The role of DNA polymerase beta gene in genome stability maintenance

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1. INTRODUCTION

Life is based on a delicate balance between mutation and repair and genomic stability and instability. Without such a precious balance, evolution and species diversification could not occur. This concept holds for the general population, whereas at individual level, survival is guaranteed by the capacity of preserving genome stability. Although all living organisms are exposed to environmental damaging agents, the majority of damage to DNA origins endogenously. Cellular aerobic metabolism generates reactive oxygen species (ROS) responsible for oxidation damage of many cell structures. Moreover, oxidation, spontaneous deamination and methylation of DNA bases constitute a potential mutation source. Lesions originated by these mechanisms are mostly blocking and potentially cytotoxic lesions (e.g. replicative DNA polymerases are unable to proceed throughout them). Cells have developed an ingenious network of mechanisms for tolerating and repairing DNA damage. DNA repair embraces the direct reversal of damage (for example, the removal of O6-Methylguanine by O6-Methylguanine-methyltransferase) and the excision of damaged bases by different mechanisms such as base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) [1]. These mechanisms have as common feature the excision of the damaged strand, the insertion of nucleotides to fill the gap and the ligation of the newly synthesized fragment. If not repaired, DNA damage could interfere with DNA replication and transcription. To mitigate this deleterious consequence, cells evolved a damage-tolerance mechanism (translesion synthesis, TLS) involving specialized low-fidelity DNA polymerases able to bypass DNA lesions [2]. Although TLS allows replication to
continue, it often introduces mutations into the newly synthesized strand. Defects or imbalances in DNA repair mechanisms and, in some cases, TLS could lead to accumulation of alterations in the genome. Some of these mutations could produce a proliferative advantage for the mutated cell, thus leading to clonal expansion and eventually carcinogenesis.

1.1 DNA DAMAGE AND REPAIR: BER PATHWAYS

The biological responsiveness to genetic insult involves different cellular processes such as DNA repair, damage tolerance, genes transcriptional regulation, signalling pathways and, finally, apoptosis [3]. Cells coordinate all these processes to counteract DNA damage and survive or, ultimately, initiate programmed cell death if the genomic insult is too large to be met (Fig.1).

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Fig.1 DNA damage, repair mechanisms and consequences (From Hoeijmakers J.H. Nature 2001)
Different lesions are repaired by different and sometimes overlapping mechanisms. BER is the main enzymatic pathway for the repair of structurally non-distorting and non-bulky lesions, such as oxidised, alkylated, deaminated bases and apurinic/apyrimidinic (AP) sites [4] (Fig. 2).

BER is a multienzymatic and highly coordinated process initiated by lesion-specific enzymes, called DNA glycosylases, able to recognize and remove the modified base by the cleavage of the N-glycosydic bond between the target base and the sugar moiety. This activity leads to the release of the free damaged base and gives rise to an AP site. This is a mutagenic and cytotoxic lesion which can also arise spontaneously or by radiation and chemicals. A significant redundancy characterizes the first step of BER, since each DNA glycosylase specifically repairs a subset of target bases (for a review see [4-7]).

DNA glycosylases have been classified as monofunctional and bifunctional on the base of the chemical reaction they catalyse [8-10].

By a base flipping mechanism, the modified base is extruded from the DNA base stack into the active site pocket of the monofunctional DNA glycosylases. Hydrolytic cleavage of the N-glycosylic bond leads to the base release without generation of covalent intermediates or phosphodiester backbone interruption. The resulting AP site is subsequently converted to ssb by an AP endonuclease which hydrolyses the phosphodiester DNA backbone at 5’ to the AP site leading to nicks with 3’-hydroxyl (3’OH) and 5’-deoxyribose 5-phosphate (5’dRp) termini [11].

The base flipping mechanism is also operated by bifunctional DNA glycosylases but, in this case, displacement of the modified base occurs by a nucleophilic reaction. A covalent Schiff base intermediate results from the reaction between the aldehyde group of sugar moiety and the ε-aminogroup of a lysine residue of the active site of the enzyme. After hydrolysis of the modified base, the associated lyase activity of the bifunctional DNA glycosylases
Fig. 2 Mechanism for BER (From Hoeijmakers J.H. Nature 2001)
leads to a single strand break (ssb) generation by the cleavage of DNA phosphodiester backbone via a \( \beta \)-elimination reaction which leaves 3’-[2,3-didehydro-2,3-dideoxy-ribose] (3’ddR5p) and 5’ phosphate termini. The 3’ddR5p terminus is removed by the 3’ phosphodiesterase activity of the AP endonuclease 1 (APE1); this \( \delta \)-elimination reaction gives rise to a 3’ hydroxyl residue.

After the cleavage of the phosphodiester bond, BER can proceed through two different subpathways: the short-patch BER (SP-BER) and the long-patch BER (LP-BER) [12]. In SP-BER, DNA polymerase \( \beta \) (Pol \( \beta \)) catalyses the resynthesis of a single nucleotide at the site of the lesion and the removal of the 5’dRp moiety [13-15] (Fig.3). The 5’dRp elimination is the rate limiting step and it is necessary to allow the ligation reaction catalysed by the X-ray repair cross-complementing 1 (XRCC1)/DNA ligase III (LIGIII) complex, which completes the repair process [16].
In LP-BER, either Pol β or the replicative DNA polymerases (Pols) δ/ε can catalyse the resynthesis of a repair patch spanning two to seven nucleotides in length [17]. The lesion-containing strand is displaced by the action of a flap-endonuclease, FEN1, which recognizes and cleaves this flap structure [18]. The newly synthesized strand is resealed by the action of DNA ligase I (LIGI) [19]. This pathway requires also auxiliary proteins, such as the replication factor C (RFC), the proliferating cellular nuclear antigen (PCNA) and a “nick-sensor” protein named Poly (ADP-ribose) polymerase I (PARP1) [20-23].

LP-BER is thought to have evolved for the repair of 5’-abasic fragment that cannot be processed by the lyase activity of Pol β, such as oxidized or reduced dRp sugars, thus requiring strand displacement synthesis.

It has been hypothesized that protein interactions may play a role in DNA polymerase selection in vivo and in determining BER efficiency and subpathway selection [for a review see ref. 24].

The relative involvement of the subpathways of BER appears to depend on many factors, such as the type of DNA glycosylase that initiate BER (the termini produced by bifunctional DNA glycosylases as those involved in oxidised base removal [25]), the oxidative state of the AP site (oxidised or reduced AP sites are repaired via LP-BER, since they are resistant to the β-elimination reaction) [26-27], and the cell cycle phase (since LP-BER and DNA replication share several proteins, LP-BER is likely to be involved in repair events occurring at replication [28-29]) [for a review see ref. 30].

Recently, an APE1-independent pathway was discovered [31]. This process, involves the activity of the endonuclease VIII-like 1 (NEIL1), a bifunctional DNA glycosylase. This enzyme catalyzes a β,δ-elimination reaction at the AP site, leading to generation of a ssb with a 3’-phosphate residue. This termini is processed by a polynucleotide kinase/phosphate (PNK), characterized by a 3’-phosphatase and a 5’-kinase activity. Following the PNK-catalysed
step, the repair pathway proceeds through Pol β-mediated synthesis and LIG I- or XRCC1/LIG III-catalysed ligation. Both SP-BER and LP-BER are characterized by physical and/or functional protein-protein interactions which can involve not only BER proteins but also proteins associated with other pathways of DNA metabolism [24]. These interactions could favour subpathway selection, recruitment of proteins and auxiliary factors at site of the lesion, stabilization of repair proteins and complexes, protection of highly reactive DNA repair intermediates and regulation of enzyme activity.

APE1 appears to be a crucial protein in regulating BER since it interacts with several up- and down-stream proteins. For example, APE1 activity is needed to facilitate DNA glycosylase turnover. DNA glycosylases display higher affinity for the produced AP site than for the damaged base [32-38]. APE1 has been shown to stimulate the displacement of two DNA glycosylase (TDG and OGG1) from the repair intermediate (i.e. AP site) [32-33;35-36] This mechanism, that, in general, does not require physical interaction between the two proteins, guarantees the AP-site protection from spontaneous degradation or unwanted chemical reactions, according to the “Passing-the-Baton” model of BER. Several lines of evidence has contributed to consider BER as an highly coordinated process, catalysed by enzymes that receive the damaged DNA substrate and pass their DNA product to the next enzyme in the pathway. An efficient image to explain this model is that of a baton passed from one runner to the next in a relay [39].

Another important interaction for BER coordination exists between APE1 and Pol β [39-40]. Although these proteins do not form a stable complex [41], it has been found that APE1 stimulates the 5'-dRp lyase activity of Pol β, by binding the incised AP-DNA product [39,42]. Moreover, it appears that APE1 can act as proofreading exonuclease during the Pol β-mediated polymerisation step. This APE1 activity would be necessary since Pol β does not exhibit an associated exonuclease activity and is a relatively inaccurate DNA polymerase [43].
While in most interactions APE1 modulates other proteins activity, some proteins can alter the activity of APE1. Among them XRCC1 and PARP1. In vitro experiments showed that the physical association between XRCC1 and APE1 resulted in an increase in both AP-endonuclease and 3'-phosphodiesterase activity of APE1 [44]. APE1/PARP1 competition for binding of the APE1-cleaved abasic site BER intermediate would be responsible for the mutual function modulation [45].

