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Scientific Background on the Nobel Prize in Chemistry 2015

MECHANISTIC STUDIES OF DNA REPAIR

compiled by the Class for Chemistry of the Royal Swedish Academy of Sciences

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The Royal Swedish Academy of Sciences has decided to award Tomas Lindahl, Paul Modrich and Aziz Sancar the Nobel Prize in Chemistry 2015 for their “Mechanistic studies of DNA repair”

Damage to the genetic material poses a threat to all organisms. To counteract this threat, cells have evolved a series of intricate DNA repair pathways that correct DNA lesions affecting base pairing or structure of DNA. Today we understand the molecular mechanisms of these pathways in great detail, in large part due to the pioneering studies by Lindahl, Modrich and Sancar that opened up the field.

Background

The human genome encodes the information needed to create a complete human being. During every cell division, more than three billion DNA base pairs are replicated and copies of the genome are transferred to the daughter cells. Although very efficient, the DNA replication machinery responsible for this task still makes occasional mistakes. Given the size of the human genome and the large number of cells in a human body (about 3.7×10^{13}) mistakes will inevitably accumulate during the lifetime of an individual. Most of these errors will remain silent, but they can also cause serious diseases.

Despite its essential role in storing genetic information, the DNA molecule has limited chemical stability and is subject to spontaneous decay [1]. Processes such as hydrolysis and oxidation occur at significant levels *in vivo*, in part due to reactive metabolites continuously generated in various physiological processes. In addition, external factors like radiation and genotoxic chemicals will further stimulate of DNA damage formation.

The inherent instability of DNA constitutes both an opportunity and a threat. DNA lesions can block important cellular processes such as DNA replication and transcription, cause genome instability and impair gene expression. Lesions can also be mutagenic and change the coding capacity of the genome, which can lead to devastating diseases and conditions associated with genome instability, including cancer, neurodegenerative disorders and biological ageing. At the same time, without mutations Darwinian evolution would not be possible. Furthermore, mutagenic chemicals and radiation can also have a healing effect; they can for instance be used to treat cancer, by introducing DNA lesions that halt cell proliferation and stimulate programmed cell death.

The cell has developed ways to counteract DNA lesions and to keep DNA mutations at a tolerable level. A number of different DNA repair mechanisms correct lesions and safeguard the integrity of the genome. Four fundamental DNA repair pathways delineated by this year's Nobel Prize laureates will be discussed here.

Photoreactivation – the first DNA repair mechanism

In the 1920s, the American geneticist Hermann Muller (Nobel Prize in Physiology or Medicine, 1946) found that X-rays could mutate and kill cells [2]. Later on, other types of agents, including ultraviolet (UV) light, were also shown to affect cell viability and mutation levels. The cellular target for the lethal effects of X-rays and UV-light was unknown at this time, and there were no identified cellular mechanisms that could repair the lesions once they occurred. A breakthrough came in the late 1940s, when Albert Kelner studied bacteria and their recovery in response to damage caused by UV-light. Kelner found that visible light could dramatically stimulate growth recovery after a growth arrest caused by UV exposure [3], [4]. The phenomenon was termed *photoreactivation* and it pointed to the existence of a light-dependent cellular mechanism that could correct UV-induced cellular damage.

Oswald Avery and co-workers had demonstrated in 1944 that DNA is the material of heredity [5] and in the 1950s it became clear that UV-induced damage most likely was targeted to DNA. At this point, Renato Dulbecco (Nobel Prize in Physiology or Medicine, 1975) suggested that photoreactivation was an enzymatic reaction dependent on visible light [6]. The correctness of this assumption was demonstrated by Stanley Rupert, who in a series of reports showed that DNA could be reactivated by visible light in the presence of a cell-free extract from *Escherichia coli* or *Saccharomyces cerevisiae* [7, 8]. The observation of this enzymatic activity, known as the photolyase, was of profound importance, since it demonstrated for the first time the existence of DNA repair enzymes that could rescue UV-irradiated DNA. At first, the photolyase was just an activity in an extract, but in 1978 Aziz Sancar could clone the *E. coli* photolyase gene and amplify the gene product *in vivo* [9]. Sancar was at the time a PhD student of Rupert's, but instead of continuing to characterise the photolyase, he wrote his PhD dissertation and graduated. It would take another six years, before Sancar returned to photolyase research.

