MEASURING DNA REPAIR

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ABSTRACT
During the life of a cell, its DNA is subjected to numerous insults that affect its structure and lead to mutations or cell death. To cope with the problem cells have developed mechanisms to repair the damage and to restore the genetic information encoded in DNA. The ability of cells to survive genotoxic stress is directly connected with their ability to repair DNA. For this reason repair capacity and repair rates are important biomarkers with prognostic and diagnostic value that are used in environmental research and medicine. In the present paper we briefly describe the major types of DNA damage, the different repair pathways that have evolved to repair damaged DNA, and review the methods used to measure the repair rates and repair capacity of cells and organisms.

Keywords: DNA repair, DNA damage, DNA repair pathways, DNA repair assays

Abbreviations used: SSB: single strand break; DSB: double strand break; cisplatin: cis-diaminedichloroplatinum(II); BER: base excision repair; AP: apurinic or apyrimidinic; NER: nucleotide excision repair; TCR: transcription coupled repair; GGR: global genomic repair; NHEJ: nonhomologous end joining; HR: homologous recombination; ICL: interstrand crosslink; PCR: polymerase chain reaction; HCR: host cell reactivation assay; BrdU: Bromo-deoxyuridine; UDS: unscheduled DNA synthesis;

Introduction
From chemical point of view DNA is well suited to preserve the genetic information. Both the phosphodiester bonds that provide the backbone of the DNA chains and the N-glycoside bonds that provide the attachment of the four bases to the backbone chains are quite stable under physiological conditions. The bases themselves are also well protected being stacked upon one another in the inner part of the DNA double helix. Nevertheless, in the course of cell life, DNA is often broken or modified in some way and any modification of the chemical structure of DNA is considered as damage. Damages could block the processes of transcription and replication. In addition, during the process of DNA replication of the damaged DNA the genetic information could be compromised thus turning the lesions into mutations, which present a risk for the very existence of the cells and organisms. To cope with such events and to preserve the genetic information intact, cells and organisms have developed pathways to repair the different DNA lesions. Although during the last years enormous progress has been made in the understanding of the different repair pathways, there are still steps and processes we are uncertain about. For instance, in many cases we do not know how the interplay between the different repair pathways is regulated, etc. Fortunately, for many purposes it is not necessary to know the precise repair mechanism, but rather the repair rate, repair capacity and repair specificity of the cells and tissues. For this reason in the present review we describe the methods for measuring repair end points and do not dwell in details on the molecular mechanisms of the individual repair pathways.

DNA lesions
Some agents inflict well defined specific lesions on DNA. However, most of the agents inflict multiple types of lesions, some of which are later turned into yet other types. For this reason the lesion spectra are complex and it is often impossible to deal with one specific type of lesion at a time. Nevertheless, for the sake of clarity here we will give a short description of the lesions separately. The different types of lesions are described in greater details in other materials (25).

Single strand breaks
Single strand breaks (SSB) are the most common and the simplest type of lesions in DNA. They are formed by endogenous and exogenous agents that by different mechanisms cut the phosphodiester bond. In case when the phosphodiester bond is being hydrolyzed, the resulting 5’ end is phosphorylated and the 3’ end contains deoxyribosyl moiety with a free 3’-oh group. Such lesions do not call for activation of any specific repair pathway. They are immediately repaired by ligation. In the cases when 5’-OH and 3’-phosphodeoxyribosyl groups are formed, some processing is needed before ligation. In this case when 3’ and 5’ specific endonucleases digest short stretches from both sides to give 5’-phospho and 3’-deoxyribosyl groups, DNA is being repaired by gap-filling DNA synthesis. SSBs are also formed in the course of repair of other types of DNA damage. Damaged bases are usually removed from the phosphodiester backbone to form apurinic and apyrimidinic sites (AP sites), which are then converted into SSBs by cellular AP endonucleases. In the course of repair of different adducts
and crosslinks a transient formation of SSBs is also observed, which could be used to monitor the process.

