus of a DNA strand to which polymerase can bind (Nicholas and Sancar, 1992; Shivji et al., 1992).

The participation of PCNA in NER suggests that another DNA polymerase accessory factor, RFC, be also involved. The multisubunit RFC factor acts as a molecular matchmaker for PCNA (O'Donnel et al., 1993; Sancar and Hearst, 1993). RFC assists PCNA to load onto gapped DNA templates in an ATP-dependent manner, creating a sliding clamp for polymerase δ and polymerase α (Hubscher and Spadari, 1994; Kelman and O'Donnell, 1994; Podust et al., 1994; Cullmann et al., 1995). The gap is filled in a precise manner without enlargement in either direction, the 3′ or 5′ and hence the repair patch exactly matches the excision patch (Figure 3) (Huang et al., 1992).

Finally, sealing (ligation) of the newly synthesized DNA to the existing DNA completes the NER process. The repair patch is then sealed by any of the four distinct ATP-dependent DNA ligases, most likely ligase I (Figure 3) (Lindahl and Barnes, 1992; Petrini et al., 1995) because mutations in the gene for DNA ligase I can give rise to a UV-sensitive phenotype (Barnes et al., 1992). To ensure the removal of damaged sites without disruption to the rest of the genome it is very necessary that the events be carried out in the correct order.

A plant homologue of human *ERCC1* gene has been reported from male germline cells of lily (Xu et al., 1998). The protein encoded by the plant gene is also reported to correct significantly the sensitivity to the cross-linking agent mitomycin C in ERCC1-deficient Chinese hamster ovary cells (Xu et al., 1998). These results suggest that the NER mechanism is conserved in yeast, animals, and higher plants.

V. DNA DOUBLE-STRAND BREAK REPAIR

DNA double-strand breaks are created by ionizing radiation (Dizdaroglu, 1992), chemical agents and can also occur as intermediates in certain somatic recombination such as V(D)J recombination, and perhaps during transposition reactions (Lieber, 1991). V(D)J recombination is the genomic rearrangement that creates antigen-receptor diversity in vertebrates (van Gent et al., 1995). Failure to rejoin breaks can lead to loss of portions of chromosomes or to rearrangements. If genes inactivated by such events encode essential cellular components, then cell death will occur.

A. Proteins Involved in DNA Double-Strand Break Repair

The DNA-dependent protein kinase (DNA-PK) is required for normal

double-strand break rejoining activity in mammalian cells. It is a heterotrimer consisting of a p450 catalytic subunit and Ku86 and Ku70 regulatory subunits (Anderson, 1993; Tuteja et al., 1994; Tuteja and Tuteja, 2000). This DNA-PK holoenzyme is activated after binding to DNA strand breaks and is able to phosphorylate many protein substrates *in vitro*, although its physiological targets are unclear (Anderson, 1994; Hartley et al., 1995). Mammalian cells that are deficient in catalytic subunit of DNA-PK or Ku protein show highly characteristic defects. The most noticeable of these is sensitivity to ionizing radiation that induces double-strand DNA breaks (Rathmell and Chu, 1994; Taccioli et al., 1994; Boubnov et al., 1995). In addition to having a defect in general double-strand break rejoining, mutants with defects in components of DNA-PK are also unable to perform correct recombinational V(D)J rejoining of maturing immunoglobulin genes (Blunt et al., 1995). The end-binding activity of Ku may serve as an entry site for other proteins and the phosphorylation activity of DNA-PK may activate or inhibit other repair factors (Tuteja and Tuteja, 2000). The repair of double-strand breaks by homologous recombination with another allele can be achieved with high fidelity, whereas repair by nonhomologous end joining may result in lost or changed genetic information. The balance between these two pathways is apparently influenced by the relative amounts of RAD52 and Ku (Van Dyck et al., 1999).

Recently nibrin, a novel DNA double-strand break repair protein, has been identified and it has been shown that it is directly involved in the processing of DNA double-strand breaks caused by ionizing radiation (Varon et al., 1998; Digweed et al., 1999).