Dark repair – the discovery of nucleotide excision repair

In addition to photoreactivation, UV damage can also be repaired in a light-independent process (known as "dark repair"). That UV-irradiation of DNA introduced thymine dimers *in vitro* was reported in 1960, but the *in vivo* relevance of this finding was unclear [10]. A couple of years later, Jane Setlow and Richard Setlow demonstrated that thymine dimers inactivated transforming DNA in the bacterium *Hemophilus influenzae* and that this type of lesion was responsible for the biological effect of UV-radiation [11, 12]. This realization made it possible to study the precise molecular consequences of thymine dimers and investigate how cells deal with

them. In 1963, Richard Setlow reported that thymine dimers inhibit DNA synthesis and that these lesions are excised from DNA in wild-type UV-irradiated bacteria, but not in an UV-sensitive, mutant *E. coli* strain [13]. In 1964, he made the seminal discovery that thymine dimers disappeared from the irradiated, high molecular weight genomic DNA shortly after exposure to UV and instead appeared in low molecular weight fractions. Richard Setlow and his colleague William Carrier correctly interpreted this result as thymine dimers being excised (removed) from the DNA, hence the name *excision repair* [14]. Around this time, Richard Boyce and Paul Howard-Flanders also published observations with conclusions similar to those reached by Richard Setlow [15]. The emerging mechanisms of what later became known as *nucleotide excision repair* (NER) was further elucidated in crucial work by Philip Hanawalt and David Pettijohn, who found that UV-irradiation stimulated DNA repair synthesis even outside the S-phase, i.e. independent of genome replication [16]. The groundbreaking contributions of these early pioneers clearly indicated the existence of repair mechanisms that could correct UV-induced lesions, but the precise molecular mechanisms underlying NER remained obscure.

The molecular mechanisms of NER

The identification of the enzymes responsible for NER was greatly assisted by genetics analyses. Earlier bacterial work had identified *uvrA*, *uvrB*, and *uvrC* genes in a search for mutations that impaired NER and hindered growth resumption after UV irradiation [17]. Work *in vivo* [18-20] and in *E. coli* extracts [21] by Erling Seeberg and others indicated that the *uvr* gene products functioned by endonucleolytic cleavage of irradiated DNA, but this could not be examined in detail due to the lack of purified proteins.

In the 1970s, identification of proteins was a major challenge, since DNA sequencing techniques were limited and a given gene locus could encode more than one protein. Aziz Sancar, now working with W. Dean Rupp at Yale School of Medicine, developed the elegant Maxicell technique, which relies on a UV-repair deficient bacterial strain [22]. After transformation of a plasmid DNA of interest, the hypersensitive bacteria can be UV-irradiated, which causes breakdown of the larger chromosomal DNA, whereas the plasmid molecules that have not been hit by UV can continue to replicate and express proteins. In combination with radioactive amino acid incorporation, the technique allows for labelling and detection of plasmid-encoded proteins in the absence of a chromosomal background. The Maxicell technique was soon applied to a wide variety of protein identification projects and allowed Sancar to rapidly identify the proteins encoded by the *uvrA*, *uvrB* and *uvrC* genes [23-25].

In a groundbreaking work published in 1983 [26], Sancar used the purified UvrA, UvrB, and UvrC proteins to reconstitute essential steps in the NER pathway. The three proteins acted specifically on damaged DNA. With UV-irradiated DNA as a substrate, the proteins hydrolysed two phosphodiester-bonds on the damaged DNA strand. The incisions were performed at

