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DNA Repair: Mechanisms and Measurements

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Abstract The early findings that significant amounts of modifications are induced to the cellular DNA both spontaneously and as a consequence of metabolism and environmental exposures have led to the discovery of the existence of multiple highly efficient repair mechanisms to maintain the integrity of DNA.

There is increasing evidence of considerable variation between individuals with regard to activity of DNA repair mechanisms and there are examples of diseases that have deficiencies in DNA repair as a risk factor. It is anticipated that detailed understanding of the DNA repair mechanisms will lead to insights for prevention of diseases, for diagnosis, into resistance to chemotherapeutic agents, and for development of therapeutic tools. DNA repair is also considered to be an important factor in aging.

This chapter briefly summarizes the known DNA repair pathways and reviews the approaches for clarifying and measuring DNA repair in vivo and in vitro.

Keywords Direct repair · Base excision repair (BER) · Nucleotide excision repair (NER) · Transcription-coupled repair · HR · NHEJ · DNA repair polymorphisms · DNA repair knockout · Inborn DNA repair defects · Incision assay · Base modifications

1 Introduction

Since DNA is the carrier of genetic information and spontaneous mutations occur only at low frequency, cellular DNA was initially regarded as an essentially stable entity. It is now clear that modifications are induced at high rate to the cellular DNA. This occurs both spontaneously as a consequence of metabolism, and from environmental exposures. During the last decade the existence of efficient repair mechanisms for maintaining the integrity of DNA have been discovered.

By the beginning of 1900 the term gene (derived from genesis) had already been established and the relationship between high-energy radiation and skin cancer discovered. Nobel price winner H. J. Muller (in 1946) established both the concept of induction of mutations and hints of gene repair mechanisms, from studies on Drosophila. This was long before the discovery of DNA as the carrier of genetic information [1]. Undoubtedly, the discovery of the genetic code in DNA moved the focus from the repair mechanisms to transcription and gene function. It took another decade before the importance of DNA repair again attracted attention.

One of the first important insights in the field of DNA repair was made after isolation of *E. coli* mutants with exceptional sensitivity to UV light [2] and subsequent mapping of mutations in genes designated *uvr A*, *B*, and *C* [3]. This led to the model of nucleotide excision repair [4, 5]. Later the base excision repair pathway was exposed when Lindahl and coworkers [6, 7] discovered a series of bacterial enzymes that recognized and removed modified nucleotides by a DNA *N*-glycosylase reaction.

The initial 1966 description of four DNA repair steps [8] still represents the overall model of DNA repair; however, the scheme is now much more complex with at least six repair pathways, each consisting of several proteins (Table 1).

For a period it was believed that DNA repair was confined to the nuclear DNA, as an early finding showed the absence of repair of UV-induced damage in mitochondrial DNA [9]. However, several reports later showed that mitochondria can repair a variety of DNA lesions including strand breaks, alkali-sensitive sites, Fpg-sensitive sites (reviewed by [10]), and mismatches [11]. The significance of mitochondrial DNA repair is not yet clear. Few defects in mitochondrial DNA repair and/or replication have been reported. Two diseases have so far been attributed to alterations in the major mitochondrial DNA repair enzyme polymerase γ: progressive external ophthalmoplegia and Alper's syndrome [12].

Table 1 The first model of DNA repair suggested in 1966 was divided into four general steps. This scheme corresponds very nicely to the present models of DNA repair, though numerous details have been added. From recent reviews we have made a list of the proteins known to be involved in the different repair pathways

	DNA repair in 1966 [8]	DNA repair in 2003 [15–17, 21, 25]
Step 1		
Damage recognition and initiation of repair 	One of the two DNA strands are interrupted: 1. by radiation induced chain break 2. by enzymatic excision of damaged bases 3. by a recombination enzyme	**BER**: OGG1, NEIL1, NEIL2, MYH, NTH, APE1, UNG, MPG, UV-endonuclease, XRCC1, PARP, PNK **GGR-NER**: XPC, hHR23B, TFIIH, XPG, XPA, RPA **TCR-NER**: Pol II, CSB, CSA, TFIIH, XPG, XPA, RPA **HR**: strand break **NHEJ**: strand break **MMR**: MSH2/6, MSH2/3, MLH1/PMS2, MLH1/PMS1
Step 2		
Procession of repair patch	Nucleotides (in a limited distance) are released, presumably through the action of an enzyme	**BER**:DNA pol β, XRCC1, DNA pol δ, DNA pol ε, PCNA, FEN1 **NER**: ERCC1, XPF **HR**: RPA, RAD50/MRE11/NBS1, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3 **NHEJ**: KU70/KU80, DNA-PK_{CS}, Artemis **MMR**: exonuclease 1, FEN1, RPA, PCNA, RFC
Step 3		
Synthesis of new DNA 	DNA helices are reconstructed by a DNA repair polymerase	**BER**: DNA pol β, XRCC1, DNA pol δ, DNA pol ε **NER**: DNA pol δ, DNA pol ε **HR**: Holliday junction resolvases, BRCA1, BRCA2, **NHEJ**: – **MMR**: DNA pol δ, DNA pol ε

Table 1 (continued)

	DNA repair in 1966 [8]	DNA repair in 2003 [15–17, 21, 25]
Step 4 Ligation of the DNA strand	Phosphodiester backbone is joined when the last nucleotide is inserted into the gap	**BER**: DNA ligase 1, DNA ligase 3 **NER**: DNA ligase 1 **HR**: DNA ligase 4 **NHEJ**: XRCC4, DNA ligase 4 **MMR**: DNA ligase?