**Pyrimidine dimers**

Irradiation of DNA with UV light with wave length at about 260 nm, where the absorption maximum of DNA is, causes formation of photoproducts. Since UV radiation is a part, although minor, of the sun light spectrum and all living organisms are permanently exposed to it, the effect of the UV irradiation on DNA is of considerable interest and has been most profoundly studied. UV irradiation causes two major types of photoproducts – cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts. When DNA is illuminated with UV light, adjacent pyrimidines and most readily adjacent thymines, stacked on each other, take part in a photoreaction in the course of which their respective 5,6 double bonds are mutually saturated and a four-member cyclobutane ring is formed linking the two pyrimidine molecules. The pyrimidine cyclobutane dimers effectively block both DNA transcription and replication, although they do not cause significant conformational distortion of the DNA double helix. The second class of dimers is the pyrimidine-pyrimidone (6-4) photoproducts. They are formed between C6 position of a pyrimidine and C6 of the adjacent pyrimidine. This photoproduce causes a major distortion of the DNA double helix. It could be distinguished from the cyclobutane dimers by its lability to alkali, to which the cyclobutane dimers are insensitive. Several other photoproduets have been identified, but in negligible amounts and will not be described here.

**Modifications and adducts**

Many chemical compounds react with DNA giving different end products. Oxidizing agents - either product of the cellular metabolism or of environmental origin, oxidize predominantly purine bases giving rise to the corresponding 8-oxopurines, or alternatively open their imidazole rings to form formamidopyrimidines. Alkylating agents also readily react with DNA. These include wide variety of chemicals, some mono- and some bi-functional. Many atoms in all four bases represent targets for electrophilic alkylating compounds and as a result of the respective reactions different amounts of N-, and O-alkylated bases, as well as alkylphosphates are formed in DNA. Because of their genotoxic effect many mono- and bi-functional alkylating agents such as N-methyl-N’nitro-N’-nitrosoguanidine and N-mustards are used in cancer therapy.

Other chemicals such as monofunctional trioxsalens, N2-acetyl-2-aminofluorene, 4-nitroquinoline 1-oxide and others also readily react with DNA bases forming a class of modifications collectively referred to as “bulky adducts”. As a rule they cause significant distortion in the DNA structure because modified bases could not accommodate within the regular double helix. Intrastrand bridges formed by bifunctional agents that react with two bases on the same DNA chain could also be included to this type of damage. For instance cisplatin forms bridges between 1,2 d(GpG) and 1,3 d(GpXpG) guanine bases on the same DNA strand, which cause a significant distortion of the double helix.

**Mismatches**

For conformational and thermodynamic reasons guanines are paired with cytosines and thymines with adenes in double strand DNA. Thus, complementary GC and AT base pairs are formed. Mismatches arise when some of the bases are paired with other than the normal complementary base. Mismatches are highly mutagenic, because in the course of DNA replication, one of the DNA daughter molecules will contain normal DNA, while the other will contain a point mutation. Mismatches are usually generated in the course of DNA replication when for different reasons the correct base in the parental strand is complemented by a wrong base in the newly synthesized strand to form a mismatch.

**Double strand breaks**

The damages affecting both strands of DNA are the double strand DNA breaks (DSB) and interstrand cross-links (ICL). Unlike the lesions that affect only one strand and could be repaired by using the undamaged strand as a template, lesions that affect both DNA strands are much more difficult to repair. DSBs are formed by ionizing radiation and different chemical reagents with clastogenic effect. As a rule these agents primarily form SSBs, but when many SSBs are inflicted in close proximity (clustered damage) some happen opposite to each other on the two DNA strands which lead to DSB formation. DSBs are the most dangerous lesions because any DSB generates a new chromosome with recombinogenic, unprotected by telomeres ends, which lead to chromosome rearrangements and is a direct threat to genome stability. Eukaryotic cells tolerate only a few DSBs per genome, after which they trigger apoptosis.

**Interstrand crosslinks**

Interstrand crosslinks are formed when bifunctional agents such as nitrogen and sulphur mustards, psoralens, cispatin, etc., react with bases on the opposite DNA chains. Also, many complex organic compounds such as benzopyrenes, as well as antibiotics such as mitomycin C, intercalate in the DNA double helix to form interstrand bridges. This form of damage is difficult to repair and usually more then one repair pathway is involved in the repair process.

**DNA repair pathways**

**Reversal of damage**

There are cases when lesions are repaired by reversing the damage. Such is the repair of SSBs by simple resynthesis of DNA and religation. Another example is the reversal of pyrimidine cyclobutane dimers by photoreactivation. In this case the dimers are enzymatically monomerized after illumination. The enzymes that carry out the reaction are collectively known as photolyases, because they are activated by long wave UV, or visible light (300-600 nm). They are widely distributed in nature and have been isolated from a number of bacterial and eukaryotic cells. Nevertheless, photolyases have
not been discovered in mammals, which lead to the conclusion that this activity has been lost in evolution.