VI. DISORDERS OF DNA REPAIR

Three rare, autosomal recessive disorders are known to be based on NER deficiency; the prototype NER disease, xeroderma pigmentosum (XP) (Cleaver, 1968), Cockayne's Syndrome (CS) (Nance and Berry, 1992) and photosensitive form of trichothiodystrophy (TTD) (Itin and Pittelkow, 1990; Stefanini et al., 1993a; Stefanini et al., 1993b). These diseases show distinct clinical phenotypes. The hallmarks of XP are sun sensitivity, pigmentation changes, and an increased incidence of skin cancer (Kraemer et al., 1987). CS exhibits growth and mental retardation and a characteristic bird-like face as well as neurodegeneration and retinal degeneration (Nance and Berry, 1992). TTD is characterized by sulfur-deficient brittle hair together with growth and mental retardation (Itin and Pittelkow, 1990). Neither CS nor TTD is cancer prone (Lehmann, 1995).

Ataxia Telangiectasia (AT) is an autosomal recessive disorder characterized by cerebellar ataxia, high incidence of cancers and hypersensitivity to ionizing radiation but not to UV (Swift et al., 1991). In addition, there are immune deficiencies, both cellular and humoral, and a greatly elevated incidence of tumors, especially of the lymphoreticular system. Increased chromosome breakage is observed in cultured lymphocytes and fibroblasts from AT patients, adding AT to the list of 'chromosome breakage' or 'chromosome instability' syndromes (Shiloh, 1997; Lehmann and Carr, 1995).

Nijmegen breakage syndrome (NBS) is also an autosomal recessive 'chromosomal instability' syndrome characterized by microcephaly, growth retardation, immunodeficiency and cancer predisposition. Cells from NBS patients are hypersensitive to ionizing radiation with cytogenetic features indistin-

guishable from ataxia telangiectasia (Digweed et al., 1999). The immunologic characteristics of NBS encompass both developmental defects in tissues where lymphocytes develop and cellular defects in the responses of these cells to stimuli (Chrzanowska et al., 1995).

The focus of the present review is to describe the photosensitive syndromes: xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy in greater detail in the following sections.

A. Xeroderma Pigmentosum

Xeroderma pigmentosum (XP) is an extremely rare, autosomal recessive disease. The skin of the patients is normal at birth but develops progressive atrophy, irregular pigmentation and later basal cell and squamous cell carcinomas. The sun exposure of XP patients generally results in progressive degenerative alterations of the skin and eyes and the mean age of onset of these symptoms is about two years (Kraemer, 1997). The disease is characterized by extreme sensitivity to sun exposure, a high incidence of skin cancer and frequent neurological abnormalities. This disease involves DNA repair and replication deficiencies that predispose homozygous individuals to a 1000-fold increase in non melanoma and melanoma skin cancers. Two major forms of XP are known: one form is caused by a defect in excision repair and the other lacks the capacity to replicate damaged DNA. The diagnostic features of XP are dry scaly skin (xeroderma), abnormal pigmentation on sun-exposed areas of the skin (pigmentosum) photosensitivity and marked predisposition to skin cancer (Cleaver and Kraemer, 1994; Woods, 1998). XP patients are extremely sensitive to solar exposure due to the DNA damaging effect of the UV component of sunlight (Cleaver and Kraemer, 1994). It is characterized by photodermatoses including skin cancer at an early age, and in some cases neurological abnormalities (Robbins et al., 1974; Chu and Mayne, 1996). A large group of XP patients exhibit a high risk toward the development of cutaneous malignancies (basal cell carcinomas, squamous cell carcinomas, and melanomas).

Up to 50% of XP patients have associated symptoms of the De Sanctis-Cacchione syndrome (DSC), which includes immature sexual development, growth retardation and neurological abnormalities of mental retardation, microcephaly and sensorineural deafness (DeSanctis and Cacchione, 1932). DSC describes mainly neurologic and developmental abnormalities of individuals with a freckling (pigmentosum) and parchment (xeroderma)-like skin. It was later discovered that all DSC patients belong to XP, and the DSC designation has gone out of use.