Abbreviations: *APE1* apurinic/apyrimidinic endonuclease 1, *BER* base excision repair, *BRCA1* breast cancer 1, *BRCA2* breast cancer 2, *CSA* Cockayne syndromeA, *CSB* Cockayne syndrome B, *DNA-PK*$_{CS}$ DNA-dependent protein kinase catalytic subunit (XRCC7), *ERCC1* excision repair cross-complementing rodent repair deficiency, complementation group 1 *FEN1* flap structure-specific endonuclease 1 (DNase IV), *GGR* global genome repair, *hHR23B* human Rad 23 homologue B, *HR* homologous recombination, *KU70* Ku protein 70 kDa (XRCC6), *KU80* Ku protein 80 kDa (XRCC5), *MLH1* Mut L homologue 1, *MMR* mismatch repair, *MPG N*-methylpurine-DNA glycosylase, *MRE11* meiotic recombination 11 homologue, *MSH2* Mut S homologue 2, *MSH3* Mut S homologue 3, *MSH6* Mut S homologue 6, *MYH* Mut Y homologue, *NBS1* Nijmegen breakage syndrome 1, *NEIL1* Nei endonuclease VIII like, *NEIL2* Nei like 2, *NER* nucleotide excision repair, *NHEJ* non-homologous end joining, *NTH* endonuclease III, *OGG1* 8-oxoguanine DNA N-glycosylase, *PARP* poly (ADP-ribose) polymerase, *PCNA* proliferating cell nuclear antigen, *PMS1* postmeiotic segregation increased 1, *PMS2* postmeiotic segregation increased 2, *PNK* polynucleotide kinase, *Pol* polymerase, *RAD50* human radiation sensitive gene 50 homologue (S. cerevisiae), *RAD51* human radiation sensitive gene 51 homologue (S. cerevisiae), *RAD51B* human RAD51 paralogue B, *RAD51C* human RAD51paralogue C, *RAD51D* human RAD51 paralogue D, *RFC* replication factor C, *RPA* replication protein A, *TCR* transcription coupled repair, *TFIIH* transcription factor IIH, *UNG* uracil-DNA glycosylase, *XPA* Xeroderma pigmentosum complementation group A, *XPC* Xeroderma pigmentosum complementation group C, *XPF* Xeroderma pigmentosum complementation group F, *XPG* Xeroderma pigmentosum complementation group G, *XRCC1* X-ray repair complementing defective repair in Chinese hamster cells 1, *XRCC2* X-ray repair complementing defective repair in Chinese hamster cells 2, *XRCC3* X-ray repair complementing defective repair in Chinese hamster cells 3, *XRCC4* X-ray repair complementing defective repair in Chinese hamster cells 4.

It is anticipated that detailed understanding of the DNA repair mechanisms will lead to insights for prevention of diseases, for diagnosis, into resistance to chemotherapeutic agents, and for development of therapeutic tools. This chapter briefly summarizes the known DNA repair pathways and reviews the approaches for clarifying and measuring DNA repair in vivo and in vitro. Excellent comprehensive reviews have recently been published on repair mechanisms and chromatin structure [13, 14], on the chemistry and biology of DNA repair [15], on the chemistry of glycosylases [16], and on how DNA repair mechanisms may relate to cancer and ageing [17]. In this chapter we also describe the current available methods for estimation of DNA repair, qualitatively and quantitatively.

2
DNA Repair Mechanisms

2.1
Repair of the Precursor Pool

The presence of the oxidized nucleotide triphosphate (e.g., 8-oxodGTP) in the DNA synthesis precursor pool has been shown to be an important part of the potential mutagenic risk of oxidative stress. This is pointed out by the existence of specific 8-oxodGTP triphosphatases (MutT protein in *E.coli* and hMTH1 in humans) that eliminate 8-oxodGTP from the nucleotide precursor pool, and thereby prevent the insertion of the oxidized deoxynucleotide triphosphate opposite dA or dC residues during DNA replication [18, 19]. Both enzymes have also been shown to degrade 8-oxoguanosine triphosphate (8-oxoGTP), which can otherwise be incorporated in RNA opposite C or A [20].

2.2
Direct Repair

Direct repair is a single-step reversal of a lesion in the DNA by a single enzyme. One example is the O^6-methylguanine-DNA methyltransferase (MGMT), which repairs DNA by a "suicide" mechanism involving the transfer of the methyl-group from O^6-methylguanine (or other alkylated substrates) to a specific cysteine residue in the enzyme [21]. This type of repair is especially important with regard to alkylating agents. Another example is the DNA photolyase, which specifically repairs UV-light-induced pyrimidine dimers by the use of energy from blue light [22]. The principal item of the repair mechanism is the transfer of one electron from the photolyase enzyme onto the pyrimidine dimer, which breaks the linkage between the pyrimidine molecules. Subsequently, the electron is returned to the oxidized molecule, whereby the enzyme and the biologically intact DNA are recreated [23]. Photolyase activity has been demonstrated in cell extracts of a large number of higher eukaryotes (reviewed

in [24]) and a mammalian DNA photolyase has been cloned [22]; however, it is still unclear whether human cells possess significant photolyase activity [25].

2.3 Base Excision Repair

The mammalian process of base excision repair (BER) deals with most single base damages and abasic sites and acts on both oxidative and non-oxidative base modifications. The modified bases are removed by a subset of repair proteins called DNA glycosylases. Each DNA glycosylase enzyme is specific for one or a few altered bases in the DNA and catalyzes its removal.