The high level of coordination of BER enzymes is also guaranteed by proteins such as XRCC1. This protein is devoid of any known catalytic activity but interacts with several proteins (DNA glycosylases, APE1, Pol β, LIGIII, PNK, PARP1, PCNA), operating as a molecular scaffold that recruits proteins, increasing repair efficiency [24,39,44]. Crucial for SP-BER is the interaction between XRCC1 and Pol β [48]. In vitro experiments showed that XRCC1 can inhibit DNA strand-displacement synthesis by Pol β thus promoting SP-BER [49]. XRCC1 interacts also with PARP1, a protein which binds to ssb generated directly by irradiation or indirectly as repair intermediates [22]. PARP1 acts as a “molecular nick sensor”. When bound to DNA nicks, PARP1 catalyses poly(ADP-ribose) units addition from NAD+ to several acceptors, including PARP1 itself and proteins involved in chromatin architecture and DNA metabolism [46-47]. This covalent modification leads to PARP1 dissociation from DNA with subsequent facilitated access to the break by other repair enzymes.

A physical interaction between XRCC1 and PARP1 occurs and it is characterised by a negative regulation of PARP1 activity by XRCC1 and an ADP-ribosylation of XRCC1 by PARP1. In vivo localization experiments support the hypothesis that this interaction is necessary to the recruitment of XRCC1 and other associated BER proteins to the ssb for repair [50-51].

Among the other protein-protein interactions, it has been observed a stimulatory effect of PARP1 on DNA strand-displacement synthesis by Pol β, in the presence of FEN1, suggesting an involvement of PARP1 mainly in LP-BER [23].
Many other interactions are involved in BER regulation and in linking BER to other pathways, such as NER, MMR and damage signalling [24].

As an example of cross-talk between difficult DNA repair pathways, it has been shown that two DNA glycosylases interact with NER proteins. NER is a flexible DNA repair mechanism able to remove a plethora of structurally unrelated DNA lesions. Helix-distorting lesions, such as cyclobutane pyrimidine dimers (CPDs), (6-4) photoproducts (6-4 Ps), polycyclic aromatic hydrocarbons (PAHs) and crosslinks induced by anti-tumour drugs, are typical substrates of NER. After damage recognition, the lesion-containing strand is incised on either side of the lesion and a 25-30 nucleotides fragment is excised. The resulting gap is filled by DNA repair synthesis, followed by strand ligation [52-53]. Thirty polypeptides participate to this mechanism. Among them, a damage recognition factor (XPC) and a 3’ structure-specific endonuclease (XPG) have been found to form complexes with thymine DNA glycosylase (TDG) and thymine glycol (Tg) DNA glycosylase (NTH1), respectively. XPC promotes TDG turnover by competition for AP site product binding [54]; XPG promotes NTH1 binding to its substrate, increasing both its base excision and AP lyase activity [55-56].

Similarly to what observed for NER and BER proteins interaction, two DNA glycosylases have been found to physically interact with MMR proteins. MMR is a post-replicative repair mechanism involved in correction of mispaired bases (mismatches) and of the insertion/deletion loops caused by slippage during replication of repetitive sequences or during recombination [57]. MMR consists of four steps: mismatch recognition, recruitment of several factors, degradation of the newly synthesized mismatch-containing strand, resynthesis of the excised tract. The first step of MMR pathway is catalysed by two protein complexes (MutSα and MutSβ), characterized by a different binding specificity. Both of these complexes contain the MSH2 protein, which interacts with MSH6 protein in MutSα and with MSH3 in MutSβ [58]. The MutSβ
complex interacts with the adenine DNA glycosylase (MYH), leading to an increase in substrate-binding and glycosylase activity of MYH [59]. Another interaction has been reported between the thymine and uracil DNA glycosylase (MED1) and the MMR components [60]. It has been suggested that this interaction could be involved in a damage-specific response [61]. Finally, both in vitro and in vivo data have shown the existence of an association between APE1 and the “guardian of the genome”, p53 [62]. p53 is a protein involved in the damage-signalling pathway [63]. Similarly, it has been reported an interaction between p53 and Pol β. It has been suggested that these interactions could account for an enhancement of BER activity [64-66].

To increase the complexity of this network, it should be taken into account the crucial role of post-translational modifications of BER proteins in regulating the interaction affinities and/or the enzymatic activities, thus influencing proteins recruitment, subpathway selection and repair efficiency [for a review see ref. 24]. These modifications would not be necessary if a preformed repairosome complex was always assembled into the cell. But the in situ analysis of the repair process has shown that BER proteins (or different complexes) sequentially accumulate at the damage site [67].

All these lines of evidence contribute to clarify that BER is a dynamic process, characterised by multiple and sequential protein interactions that occur in concert with other DNA processing mechanisms.

1.2 BER AND HUMAN DISEASE

For many years no direct relationship between BER defects and disease has been reported. For example, experiments with mice defective in DNA glycosylases showed that inactivation of these enzymes is well tolerated, leading only to a slight increase in mutation frequency. These data were in contrast with that obtained in E.coli and yeast, where a defect in DNA glycosylases leads to
elevated mutation frequencies. Recently, these contrasting lines of
evidence begin to be clarified [for a review see ref. 4]. Studies
have demonstrated that 8-oxoG DNA glycosylase (ogg1) knock out
ageing mice develop lung cancer [68] and, in humans, defects in
OGG1 activity are associated with elevated lung cancer risk [69].
The analysis of ageing mice defective in uracil DNA glycosylase
(UNG) has shown an increased incidence of B-lymphomas [70].
Moreover, studies have demonstrated the association between
defects in MYH and adenomatous colorectal polyposis and
colorectal cancer risk [71-72].
In contrast to the mild phenotype of DNA glycosylase knock out
mice, defects in down-stream BER proteins (APE1, Pol β, XRCC1)
are lethal in mice. This could be due to the involvement of these
enzymes in developmental processes and/or to the persistence of
“unattended” BER intermediates, which are the substrate for these
proteins. This last hypothesis is supported by recent evidence which
shows the highly mutagen potential of the BER intermediates [73].
Finally, small-scaled epidemiological studies have linked BER
gene polymorphisms and several types of cancer [74].
Since BER works in concert with other repair processes (see
above), severe phenotypes could be identified by combining DNA
repair gene knock out.
The “passing-the-baton” model for BER gave rise to the hypothesis
that an imbalance in BER proteins could cause a loss of
coordination, thus exposing cell to the potentially cytotoxic or
mutagenic effects of unattended BER intermediates. This would
potentially result in increasing the spontaneous mutation frequency
and thus cancer risk. The first evidence supporting this hypothesis
was obtained in yeast, by overexpression of 3-methyladenine DNA
glycosylase (MAG1) relative to the AP endonuclease. Such an
imbalance produced a strong mutator phenotype [75]. More
recently, Hofseth and coworkers have examined the activity of two
BER enzymes in tissues from noncancerous colons of ulcerative
colitis patients [76]. Ulcerative colitis is a chronic inflammatory
disease associated with chromosome instability (CIN) and
microsatellite instability (MSI) [77-78]. The correlation between ulcerative colitis and increased colon cancer risk has been reported [79]. Several type of cancer are characterised by CIN, i.e. by numeric and structural alterations of chromosomes, caused by defects in DNA repair pathways or in mitosis process. In humans it has been demonstrated that defects in MMR generate a mutator phenotype [80] and are a risk factor for cancer susceptibility [81-82]. In particular, a defect in MMR causes MSI, an accumulation of mutations at repeated sequences (microsatellite DNA) of target genes. It has been shown that ulcerative colitis patients have increased 3-methyladenine DNA glycosylase (AAG) and APE1 enzymatic activity and this “adaptive imbalanced” increase is associated with MSI. Although the mechanism of this association is still unclear, this is the first evidence that imbalances in BER proteins may have DNA damaging effects, contributing to carcinogenesis in chronic inflammation diseases [76].

1.3 DNA POLYMERASE BETA

Pol β is a member of the X-family of polymerases. It has been extensively characterized and employed as model for the study of the DNA synthesis mechanism because of its small size (335 aminoacids residues, 39kDa) and lack of accessory proteins. Human Pol β is encoded by a 34 kb single-copy gene, mapped to the short arm of chromosome 8 (8p12-p11) and composed of fourteen exons (Fig.4). Although Pol β appears to be evolved separately from other DNA/RNA polymerases of known structure, it shares many structural and mechanistic features with other polymerases [83].
Pol β is folded into two distinct domains each associated with a specific functional activity: the amino-terminal (8kDa) and the carboxy-terminal (31kDa) domain, connected by a protease sensitive hinge region (Fig.5). The lyase activity of Pol β (see before) is mediated by the 8kDa domain, encoded by exons I-IV [84-85]. In addition to this dR p-lyase function, the N-terminus domain carries a single strand DNA binding activity necessary to direct Pol β to short gaps possessing a 5'-phosphate termini [41]. If the gap size spans from 1 to 6 nucleotides Pol β catalyses DNA synthesis, coordinated by the 8kDa domain, in a processive fashion. The presence of a gap larger than 6 nucleotides impedes Pol β-mediated DNA synthesis, even if the enzyme binds at the 5’ termini [86]. Like several DNA polymerases, Pol β polymerase domain is composed of three subdomains: the catalytic, duplex DNA binding and nascent base pair binding subdomain [87]. According to the
nomenclature that utilizes the architectural analogy to a right hand, these subdomains are also called palm, thumb and fingers, respectively [88] (Fig.6). The catalytic subdomain coordinates two divalent metal cations that assist the nucleotidyl transferase reaction and is spatially situated on opposite side of the other two subdomains [89].