Another well studied and universal reversal reaction is the dealkylation of O6-alkylguanine and O4-alkylthymine carried out by specific DNA alkyltransferases. The alkylating agents usually produce a plethora of N- and O- alkylated bases. Both in prokaryotic and eukaryotic cells enzymatic activities are present that could effectively remove the alkyl groups from the O-alkyl derivatives. Evolutionally this shortcut repair pathway has probably developed to ensure immediate and effective repair of these lesions because O6-alkylG mispairs with T, and O4-alkylT mispairs with G, which makes them very potent mutagenic agents.

**Base excision repair**

This pathway is present in all prokaryotic and eukaryotic cells. By BER relatively minor modifications of the bases arising mostly by endogenous factors such as deamination of cytosine, or oxidation of guanine are repaired. In the BER pathway the damaged base is excised as a free base. The process begins with the activation of specific DNA glycosylase that cuts the damaged base from the sugar-phosphate backbone of DNA giving abasic AP (apurinic or apyrimidinic) site. The formed AP sites are further processed to single strand DNA breaks, or nicks by the action of AP endonucleases, which hydrolyze the phosphodiester bond immediately 5’ to the AP site. Then cellular exonucleases remove the remaining 5’- sugar thus clearing the way an undamaged nucleotide to be inserted by DNA polymerase. Finally the DNA integrity is restored by ligation of the repaired strand (70).

**Nucleotide excision repair**

The most universal and wide spread repair pathway both in prokaryotic and eukaryotic cells is the nucleotide excision repair (NER) (22, 23, 60). By NER a wide variety of lesions are repaired such as pyrimidine dimers, bridges, bulky adducts, etc. There are two NER sub-pathways: transcription coupled repair (TCR) and global genomic repair (GGR). TCR is activated in the cases when the damage is within the actively transcribed DNA. GGR is recruited when the damage is outside the transcribed regions of the genome. The two pathways differ in the first step of damage recognition. It is believed that in TCR the signal for activation of repair is the stalled transcription machinery. For this reason the efficiency of repair in this case depends on the effectiveness of the damage to block transcription. In the GGR the efficiency of damage recognition or “sensing” depends on the structural changes in the DNA helix. For this reason damages that bring about prominent kinks, unwinding, or bulges in DNA are more effectively repaired by this pathway than damages which have lesser effect on the double helix (3). The NER pathway has been fully reconstructed in vitro both in bacteria and eukaryotic cells, and the individual protein factors and enzymes are isolated and characterized. In bacteria the damage is recognized and repaired by a complex of 3 proteins UvrA, UvrB and UvrC called UvrABC complex. In eukaryotic cells the pathway is much more complex. In human cells the damage is recognized by the XPC-HR23B complex, then the basal transcription factor TFIHH is recruited, which contains helicase activity that unwinds the DNA double helix around the damage. The single strand DNA is stabilized by RPA, a single strand DNA binding protein, and XPA verifies the presence of the damage. Then XPG and XPF, which possess endonuclease activity, introduce nicks on both sides of the damage, thus excising it. After this dual incision, the gap is filled by DNA pol δ. Finally, the ligation is carried out by XRCC9/DNA ligase IIIα (22, 23, 60).

**Mismatch repair**

Both prokaryotes and eukaryotes are able to repair mismatched base pairs in their DNA. Mismatches are mostly generated as replication errors during DNA replication. In this case the correct base is on the parental strand and the wrong base is on the newly synthesized DNA strand. The repair of mismatches is carried out by mismatch repair pathway, which shares many steps with BER and NER. The wrong base is excised either as a part of a short oligonucleotide (short patch repair), or as a part of a DNA fragment several kb long (long patch repair) and then the gap is filled by repair DNA synthesis. The problem in mismatch repair is how to distinguish which one of the two bases forming the mismatch is wrong and which is correct, since the wrong base is also normal in the sense that it is not damaged or modified in any way. This is achieved by the so called methyl-directed mismatch repair. At the time of its synthesis DNA is not methylated. It acquires its specific methylation pattern several hours after replication. For this reason some time after replication the parental DNA strand is methylated, while the newly synthesized DNA strand is not. “Mut” proteins recognize the strand that is methylated thus identifying it as the parental strand and excise the mismatched base from the opposite strand. In Escherichia coli (E. coli) 3 Mut proteins, designated as MutS, MutL and MutH have been identified as key members in the strand specific, methyl-directed mismatch repair system. Human homologs of MutS and MutL but not of MutH have been found (38).