precise locations relative the UV adduct, one at the 8th phosphodiester bond 5' to the lesion and a second at the 4th or 5th bond 3' to the same lesion, thus generating a 12-13 nt long fragment. Later, Sancar could show that the rate of the reaction is stimulated by UvrD (DNA helicase II) and DNA polymerase I (Pol I), which catalyses the removal of the incised strand and synthesis of the new DNA strand, respectively [27]. Finally, DNA ligase catalyses the formation of two new phosphodiester bonds and thus seals the sugar-phosphate backbone. In the 1983 paper, Sancar described the UvrA, B, and C proteins as working together in a single complex (an excinuclease), but later findings by him and also others have modified this view. We know today that Uvr proteins associate with DNA lesions in a step-wise fashion (Figure 1) [28]. First, a complex of two UvrA and one UvrB subunit ($UvrA_2B$) tracks along the DNA. The UvrA subunits are responsible for the initial damage recognition on double-stranded DNA, which causes the $UvrA_2B$ complex to halt. At this point, the UvrB helicase activity is activated, leading to local unwinding of DNA around the lesion (about 5 bp), kinking of the template and further recognition of the damaged strand by UvrB. As a consequence, the UvrA proteins dissociate from UvrB and a single UvrC subunit binds to the remaining UvrB-DNA complex. UvrC activates UvrB, which makes the 3' incision, which is followed by UvrC catalysed incision at the 5' side of the lesion. The UvrD helicase displaces the damaged DNA strand, after which only UvrB remains bound to the gapped DNA. At this point, Pol I associates with DNA, fills the gap and UvrB is released. Finally, the repaired patch is ligated.

We know today that thymine dimers are just one of numerous types of lesions that interfere with normal base pairing and thus distort the helical structure of DNA. NER can recognise these problems and correct them by its cut-and-patch mechanism. The overall mechanisms of NER in mammalian cells are closely related to those characterised in bacteria. The mechanisms of damage recognition, incision on both sides of the lesion, removal of the damaged oligomer and resynthesis of the gap are very similar between the two systems. However, even if the overall strategy of repair is the same, the proteins responsible are distinct. Whereas damage recognition and dual incision is carried out by only three proteins in *E. coli* more than fifteen proteins are required to carry out the same function in human cells.

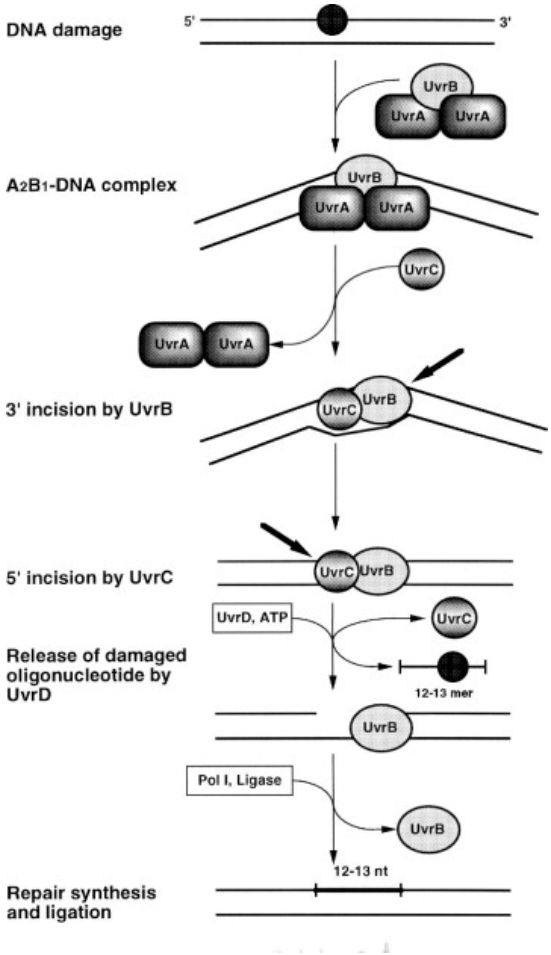

Figure 1. (Petit and Sancar, Biochimie 1999, 81:15-25) A simplified model for nucleotide excision repair in *E. coli*. First, a heterotrimer of UvrA and UvrB is located to the DNA damage. Next, DNA is kinked and partially unwound by UvrB through an ATP-dependent reaction. UvrA leaves and UvrC binds to the UvrB-DNA complex, activating UvrB that makes the 3' incision, which is followed by an UvrC dependent 5' incision. The excised oligomer is released by the UvrD helicase. Pol I fills the gap and releases UvrB at the same time. Finally, the repaired patch is ligated.