Pol β catalyses a two-step nucleotide-binding mechanism: the polymerase-DNA complex binds the incoming nucleoside triphosphate (dNTP), placing it within the active site; subsequently, conformational changes occur, leading to the alignment of the
catalytic atoms [90]. According to molecular modelling, these conformational events comprise both DNA and protein residues and are not concerted, occurring in a systematic series of events [91-93]. In particular, structural studies revealed that the finger subdomain repositions itself to “sandwich” the nascent base pair between the growing DNA terminus and the polymerase [94]. This active closed conformation differs from the inactive open conformation, characterised by a similar arrangement of the polymerase subdomains but various positions of the lyase domain [95-96]. This “induced-fit” mechanism explains how DNA polymerases discriminate between correct and incorrect nucleotide. It provides evidence for a geometric selection mechanism by which the polymerase active site imposes geometric constraints that favour base-pairing that comply with these restraints [97]. While binding of a correct dNTP leads to conformational changes that result in an activated complex, the incorrect dNTP binding results in a poor fit, i.e. an suboptimal “activated” complex [98]. According to the induced-fit model, a rate-limiting conformational change must limit correct nucleotide insertion [99-100]. Kinetic studies have shown that insertion by A-, B- and Y-family polymerases is limited by a conformational change [101-105]. Nevertheless, there is no evidence for a rate-limiting conformational change for Pol β, i.e. subdomain motions are too rapid to limit nucleotide insertion [92,106-107].

Although DNA replication and repair synthesis are characterized by high fidelity, an incorrect dNTP is occasionally inserted with efficiency varying among different polymerases. The efficiency by which Pol β inserts an incorrect nucleotide and extends the mispair depends on the identity of the mispair and sequence context [108-111]. The lack of any associated proofreading activity confers to Pol β a highly mutagenic DNA synthesis when copying undamaged DNA. For misincorporation of dGMP opposite template thymine an error rate between $10^{-1}$ and $10^{-5}$ has been reported for Pol β [111-113]. Moreover, reconstitution of single BER reactions with purified proteins has shown a minimum fidelity of the single
nucleotide gap filling reaction catalysed by Pol β, compared to the overall BER efficiency. This evidence suggests that Pol β insertion efficiency and accuracy may be enhanced during BER process by protein-protein interactions or by the presence of the 5’ dRp group, which is excised only after polymerisation reaction catalysis [114]. Because of its high error-rate, Pol β is considered an error-prone DNA polymerase able to increase the spontaneous mutation frequency if overexpressed in mammalian cells [115]. Several lines of evidence have shown that Pol β expression level is crucial for genetic stability and tumorigenesis. Pol β gene is characterized by an expression profile of ~6 mRNA molecules per cell throughout the cell cycle [116] and it is inducible under stress circumstances [117-118]. Pol β overexpression has many dramatic consequences such as gene mutations [119], MSI [111,120], CIN and tumorigenesis [121-122]. Moreover, overexpression of Pol β in cancer cells can enhance resistance to chemotherapeutic agents [115]. The mechanism by which overexpressed Pol β increases mutation frequency is still unclear and contrasting data have been presented. It has been proposed that overexpressed Pol β participates to cellular functions that do not normally require it, such as DNA replication and DNA repair via the DNA synthesis step of the nucleotide excision repair pathway [119,123]. Nevertheless, a lack of correlation between mutation rates and specific levels of Pol β overexpression has been also reported, suggesting a mechanism of mutagenesis which is not characterised by a competition by Pol β over other replicative DNA polymerases [120]. It seems possible that overexpression of Pol β induces imbalances in nuclear organization of proteins involved in DNA replication (PCNA, FEN1), leading to disruption of coordinated interactions during replication and to an increase in polymerase dissociation [120]. The analysis of tumour samples, compared to matched normal tissues, by both cDNA and protein arrays revealed that Pol β is overexpressed in tumour tissues by 2-fold or more in 50 of the 152 samples analysed [124]. The tumours studied belong to different tissues types and among these, uterus, breast, ovary,
stomach and skin tumours were characterised by high frequency of Pol β overexpression. The authors propose that Pol β and other specialized polymerases overexpression in tumours could correlate with an increased mutation frequency and contribute to generate a “mutator phenotype”. The mutator phenotype hypothesis predicts that an early event in tumorigenesis is the generation of a mutation that controls the fidelity of DNA replication and/or the efficiency of DNA repair [124]. This initial mutation can increase the possibility that additional genes involved in genome maintenance accumulate alterations, resulting in a cascade of mutations in several genes that under selective pressure may result in the development of malignant cancer cells. From this point of view, genes for specialized polymerases should be considered as proto-oncogenes. A smaller percentage of tumours analysed by cDNA and protein arrays was characterised by Pol β underexpression (12-20%) [124]. A previous study has shown that Pol β haploinsufficiency results in genomic instability [126]. In particular, half the gene dosage does not contribute to tumorigenesis in young mice but it leads to an increase in the mutagenic response to carcinogen exposure. Moreover, several studies have shown that Pol β mouse defective cells [14] are hypersensitive to MMS and less efficient in the rejoicing of induced ssb, compared to wild type (wt) mouse fibroblasts. Moreover, they show increased induced sister chromatid exchange and mutation frequency [127-129]. The inability to tolerate carcinogen exposure has important human health implications. Polymorphisms that alter Pol β function on one chromosome could impact the cell through haploinsufficiency. Not only imbalanced Pol β expression but also deletions of chromosome 8p characterize many types of cancer. Losses in this area are associated with more aggressive cancer forms [130-139]. Mutations in Pol β gene have been found in colon, prostate and in gastric cancer tissues [140-144]. Functional analysis of two of these mutations showed a decrease in DNA synthesis fidelity in Pol β mutated proteins and their ability to induce permanent cellular transformation in mouse cells [145].
It is becoming increasingly clear that, together with gene mutations and alterations of gene expression, also the reduction of fidelity of transmission of genetic information as it flows from DNA may have deleterious consequences on genome stability. Errors in epigenetic processes, such as methylation, transcription, RNA processing and translation can affect the normal phenotype. An epigenetic process which has a crucial role in maintaining the normal flow of genetic information is pre-mRNA splicing. It is carried out by a multiprotein complex, the spliceosome, which selects the correct splice sites in order to produce mature mRNA molecules [146-148]. In particular, alternative splicing is implicated in regulating the temporal and spatial expression of many genes, by selection of different splice sites and the consequent production of splice variants [149]. Several lines of evidence show how splice variants can be associated with human pathologies and, for this reason, must be considered not only the products of a legitimate alternative splicing process [150]. Multiple Pol β splice variants have been identified in many cell types, including cancer cells [151-152]. Little is known about the function of these splice variants and the mechanism by which they are produced (regulated or aberrant splicing?). Their presence even in normal tissues leads to hypothesise that they serve some function in the cell. There have been reports that a Pol β splice variant missing the exon 11 codes for a dominant-negative protein [153], found in normal and tumor colorectal tissue [141,154], testis tumor [154], lung tumor [155], and normal blood samples of Werner patients [156]. It can not be excluded that Pol β splice variants play a role in human disease, including cancer.

All these lines of evidence give rise to the hypothesis that the status of Pol β gene in normal tissues may be of predictive value.

1.4 GASTRIC CANCER (GC)

Despite a dramatic reduction in incidence and mortality rates, GC still represents the fourth most common cancer in the world and the
second leading cause of cancer death [157]. Undoubtedly, several factors, such as nutritional, infectious and genetic ones, play a role in the multifactorial and multistage process of gastric carcinogenesis [158-160].

Diet has been identified as an important factor in the etiology of GC. In particular, several studies have indicated that a high consumption of foods rich in salt, nitrite and preformed nitroso-compounds is associated with an increased GC risk [For a review see ref 159]. In contrast, an high consumption of fresh fruit and raw vegetables and an high intake of antioxidants (such as vitamins C and E, carotenoids and polyphenols) are associated with a reduced GC risk [162-163]. In humans, the free-radical scavenging properties of the latter compounds are responsible for the inhibition of carcinogenic N-nitroso compounds production in the gastric microenvironment.

Domestic refrigeration and reduced salt consumption may explain the decreasing temporal trend of GC.

Over nutritional factors, familial history and infectious agents must be taken into account in considering GC etiology. Recent molecular analysis showed the presence of many genetic alteration in gastric carcinogenesis, such as p53 [164], β-catenin [165], E-cadherin [166], trefoil factor 1 [167] and c-met [168]. Moreover, functional polymorphisms of interleukin 1β (IL1B), IL1 receptor antagonist (IL1RN) and GST genes have been associated with an increased GC risk, even if ethnic variability in allele frequencies can lead to collect contrasting results [169-172].