A fraction of XP patients display progressive neurological abnormalities. The neurological abnormalities correlate with postmortem findings of premature neuronal death in the central and peripheral nervous systems. 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 (Robbins et al., 1991).

XP cells are hypersensitive to ultraviolet (UV) radiation owing to a defect in the NER pathway. DNA repair synthesis after UV-irradiation, as measured by tritiated thymidine incorporation (autoradiographically) is severely reduced in XP (Cleaver, 1968). A series of mutagenesis studies on these cells revealed that XP cells are hypermutable after UV challenge. The spectrum of UV-induced mutations in XP cells is also different from that in NER-proficient cells.

XP patients are clinically very heterogeneous. Consequently, heterogeneity in severity of the repair defect and of symp-

toms such as sun sensitivity and neuronal abnormalities is found. The symptoms vary from severe neurological implications and a high number of skin tumors to mild pigmentary abnormalities without signs of neurological abnormalities. This variation can be due to differences in genetic background of affected persons or to allelic variation. It has been shown by cell fusion studies that XP is a multigenic disorder (De Weerd-Kastelein et al., 1972). Cell fusion experiments based on complementation of repair synthesis have recognized the presence of seven XP groups which exhibit various defects in the initial steps of the DNA NER pathway (XP-A through XP-G, each carrying a mutation in a different gene) that are characterized by varying levels of UV sensitivity and corresponding deficiencies in repair (Vermeulen et al., 1991; Hoeijmakers, 1993; Cleaver, 2000). With the exception of XP-E, all the XP proteins appear to be absolutely required for nucleotide excision repair using the *in vitro* assay. It has been shown using *in vivo* assay that XP-A cells have minimal repair activity, because XP-A is involved in the recognition step of NER (Cleaver and States, 1997). XP-E cells have substantial repair activity, consistent with the partial requirement of XP-E protein in NER. Cells in several other complementation groups have significant levels of repair *in vivo* (typically 20 to 50% in groups C and D), although the proteins are absolutely required *in vitro.* Of the seven-complementation groups, the A, B, D, and G forms manifest neurological symptoms. A possible explanation for the onset of neurological abnormalities in XP patients is that defective DNA repair in nerve cells of endogenous (oxidative) NER lesions induces neuronal death (Reardon et al., 1997).

Whereas the features of TTD have not been found in combination with XP, mutations in three of the XP genes, XP-B, XP-D, and XP-G can give rise to combined symptoms of XP and CS (XP/CS). In addition to these groups, there is a heterogeneous group called XP variant (XP-V) in which cells have normal excision repair but are defective in the so-called post-replication repair (Boyer et al., 1990). These cells are only slightly more sensitive than normal cells to the killing action of UV light radiation (Cordonnier and Fuchs, 1999). The spectrum of mutations is also different from that in normal cells (Wang et al., 1993). 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. The extracts from XP-V cells were also found to be defective in translesion synthesis (Cordonnier et al., 1999). There are some specialized enzymes that can bypass DNA damage and extend replication forks through damaged sites. DNA polymerase ζ in the yeast *Saccharomyces cerevisiae* is composed of a catalytic subunit Rev3 (in the same family as POL α, δ, and ε) and an accessory factor Rev7, which together have the ability to bypass pyrimidine dimers and other adducts in DNA (Lawrence and Hinkle, 1996; Gibbs et al., 1998). The enzyme often incorporates incorrect nucleotides during bypass of damage. Another important human DNA polymerase in this family is designated DNA polymerase η. This enzyme is homologous to yeast Rad30 (Johnson et al., 1999b) and consists of 713 amino acids. POL η can bypass thymine-thymine dimers, and usually two A residues are correctly inserted opposite the lesion. Recently, it has been shown that the XP-V cells lack this particular bypass activity because of inactivating mutations in the POL η gene (Johnson et al., 1999a; Masutani et al., 1999a; Masutani et al., 1999b). This explains previous observations of a defect in bypass of damage in XP-V cells and cell extracts

(Cordeiro-Stone et al., 1997; Svoboda et al., 1998; Cordonnier et al., 1999) and accounts for the marked hypermutability of XP-V cells in response to UV light and some chemical agents. POL η is missing in most or all individuals with XP-V, indicating that it is not essential for life. In XP-V cells, NER can remove a large fraction of UV-induced thymine-thymine dimers, but because pol η is missing, any remaining dimers are more likely to be bypassed by polymerases such as pol ζ that incorporate incorrect residues.