**Non-homologous end joining**

To repair DSBs two recombination pathways are used – non-homologous end joining (NHEJ) and homologous recombination (HR) (39, 61, 68, 71). Non-homologous end joining is a widely used pathway to repair DSBs by directly joining the two DNA ends. Although it has been reported that some bacteria also have end-joining pathways, this pathway is specific for eukaryotes including humans, where it plays an important role in V(D)J recombination during the process of B-cell and T-cells production in the course of the immune response (40, 62). NHEJ typically utilizes short homologous overhangs at the ends of the DSBs. When such overhangs are absent, steps are taken to digest some DNA in order to prepare such ends. This could alter the information encoded in DNA, which would lead to mutations. The major proteins of this pathway in the higher eukaryotic cells are the XRCC4/LIG4 complex and the DNA-dependent protein kinase (DNA-PK)
which consists of DNA end-binding heterodimer Ku70-Ku80, and the catalytic subunit DNA-PKcs (68).

**Homologous recombination**

NHEJ in eukaryotic cells is usually used in quiescent cells and throughout the cell cycle of cycling cells, while homologous recombination is used to repair DSBs in S and G2 phases when sister chromatids are available (57, 61). Also, ICLs are repaired by a pathway including HR (20, 45). HR is a complex process preserved throughout evolution from bacteria to humans. In order to take place a DNA molecule that shares regions of homology with the damaged DNA molecule is used as a donor DNA. First, the DNA ends of the DSB are processed by specific 5'-exonucleases to form naked 3' tails. Then these single stranded tails invade the double strand donor DNA molecule to form a cruciform structure called Holliday junction. The Holliday junction can migrate along DNA until finding a homologous region. In the Holliday junctions each broken DNA strand is aligned to an intact complementary DNA strand from the donor molecule that serves as a template to repair the break. The junction is resolved by cutting the two crossovers by exonucleases called resolvases, thus creating two new DNA molecules, each containing one repaired strand from the damaged DNA and a complementary strand from the native DNA (39, 71).

In *E. coli* the homologous recombination pathway used to repair DSBs is mediated by RecA and RecBCD. These enzymes bind to the DSB ends, and by their exonuclease activity degrade one of the DNA strands to produce a 3' tail. RecA binds to the single strand DNA to facilitate the strand invasion reaction and the Holliday structure formation. RuvABC catalyses the following branch migration in search of homology with the duplex DNA and finally the resolution is carried out by RuvC. Different sets of proteins take part in the HR of eukaryotic cells, but the general mechanism is the same. The MRN complex (Mre11, Rad50 and Nbs1) is the first to arrive at the broken site. This complex has several tasks among them to process the double strand ends to produce 3'-overhanging single strand ends necessary for the following steps of the recombination reaction. However, since eukaryotic DNA is not nked but complexed with histones to form nucleosomes, chromatin rearrangement is taking place in the vicinity of the breaks to grant access of the enzymes taking part in HR to the broken DNA ends (19). This is connected with a massive phosphorylation of the histone variant H2AX several kb on both sides of the DSB, which is a hallmark of DSB presence (55). RPA then binds to the single strand DNA tails to facilitate the strand invasion reaction to form the four-way Holliday structure, which in mammalian cells is carried out by the recombinase Rad51 along with its paralogs XRCC2, XRCC3, Rad51B, Rad51C and Rad51D (68). The obtained single-strand gaps are filled by DNA synthesis and the Holliday junctions are resolved by the Rad51C/XRCC3 complex (41).

Finally, it must be stressed that especially in the cells of higher organisms all repair pathways work as network, sometimes complementing each other and sometimes taking over each other. Thus ICLs and sometimes bulky adducts are repaired by the consorted action of NER and HR; HR and NHEJ are applied alternatively to repair DSBs depending on the phases of the cell cycle; BER, NER and mismatch repair work often together, etc (51, 57, 61, 74).