Mutations in the NER system is linked to a number of human genetic disorders, including *Xeroderma pigmentosum* (XP), which is characterised by hypersensitivity to UV-radiation and a very high risk for skin cancer. Many of the genes encoding mammalian NER factors were first identified by James Cleaver, who carefully identified XP causing mutants in affected humans [29]. Over the years, the mammalian NER system and its mechanisms have been characterised in detail by a number of different laboratories. Among the many notable contributions is the reconstitution of human NER with purified factors by Sancar [30] and Richard D. Wood [31].

The photolyase and its reaction mechanism

In 1982, Sancar took up a faculty position at the University of North Carolina, Chapel Hill. At this point he also returned to his PhD project, the photolyase. Between 1984 and 1989, Sancar published a series of papers in which he described the mechanisms for photolyase function, including the identification of two chromophores present in the *E. coli* enzyme [32-34]. Sancar demonstrated that photolyase, can convert the energy of an absorbed photon into chemistry that

produces a localised free radical that initiates thymine dimer splitting. One of the two chromophores in the photolyase is the fully reduced flavin–adenine dinucleotide (FADH-). This chromofore functions as catalytic cofactor and upon excitation, it performs the actual repair, when the excited flavin cofactor transfers an electron to the pyrimidine dimer to generate a charge-separated radical pair (FADH· + Pyr<>Pyr·–). The anionic ring of the dimer is split by a cycloreversion, and the excess electron returns to the flavin radical to restore the catalytically competent FADH– form and close the catalytic photocycle (Figure 2). The reaction is light-activated, since the flavin cofactor may be excited by direct photon absorption or by resonance energy transfer from another chromophore, which is an antenna pigment (methenyltetrahydrofolate or deazaflavin) that harvests sunlight and enhances repair efficiency.

Photoreactivation was the first form of DNA repair identified, but this process is not conserved in mammalian cells, which instead rely on NER for correction of UV damage. However, the photolyase has mammalian homologues, which are used to help setting the circadian clock, i.e. the regulation of biological processes in response to light [35].

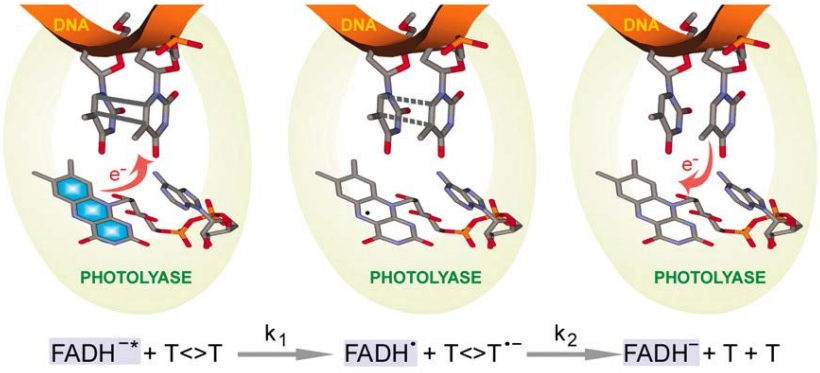

Figure 2. (Kao et al., Proc Natl Acad Sci USA 2005, 102: 16128-32) Repair of damaged DNA by photolyase. Photolyase acts through an electron-transfer radical mechanism. The key catalytic reactions, charge separation (*k* 1) and ring splitting (*k* 2), are given at the bottom.

DNA is an unstable molecule

In the early 1970s Tomas Lindahl demonstrated that DNA has limited chemical stability even in the absence of external physical assaults. Under physiological conditions DNA is subject to a number of chemical reactions such as hydrolytic deamination, oxidation and non-enzymatic methylation. These reactions modify the bases of DNA and as a consequence increase the risk for mutations. Tomas Lindahl used the term DNA decay to describe these processes and elegantly demonstrated that under physiological conditions, spontaneous hydrolytic DNA

depurination occur at significant levels [36] and stimulate cleavage of DNA chains [37]. Perhaps the most fascinating discovery was the demonstration of high levels of spontaneous cytosine deamination under physiological conditions, which leads to the formation of uracil. Since uracil forms base pairs with adenine, cytosine deaminiation is a highly mutagenic process, with important long-term consequences. High levels of cytosine deamination pose a risk of depleting the genetic material from cytosine-guanine base pairs and replacing them with thymine-adenine [38].