The process of BER is divided into two overall biochemical pathways. These are termed "short patch" or "single nucleotide" BER involving replacement of one nucleotide, and "long patch" BER pathway with gap filling of several nucleotides. The repair pathways are not completely clarified as yet, and controversy still exists regarding the involved DNA polymerases, DNA ligases, and accessory proteins. Most of the present work concerning the different BER pathways are in vitro studies of reconstituted pathways with purified proteins, which may not reflect the in vivo mechanisms and do not take into account the possible regulatory mechanisms. Table 1 is based on recent reviews [15–17, 26, 27] and lists the proteins known to be involved in BER.

BER of damaged bases are initiated by a glycosylase that recognizes and removes the damaged base leaving an abasic site. There are two functional types of glycosylases named mono- and bi-functional DNA glycosylases. Mono-functional or simple DNA glycosylases hydrolyze the base-sugar (*N*-1′C) glycosylic bond to release the modified or damaged base from DNA and thereby generate an unmodified abasic (AP) site. Successive incision by an AP endonuclease makes a nick in the DNA strand. Bi-functional DNA glycosylases work by a two-step incision mechanism. Besides hydrolysis of the *N*-glycosylic bonds of their substrates, they also contain a β-lyase activity that cleaves the resulting AP-sites. The completion of the base excision repair process after removal of the damaged base can diverge in at least four pathways. As yet, there is little understanding of the potential mechanisms coordinating the different repair pathways when they are functional in the same cell.

There is strong evidence from studies in mammalian cell extracts that DNA polymerase β (pol β) is the major DNA polymerase participating in single nucleotide gap filling during short patch BER [28–31]). After incision of the DNA strand by an endonuclease or a glycosylase, DNA pol β catalyzes an β-elimination of the 5′ deoxyribose-5-phosphate by an intrinsic dRPase activity and subsequently fills the resultant one-nucleotide gap. The nick is sealed by DNA ligase III. The pathway is dependent on the XXRC1 protein [32]. However, the existence of a pol β-independent single nucleotide repair patch pathway for removal of 8-oxodG in DNA in pol β knock out mouse cell extracts has been established by Dianov et al. [33]. The repair gap in these cells is filled by DNA polymerase δ or ε. It is not clear whether this mechanism operates simultane-

ous with the pol β-dependent single nucleotide BER pathway in normal cells or only as a back up when pol β is deficient. A DNA pol β-independent long patch (2–6 nucleotides) base excision repair pathway has also been reported as a possible back-up system in pol β knockout mouse cell extracts [34, 35]. Both DNA polymerase δ and ε were able to replace nucleotides at the lesion site.

Long patch BER differ from short patch BER and from NER by both the repair patch size and the enzymes involved. Klungland and Lindahl [36] described the 2–6 nt long repair patch BER after reconstitution of repair of a reduced or oxidized abasic site. Human AP endonuclease, DNA polymerase β, DNA ligase III or I, and the structure-specific FEN1 (flap endonuclease/DNase IV) showed to be essential for the repair process. In addition, PCNA could promote the long patch pathway by stimulation of FEN1. Pol δ could substitute for pol β. Long patch BER has also been described as gap filling of up to 13 nucleotides [32, 34, 37, 38]. This pathway was shown to require AP endonuclease, FEN1, PCNA, DNA polymerase δ or ε, and DNA ligase I.

2.4 Nucleotide Excision Repair

The nucleotide excision repair (NER) pathway is involved in the removal of a wide range of bulky DNA adducts and DNA cross-links. In addition, the NER enzymes may recognize many diverse DNA lesions that do not disturb the DNA helix, including 8-oxodG and other modified bases. Under normal conditions NER does not contribute significantly (maybe as little as 0.1% [39]) to the removal of 8-oxodG in mammalian cell extracts, not even when the pol β-dependent BER pathway was disrupted [33]. It is, however, a common assumption that NER works as a back up system for BER, i.e., when BER is deficient NER can repair the base modifications.

The NER pathway consists of two different sub-pathways known as global genome repair (GGR) and transcription-coupled repair (TCR). Most genes involved contribute to both sub-pathways.

In GGR-NER the initial recognition step is believed to occur via the binding of XPC protein at the site of the lesion [40] in complex with the human homologue of the yeast RAD23B protein (HHR23B) [41]. Subsequent to the recognition of the DNA damage, the basal transcription initiation factor IIH (TFIIH) and the XPA protein are recruited to the lesion where they initiate the local opening of double-stranded DNA around the lesion [42]. XPA and replication protein A (RPA) are then thought to form a pre-incision complex. The first DNA cut is made by the endonuclease XPG five to six nucleotides downstream (3′) from the lesion. Then the XPF-ERCC1 endonuclease complex cleaves the 22nd, 23rd or 24th bond upstream (5′) of the damage and thus releases a 27–29-mer [43]. The gap is filled by DNA polymerase δ and/or ε. DNA replication proteins like PCNA, RPA, and replication factor C (RF-C) support the de novo DNA synthesis in NER. Finally, the 3′ nick is closed by DNA ligase I. Efficient GGR has been shown to require the p53 tumor suppressor protein [44, 45] probably with

the p48 protein as the link between p53 and the nucleotide excision repair apparatus [46].