1. Genes Responsible for Xeroderma Pigmentosum Defects

XP-A gene codes for a protein that is central to NER and binds to DNA, with a preference for damaged DNA due to a variety of UV light and chemical damage (Wood, 1995). It also acts as an anchor for other repair proteins to attach and carry out excision and replacement synthesis. The damage recognition ability of XP-A is due to the presence of a DNA-binding domain, which combines a zinc finger, and a single-strand-binding region, which may infiltrate small single-stranded region, caused by helix-destabilizing lesions. Mutations in XP-A that are within the DNA binding site produce more severe central nervous system disorders, than mutations in the C-terminal region of the protein (Cleaver and States, 1997). On the other hand, mutations in two members of the TFIIH complex, the XP-B and XP-D genes are generally very severe with both skin and central nervous system disorders. Because these two genes act also as helicases, they act in opening up the DNA to initiate transcription as well. A deficiency in one of these helicases can also produce the symptoms of CS and in some cases, TTD as well (Marionnet et al., 1995; Weeda et al., 1997b). Because XP-D is part of the TFIIH complex, it binds to the promoters of the genes and facilitates the initiation of transcription and is also involved in the repair of damaged DNA. A number of mutations have been found in XP-D when compared with XP-B in which only a few mutations have been detected (Taylor et al., 1997; Sebastiaan Winkler and Hoeijmakers, 1998). Apparently, XP-D tolerates more mutations, suggesting that its tertiary structure is less critical for its activity in basal transcription than that of XP-B. Each of the XP-D mutation has a different effect on XP-D activity and structure and most of the mutations are clustered in the carboxy terminus of the protein (Coin et al., 1998). The DNA unwinding activities of the separate wild-type XP-B and XP-D subunits with intact TFIIH were compared. It was found that the 3′ to 5′ DNA helicase activity of XP-B is weaker in the context of TFIIH than that of the isolated protein, but the 5′ to 3′ unwinding capacity of XP-D appeared to be more potent when the protein is complexed with the other TFIIH components. These data suggest that some TFIIH subunits increase the unwinding activity of XP-D. It has been shown that the helicase activity of XP-D is stimulated ~10-fold by its interaction with the p44 subunit of TFIIH (Coin et al., 1998). All of the C-terminal mutations of XP-D result in the same biochemical defect: decreased helicase activity and concomitant loss of detectable interaction with p44. An inactivating deletion or truncating mutation in XP-D is incompatible with the essential transcription function of the protein (de Boer and Hoeijmakers, 2000).

The moderate UV sensitivity and intermediate repair synthesis typical of XP-F patients could be due to the anticipated dual function of the XP-F/ERCC1 complex in NER and repair of interstrand crosslinks (Davies et al., 1995). All XP-F cells exam-

ined so far have a detectable, but greatly reduced amount of XP-F protein (Yagi et al., 1997). A null allele for XP-F or ERCC1 and the consequential defect in crosslink repair may be incompatible with life.

A patient with the clinical features of both xeroderma pigmentosum and Cockayne's syndrome has been reported and this individual has unusual cellular responses to UV light. It has been shown that the cultured fibroblasts and lymphocytes of this patient are extremely sensitive to irradiation with UV but the nucleotide excision repair is 30 to 40% that of the normal (Broughton et al., 1995). This particular deficiency has been assigned to the XP-D complementation group and two causative mutations in the XP-D gene have been identified. The allele inherited from the patient's mother has a glycine to arginine change at amino acid 675 and the allele inherited from his father contains a –1 frameshift at amino acid 669. Both these mutations are present in the C-terminal 20% of the 760 amino acid XP-D protein (Broughton et al., 1995).