**Methods to measure DNA repair**

Generally, there are two ways to measure repair rates. One is to monitor the removal of the DNA damage; the other is to monitor the restoration of the activity of the damaged DNA.

Techniques have been developed to monitor removal of specific lesions such as pyrimidine dimers by using T4 endonuclease V. This enzyme can remove the thymidine cyclobutane dimers and then cut the obtained abasic sites to produce SSBs at the sites of the dimers (6). For this reason DNA fragments that contain cyclobutane dimers are cut by the enzyme, while the fragments without dimers remain intact. By determining the amount of intact DNA fragments after treatment with T4 endoV and alkaline electrophoresis, the course of repair of the cyclobutane dimers can be monitored. Such techniques have limited use, since they could monitor the repair of a single type of lesions. More wide application have techniques such as DNA alkaline elution, comet assay, etc., that can monitor the repair of different types of lesions.

**Alkaline elution**

By alkaline elution techniques strand breaks appearance and disappearance is monitored and used to measure the repair rates of many different lesions that are converted *in vivo* or *in vitro* into single strand breaks. As pointed out above, most of the damaged bases could be recognized by specific glycosylases that remove them by hydrolysis of the respective N-glycoside bonds. Thus AP sites are created, which could be transformed into SSBs either by using specific enzymes, or by alkalization. SSBs are also transiently formed during the course of NER, mismatch repair and HR. Single strand breaks could be measured by a variety of alkaline elution techniques. In all of them cells are lysed on a support, washed with high salt buffer to wash out most of the proteins and then washed with alkaline solutions. This causes denaturation of DNA and short fragments formed as a result of the presence of SSBs are eluted preferentially. From the ratio between the eluted and retained DNA fractions, an approximate number of breaks per unit of DNA can be calculated. The method is applicable with rather high number of breaks in order to generate detectable amount of relatively short DNA fragments. This approach has been used to monitor and compare repair kinetics of different normal and malignant cells (21) and has even been automated (7).

**Agarose gel electrophoresis**

DNA breaks appearance and disappearance can also be studied by electrophoresis. Although denaturing conditions are sometimes used to determine SSBs, most often agarose gel electrophoresis is applied to detect DSBs both in total DNA and within defined DNA regions. The most widely used method for
determination of the repair kinetic of random DSBs induced by ionizing radiation or by radiomimetic chemicals is the pulse field gel electrophoresis (PFGE). PFGE is a variation of the standard neutral agarose gel electrophoresis by introducing an alternating voltage gradient for better resolution of larger DNA fragments. There are several modifications of PFGE described in the literature based on resolution capabilities of the electrophoresis (10). For determination of the repair kinetic of defined DNA regions DNA isolated from cells at different times after the treatment is digested with restriction endonucleases to generate the fragments of interest and electrophoresed under neutral conditions. Gels are transferred to nylon membranes by blotting and the filters are hybridized with labeled probes (42). The relationship between the DNA fraction and the number of DSBs in it is given by the Poisson equation:

\[ F = e^{-L \cdot B} \]

where \( F \) is the ratio between the amount of the DNA fraction after the treatment relative to the untreated control, \( L \) is the length of the fraction in kbp and \( B \) is the number of breaks per 1kbp.

ICLs could also be monitored by agarose gel electrophoresis. In this case DNA is subjected to a round of denaturation/renaturation before subjecting to electrophoresis. Under these conditions the DNA fragments containing ICLs migrate as double strand fragments because the two strands could not be separated and rapidly renature. The fragments without crosslinks migrate as single strand DNA (2, 66).

Comet assay
In eukaryotic cells the DNA breaks could be monitored by the single cell gel electrophoresis, or “comet assay”. It was first introduced by Ostling and Johanson (47) and since has been widely used to monitor the effect of a broad variety of agents (24, 44, 46). Two varieties of the technique are used – neutral and alkaline comet assay. The first measures only DSBs, while the second can measure SSBs as well. Briefly, cells are mixed with low melting agarose and poured on microscope coverslips. The encapsulated cells are treated with high salt and detergent to lyse the cells and to dissociate the histones, and with alkali to denature DNA. Then electric current is applied to perform electrophoreses, the samples are stained with ethidium bromide, or another DNA binding fluorescent dye, and observed under fluorescent microscope. In the case of unbroken DNA, it forms a symmetrical halo around the nucleus. However, in the case when DNA is broken, the DNA fragments migrate away from the nucleus forming a picture resembling a comet with a tail. The amount of DNA in the tail is indicative for the number of breaks and could be used to monitor their repair. This assay is very sensitive and could be used for very low number of breaks. It has been used to study the effect of different environmental agents on DNA. At present different DNA damage and repair software scoring systems are commercially available by which the comet tails moments (product of the tail length and the amount of DNA in the tail) are normalized and the number of breaks is quantified.