TCR refers to a repair pathway that preferentially and rapidly repairs lesions on the transcribed strand of active genes (less than 1% of human DNA) [47, 48]. In TCR-NER the ability of a lesion to block RNA polymerases seems critical. The stalled polymerase recruits at least two TRC-specific factors: CSA and CSB. The remainder of the TCR-NER pathway (i.e., unwinding, incision, excision, DNA synthesis, and ligation) may be identical to the GGR-NER pathway. The breast and ovarian cancer susceptibility gene BRCA1 is involved in TCR [49, 50]; however, the exact biological function of the protein remains uncertain (for review see [51]).

2.5 Mismatch Repair

A special repair system designed to recognize and eliminate mismatches from DNA [52] differs from the other repair systems as many of the mismatches recognized are composed of normal nucleotides, but the DNA structure is abnormal because of base mispairing. The mismatch repair in mammalian cells recognizes all possible normal base mismatches, 1–12 base loops, and certain pairs involving modified bases like O^6-methylguanine:T, O^6-methylguanine:C, and O^4-methyladenine:A. The human mismatch repair system consists of the MutS complex, which is thought to bind to a mismatch and bi-directionally searches along the DNA for a nick in one of the strands that will signal the initiation of exonucleolytic degradation [53]. It is presumed that the nicks of Okazaki fragments on the lagging strand and the 3′-terminus of the leading strand provide the necessary signal for initiating the mismatch repair reaction [21]. The resulting single-strand gap that can cover up to 1,000 nucleotides is filled by DNA polymerase and closed by DNA ligase.

2.6 Double Strand Break Repair: Homologous Recombination and End Joining

When strand breaks remain open at a lesion site, or when non-repaired damage blocks the progress of a DNA replication fork to produce a daughter strand gap, a complex cascade of reactions is triggered to stop the cell cycle machinery and recruit repair factors. When, after replication, a second identical DNA copy is available, homologous recombination (HR) seems to be preferred; otherwise cells rely on non-homologous end joining (NHEJ), which is more error-prone [17].

An essential component of the HR repair pathway is the strand-exchange protein, known as RecA in bacteria or Rad51 in yeast. Several mammalian genes have been implicated in recombinational repair on the basis of their sequence homology to yeast Rad51; one of these is XRCC2, which has been shown to be essential for the efficient recombinational repair of DNA double strand breaks

(DSB) between sister chromatids in hamster cells [54]. Human RAD52 and RPA in a physical interaction have also been shown to be essential for homologous recombination [55], and so has the XRCC4 gene, which is required for the repair of DSB in mammalian cells [56].

The NHEJ reaction links ends of a DSB together without any template, using the end binding KU70/80 complex. DNA-dependent protein kinase catalytic subunit (DNA-PK_{CS}) and the nuclease Artemis bind to the KU-complex and trim the overhangs at the double strand break before ligation by the XRCC4-DNA ligase4 complex (reviewed by [57, 58]).

3 Measuring DNA Repair

3.1 Structural Analyses

The structural analyses of DNA repair enzymes are not important in a quantitative aspect but have provided very useful qualitative insights. First of all, their existence is directly proved but the structure is also a foundation for detailing the mechanisms of the repair pathways. Several repair proteins have been identified by various methods and the functional description and assembly of protein complexes have been investigated.

In recent years several high-resolution crystal structures of DNA repair glycosylases bound to their substrates, substrate analogues, or products have been solved. DNA glycosylases are probably the best understood DNA-repair enzymes in terms of damage recognition and catalysis, and several reviews covering this topic have appeared recently [15, 58–61]. Crystal structures of the repair enzymes can show how the substrate bases are exposed to the active site of the enzyme and suggest the mechanisms of catalytic specificity.

Structures of multiple DNA glycosylases have been determined, and though they share little sequence homology they can be placed in two main structural families (reviewed by [59]). The first consists of the uracil DNA glycosylase (UNG), the G:T/U mismatch DNA glycosylase (Mug), the thymine DNA glycosylase (TDG), and the single-strand specific monofunctional DNA glycosylase (SMUG). They share two common motifs, one involved in pyrimidine binding and one involved in glycosidic bond hydrolysis. The second and better-defined family of DNA glycosylases includes human 8-oxoguanine DNA glycosylase (hOGG1), human mutY homologue (MYH), human endonuclease III homologue (hNTH), and methylated DNA-binding domain protein 4(MBD4), along with a number of proteins from other organisms. The crystal structure has revealed that these proteins contain a conserved central α-helical domain. The hallmark is a helix-hairpin-helix (HhH) motif followed at a specified distance by an invariant aspartic acid residue that is essential for the catalytic activity of the enzyme [62].

A general strategy for all glycosylases is to flip the target out of the double helix and into an active site pocket of the protein where catalysis takes place. Recognition of the damaged base in the pocket is accomplished through π-stacking, and through hydrophobic and hydrogen bonding interactions [15]. However, it remains to be shown how these enzymes recognize the damaged base in the DNA before it is flipped out.

The mechanistic steps for eukaryotic NER involve the concerted action of at least six proteins or protein complexes. Recognizing damaged DNA is the first step and is mechanistically highly interesting. Many chemically distinct DNA lesions have to be recognized, and at the same time inappropriate repair must be avoided at other sites. The two sub-pathways, GGR and TCR, employ different methods of damage recognition and different enzymes are involved. For TCR the stalled RNA polymerase seems to be the signal that initiates repair, whereas for GGR the XPC/HR23B protein complex is thought to be responsible for the damage recognition (reviewed by [17]). Scanning force microscopy (SFM) at nanometer resolution has revealed that damage recognition by the XPC/HR23B complex induces a bend in DNA upon binding to a single damaged base at a defined position [63]. The authors expect this distortion to be an important feature required for subsequent assembly of an active NER complex. They also suggest from their experiments that the interaction of XPA-RPA with DNA requires both bending and unwinding of the DNA helix, probably achieved after binding of TFIIH and XPG to the DNA-XPC/HR23B complex.