Discovery of base excision repair

Based on his observation that uracil is frequently formed in DNA, Tomas Lindahl came to the conclusion that there must exist an enzymatic pathway that can handle this and other types of base lesions. In a now classic study, he single-handedly identified the *E. coli* uracil-DNA glycosylase (UNG) as the first repair protein [39] and two years later a second glycosylase, specific for 3-methyladenine DNA [40]. We know today that UNG is the founding member of a large family of proteins that orchestrate *base excision repair* (BER). The identification of UNG relied on careful analysis of enzymatic release of uracil as a free base from DNA *in vitro*. Lindahl demonstrated that the enzyme was specific to DNA and did not act on deoxymononucleotides or any form of RNA. He also showed that the DNA backbone remained intact in the process, which immediately suggested the involvement of another category of enzymes, the apurinc/apyrimidinic endonucleases. An *E. coli* activity specific for apurinic sites had been identified just two years earlier by Walter Verly [41, 42]. Lindahl could outline the basic concepts for BER already in the 1974 paper and in a series of papers published in the years to follow he and others could verify the proposed model. Through continued studies, Lindahl could reconstitute the entire BER with purified enzyme, both from *E. coli* [43] and human cells [44]. The process is initiated when a DNA glycosylase recognises and hydrolytically cleaves the base-deoxyribose glycosyl bond of a damaged nucleotide (Figure 3). Mammalian cells contain number of different DNA glycosylases, which act on various forms of base modifications [45]. Once a damaged nucleotide has been identified, the DNA glycosylase kinks the DNA and the abnormal nucleotide flips out [46]. The altered base interacts with a specific recognition pocket in the glycosylase and is released by cleavage of the glycosyl bond. The DNA glycosylase itself often remains bound to the abasic site until being replaced by the next enzyme in the reaction cycle, the apurinic/apyrimidinic (AP) endonuclease, which cleaves the DNA backbone at the 5′ side of the abasic position. The AP endonuclease also associates with DNA polymerase β (pol-β), to fill the gap [47]. In addition, pol-β harbors a lyase activity, which excises the 5′-terminal base-free sugar phosphate residue in a nonhydrolytic elimination process [48]. However, the repair of oxidatively damaged nucleotides has no requirement for such a pol-β-associated lyase activity, because the DNA glycosylases concerned possess endogenous AP lyase activity themselves. In a final step, DNA ligase III/XRCC1 heterodimer interacts with pol-β, displaces the polymerase, and catalyses the formation of a new phosphodiester bond [44].