Two cases with the combined features of XP and CS have been assigned to the XP-D complementation group (Lafforet and Dupuy, 1978; Broughton et al., 1995). It was observed that despite their extreme UV sensitivity, the cells from these patients appeared to incise their DNA as efficiently as normal cells. When irradiated plasmids were introduced into nonirradiated XP-D/CS cells, the ectopically introduced damage triggered the induction of breaks in the undamaged genomic DNA (Berneburg et al., 2000). XP-D/CS cells thus have a unique response to sensing UV damage, which results in the introduction of breaks into the DNA at sites distant from the damage. It has been proposed that it is these spurious breaks that are responsible for the extreme UV sensitivity of these cells (Berneburg et al., 2000).

B. Cockayne's Syndrome

The major clinical features of Cockayne's syndrome (CS) are progressive leukodystrophy, dwarfism, progressive microcephaly, loss of adipose tissue, mental retardation, retinal atrophy, gait defects, cataracts, dental caries, acute sun sensitivity and progressive growth retardation (Leech et al., 1985; Lehmann, 1995). The majority of patients present between the ages of 3 to 5, often with sensorineural deafness, initially masquerading as mild developmental delay, but later including developmental delay. Usually at this time, growth deficiency, particularly with loss of adipose tissue, is becoming apparent, as is a characteristic facial appearance with sunken eyes. Neurological signs are mental retardation (intelligence decline and behavior problems), loss of gait and speech capacity, hyper-reflexia, occasionally seizures and sensorineuronal hearing loss (Otsuka and Robbins, 1985; Cruz Martinez and Anciones, 1991). These neurological features might be explained by calcification of basal ganglia and more likely by dysmyelination of the central and peripheral nervous system (Nance and Berry, 1992). The individuals affected with CS display impaired sexual development and postnatal growth failure and this condition is termed cachectic dwarfism. The main causes of death in these patients are pneumonia and respiratory infections and the mean age of death is about 12 years (Nance and Berry, 1992). Very rarely, individuals have clinical features of both XP and CS. XP complementation group G contains some patients with the combined features of XP and CS.

The NER occurs normally both *in vivo* and *in vitro* in CS cells. The repair defect in CS cells is manifested as retarded or absent recovery of DNA (Lehmann et al., 1979) or RNA synthesis (Mayne and Lehmann, 1982)

after an UV challenge that normally recovers within a few hours. Therefore, it is clear now that CS cells are defective in an important subpathway of NER. Following DNA damage, it is very important for the cell to remove damage from actively transcribed regions of DNA, and in human cells repair is more rapid in transcribed and in untranscribed regions. Furthermore, it is the transcribed strand that is repaired most rapidly and this preferential repair is referred to as transcription-coupled repair. It seems that transcription-coupled repair may in fact act upstream of both nucleotide- and base-excision repair. The repair defect in CS was shown to be based on an impaired preferential repair of the transcribed strand (Venema et al., 1990; van Hoffen et al., 1993;).