Epidemiological studies provided a positive correlation between *Helicobacter pylori* (*H. pylori*) infection and GC risk [161, 175-177]. *H. pylori* is a non-invasive, non spore-forming and spiral-shaped Gram-negative rod bacteria. It induces infiltration of the gastric mucosa by neutrophils, macrophages and T and B lymphocytes. This induced immune and inflammatory response is unable to clear the infection and leaves the host prone to complications resulting from chronic inflammation [178]. This is the initial phase of the pathogenetic model which describes gastric
carcinogenesis as a multistep process [158]. The high consumption of salt or the chronic infection with *H. pylori* causes an increase of pH, leading to bacterial invasion; this event leads to an increase in the reduction of nitrate into nitrite and the formation of mutagenic and carcinogenic compounds. *H. pylori* injects bacterial proteins into the cytosol of the gastric host cell and regulates the intracellular signal transduction [179-181]. Chronic gastritis induced by persistent gastrointestinal infections may remain asymptomatic or evolve into more severe diseases, such as peptic ulcer or atrophic gastritis. Determinants of disease outcome are bacterial, environmental, population-related and individuals host factors. Among bacterial factors, vacuolating cytotoxin gene A (vacA), cytotoxin association gene A and E (cagA, cagE) and lipopolysaccharide (LPS) have been extensively studied as candidates for the inflammatory response induced in gastric mucosa, which is closely associated with the development of gastric cancer.

In acute and active gastritis caused by *H. pylori* infection, the initial inflammatory response to the pathogen is carried out by neutrophils. In activated neutrophils, NADPH oxidase transfers an electron from NADPH in cells to oxygen inside and outside cells. The oxygen molecules that receive an electron become superoxide radicals (O$_2^-$), which is rapidly converted to hydrogen peroxide (H$_2$O$_2$), by spontaneous dismutation or enzymatic superoxide dismutase (SOD) activity. Hydroxyl radicals (OH) are formed non-enzymatically in the presence of Fe$^{2+}$ as products of a secondary reaction. Moreover, in the presence of chloride ions, a potent oxidant, i.e. hypochlorous acid (HOCl), is formed from H$_2$O$_2$. It has been demonstrated that reactive oxygen species (ROS) production is enhanced by *H. pylori* infection [182] and, in particular, by cagA-positive *H. pylori* species [183]. *H. pylori* seems to be resistant to the antimicrobial action of ROS, probably because of bacterial antioxidant species production [178]. The excessive ROS production induces oxidative stress to the gastric mucosa and may damage cellular components, such as fatty acids, proteins and
DNA. NH$_3$ derived from $H. pylori$ reacts with HOCl to yield a lipophilic compound, i.e. monochloramine (NH$_2$Cl), which penetrates biological membranes, oxidizing intracellular components [184]. Recent studies have demonstrated that gastric epithelial pit cells are able to produce ROS via activation of non-phagocytic NADPH oxidase in response to $H. pylori$ [185]. Cell proliferation may be affected from activation of redox-sensitive transcription factors in response to ROS [186]. Lipid peroxidation mediated by ROS is considered an important cause of cell membrane destruction and cell damage [178]. ROS attack phospholipids of membrane producing fatty acids radicals and hydroperoxides, leading to the perturbation of cellular and organelar membrane function. Moreover, it has been shown that $H. pylori$ directly reduces intracellular concentration of glutathione (GSH), an important endogenous antioxidant, and impairs its metabolism in the gastric epithelial cells [187-189]. The consequently increased oxidative stress may induce DNA lesions, such as oxidated bases and single strand breaks, which are mainly repaired via BER. The pivotal role of BER in protection of free radical attack has been demonstrated from Harris and coworkers in a study conducted on patients affected from ulcerative colitis (UC) [190]. They showed an increased activity of 3-methyladenine DNA glycosylase (AAG) and APE1 in inflamed epithelial areas of UC patients. What is surprising is that UC patients with MSI have the largest increase and imbalance in levels of AAG and APE1. Thus, the overexpression of these two BER enzymes may contribute to MSI, by a yet-to-be-defined molecular mechanism. The authors suggest that the adaptive increase in AAG and APE1 activity may reflect a defense mechanism to repair ROS-induced DNA damage. But, paradoxically, this adaptive imbalance in BER enzymes may have DNA damaging effects (such as MSI in genes involved in tumorigenesis) and contribute to carcinogenesis in chronic inflammation.

Among European countries, Italy has one of the highest death rates for stomach neoplasms, with marked internal variability. In
particular, gastric cancer incidence and mortality are highest in the central-northern regions and lowest in southern Italy [173]. Even in this case diet has been identified as an important factor in the GC etiology, but other factors, such as founder effect cannot be excluded.
2. RESULTS

2.1 GENOMIC DNA ANALYSIS

Genomic DNA from 5 gastric tumours and 3 gastric cancer cell lines was screened for Pol β somatic and germline mutations, by amplifying and sequencing exons 1-14, exon α and splicing junctions. Any mutation was found neither in exons sequences nor in splicing junctions.

2.2 cDNA ANALYSIS

We analysed cDNA molecules obtained by RT-PCR on mRNAs extracted from normal and tumour tissues of 20 patients, from 3 gastric cancer cell lines and from biopsies of 2 healthy patients. Primers used to amplify Pol β cDNA were located at the beginning of exon 1 and after the end of exon 14. Except for healthy patient tissues, a PCR product of Pol β was obtained (the tissues from healthy patients were extremely small and this might have affected quantity and quality of recovered RNA). On agarose gel, two bands were detectable: a product of 1009 bp, corresponding to full length Pol β cDNA; a lower molecular weight product (900<bp<1009), corresponding to cDNA molecules which had lost one exon at least (variant Pol β cDNA) (Fig.6). Both normal and tumour tissues were characterized by these two cDNA forms. Also in gastric cancer cell lines we found the two amplification products. In particular, PCR-amplified Pol β cDNA from AGS gastric cell line revealed multiple bands.

RT-PCR products were used as substrate in a second PCR, nested-PCR, performed with primers designed to amplify contiguous 200 bp DNA fragments of the coding region of the human full length Pol β cDNA (Fig.7). Sequence of each primer was complementary to that of two adjacent exons; in presence of exon skipping the expected amplification product is <200 bp. Each couple of primers
permitted to identify which exon skipped. Nested-PCR analysis confirmed a wide variety of Pol β cDNAs, both in normal and tumour tissues of all 20 patients analysed (Fig.8), in gastric cell lines and in gastric tissue from one healthy patient (the second one could not be amplified probably because of RNA quality and quantity).

Fig.6 Example of products of RT-PCR performed on mRNA extracted from normal (lane 2) and tumour (lane 3) tissues of GC patients and from AGS gastric cell line (lane 4). Lane 1: DNA ladder

Fig.7 Nested-PCR scheme
Exon 2, 5, 6, 9 and α were the most often omitted from cDNA (Fig. 8). In the major part of samples analysed, wt and variant form of Pol β cDNA coexisted. Pol β cDNA missing exon 2 (Ex2Δ cDNA) was the most prevalent splice variant, being observed in normal and tumour tissues of all 20 samples from patients and in gastric cell lines.

The loss of exon 2 with the retention of exon 3 would be predicted to produce a 26 amino acid product that would include the first 20 aminoacids of the ssDNA binding dRpase domain of Pol β [190]. Surprisingly, in 4 individuals (# 17, 35, 53 and 104) the mRNA Ex2Δ was the predominant form, both in normal and tumour tissues, while in 3 individuals (# 12, 16 and 32) the exon 2 skipping was the most represented event only in tumour tissues (Fig.9).
In 17 of the 20 individuals (85%) and in normal but not in tumour tissue of one individual Pol β cDNA missed exon α. Previous studies showed that the exon α-encoded 35-aminoacid insertion falls in the DNA binding region (α-helix H) of Pol β [96,192]. Although this region is critical for functionality of the enzyme [193], activity gel analysis of extracts from E.coli cells expressing the Pol β isoform cDNA revealed that the 42 kDa polypeptide is capable of DNA polymerase activity [151]. Our nested-PCR strategy doesn’t permit to discriminate between exon 5 and exon 6 loss, since these exons have the same size. Thus, in 5 of 20 samples (25%) and in the AGS cell line exon 5 or 6 skipped. The resulting product would be a 97 amino acids or a 112 amino acids protein for exon 5 or exon 6 skipping, respectively.

Besides exon 2 and α skipping, the loss of exon 5 in combination with exon 6 was frequently detected (55%). This skipping event is predicted to produce a 97 amino acids product.

Finally, in 4 of 20 samples (20%), in normal mucosa of patient #27 and in tumour tissue of patient #119 we found exon 9 skipping. In this case a 171 amino acids protein is expected.

All truncated cDNAs were characterized by the loss of entire and not partially deleted exon sequence.

It must to be noted that this nested-PCR strategy does not allow to detect those splice variant which originate from loss of that exons that are template for nested-PCR primers annealing (e.g. exon 1, 4, 5, 6, 9).
7, 10, 13, 14). Thus, the occurrence of these variants was tested by cDNAs cloning and sequencing (see paragraph 3.4)

**2.3 QUANTITATIVE-PCR (Q-PCR) ANALYSIS**

We performed Q-PCR analysis on mRNA from normal and tumour tissues of patient #17 and 53 to determine the relative ratio between wt Pol β mRNA and Ex2Δ mRNA quantity. By using primers and probe specific for wt or Ex2Δ mRNA (see Material and Methods), we were able to confirm data previously obtained by Nested-PCR. Both in normal and tumour tissues of patients #17 and 53 the Ex2Δ Pol β mRNA was more represented than wt Pol β mRNA if not the only form present (see T17 and N53 in Fig.10). This finding let us hypothesise that in the gastric tissue of these individuals a decrease in BER capacity would occur due to the almost exclusive presence of Ex2Δ variant (lacking of catalytic activity). These tissues could, thus, be considered as Pol β null tissues.