3.2
Common Polymorphisms

When quantitative assays for specific repair activities in vivo are not available, the "experiments of nature" in the form of genetic polymorphisms can be of value. Very often a change in the genetic sequence leads to decreased activity, provided its function is not essential to the development and survival of the fetus. The study of common genetic polymorphisms can therefore be used to obtain useful information about the DNA repair mechanisms and their importance in vivo.

Polymorphisms in several DNA repair genes have been identified, but little is known about their phenotypic significance. A polymorphism in itself does not uncover a repair defect and cannot be regarded as quantitative. However, the polymorphisms may explain the inter-individual variations in DNA repair capacity observed in humans and may be associated with susceptibility to cancer (for a review, see [64]). Some examples of the work in this area will be summarized below.

A variant allele of XRCC1 in exon 10 (Arg399Gln) may be associated with higher DNA adduct levels in lymphocytes [65], although another study showed that the homozygote variant genotype (399 GG) apparently had a protective effect against bladder cancer relative to carriers of either one or two copies of the common allele [66]. No significant interaction was found with smoking. Different explanations for the discrepancy between the two studies can be

found, e.g., another polymorphic gene might be in linkage disequilibrium with XRCC1 or the variants might be more likely to undergo apoptosis or senescence and therefore have a decreased risk of cancer.

The Ogg1 Ser326Cys polymorphism has been investigated in relation to breast cancer risk in 859 postmenopausal women (425 cases and 434 controls) but no association between genotype and breast cancer risk was found [67].

In DNA ligase I a single nucleotide polymorphism (A to C) in exon 6 was evaluated in a case control study with 530 cases and 570 controls with regard to susceptibility to lung cancer. The polymorphism was found to be very common, but there was no association with the risk of lung cancer, neither in adenocarcinomas nor in squamous cell carcinomas [68].

The XPD polymorphism found in exon 6 (C to A at nucleotide 22541) showed a mild protection against basal cell carcinoma for the CC genotype, whereas the polymorphisms (Asp312Asn) and (Lys751Gln) showed no correlation between genotype and risk of basal cell carcinoma in the study group (N=189) [69].

An XPA polymorphism (A to G) in the 5′ non-coding region of the XPA gene was recently evaluated in 695 lung cancer cases and 695 controls [70]. The presence of one or two copies of the G allele was associated with a reduced lung cancer risk; however, as in the above examples the functional significance is unknown.

A recent study by Mort et al. [71] showed that the NER genes XPD, XPF, XPG, and ERCC1, and the BER gene XRCC1 are not important genetic determinants in colorectal carcinogenesis, while a modest association between colorectal cancer and a polymorphism in the recombination repair gene XRCC3 gene was found.

A thorough review of markers of DNA repair and susceptibility to cancer in humans from 2000 [64] has summarized the results from 64 epidemiologic studies that addressed the association of cancer susceptibility with a putative defect in DNA repair capacity. The authors concluded that the vast majority of studies showed a difference between cancer case subjects and although this observation is compatible with a chromosomal instability due to the cancer itself, it was notable that impaired mutagen sensitivity was also observed in healthy relatives of cancer subjects. There were a variety of functional tests used that only indirectly addressed DNA repair and these showed high variability in their expression. Finally, the issue of confounding was almost totally unexplored.

It is obvious that more studies are warranted, with a precise estimate of the DNA repair capacity in addition to the genotype-cancer association. It is believed that the study of inter-individual variability in DNA repair will greatly contribute to our understanding of human carcinogenesis.

3.3
Naturally Occurring DNA Repair Knockouts

Inborn deficiencies in DNA repair have also provided useful information regarding the importance of DNA repair in vivo. However, as many of the

involved proteins are not limited to playing a role in DNA repair, it can be very difficult to determine the contribution of the repair deficit versus the other possible deficits, e.g., insufficient transcription. For example, gene expression profiling by a micro-array technique of primary fibroblast cell cultures from young and old humans and from patients with Werner syndrome has shown that the expression profile of Werner cells closely resembles that of old normal fibroblasts, even though Werner cells only have a single genetic defect that leads to production of a single mutated protein [72].

Known DNA repair-deficient syndromes mainly affect the nucleotide excision repair pathway and the mechanisms for strand break repair. No human disorders caused by inherited BER deficiencies have been identified. The most likely explanations are based on the generated mice knock out models. Deficiency of a single glycosylase may not cause an overt phenotype as the substrates can be repaired by other glycosylases or by other repair systems. In contrast, knock out of BER core proteins often induces embryonic lethality.

Many insights into the importance and the mechanism of human NER have come from the study of cells from patients with hereditary disorders. Xeroderma Pigmentosum (XP) patients are abnormally sensitive to sun exposure, presenting with skin melanomas at an early age, but are also prone to other malignancies caused by known carcinogens due to mutated XP genes [73, 74]. The XP type C patients are only deficient in GGR-NER but proficient in TCR. XP type A patients are completely deficient in NER and display progressive neurological dysfunction in addition.