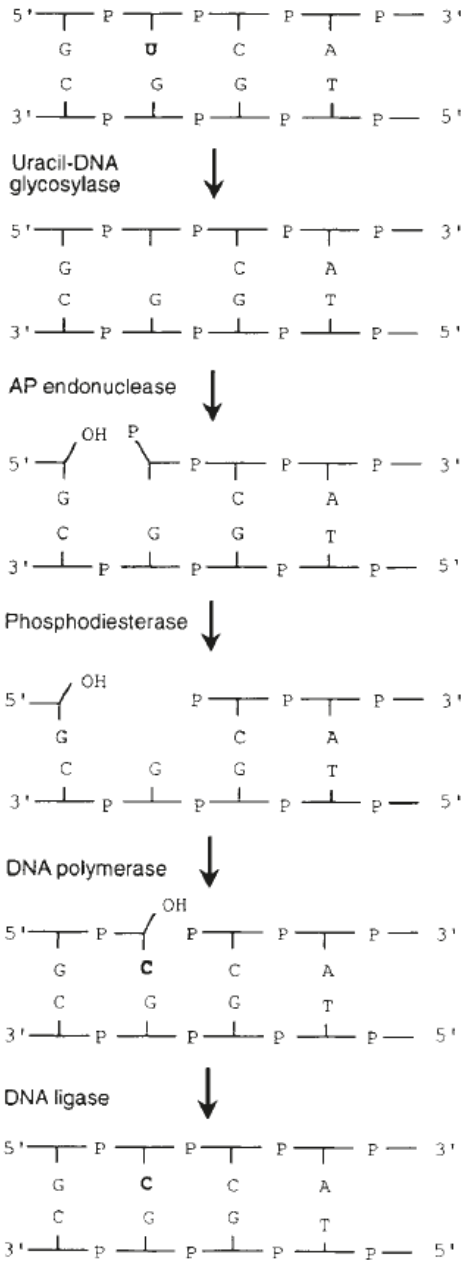

Figure 3. (Lindahl, Nature 1993, 362:709-715) The base excision repair pathway for removal of endogenous damage from cellular DNA. The scheme shows the removal of a deaminated cytosine residue (uracil). The details of the reaction are given in the text.

We know today that BER corrects many different forms of lesions that affect the bases without causing gross structural perturbation in the DNA structure. Lesions like these present a special challenge to the replication machinery, since they may cause miscoding, i.e. the chemically altered base has the potential to base pair with an erroneous base, either during DNA replication or transcription. To date, more than 100 different types of oxidative lesions have been identified and the vast majority of these are corrected by BER. The lesions corrected by BER may also be formed by exogenous factors, including ionizing radiation.

Mismatch repair

As noted above, the DNA replication machinery is not error-free. There is always the possibility that an incorrect nucleotide is introduced during synthesis of a new DNA strand. As a result, a non-Watson-Crick base pair is formed, which distorts the double-stranded DNA helix. These types of errors are known as mismatches and they have the capacity to change the sequence of DNA, i.e. to introduce mutations. As a first line of defence against mismatches, replicating DNA polymerases contain a 3′ to 5′ exonuclease activity that allows them to proofread the newly synthesized DNA strand [49]. The exonuclease activity can correct mistakes during DNA replication by reversing the direction of the polymerase and excising incorrectly introduced nucleotides. Even if proofreading efficiently corrects most mistakes made during DNA synthesis, some non-Watson-Crick base pairs still remain. To correct these errors, cells use mismatch repair. It is estimated that that replicative DNA polymerases with proofreading *in vitro* have error frequencies of about 5×10^{-5}. Mismatch repair lowers this frequency significantly and the mutation rate in for example human germ cells is estimated to be close to 1×10^{-8} per base pair per generation [50].

The early studies of mismatch repair were mainly carried out by geneticists interested in recombination. Robin Holliday had hypothesised that heteroduplexes between different DNA strands would be formed during recombination [51]. The mismatches formed in this way had to be corrected by some sort of cellular system, leading to gene conversion. Indeed, transfection of heteroduplex molecules of lambda phage DNA into *E. coli* led to repair of the mismatches, but the enzymatic system required for this effect was unknown. An important step was taken in 1976, when Robert Wagner and Matthew Meselson reported that repair of two or more close sites on the same heteroduplex DNA molecule occur more often on the same strand than on the opposite strand [52]. This observation led them to propose that mismatches are repaired by a strand-specific mechanism directed towards one strand, which allows for repair of long DNA tracts. They also speculated that strand-specific mismatch repair could be used to correct non-canonical base pairs formed during DNA synthesis. The repair machinery could be guided to the newly replicated strand either via a special relation to the replication machinery or directed by under-methylation of the newly synthesised strand. In *E. coli* DNA is normally methylated on both strands at GATC-sites, but during DNA synthesis the nascent strand is unmethylated for a period of time [53], which could allow the mismatch repair machinery to distinguish newly replicated DNA from the template DNA strand. In support of this notion, loss of cellular DNA methylase, the enzyme that adds a methyl group to the adenine of the sequence GATC, was already in 1975 shown to cause higher mutation rate in *E. coli* [54]. The genetics of mismatch repair was further clarified when Barry W. Glickman and Miroslav Radman could demonstrate

that *mutH*, *mutL*, *mutS* and *uvrD* genes were all required for the methylation-instructed DNA mismatch correction [55].