1. Genes Defective in Cockayne's Syndrome

Complementation analysis has disclosed three groups in CS: A, B, and C (Tanaka et al., 1981; Lehmann, 1982). Groups A and B comprise of the classical CS patients and group C contains the patients with combined XP and CS symptoms. A few CS patients have also been assigned to the rare XP groups B, D, or G. Two of the complementation groups of CS (CS-A and CS-B) exhibit no defect in overall genomic NER, but they are severely deficient in transcription-coupled repair of certain lesions produced by ionizing radiation (Leadon and Cooper, 1993). The involvement of CS genes in transcription-coupled repair has led to the suggestion that CS might also be a 'transcription syndrome'. Two candidates of transcription-repair coupling factors are CS-A and CS-B, respectively, encoded by the *CS-A* and *CS-B* genes (Troelstra et al., 1992; Henning et al., 1995). Mutations in either of the two nonessential genes, *CS-A* or *CS-B* result in defective transcription-coupled repair and are the genetic defect in over 90% of CS patients. The protein sequence of *CS-A* contains the consensus of five "WD-repeats" (Henning et al., 1995). This type of motif is present in a number of proteins, which are believed to possess a regulatory rather than a catalytic role in many cellular functions, including cell division, signal transduction, messenger RNA modification, and transcription (Neer et al., 1994). Many of these WD-repeat proteins reside in multiprotein complexes. The CS-A and XP-G proteins interact with components of TFIIH and CS-B interacts with CS-A and XP-G (Henning et al., 1995). The product of the *CS-B* gene contains a region of multiple ATPase/putative helicase motifs (Troelstra et al., 1992; Guzder et al., 1996), because it contains the seven conserved domains which define a large superfamily of DNA and RNA helicases (Gorbalenya and Koonin, 1993). Extracts from CS-A and CS-B cells show decreased levels of transcription from the adenovirus major late promoter *in vitro* and the defect is corrected by transfection with the corresponding CS gene (Chu and Mayne, 1996). Thus, although the *CS-A* and *CS-B* genes are not essential for transcription, mutations in these genes might affect transcription indirectly. *CS-B* knockout mice display only mild CS symptoms, but completely repair deficient *CS-B/XP-A* double mutant mice suffer from severe growth failure and die before weaning (de Boer and Hoeijmakers, 1999).

It has been shown that DNA damage produced by ionizing radiation (not ultraviolet radiation) is subject to transcription-coupled repair in normal human cells and in XP-A mutant cells but not in CS-B mutant cells (Leadon and Cooper, 1993). It might be possible that all patients with CS are deficient in the transcription-coupled repair of different types of oxidative lesions. Oxidative damage, including thymine glycols,

is shown to be removed by transcription-coupled repair in cells from normal individuals and from XP-A, XP-F and XP-G patients who have NER defects but not from XP-G patients who have severe CS. It seems that transcription-coupled repair of oxidative damage requires an XP-G function distinct from its NER endonuclease activity. The *XP-G* gene in the three documented cases of combined XP-G/CS has been examined. An unexpected common mutational pattern in these three patients has been reported suggesting an important second XP-G function responsible for the CS clinical phenotype (Nouspikel et al., 1997). These three XP-G/CS patients had mutations that would produce severely truncated XP-G proteins. In contrast, two sibling XP-G patients without CS are able to make full-length XP-G, but with a missense mutation that inactivates its function in NER. These results suggest that XP-G/CS mutations abolish interactions required for a second important XP-G function and that it is the loss of this second function, that leads to the CS clinical phenotype (Nouspikel et al., 1997).

It has been shown that defective transcription-coupled repair of oxidative damage contributes to the developmental defects associated with CS (Cooper et al., 1997) and XP-G is required for transcription-coupled repair of such damage and probably this represents the second XP-G function. It has been suggested that transcription-coupled repair of oxidative damage is independent of the XP-G incision function in NER, and even of NER altogether. Instead, glycosylases and AP endonucleases in a BER pathway presumably initiate this activity. This pathway requires at least a transient interaction between XP-G and TFIIH, and perhaps other proteins, to displace RNA polymerase II stalled at a lesion (Donahue et al., 1994; Habraken et al., 1996). Recently, transcription-coupled repair of oxidative lesions has been tested by Le Page et al. (2000). It has been shown that CS cells, including CS-B, XP-B/CS, XP-D/CS, and XP-G/CS not only lack transcription-coupled repair but also cannot remove 8-oxoG in a transcribed sequence, despite its proficient repair when not transcribed. The XP-G/CS defect uniquely slows lesion removal in nontranscribed sequences. This defective transcription-coupled repair leads to a mutation frequency at 8-oxoG of 30 to 40% compared with the normal 1 to 4%. The transcription by RNA polymerase II is also blocked by unrepaired 8-oxoG. These data demonstrate that transcription-coupled repair is required for polymerase release to allow repair and that CS results from defects in transcription-coupled repair of oxidative lesions (Le Page et al., 2000). They also showed that 8-oxo-guanine (or 8-oxo-guanine plus a ligand) is an obstacle to the movement of RNA polymerase II, and that the arrested polymerase must be released to give the repair enzymes access to the site. Therefore, not only is the transcription-coupled repair is missing in CS, but the blocked RNA polymerase II prevents lesion recognition and repair by the global BER pathway as well.