Cockayne syndrome (CS) patients with mutation in the CSA or CSB gene are completely deficient in TCR-NER, but proficient in GGR. This rare hereditary disease is characterized by postnatal growth failure and early onset of severe neurobiological abnormalities, but no cancers [75]. This may be explained by the fact that the TCR defect causes CS cells to be particularly sensitive to lesion-induced apoptosis, thereby protecting against tumor genesis [17]. This syndrome emphasizes the importance of specific repair of actively transcribed DNA.

Photosensitive trichothiodystrophy (TTD) patients have defects in the XPD or XPB gene and cannot repair cyclobutane dimers (CPD). The phenotype is characterized by many of the symptoms common to the CS patients but with the additional characteristics of brittle hair and nails, and scaly skin. Why mutations in XPD and XPB can give rise to both XP and TTD is explained by the dual functions of the proteins in NER and transcription. The special hallmarks of TTD are thus due to reduced transcription and expression of matrix proteins [76].

Fanconi's anaemia (FA) is an autosomal disorder with bone marrow failure, variable presence of developmental abnormalities, hypersensitivity to DNA cross-linking agents, and a very high incidence of cancer. Different complementation groups have been cloned, but the exact pathway remains uncertain. However, data suggest that the syndrome is linked to mutations in XRCC9 and XRCC11 and therefore to defects in the homologous recombination repair pathway (summarized in [26]).

Nijmegen breakage syndrome (NBS) is associated with defects in NBS1 involved in homologous recombination. The patients display an excessively high risk for the development of lymphatic tumors, immunodeficiency, and chromosomal instability as well as microcephaly and growth retardation [77].

The clinical phenotype of ataxia telangiectasia (AT) syndrome is more striking due to ataxia, cerebral degeneration, and dilated blood vessels in the eyes, but shares many of the characteristics from NBS (chromosome instability, radiation sensitivity, and increased cancer risk). The gene responsible for AT is named ATM and the protein product is a protein kinase, likewise involved in double-strand break repair.

Members of the RECQL gene family are responsible for the syndromes Werner syndrome (mutation in WRN), Bloom syndrome (mutation in BLM), Rothmund–Thomson syndrome (mutation in RECQL4), and RAPADILINO (mutation in RECQL4) (summarized in [78]). All polypeptides encoded by RECQL genes share a central region of seven helicase domains, however, the precise function of the enzymes are unknown. As the patients (at least in Bloom, Werner, and Rothmund–Thomson syndromes) are cancer prone and display chromosomal instability, the most likely affected genome maintenance mechanism is homologous recombination [17].

Hereditary non-polyposis colorectal cancer (HNPCC) is due to defects in mismatch repair (MMR) and thus to deficiencies in removing nucleotides mispaired by DNA polymerases as well as insertion/deletion loops (1–10 bases). This dramatically increases the mutation rate. The affected genes are MLH1 (60%), MSH2 and MSH6.

3.4 Generated Knock Out Mice

The ability to "knock out" a specific gene has proved very useful in elucidating complex DNA repair enzymes and in establishing the role of individual proteins in the suppression or generation of cancer. The conventional target gene replacement technologies used to generate knock out strains are both technically difficult and time consuming. Nevertheless, 148 mice strains are listed in the 5th version of the "Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage" [79]. They are listed in seven tables that represent defects in direct repair, BER, NER, MMR, strand break repair, trans-lesion synthesis, and finally in a table of mouse strains with mutations in genes that influence other cellular responses to DNA damage.

It is beyond the aim of this chapter to review the huge amount of data published regarding these mouse strains. However, a very generalized picture is that many of the knock outs give strikingly normal phenotypes while others are embryonically lethal. The former reflects the overlap in the repair pathways and maybe also a strong selection as the fertility of the knock out mice is often severely lowered. At the other end, the lethal phenotype limits the amount of

information gained from the knocked out gene. Still, the method has been useful in revealing the many repair mechanisms.

3.5 RNA Interference

The discovery of small interfering RNAs (siRNA) was in 2002 nominated as the scientific "Breakthrough of the year" by the editors of Science [80]. Providing researchers with a powerful tool for gene regulation, siRNAs have opened a new door for genetic research and therapeutics, and RNA interference (RNAi) is rapidly becoming a chosen technique for down-regulating the expression of a specific gene.

RNA interference (RNAi) was initially proven to be a method of knocking down gene activity by the introduction of a long double-stranded RNA (dsRNA) molecule in invertebrates such as Drosophila and *C.elegans* [81–83]. However, in most mammalian cells the introduction of long dsRNA initiates a cellular interferon response that ultimately causes cell shutdown and leads to apoptosis. Instead, the introduction of small 21–23 nucleotides sequence-specific RNA duplexes was found to initiate post-transcriptional gene knockdown, apparently without triggering this non-specific effect in mammalian cells [84].

The RNAi process can occur in eukaryotes by introducing short (or small) interfering RNA (siRNA) duplexes directly or by expression of long double-stranded RNA (dsRNA) as hairpins or snap-back RNAs and subsequent cleavage into siRNAs, facilitated by an enzyme called Dicer (reviewed by [85]). The siRNA associates with an intracellular multi-protein RNA induced silencing complex (RISC). This complex recognizes and cleaves complementary cellular mRNA. The cleaved mRNA is targeted for degradation, ultimately leading to knock down of post-transcriptional gene expression in the cell [85].

The possibility of engineering cells to provide limiting levels of an essential gene product instead of a complete knock out is a major advantage of this technology. In addition, the RNAi methods are much easier to apply than the conventional gene knock out technology and offer a rapid path for probing gene function and, importantly, the procedure makes it possible to simultaneously suppress several genes. The specificity of RNAi may also make it possible to silence a disease-causing mutant allele specifically and the technology is hoped to become a tool to create RNAi-based therapeutics.