![Fig.10 Comparison by QPCR of wt Pol β mRNA and Ex2Δ Pol β mRNA level in normal and tumour tissues of 2 GC patients](image-url)
2.4 CLONING AND SEQUENCING OF POL β cDNAs

To better characterize the Pol β splicing profile and study in detail the Ex2Δ Pol β variant, we cloned cDNA molecules obtained from patients in a mammalian expression vector (pcDNA4/HisMax, Invitrogen). This vector contains the cytomegalovirus promoter that permits to obtain high expression levels of recombinant protein. The PCR product cloned in this vector is expressed as a fusion protein containing a polyhistidine (6xHis) tag at the N-terminus. We sequenced 23 Pol β variant cDNAs and confirmed that the exon 2 (17/23) and the exon α (22/23) skipping were the predominant events (Fig.11). Exon 5, 6 and 9 occurred at similar frequency (5/23).

This approach revealed also those splicing variants that by nested-PCR strategy could not be detected. These splicing variants were characterised by the loss of exon 3 (2/23), 4 (5/23), 7 (2/23), 8 (2/23) and 10 (2/23). Moreover, we found the exon 11-deleted Pol β variant (6/23), previously characterized as dominant negative of wt Pol β [194-195]. In 17/23 cDNAs sequenced exon 2 was lost in combination with exon α. In 5/23 samples more than 3 exons were lost in combination.

![Fig.11 Results of sequencing of vector molecules containing variant cDNAs obtained from GC patients](image-url)
2.5 CHARACTERIZATION OF EX2Δ POL β VARIANT-TRANSFECTED CLONES

Pol β null and isogenic wt mouse fibroblasts were transfected with the expression vector containing the Ex2Δ Pol β cDNA (pcDNA4-Ex2Δ) or the wt Pol β cDNA (pcDNA4-wt, as positive control). Stably transfected clones were isolated and screened for expression of wt/Pol β variant by RT-PCR, western blot and QPCR. Three clones were selected: a clone of Pol β null cells expressing human wt Pol β (Pol β null/wt Pol β); a clone of Pol β null cells expressing the Ex2Δ Pol β variant (Pol β null/Ex2Δ Pol β) and a clone of wt cells expressing Ex2Δ Pol β variant (wt/Ex2Δ Pol β). Since Ex2Δ Pol β mRNA contains a premature stop codon in exon 3, we considered that mechanisms responsible for the elimination of mRNAs containing premature translation termination codons (PTCs), such as NMD (Nonsense Mediated mRNA Decay) and NAS (Nonsense Associated altered Splicing) [195-197], could occur in transfected cells. Thus, by performing RT-PCR, we verified the presence of the human Ex2Δ Pol β mRNA, both in wt and Pol β null mouse fibroblasts (Fig.12, lane 3, shows the presence of Ex2Δ Pol β mRNA in Pol β null/Ex2Δ Pol β).

Fig.12 RT-PCR on mRNA from Pol β null/Ex2Δ Pol β (lane 3). Lane 1: DNA ladder; lane 2: HeLa cells (positive control)
Western blot experiments were performed with AGS and transfected clone cell extracts to detect translation of the splicing variants. Polyclonal antibody specific for Pol β allowed us to detect clearly wt Pol β in AGS (Fig.13A) and Pol β null/wt Pol β (Fig13B, lane 2). For exposure time >1 min several bands could be observed both in AGS (Fig.13A, lane 2) and Pol β null/wt Pol β (data not shown) extracts, thus making difficult to assign these signals to Pol β variants presence.

![Western blot results](image)

**Fig.13** Results of Western blot performed on whole cell extracts from AGS (A) and transfected or untransfected Pol β null and wt cells (B). (A) Two exposure time (lane 1: 1 min; lane 2: 30 min) are shown for the western blot of AGS cell extracts. (B) lane 1: wt fibroblasts (positive control); lane 2: Pol β null/wt Pol β; lane 3: Pol β null fibroblasts (negative control)

By using the polyclonal antibody specific for Pol β the Ex2∆ variant was not detected (probably for the small size of the epitopes) neither in Pol β null/Ex2∆ Pol β nor in wt/Ex2∆ Pol β . Thus, the same western blot experiment was repeated by blotting with an antibody which recognizes the 6xHis tag coded by the expression vector used. Unfortunately, since this antibody is highly
a-specific, we again didn’t obtain clear evidence of \( \text{Ex2}\Delta \) Pol \( \beta \) variant synthesis. It will be therefore interesting to perform western blot by using an antibody specific for the 8 kDa domain of Pol \( \beta \) (collaboration with Prof. S.H. Wilson, NIEHS, Research Triangle Park, NC).

We can exclude that the \( \text{Ex2}\Delta \) Pol \( \beta \) variant was not detectable owing to a too low expression level, since by QPCR analysis it showed a 15-fold increase in expression level compared to a Pol \( \beta \) null clone expressing human wt Pol \( \beta \), detectable by western blot (data not shown).

By QPCR we compared the expression level of \( \text{Ex2}\Delta \) Pol \( \beta \) mRNA in transfected clones to that observed in normal and tumour tissues of individual #17 and 53 and we found similar levels. Therefore the \textit{in vitro} mouse cell system is a good model for the \( \text{Ex2}\Delta \) Pol \( \beta \) variant detected in human tissues.

**2.6 GAP-FILLING ASSAYS**

To characterize the functional activity of \( \text{Ex2}\Delta \) Pol \( \beta \) variant and obtain also an indirect evidence of its synthesis in mammalian cells, whole cell extracts of transfected clones were tested in a Pol \( \beta \) repair synthesis assay. By using a DNA substrate containing a single-nucleotide gap (a typical BER intermediate substrate for Pol \( \beta \)), we could test the polymerase activity of wt/\( \text{Ex2}\Delta \) Pol \( \beta \) in whole cell extracts from transfected clones. The reaction was allowed to proceed for different incubation times (2, 5 and 10 min).

Pol \( \beta \) null/wt Pol \( \beta \) cells (Fig.14, lane 3) were able to perform the 1 nucleotide (nt) DNA repair synthesis reaction, even if (as expected) at lower efficiency than wt fibroblasts (lane 1). In contrast, Pol \( \beta \) null cell extracts were impaired in performing the gap-filling reaction, as expected (Fig.14, lane 2).

Surprisingly, wt/\( \text{Ex2}\Delta \) Pol \( \beta \) cell extracts (Fig.15A, lane 2) showed a decreased efficiency (50%, Fig.15B) in Pol \( \beta \)-mediated gap-filling reaction when compared to untransfected wt fibroblasts (Fig.15A, lane 1).
Overall, these lines of evidence indicate that the expression of human Pol β in defective mouse fibroblasts leads to a functional complementation of their defect. In contrast, Pol β null/Ex2Δ Pol β cells maintain the inability to perform 1nt gap-filling reaction which characterize Pol β null cells. Finally, the Ex2Δ Pol β variant causes a decrease of BER efficiency when expressed in wt mouse fibroblasts, suggesting a potential dominant negative activity of this variant.

Fig. 14 Gap-filling assay performed with 1) wt 2) Pol β null 3) Pol β null/wt Pol β 4) Pol β null/Ex2Δ Pol β whole cell extracts
Fig. 15 (A) Gap-filling assay performed with 1) wt and 2) wt/Ex2Δ Pol β whole cell extracts (B) These data refer to three experiments of gap-filling performed with three independent cell extracts of wt and wt/Ex2Δ Pol β fibroblasts.
2.7 CELL GROWTH RATE ASSAYS

Since Pol β null mouse fibroblasts are hypersensitive to the killing effect of alkylating agents, cell growth rate assays in transfected and untransfected wt/Pol β null cells were performed after treatment with a methylating agent, such as methyl methansulphonate (MMS). In particular, MMS treatment results predominantly in an accumulation of alkylated lesions, such as N7-methylguanine (83% of induced lesions), N3-methyladenine (8.3%) and abasic sites that arise as BER intermediates [199]. Several studies have shown that fibroblasts from Pol β null mice present increases in chromosomal damage, apoptosis and necrotic cell death in response to monofunctional DNA-methylating agents [14,127]. This sensitivity is due to the lack of Pol β-dependent BER (SP-BER) and the lower efficiency of the alternative Pol β-independent BER pathway (LP-BER)[27]. It has been demonstrated that the dRp lyase activity of Pol β is sufficient per se to reverse MMS-induced hypersensitivity in Pol β null cells [198], indicating that the persistence of the 5’dRp moiety is responsible for the increased cytotoxicity. Thus, Pol β null cells represent the optimal system to investigate the biological role of Pol β variants in vivo. Different MMS doses were selected to treat wt and Pol β null transfected/untransfected fibroblasts due to their different sensitivity to alkylation damage. When treated with MMS doses ranging from 0 to 2 mM, Pol β null/wt Pol β cells were more resistant (D37%=2.2 mM) to MMS than Pol β null cells (D37%=1.6 mM) but more sensitive (as expected) than wt cells (D37%=5.8 mM) (Fig.16). In contrast, Pol β null/Ex2Δ Pol β and untransfected Pol β null cells were similarly hypersensitive to the cytotoxic effect of MMS (D37%=0.58 mM and D37%=0.63 mM, respectively) (Fig.17).
In this range of MMS doses the survival of wt/Ex2Δ Pol β (D$_{37\%}$ = 2 mM) was similar to that of wt cells (D$_{37\%}$ = 2.3 mM).