There is a modification of the alkaline comet assay that could be used to measure the repair of ICLs as well (43, 72). To this end, cells treated with crosslinking agents are then treated with DNA breaking agents, such as IR or chemicals at different times after the crosslinking. When these cells are subjected to alkaline single cell gel electrophoresis, no comets are obtained because ICLs hold together the DNA fragments generated by the DNA breaking agent. With the elimination of ICLs during the course of ICL repair, the DNA fragments are liberated and comet tails are formed. Thus in this case the comet tail length is in reverse relationship with the number of ICLs.

**Unscheduled DNA synthesis**
The non-replicative repair DNA synthesis is called unscheduled DNA synthesis (UDS). Regardless of the type of lesion and of the particular mechanism by which repair is carried out, the final step of any repair pathway includes gap filling DNA synthesis. In this way the repair rates are roughly proportional to UDS rates. Since first detected by Cleaver in the early 60s of last century (15), UDS has become a valuable quantitative tool to measure DNA repair both in vivo and in vitro. UDS is usually measured by determining the incorporation of labeled nucleotides under conditions of inhibition of the replicative DNA synthesis. Later a number of ways have been developed to measure repair DNA synthesis more accurately. Thus, for instance cells could be exposed to UV light, treated with hydroxyurea to block the replicative DNA synthesis and then cultured for different times in media containing BrdU. BrdU is incorporated in the regions that undergo repair and they could be then isolated and characterized by immunoprecipitation of the BrdU containing DNA fractions with anti-BrdU antibody. In this way the repair rates in different regions of the genome could be determined (11, 12).

**PCR assay**
A convenient way to determine the presence of lesions in DNA is by PCR. Practically all DNA lesions represent blocks for the DNA polymerase during DNA synthesis and for this reason DNA fragments that contain such lesions would not give product when used as templates in PCR. This scheme has been used in different experiments to monitor DNA repair of UV or chemically produced DNA lesions. To this end, primers flanking the DNA sequence of interest are constructed of UV or chemically produced DNA lesions. In this way the repair rates in different regions of the genome could be determined (11, 12).

**Mass spectrometry**
The most direct and specific method to determine different types of DNA modifications is the mass spectrometry. Given
the proper controls for calibration, all types of modified bases could be quantitatively determined by this method. To this end, DNA is isolated at different times after treatment and analyzed for the modification of interest. The problem with this approach is the use of such conditions for DNA hydrolysis that would not change the chemical nature of the products. This could be done enzymatically by endo- and exonucleases. Thus, using combinations of liquid chromatography and mass spectrometry, or gas chromatography and mass spectrometry the formation and repair of different products obtained after UV irradiation and oxidation have been determined (9, 18, 32).

Immuno-assays
Antibodies have been raised against different base modifications and used to monitor their repair both in batch experiments by immunoprecipitation, and in single cells by fluorescent microscopy (1, 16). However, the role of such antibodies raised directly against the modified bases is limited because in some cases their specificity is not adequate, and in many cases the modifications are not antigenic at all. Recently indirect antibodies are used that are not raised against the modifications themselves. It has been established that the repair of most of the lesions, regardless of the particular repair pathway, is carried out by discrete protein aggregates that assemble at the site of damage and disassemble after the damage is repaired. When fluorescent antibodies are used against members of these aggregates, they are visualized in vivo as transient foci, which appear shortly after the infliction of the damage and disappear after the damage is repaired. For instance DSBs repair could be monitored by following the foci appearance and disappearance using specific antibody against the phosphorylated histone variant H2AX (γ-H2AX). It has been established that shortly after a DSB is produced, H2AX in the vicinity of the break is phosphorylated and stays thus modified until the break is repaired, which permits this molecule to be used as a reliable biomarker for DSBs (35). This approach is widely used, antibodies against γ-H2AX are commercially available and the sensitivity of the method is such that it could detect even 1 DSB per cell. Another example is the use of anti-Rad51 antibody to follow the repair of DSBs by homologous recombination (65), the use of anti-PCNA to monitor repair by NER (5), etc.