It has been proposed recently, that CS could be characterized as a disease of excessive cell death by apoptosis (Hanawalt, 2000). This affects the rapidly metabolizing cells, such as neurons, that generate high levels of reactive oxygen species. This apoptosis model could also explain the problems of stunted growth and neurological deterioration. It might also explain why CS patients are not prone to skin cancer because dead cells do not form tumors (Hanawalt, 2000).

C. Trichothiodystrophy

Trichothiodystrophy (TTD) is a rare, autosomal recessive disorder characterized by

sulfur-deficient brittle hair and nails, mental retardation, impaired sexual development, and ichthyosis (Itin and Pittelkow, 1990; Tolmie et al., 1994). Scanning electron microscopy shows incomplete cuticles and biochemical analysis shows that TTD hair is severely deficient in cysteine-rich proteins present in the cuticle cells. This has led to the speculation that the brittle hair is caused by decreased expression of genes encoding the cysteine-rich proteins. TTD hairs are dry and sparse, and the hair shaft breaks easily. The specific reduction in cysteine-rich matrix protein expression in hair of TTD patients indicates a defect in a late stage of hair keratinocyte differentiation (Gillespie and Marshall, 1983). Reduced cysteine-rich matrix protein contents affect the integrity of the hair shaft, because intermediate keratin filaments are not crosslinked properly. A similar defect may also explain hypoplastic and easily breakable nails of TTD patients (Itin and Pittelkow, 1990) as well as the ichthyosis.

Photosensitivity has been reported in approximately 50% of the cases, but no skin cancer is associated with TTD. Virtually all photosensitive TTD patients have a deficiency in the NER of UV-induced DNA damage that is indistinguishable from that of xeroderma pigmentosum complementation group D patients. In addition to brittle hair, these patients can have impaired intelligence, decreased fertility and short stature. Those TTD patients with impaired intelligence appear to have neurological deficits quite similar to CS, including ataxia, spasticity and microcephaly. In a few cases, calcification of the basal ganglia and dysmyelination have been reported, which could be the cause of clinical features (Chen et al., 1994; Tolmie et al., 1994). Clinical manifestations and their severity vary extensively between TTD individuals. It was discovered that TTD is associated with deficient NER (Yong et al., 1984).

1. Genes Responsible for Trichothiodystrophy

The patients fall into three clinical forms corresponding to mutations in three genes. Complementation studies on about 20 UV-sensitive TTD families have shown that the NER defect in all but two families can be assigned to the XP-D complementation group (Stefanini et al., 1993a; Stefanini et al., 1993b; Broughton et al., 1994). The defect can also be assigned in some families to the *XP-B* gene (Vermeulen et al., 1994) and in a single individual to a completely new nucleotide excision repair gene designated TTD-A (Stefanini et al., 1993b). Nucleotide-sequence analysis of the XP-D cDNA from three TTD cell strains (TTD1V1, TTD3VI, and TTD1RO) revealed mutations within the region from amino acid 713-730 and within previously identified helicase functional domains (Takayama et al., 1996). The various clinical presentations and DNA repair characteristics of the cell strains can be correlated with the particular mutations found in the ERCC2 locus. Mutations of Arg658 to either His or Cys correlate with TTD cell strains with intermediate UV-sensitivity, mutations of Arg722 to Trp correlates with highly UV-sensitive TTD cell strains, and mutation of Arg 683 to Trp correlates with XP-D. Alleles with mutation of Arg616 to Pro or with the combined mutation of Leu461 to Val and deletion of 716-730 are found in both XP-D and TTD cell strains (Takayama et al., 1996). Recovery of normal DNA repair and mutagenesis in trichothiodystrophy cells after transduction of the *XP-D* human gene has been reported (Marionnet et al., 1996; Quilliet et al., 1996). The causative mutation in two mild TTD patients (TTD6VI and TTD4VI) was assigned to *XP-B* gene. It was found to be a single base substitution resulting in a

missense mutation (T119P) in a region of the XP-B protein completely conserved in yeast, *Drosophila*, mouse, and man (Weeda et al., 1997b).