By increasing MMS dosage up to 5 mM, wt/Ex2Δ Pol β showed an higher MMS sensitivity (D$_{37\%}$ = 3 mM) than wt untransfected cells (D$_{37\%}$ = 5.7 mM), in agreement with gap-filling assay results, that showed a lower Pol β repair synthesis activity in this clone.

To confirm these data it will be necessary to repeat MMS treatment.

**Fig. 16** Results of growth rate assay performed with Pol β null/wt Pol β (0-2 mM MMS) and wt/Ex2Δ Pol β (0-5 mM MMS). Untransfected wt and Pol β null are shown for comparison.
Fig. 17 Results of growth rate assay performed with Pol \( \beta \) null/Ex2Δ Pol \( \beta \) and wt/Ex2Δ Pol \( \beta \). Untransfected wt and Pol \( \beta \) null are shown for comparison.
3. DISCUSSION

3.1 GENOMIC DNA ANALYSIS

DNA polymerase β is the main DNA polymerase involved in SP-BER. Several studies have shown that somatic and/or hereditary mutations in Pol β gene or alterations of Pol β expression level could lead to genomic instability. Thus, it has been proposed to consider Pol β (and also other specialized polymerases) as a proto-oncogene, which could contribute to generate a “mutator phenotype” [124,125].

We screened for Pol β mutations in normal and tumour tissues of patients suffering from GC. Gastric cancer is the fourth most common cancer in the world and the second leading cause of cancer death [157]. Nutritional, infectious and genetic factors are involved in the complex etiology of GC. In Italy GC risk distribution is characterized by a marked internal variability, with higher incidence and mortality in the central-northern regions than in southern Italy [173]. In particular, we analysed tissues from individuals living in Tuscany, a region where GC incidence is as high as in Japan.

GC has been described as a multistep process in which mucosa inflammation has a pivotal role [158]. Inflammatory process is characterized by a massive production of ROS, which induce DNA, protein and cell structures damage. BER is the repair mechanism mainly involved in repair of oxidative stress-induced DNA lesions. The pivotal role of BER in protection of free radical attack has been demonstrated in a recent study conducted on patients suffering from ulcerative colitis (UC) [190]. It has been shown that inflamed epithelial areas of UC patients are characterised by the over-expression of two BER enzymes and that this adaptive increase is correlated to MSI. This surprising finding has generated the paradox of DNA damaging effects (such as MSI
in genes involved in tumorigenesis) induced by adaptive imbalance in BER enzymes. We analysed genomic DNA from tissues of 5 individuals and from 3 gastric cancer cell lines. We didn’t detect any somatic and/or hereditary mutation, neither in exon sequence nor in splicing junction of Pol β gene. This finding is in agreement with previous data obtained from analysis of Pol β gene in 11 bladder tumours and 8 bladder cancer cell lines [152] and in untransformed and lymphoblastoid cells [200]. In contrast, mutations in Pol β gene have been found in colon, prostate and also in gastric cancer tissue [140-144]. In particular, Iwanaga and coworkers found missense mutations in 6 of 20 GC samples analysed. In 5 of 6 mutants, BER activity was not influenced, while one mutant was able to inhibit BER in vitro [143]. This discrepancy with our results could be explained by the different population examined and/or by the smaller size of our sample collection.

3.2 cDNA ANALYSIS

Whereas no mutation within Pol β gene could be detected by screening genomic DNA, cDNA analysis of tissues from 20 GC patients and 3 gastric cancer cell lines revealed the presence of many splicing variants. Interestingly, both normal and tumour tissues of all patients analysed were characterised by splice variants. This evidence, in agreement with previous findings [152,154], led us to discard the hypothesis that splice variants are a peculiarity of tumour tissue. Moreover, since splice variants have been detected also in other types of tissue [130,151,152-155], they cannot be considered specific of gastric tissue. By RT- and Nested-PCR we determined a splicing profile characterised by the loss of exon 2 as the predominant event (it was detected in all 20 patients and gastric cancer cell lines). Both normal and tumour tissues were characterised by exon 2 loss, but what is surprising is that both in normal and tumour tissues of some
patient the Pol β exon 2 deleted (Ex2Δ) mRNA is more represented than the wild-type (wt) form, if not the only form detected. Thus, while in other samples wt Pol β and splice variants coexist, in these cases wt protein is expected to be nearly absent. Quantitative PCR (QPCR) analysis of two of these patients confirmed this result, showing that the ratio between Ex2Δ Pol β mRNA and wt Pol β mRNA is in favour of the Ex2Δ variant form. To ascertain and better characterise the Pol β splicing pattern, we cloned cDNAs obtained from patients in a mammalian expression vector and then sequenced the variant cDNA-containing vector molecules. By this approach we confirmed that the exon 2 deleted protein is the most represented splicing variant in our samples (80% of variant cDNAs), in agreement with previously reported data of Thompson et al. [152], where cDNAs missing exon 2 represented 86% of cDNAs sequenced. Moreover, by sequencing, we found that all splice variants result from the deletion of the entire exon sequence, e.g. deletions are precisely located within the exon-exon junctions.

This finding gave rise to many questions about the Ex2Δ protein (and splice variants in general) function and the splicing mechanism responsible for splice variants production. Since we have found splice variants also in normal tissues of GC patients, we hypothesise that Pol β splicing variant could represent a post-transcriptional regulation mechanism necessary to avoid dramatic consequences due to Pol β overexpression (e.g. genomic instability) [111,115,117-122]. We propose that this mechanism could be mediated by either variant Pol β mRNA and variant proteins. We therefore could hypothesise that after stress-induced Pol β overexpression, variant Pol β mRNAs are more transcribed than wt, leading to a decrease in wt Pol β mRNA level. Moreover, variant mRNAs which are not eliminated by NMD or NAS for the presence of multiple premature termination codons, could be translated into variant protein which act as dominant negative or inhibit Pol β by substrate competition. To support our hypothesis is the evidence of a dominant negative activity carried out by the exon
11-deleted Pol β splicing variant [153], found in normal and tumor colorectal tissue [141,154], testis tumor [154], lung tumor [155], and normal blood samples of Werner patients [156]. A second evidence in favour of our hypothesis is derived from in vitro experiments with 14 kDa and 16kDa domain of Pol β [193]. It has been shown that these truncated domains of Pol β can inhibit wt polymerase activity by competing for binding substrate.

3.3 CHARACTERIZATION OF THE EXON 2-DELETED POL β VARIANT

To investigate the reliability of our hypothesis and characterize the exon 2-deleted variant, we transfected the mammalian expression vector containing the Pol β cDNA missing the exon 2 in wt and Pol β null murine fibroblasts, and isolated stably transfected clones (wt/Ex2∆ Pol β and Pol β null/Ex2∆ Pol β). Since these cells are completely defective in Pol β, they represent an optimal system to analyse the activity of a Pol β mutant/variant.

The loss of exon 2 with the retention of exon 3 creates a premature stop codon which would lead to the production of a only 26 amino acids protein. Thus, we first hypothesised that Ex2Δ mRNA transcript could be eliminated by those mechanisms, such as NMD or NAS, which recognize in truncated mRNAs a threat for cell survival. Thus, RT-PCR was performed to screen for human Pol β variant in transfected cells. We found the presence of transcript even after several months from transfection. Thus, it seems that exon 2 skipped splice variant is able to evade mechanism which could destroy it. In Skandalis et al. it has been proposed that the Ex2Δ mRNA may evade NMD detection by translation re-initiation 463 nucleotides downstream of the legitimate initiation site in exon 8. The resulting truncated protein would be of 181 amino acids and would miss the 8 kDa domain. Alternatively, a read-through of the premature termination codon has been suggested [200].

After characterizing transfected clones for wt (positive control) and variant Pol β expression level, we performed western blot
experiments to investigate if the Ex2Δ variant was translated in Pol β null cells and which would be its size. Unfortunately, the polyclonal antibody specific for Pol β recognizes efficiently wt Pol β, both in wt fibroblasts and AGS cell line and in Pol β null/wt Pol β, but not the variant form. We tried to perform the same experiment by blotting with an antibody specific for a sequence of 6xHis coded by the expression vector, but we didn’t succeed since this antibody is highly aspecific. We can exclude that the unsuccessful detection is due to a too low expression level of Ex2Δ variant. In fact, by QPCR we have verified that both Pol β null/Ex2Δ Pol β and wt/Ex2Δ Pol β clones used in our experiments express Pol β variant at higher level (~15-fold increase) than a clone expressing wt Pol β detectable by western blot. Thus, it will be helpful and interesting to perform western blot experiments with a polyclonal antibody specific for the 8 kDa domain of Pol β (these experiments are currently in progress).

A characterization of the functional activity of Ex2Δ variant was allowed by two functional assays: gap-filling and cell growth rate assay. Gap-filling assay permits to specifically test Pol β polymerase activity, since it allows to detect the Pol β-mediated 1 nt repair synthesis [198]. When performed with Pol β null/Ex2Δ Pol β whole cell extracts, gap-filling assay revealed that the Ex2Δ variant was unable to complement the Pol β-dependent repair defect of Pol β null cells. This was an expected result since the Ex2Δ variant is predicted to miss the entire 31 kDa polymerase domain. In contrast, surprisingly and unexpectedly, we found that wt/Ex2Δ Pol β whole cell extracts were partially impaired in performing the 1 nt gap-filling reaction, presenting only 50% of repair synthesis as compared with wt fibroblasts. This finding led us to deduce that Ex2Δ Pol β variant may be translated in mammalian cells. Moreover, since it should contain only the single-strand DNA binding region of the 8 kDa domain, we hypothesise that it could compete for substrate binding with wt Pol β, causing a decreased efficiency of SP-BER.
By cell growth rate assay we tested the \textit{in vivo} phenotypic effect of Ex2\(\Delta\) variant expression. Cells were treated with MMS, since it induces lesions mainly repaired \textit{via} SP-BER and \(\text{wt}\) and Pol \(\beta\) null fibroblasts have been yet extensively characterised for their different sensitivity to alkylating agents [127].