However, pitfalls also exist for this method. Two very recent reports show that a substantial number of siRNA and shRNA vectors can indeed trigger an interferon response and although the effect of this response in vivo is not yet certain, potential non-specific effects not attributable to the gene targeted must be considered [86, 87].

RNAi technology has recently been adapted for investigation of DNA repair processes. Rosenquist et al. have used siRNAs to study Neil1 glycosylase in *E. coli* [88]. An 80% reduction in Neil1 protein was achieved and this led to hypersensitivity to ionizing radiation.

Complete depletions of BRCA1 and BRCA2 proteins, which appear to act in the Fanconi anemia repair pathway of interstrand cross-links and maintenance of genome stability, leads to cell lethality. Thus, instead of gene knock out, siRNA was used to transiently deplete the expression in order to better understand the function of the proteins [89]. It was shown that BRCA2 acts late in the FA response to interstrand cross links, whereas BRCA1 must act early in the FA response and has at least one additional role in interstrand cross-link repair outside the FA pathway.

3.6 Incision Assay

The incision assay [90, 91] is a widely used tool to investigate the mechanisms and activities of DNA base excision repair. It is a functional assay that reveals the repair capacity measured by the incision activity for a synthetic oligonucleotide of 25–30 bases in length with an incorporated base damage (numerous possibilities offered by many biotech companies) at a specific residue. The oligonucleotide is commonly 5′-labeled with [γ-32P]-dATP, but can also be labeled fluorometrically [92]. It is added as a substrate to a reaction mixture containing purified enzymes, or cell or tissue extracts. The volume of the extract is typically normalized to the total protein concentration in the sample. After incubation, the DNA is precipitated by ethanol and separated on a denaturing polyacrylamide gel. Phosphor imaging (fluorescence imaging) reveals the amount of intact and cleaved oligonucleotides. The procedure is relatively simple; however, to use it quantitatively it is very important to work with pure oligonucleotides similar in length and to measure the incision product while the reaction is lineally increasing.

Initially the assay was primarily used qualitatively to investigate the specificity of partly or completely purified repair enzymes from *E. coli* [90, 93] or mammalian cells [91, 94–98]. However, the assay has now been widely used to quantify the repair activity, e.g., [99–106]. Very recently OOG1 activity was measured in protein extracts, prepared from peripheral blood mononuclear cells or lung tissue, from 68 case patients with non-small-cell lung cancer and 68 age- and sex-matched healthy controls [107]. The overall finding was a significantly lower activity of OGG1 in the lung cancer patients, which was believed to be determined by genetic factors. Thus, low OGG1 activity might be a susceptibility factor for lung cancer. Another study suggests that decreased BER activity for lipid peroxidation adducts on adenine and cytosine is associated with inflammatory related lung adenocarcinoma [108].

3.7 Indirect Measurements of DNA Repair Activity

A variety of methods exist for measuring DNA repair. An often-used approach is to measure the amounts of DNA damaged at various time points following the induction of damage.

The alkaline elution assay [109] and the DNA unwinding assay [110] in various modifications can be used to measure single strand breaks (SSB) in DNA. The estimate of repair activity is achieved by measuring SSB immediately after damage induction and after a given incubation period, thus allowing the cells to repair the induced damages. The methods are based on a faster elution through a membrane filter or a faster unwinding of broken DNA strands under alkaline conditions.

The comet assay [111, 112] is also often used to estimate repair activity [113, 114]. Again the principle is the migration patterns of damaged DNA strands versus undamaged DNA under alkaline conditions. The comet assay has the advantage that it requires only small sample quantities.

The three methods above can be combined with enzymatic nicking of the DNA by purified repair proteins to estimate repair of specific base damages or abasic sites.

DNA double strand breaks (DSB) can be measured by the same techniques as the SSB, by changing the alkaline conditions to neutral pH and thereby avoiding the denaturation of the DNA helix. The more DSB, the faster the DNA will migrate whereas SSB will not be detected under neutral conditions. When measured before and after a defined incubation period, the repair capacity of DSB can be estimated.

Various DNA adducts can be measured both in tissue, blood cells, and urine and are widely used as biomarkers of DNA damage and are also used to estimate DNA repair. When estimating repair activity from tissue or blood cells it is important to bear in mind that the amounts of altered bases or bulky adducts reflect steady state levels and that alterations are both dependent on the induction of damage as well as the repair. Thus if a given type of DNA adduct is very efficiently repaired, the levels can possibly remain unaffected by an increased load of damage induction, whereas the levels of more slowly repaired modifications will increase and then normalize. Another important issue when discussing repair kinetics is the complex biology of ROS and thus the possibility that DNA modifications may continue to arise for a long period following induction of a certain type of damage. Thus when one calculates the $T_{1/2}$ of a given type of modification it is necessary to consider this. Conventionally, normal log linear decay is used to calculate the $T_{1/2}$. In our opinion this is not correct and may give biased results, as there is a constant production of damage during the decay. Furthermore, the quantification of adducts may be biased by artificial formation [115] and much of the data must be interpreted with caution [116–120].

Specific DNA adducts are excreted into urine as modified bases and deoxynucleotides [121–123]. As endonucleases and glycosylases repair the damaged DNA, deoxynucleotides and bases respectively are liberated. Oligonucleotides and bulky DNA adducts like benzo[a]pyrene-deoxynucleotides or aflatoxin-B_1-N_7-dG are also excreted. Immuno-affinity chromatography, GC/MS, LC-MS/MS, or HPLE-EC can be used to quantify the wide range of base modifications and thus to estimate the DNA repair activity, whereas ELISA assays do not yet have sufficient proven specificity.