A mouse model for the basal transcription/DNA repair syndrome TTD has been developed (de Boer et al., 1998a). The causative XP-D point mutation of a TTD patient has been mimicked in the mouse. TTD mice reflect to a remarkable extent the human disorder, including brittle hair, developmental abnormalities, reduced life span, UV sensitivity, and skin abnormalities. The cutaneous symptoms are associated with reduced transcription of a skin-specific gene strongly supporting the concept of TTD as a human disease due to inborn defects in basal transcription and DNA repair (de Boer et al., 1998a). It has also been reported that in accordance with the cellular studies, TTD mice exhibit a modestly increased sensitivity to UV-induced inflammation and hyperplasia of the skin. However, in striking contrast to the human syndrome, TTD mice manifest a susceptibility to UV-and 7,12-dimethylbenz(*a*)anthracene-induced skin carcinogenesis, albeit not as pronounced as the totally nucleotide excision repair-deficient XP-A mice (de Boer et al., 1998b). These findings suggest that TTD is associated with cancer predisposition and support the notion that a NER deficiency enhances cancer susceptibility (de Boer et al., 1999).

The unscheduled DNA synthesis of most photosensitive TTD fibroblast lines is about 15 to 25% of the normal but in general TTD fibroblasts are less sensitive to UV-induced cell killing than XP-D cells. A subclass of TTD fibroblasts with relatively high-unscheduled DNA synthesis (40 to 55%) and near wild-type UV-survival exists. It has been shown that photosensitive TTD cells have defective cyclobutane pyrimidine dimers repair but (partially) proficient repair of 6–4 photoproducts (Eveno et al., 1995). It was also shown that cyclobutane pyrimidine dimers are the predominant mutagenic lesions in TTD cells (Marionnet et al., 1998). The UV-induced mutation spectra is different between XP and TTD cells (Marionnet et al., 1995). It was observed that although the mutation frequency of the UV-irradiated pR2 vector was much higher in TTD and XP-D cells than in normal cells, the mutation spectrum is closer between TTD and normal cells when compared with XP-D cells (Madzak et al., 1993; Marionnet et al., 1995). The cellular catalase activity is reduced in XP but not TTD cells (Vuillaume et al., 1992). The TTD repair defect differs from the XP-type XP-D repair defect, which may explain to some extent the absence of cancer development in TTD patients. The postulated transcription defect in TTD also might suppress the initiation and/or progression of the carcinogenic process.

VII. CONCLUSION AND FUTURE PROSPECTS

The study of DNA repair occupies a central place in modern biology. The field of DNA repair in eukaryotes presently covers an enormous area and strong connections are being established between repair and other areas, including DNA replication, transcription, and cell-cycle controls, and checkpoints. The crystallographic studies of DNA repair enzymes are revealing a wide range of structural motifs used to recognize specific lesions in DNA. These studies will also help to unravel the catalytic mechanism by which these lesions are repaired. The DNA repair disorders are clinically diverse. Most of the disorders cause growth retardation and a predisposition to cancer and some cause neurodegeneration and other anomalies. Some disorder phenotypes may be caused by mutations in more than one gene. On the contrary, different mutations

in same gene can cause more than one disease phenotype. Further work is in progress to determine how each gene causes a particular phenotype and what role they may have in human development and disease.

The role of excision repair in diseases other than XP, CS, and TTD still needs to be established. It is important to study the relationship between DNA damage, repair, and aging in greater detail. It will be really interesting to know whether malfunctioning of excision repair causes aging and the various ways to retard this unwanted aging process.

ACKNOWLEDGMENTS

The authors are grateful to Drs. D. Gupta, Dhruva Tuteja, and Gaurav Tuteja for help in the preparation of the figures.

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