For MMS doses ranging from 0 to 2 mM, Pol \(\beta\) null/Ex2\(\Delta\) Pol \(\beta\) showed the same sensitivity of Pol \(\beta\) null untransfected cells, while a partial complementation of Pol \(\beta\) null defect was obtained for Pol \(\beta\) null/wt Pol \(\beta\). The hypersensitivity of Pol \(\beta\) null/Ex2\(\Delta\) Pol \(\beta\) is somewhat expected since Ex2\(\Delta\) variant probably lacks dRp lyase activity which is required for protection from MMS-induced cytotoxicity [198].

By using 0-2 mM MMS, \(\text{wt}\) and \(\text{wt}/\text{Ex2}\(\Delta\) Pol \(\beta\) fibroblasts showed resistance to MMS treatment. To see the phenotypic effect of Ex2\(\Delta\) variant we chose to treat transfected and untransfected \(\text{wt}\) fibroblasts with higher MMS doses (up to 5 mM). By this approach we could detect an increased sensitivity to MMS treatment in \(\text{wt}/\text{Ex2}\(\Delta\) Pol \(\beta\) compared to \(\text{wt}\) untransfected fibroblasts.

Overall, these findings seem to indicate that in spite of its premature stop codon and predicted small size, the Ex2\(\Delta\) could be translated in mammalian cells and acts as dominant negative on \(\text{wt}\) Pol \(\beta\). Alternatively, the Ex2\(\Delta\) variant may be not translated and the Ex2\(\Delta\) mRNA could assume a functional role to negatively regulate \(\text{wt}\) Pol \(\beta\) mRNA level.

The results obtained in the mouse cell system are of relevance when considering that the expression of Ex2\(\Delta\) Pol \(\beta\) variant is a significant phenomenon in human tissues. Even if the expression of a splicing variant with dominant negative activity could be a physiological mechanism to regulate Pol \(\beta\) expression level, the exclusive presence of such a protein could sensitise an individual to endogenous and exogenous damaging agents, by decreasing the overall BER efficiency.

To give reliability to the post-transcriptional regulation mechanism mediated by Pol \(\beta\) splice variants, we analysed 2 biopsies of
healthy patients by RT- and nested-PCR. One of the two sample analysed showed the exon 2 deleted mRNA. Thus, it seems reliable that Ex2Δ variant is involved in a mechanism that allows to control Pol β expression level.

3.4 POL β SPLICE VARIANTS AND CANCER

It becomes clear that in a context where Pol β wt and splice variants coexist, genomic stability is founded on a subtle play of balance between wt and variant form. Thus, genomic instability may result from over-expression of wt Pol β or from higher expression relative to wt of a dominant negative Pol β variant. The optimal ratio between wt and variant, which guarantees normal cell survival, remains to be established.

In the same way, many questions remain to be answered. If splice variants belong to a regulation mechanism, which are the factors involved in Pol β programmed splicing and how this mechanism is carried out? Which are the events that lead to an unbalance of wt/variant ratio? Does a proneness to unbalance exist? If it would exist, probably the unbalanced ratio should characterize all tissues of an individual.

Moreover, could a dominant negative variant level have a predictive value? How does the wt/variant ratio change during cancer progression?

Future research should address these questions.
4. MATERIALS AND METHODS

4.1 CLINICAL SAMPLES

Dr. D. Palli (Epidemiology Unit, Cancer Research and Prevention Center, Florence, Italy) co-ordinated gastric samples collection within a network of co-operative projects involving several hospitals in the Florence area. The aim of this network was to identify series of GC patients (both sexes and aged less than 75 years) residing in the area and to collect for each participant 1) detailed individual information on dietary and lifestyle habits, family history in an interview with a standardised questionnaire, after an informed consent form was signed; 2) a blood sample, immediately processed, aliquoted and stored in a –80°C freezer; 3) tumour and non involved (normal) tissue samples from GC surgical specimens, immediately snap frozen in liquid nitrogen and stored in a biological bank.

4.2 CELL LINES

GC cell lines were obtained from American Type Culture Collection. NCI-N87 and SNU-16 were cultured in RPMI 1640 (Gibco) whereas AGS in F12K (Gibco). The 3 cell lines were maintained in penicillin (100U/ml), streptomycin (100 µg/ml), 10% fetal bovine serum in a 5% carbon dioxide humidified incubator. SV40 transformed wild-type and Pol β-deficient mouse embryonic fibroblasts (a gift from Dr. S.H. Wilson, NIEHS, Research Triangle Park, NC) [14] were cultured in High Glucose D-MEM (Gibco) supplemented with 10% calf serum, penicillin (100U/ml), streptomycin (100 µg/ml) and hygromycin (80 µg/ml) at 34°C in a 10% carbon dioxide humidified incubator. Wild-type and Pol β-deficient transfected clones were cultured adding zeocin (250 µg/ml and 50 µg/ml, respectively) to High Glucose D-MEM.
4.3 DNA AND RNA ISOLATION

Cells/tissues were divided and then disrupted in lysis buffer (10mM Tris-HCl pH 8.0, 10mM EDTA, 10 mM NaCl; 0.5% SDS for DNA extraction; Quiagen Rneasy Fibrous Tissue kit, for RNA extraction).

Tissue homogenate for DNA extraction was incubated with RNase (20 µg/ml), 30 minutes at 37°C and then with proteinase K (100 µg/ml) over night at 37°C.

DNA was purified by Phenol, Phenol:Chloroform:Isoamyllic Alcohol (25:24:1) and Chloroform and then precipitated in Sodium Acetate 3M and absolute Ethanol.

RNA extraction was performed by using Quiagen Rneasy Fibrous Tissue kit.

Both DNA and RNA concentrations were determined by spectrophotometer before PCR amplification.

4.4 OLIGONUCLEOTIDES

Primers for genomic DNA analysis (Table I), RT-PCR (described in 4.6 paragraph), nested-PCR (Table II) and screening of cDNA-containing vectors were from Invitrogen.

Oligonucleotides for gap-filling assay substrate construction were from MWG-Biotech AG and are described in ref. 198.

4.5 GENOMIC DNA ANALYSIS

4.5.1 PCR

The 14 exons and exon a of Pol β (149) were amplified from genomic DNA using primers complementary to introns sequence, upstream of splicing junctions. Primers sequence is shown in Table I.
Valeria Simonelli

Genomic DNA (50 ng) was added to a master mix containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 250 μM dATP, dCTP, dGTP and dTTP; 0.8 μM of each primer; 1.25 units of Taq polymerase (Perkin Elmer, Roche Molecular Systems) and between 1.5 mM and 3 mM MgCl₂. Amplification was performed for 30 cycles in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using standard conditions at the annealing temperature of 57°C. Amplification products were checked on 2% agarose gel and then purified by Quiagen QIAquick PCR purification kit.

4.5.2 AUTOMATIC SEQUENCING

Purified PCR products were sequenced using Big Dye Terminator Cycle sequencing kit according to protocols provided by the manufacturer (Applied Biosystems) and primers used for PCR amplification (Table I). The sequencing products were precipitated by Sodium Acetate 3M pH 4.6 and absolute Ethanol and then

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCAGGACGCGTCAGTACCAA</td>
<td>TACCCACTGGACTGCGGTG</td>
</tr>
<tr>
<td>2</td>
<td>ACGTCTCGTTGTTTATCT</td>
<td>AGTCTGTAAATGACACT</td>
</tr>
<tr>
<td>3</td>
<td>ATATAGGTTATCCTGGAAGTC</td>
<td>GACACGTGCTGACTCTTTC</td>
</tr>
<tr>
<td>4</td>
<td>GATTGGTAAGCATTATTCATA</td>
<td>CCATAAGGACAGATACATA</td>
</tr>
<tr>
<td>5</td>
<td>GATCATTTAATGCTCTCAAGAC</td>
<td>GAGAACAAGCATTGATAATTC</td>
</tr>
<tr>
<td>6</td>
<td>AAGACTGTTGTTTACAAGG</td>
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<td>7</td>
<td>GTGATTGTTGATTGACATTTTC</td>
<td>GTTTAGCTAGTTGGAAGATTC</td>
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<td>8-9</td>
<td>ATGGCAGCAAATGATATAA</td>
<td>CTATGAAGTTACCAAATAC</td>
</tr>
<tr>
<td>10</td>
<td>ATGGGCAATCTCATTATGTC</td>
<td>ATGGATTGATTTGAAATTGAC</td>
</tr>
<tr>
<td>11</td>
<td>ATGGATTGATCTGGAATAGC</td>
<td>GCTATTTGTTATGACATTTTC</td>
</tr>
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<td>12</td>
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<td>13</td>
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<td>14</td>
<td>CAAGCTCTTAATCTTGCACCCT</td>
<td>TAAAGGTAGATCATGAACACA</td>
</tr>
<tr>
<td>α</td>
<td>GCTTATCTGCTGTCTTTA</td>
<td>GATGGCGCGACTGTAATTTCG</td>
</tr>
</tbody>
</table>
denatured and electrophoresed in ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

4.6 cDNA ANALYSIS

4.6.1 REVERSE TRANSCRIPTION PCR (RT-PCR) AND AMPLIFICATION OF POL β cDNA

Total RNA (1 µg) was used as substrate for RT-PCR performed by Invitrogen SuperScript One-step RT-PCR with platinum Taq kit, according to protocols provided by the manufacturer. This approach permits to reverse transcribe RNA and amplify the obtained cDNA by a single reaction. Primers used for RT-PCR were located at the beginning of exon 1 and after the end of exon 14. Primers sequence is: FORWARD PRIMER (5’ to 3’). GGCATGAGCAACGGAAGGCG; REVERSE PRIMER (5’ to 3’). CCTCATTCGCTCCGGTCCTTG. Amplification was performed for 35 cycles in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) according to the protocol provided by Invitrogen. 1/10 of the amplification product was checked on a 2% agarose gel.