The most widely studied adduct in urine is the oxidation product of guanine either as the nucleoside (8-oxodG), which probably reflects the NER, or as the base (8-OH-Gua), which represents the BER activity. Even though it has been shown that only a small fraction of the 8-oxodG modifications are repaired by the NER system by human cell extracts [124, 125], possibly as low as 0.1% [39], most investigators measure the urinary level of 8-oxodG instead and not the levels of 8-oxoG. Diet has apparently no influence on excretion, either of the nucleobase or the nucleoside in the urine [126], which was the initial reason for not measuring the modified base in urine. It is also much more difficult to measure the modified base in urine because of its much lower electrochemical reactivity. The ratio of excreted modified base to modified nucleoside could be an arbitrary measure of the contribution of NER and BER, however, there are only few data available and it has not yet been clarified whether the ratio of NER:BER changes with increased oxidative stress, or how it is distributed in the population. An estimation of repair capacity in this way is therefore somewhat uncertain.

While the excretion of modified bases and nucleotides relies on functional DNA repair mechanisms, the rate of excretion is a measure of the rate of oxidation, and this rate is not changed by changes in DNA repair. The argument for this is that if there is a change in DNA repair of a given lesion, the levels in tissue DNA will increase/decrease and a new steady state between formation and excretion will occur. In this new steady state the formation and excretion will again be identical and the rate of excretion will therefore be a measure of the rate of formation of the damage, i.e., oxidative stress, independent of repair. This is valid as long as a steady state is actually achieved and as long as there is residual repair capacity [127, 128]. Presently there are no published data on urinary excretion in DNA repair-deficient humans or animals.

A final example of indirect DNA repair measurements is the estimation of DNA repair from the frequency of mutations in a single gene after induction of damage. The hypoxanthine phosphoribosyltransferase (HPRT) gene is excellent as a model gene as mutations inducing loss of function in the gene renders the cell resistant to 6-thioguanine (TG) and thus is easily detectable [129]. Furthermore the gene is carried on the X chromosome and hence only exists in one (expressed) copy in each cell. When estimating repair capacity, part of the cells must be kept in a non-selective media for a given period of time after damage induction to allow the repair systems to work and before the mutation frequencies are determined. With this system it is not possible to detect any silent mutations in the model gene.

3.8 Direct Measurement of DNA Repair Enzyme Activity

The activity of DNA repair enzymes can be estimated from different related assays. The common characteristic is the use of synthetic DNA strands or plasmids with specific incorporated or induced damage.

The repair synthesis assay developed by Wood et al. in 1988 [130] is based on a damaged double-stranded plasmid, which is incubated with cell or tissue extract in the presence of normal dNTP and one ^{32}P-labeled deoxynucleotide. The incorporation of labeled deoxynucleotides by the repair enzymes in the extract corresponds to the repair capacity. The assay, however, is quite laborious and rarely used for quantitative purposes.

3.9 Other DNA Repair Assays

In the host cell reactivation assay [131], cells in culture are transfected with a plasmid containing, e.g., a transcription-blocking damage in a specific gene. Any type of DNA damage that renders the reporter gene product inactive can be assayed. After a given incubation time allowing the cell to repair the damage, the activity of the reporter gene product reflects the repair capacity.

The gene-specific repair assay developed by Bohr and Hanawalt in 1985 [47] is a general technique to study the repair process in individual genes. The principal idea in this approach is to use a DNA repair enzyme to generate a strand break at the site of a lesion, and then to measure the frequency of DNA lesions within specific restriction fragments located within specific genes before and after incubation. The use of Southern probing for quantifying the genes makes it possible to study various genes in the same sample. Until now the assay has only been applied successfully to cell cultures, as a substantial amount of damage is necessary to detect the repair capacity.

Another approach for studying general DNA or gene repair is to detect changes in the genetic code by DNA sequencing and calculate the repair by correlating the number of mutations to the induction of damage. It is important though to notice that a mutational fingerprint is dependent on at least three components: (1) the specificity of DNA repair, (2) the distribution of DNA damage, and (3) the ability of the genetic target to reveal mutation (related to the product and the mutation selection criteria).

4 Conclusions

DNA repair mechanisms, or rather gene repair, had already been suggested a century ago, and about 40 years ago the basic four biochemical steps were suggested (Table 1). Both knowledge and interest in DNA repair have accelerated, particularly during the last decade. It is now clear that a large number of environmental chemicals are transformed in the body, ultimately to carcinogens that act by binding to DNA and thereby inducing adducts, and that such adducts can be repaired to a large extent. Maybe more interesting, it is now clear that products from normal cellular processes, e.g., reactive oxygen species and lipid peroxidation, also are capable of modifying DNA and inducing

mutations. Furthermore, it seems that the levels of such endogenously induced DNA modifications occur at much higher rates and result in higher levels of modifications than exogenously derived modifications. Add to this, the fact that endogenous lesions are mutagenic to a large extent. Even more interesting there is increasing evidence of considerable variation between individuals with regard to activity of DNA repair mechanisms and there are examples of diseases that have deficiency in DNA repair as a risk factor. The focus has long been on modification of DNA by endogenous as well as exogenous factors. Now we have to realize that how the DNA repair mechanisms function could be even more important for development of disease and ageing.

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