Direct evidence for methyl-directed repair of mismatches came in 1983. First, Paul Modrich and Matthew Meselson used heteroduplex constructs with defined states of DNA methylation to demonstrate that DNA methylation indeed directed strand-specific elimination of mismatches in *E. coli* [56]. Furthermore, Modrich developed an assay that allowed analysis of DNA mismatch repair in cell-free *E. coli* extracts [57]. In his assay, he used the classical bacteriophage heteroduplexes, but introduced mismatches within overlapping sequences recognised by two different restriction endonucleases. About 1,000 bp from the mismatch was a GATC methylation site, which could be methylated in a controlled way and thus used to monitor strand-specific correction. Using the assay, Modrich could demonstrate that the repair activity was dependent on ATP, the methylation state of the heteroduplex, and that mutations affecting *mutH*, *mutL*, *mutS*, and *uvrD* all impaired mismatch repair in cell-free *E. coli* extracts. With the help of this elegant assay, Modrich could in a series of papers isolate the products of the different repair genes to near homogeneity, identify the proteins and investigate their properties *in vitro* in great detail [58-60].

Modrich's work culminated in a groundbreaking paper published in 1989 [61], in which he could finally reconstitute DNA mismatch correction in a defined *in vitro* system. In the paper, Modrich demonstrated the requirement of DNA polymerase III, exonuclease I, and DNA ligase for mismatch repair. He then combined these factors with purified MutH, MutL, MutS, UvrD, and single-stranded DNA-binding protein. Together these factors could process mismatches *in vivo* in a strand-specific manner directed by the single, GATC sequence methylated on only one strand (hemimethylated) and located distant from the mismatch. In this and other studies, Modrich demonstrated that MutS, which recognises and binds to non-Watson-Crick base pairs, performs a core function in mismatch repair system. To confer strand-specificity, MutH binds at hemimethylated GATC sites on the nascent strand. MutL acts as a mediator, which interacts with both MutH and MutS. MutL transduces signals from MutS, which leads to activation of the latent MutH endonuclease activity causing a nick in the nascent DNA strand near the hemimethylated GATC-site. The machinery now interacts with a helicase (UvrD), which together with the MutS, MutL, and MutH proteins separates the two DNA strands towards the location of the mismatch. Displacement of the mutant strand continues past, and halts just downstream of, the mismatch. The nascent strand is then replaced by a gap-filling reaction, in which DNA polymerase III uses the parental strand as a template.

Mismatch repair in mammalian cells.

Later studies by Modrich and others have demonstrated the conservation of mismatch repair in eukaryotic cells and in 2004, Modrich managed to reconstitute human mismatch repair with only purified factors [62]. In contrast to the situation in *E. coli*, DNA methylation does not direct

strand specific DNA repair in eukaryotic cells. One possibility is that the strand specific nicks formed during DNA replication can direct strand-specific error correction. In support of this notion, mismatch repair is more efficient on the lagging strand at the replication fork [63], and a single nick is sufficient to direct strand specific repair in *in vitro* [62, 64]. Alternatively, the mismatch repair machinery may be directed by ribonucleotides transiently present in DNA after replication [65]. In other aspects, eukaryotic mismatch repair is closely related to the *E. coli* system with conserved homologues to key factors such as MutS and MutL. The importance of the mammalian mismatch repair system is underscored by the finding that defects in this pathway cause hereditary nonpolyposis colon cancer [66, 67].

Summary

Tomas Lindahl, Paul Modrich, and Aziz Sancar have made fundamental and groundbreaking discoveries on the enzymatic mechanisms of DNA repair. Lindahl demonstrated that DNA is an inherently unstable molecule, subject to decay even under physiological conditions. Guided by thia observation, Lindahl identified a completely new group of DNA glycosylases and described their role in base excision repair. Modrich transformed the field of mismatch repair from genetic observations to a detailed biochemical understanding, first in bacteria, and later in eukaryotic cells. Sancar has transformed the field of nucleotide excision repair, from genetics and phenomena in cell extracts, to a detailed molecular description of the mechanisms involved, first in bacteria, and later also in eukaryotic cells. Sancar also explained the molecular mechanisms underlying photoreactivation, the first form of DNA repair described.

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