Cell free protein extracts
Repair efficiency can be conveniently measured in cell free systems. Protocols have been developed to prepare cell free extracts from different cell lines and tissues that are able to carry different types of repair including BER, NER, NHEJ and HR repair, etc (8, 17, 26, 34, 59). Repair efficiency in these systems parallel the repair efficiency of cells in vivo and for this reason this approach has been used to measure repair efficiencies of different cells and tissues, when the in vivo approach is not possible. To this end, DNA molecules, most often plasmids, containing the desired type and number of lesions are incubated in cell free systems. The repair process is monitored by measuring the repair DNA synthesis using labeled precursors, by PCR, or by determining the efficiency of transformation of E. coli in case that the DNA construct contains a gene for antibiotic resistance (27, 73).

Mismatch repair is also measured in cell free systems as in this case the nucleotides that should be repaired are not modified. This makes it impossible to monitor repair rate by following the rate of lesion disappearance. For this reason the ability of cells and tissues to carry out mismatch repair is determined in cell free protein extracts prepared from these sources. DNA molecules containing well defined single mismatch are incubated in the extracts and then repair rates are determined by direct sequencing of DNA, or by other means (37, 49).

Host cell reactivation assay
The host cell reactivation (HCR) assay is a general method for measuring the repair that uses the restoration of the transcription and expression of the damaged DNA. In this assay exogenous DNA containing a reporter gene is damaged in a controlled way in vitro and then is transfected into the host cells whose repair parameters will be determined. The restoration of the activity of the reporter gene is monitored and from it the repair kinetics is determined. The assay is predominantly used to assess repair in higher eukaryotic cells since they can be transfected with high efficiency. A number of studies have demonstrated that DNA repair rates for different types of DNA damage can be accurately measured by using the HCR assay. Initially viral DNA vectors were used but later plasmid HCR assays have been described (29), which monitor cellular repair by measuring the transient expression of enzymatic marker genes. Originally plasmids harboring the chloramphenicol acetyltransferase reporter gene (4, 52) were used, but later the recombinant luciferase reporter plasmid pCMV/luc was applied (53). This assay has been implemented to quantify DNA repair capacity in studies of lung cancer (64, 67), skin cancer (28, 36), head and neck cancer (13), prostate cancer (31), etc. after treatment with different genotoxic agents. Later a variant of HCR assay has been developed in which the gene for the green fluorescence protein (GFP) was used as a reporter gene. A second plasmid containing undamaged reporter gene for the yellow fluorescence protein (YFP) was cotransfected to provide intrinsic control against which GFP expression to be measured (56).

By HCR assay the repair of all types of DNA damage could be measured with the exception of DSBs, whose repair is usually monitored by pulse-field gel electrophoresis, or by following γ-H2AX foci disappearance. This is because DSBs would linearize, or fragment the plasmid DNA. Recently this drawback was partially overcome by introducing the so called I-SceI endonuclease cleavage system. In this assay cells are generated that contain a copy of the 18 bp I-SceI recognition sequence flanked by donor DNA homologous regions. The cells are transfected with a plasmid containing the gene for the I-SceI restriction endonuclease and after expression it performs a double-strand DNA cut at the I-SceI site, whose...
repair can be then monitored (14, 54, 63). Using this assay system, both HR and NHEJ can be assayed for the repair of DSBs (30, 58).

Conclusions
The ability to measure repair rates and repair capacity of cells and organisms rapidly and correctly would permit the use of these parameters as biomarkers in field tests and in the clinical laboratories. Biomarkers are measurable biological parameters that reflect the individual’s risk of diseases. During the recent years a tendency has developed in different branches of medicine, but mostly in epidemiology, to use molecular biomarkers because of their advantages as economy, speed and precision. Repair parameters (rate, capacity) are major biomarkers for risk assessment upon exposure and for drug resistance. For instance, people with low levels of mismatch repair represent a risk group for colon cancer, people with reduced capacity for NER run a higher risk for skin cancer upon exposure to sun light, etc. therefore, repair capacity could serve as an informative biomarker to monitor the development of resistance towards specific agents (53, 72).

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