4.6.2 NESTED-PCR

Nested-PCR was performed on 1 µl of RT-PCR products with primers designed to amplify contiguous 200 bp DNA fragments of the coding region of the human full length Pol β cDNA. Primers sequence is shown in Table II. Amplification was performed for 30 cycles in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) using standard conditions at the annealing temperature of 60°C. 1/10 of the amplification product was checked on a 2% agarose gel.
4.7 QUANTITATIVE PCR (QPCR) ANALYSIS

cDNA for QPCR was obtained by using the High Capacity cDNA Archive kit (Applied Biosystems), according to the protocol provided by the manufacturer. Two couples of primers, one specific for wild type+variant human Pol β cDNAs and the other one specific for the exon 2 deleted human Pol β cDNA were used to perform QPCR. For wt+variant Pol β cDNAs amplification a forward primer (5’ CGCCGCAGGAGACTCTCA 3’) complementary to exon 1, and a reverse primer (5’ TCTTCTCAAAAGTTTGCGAGTTCTG 3’) complementary to exon 1-exon 2 junction were used (1-2 couple). For exon 2 deleted Pol β cDNA amplification the same forward primer was used, while a different reverse primer (5’ TGCTATAACAGATGCTGTTTCTG 3’) complementary to exon 1-exon 3 junction was used (1-3 couple). For both amplification the same probe, complementary to exon 1 was chosen. The optimal concentration of primers and probe was determined by combining different primers concentrations (from 50nM to 900nM) with a fixed probe concentration (200nM), amplifying ~100 ng of cDNA from AGS cell lines. The amplification plots were examined and the primers concentrations that gave the lowest Ct value (e.g. forward primer 900 nM and reverse primer, both for 1-2 and 1-3 couple, 300 nM) were selected. To verify that both couples of primers had the same amplification efficiency, we performed a preliminary experiment by applying the

<table>
<thead>
<tr>
<th>Exons</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4</td>
<td>ATGAGCAGAGCGAGCGCGCGCG</td>
<td>GCTCCTACTGCGAGCGATTTCTAGCTT</td>
</tr>
<tr>
<td>4-7</td>
<td>AAAAAATGCTGAAAGATGGATGAGTTTTTAGC</td>
<td>CTTGTTCAATTCTCTTCTCTTGAAATGTC</td>
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<tr>
<td>7-10</td>
<td>TCATCAAGGTTGCTGAAAAACATTATTTG</td>
<td>AAATCTGGAATGCTACGAGAAACATC</td>
</tr>
<tr>
<td>10-13</td>
<td>CTTAGAATCAACAAAAAGGCGCAAAACTG</td>
<td>CAATCTACTGATGCTGTTTCTGTTGAGAACC</td>
</tr>
<tr>
<td>13-14</td>
<td>TGGCTCTCTATCTCATGCTGAAAGGATAA</td>
<td>CGCTCGGTCCTGCTGCTGCTCCTG</td>
</tr>
</tbody>
</table>

Table II
standard curves method. Serial dilutions of cDNA from AGS (from a theoretic amount of 0.01 ng up to 1 ng) were amplified by using primers and probe for wt+variant, Ex2∆ and standardized primers and probe for the housekeeping gene (GAPDH, Assay-on-demand, Applied Biosystems) used as endogenous control. By plotting Ct values vs log of cDNA concentration and we obtained parallel curves (data not shown) that indicated an equal amplification efficiency for the two couples of primers and the housekeeping gene.

Approximately 100ng of cDNA of normal and tumour tissues from patient #17 and 53 or from transfected clones (Pol β null/wt Pol β, Pol β null/Ex2∆ Pol β, wt/Ex2∆ Pol β) were separately amplified.

To compare the relative amount of wt and Ex2∆ Pol β cDNA within the same tissue, the relative quantity (RQ) value (obtained from software of analysis) of Ex2∆ Pol β cDNA amplified with 1-3 couple was subtracted from the RQ value obtained for the amplification with 1-2 couple, to obtain the RQ for wt Pol β cDNA. Then, wt and of Ex2∆ Pol β RQ values were compared.

4.8 CLONING OF cDNAs MOLECULES IN MAMMALIAN EXPRESSION VECTOR

PCR-amplified Pol β cDNA molecules from normal and tumour tissues of one of 20 GC patients analysed were cloned in a mammalian expression vector (pcDNA4/HisMax, Invitrogen), by using one-step cloning strategy (“TOPO Cloning”, Invitrogen). After ligation, vectors were transformed into TOP10 E.coli cells (Invitrogen) by heat-shock protocol provided by the manufacturer. Cells were plated at various densities onto agar containing 100μg/ml ampicillin and incubated at 37°C over night. Colonies were picked and screened by PCR, using primers which annealed to the expression vector (Xpress and BGH primers). Plasmids were purified and sequenced.
4.9 EXPRESSION OF EX2Δ POL β VARIANT IN MAMMALIAN CELLS

4.9.1 TRANSFECTION

Vector containing human wt and Ex2Δ Pol β cDNA were transfected into wt/Pol β null mouse fibroblasts. Empty vector and vector containing wt Pol β cDNA were transfected as negative and positive control, respectively. Before transfection, vector molecules were linearized with Sca I restriction endonuclease (Biolabs) to facilitate selection of stable transfection. For transfection, lipofectamine 2000 (Invitrogen) was used, according to the manufacturer’s protocol.

4.9.2 SELECTION OF STABLE CLONES

pcDNA4/HisMax contains the resistance gene to Zeocin. The appropriate concentration of Zeocin to use for selection was determined by adding varying concentration of the antibiotic (according to Invitrogen protocol) to the growth medium. After 1 week for Pol β null and about 2 weeks for wt fibroblasts, 50 µg/ml and 250 µg/ml of Zeocin killed Pol β null and wt, respectively and were, thus, chosen to select stable integrants. After 48 hours from transfection, cells were splitted and Zeocin added. Resistant clones were picked and expanded.

4.9.3 SCREENING OF STABLE CLONES

Stable clones were screened by RT-PCR to detect transcription of human wt/Ex2Δ Pol β mRNA. Primers used were the same employed in cDNA analysis.
4.9.4 WESTERN BLOT

Whole cell extracts for western blot analysis were first of all prepared by resuspending cells (10^6 cells per 20 µl) in buffer I (10mM Tris/HCl pH 7.8 and 200 mM KCl). An equal volume of buffer II (10 mM Tris-HCl pH 7.8, 200 mM KCl, 2 mM Na2EDTA, 40% glycerol, 0.2% Nonidet P-40, 2 mM dithiothreitol, 100 µg/ml aprotinin, 50 µg/ml leupeptin, 10 mg/ml pepstatin, 5mM phenylmethylsulphonyl fluoride) was added and the suspension rotated at 4°C for 1 hour and centrifuged at 16000g for 10 minutes. The supernatant was recovered, aliquoted and stored at -80°C.

About 40 µg of whole cell extracts per cell line were tested. Both anti-Pol β polyclonal antibody (a gift from Dr. S.H. Wilson, NIEHS, Research Triangle Park, NC) and anti-HisG (Invitrogen) were used diluted 1:5000 into Phosphate-Buffered Saline (PBS) containing 0.05% Tween-20 and 5% nonfat, dry milk. They were incubated with gentle agitation for 1 hour at room temperature and then stored at +4°C over night. Incubation with secondary antibodies and detection reaction were performed according to manufacturer’s instruction (PIERCE).

4.10 GAP-FILLING ASSAY

Gap-filling assay was performed according to Sobol et al. protocol [199].

4.10.1 PREPARATION OF DNA SUBSTRATE

A single-nucleotide gap substrate was constructed by annealing 200 pmol of three oligodeoxyribonucleotides (sequence in ref. 199) in TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). Template-primers annealing was performed in DNA Thermal Cycler (Perkin Elmer) by heating for 5’ at 70°C and slowly cooling at 30°C.
4.10.2 INCUBATION WITH WHOLE CELL EXTRACTS

Forty micrograms of whole cell extracts (prepared as described above, in western blot section) were incubated as described in ref. 199. A time-dependent kinetics was performed by incubating DNA substrate and whole cell extracts for 2, 5 and 10 minutes at 37°C.

4.11 CELL GROWTH RATE ASSAY

Transfected and untransfected cells were seeded at the density of 4x10^4 cells/well in six-well dishes (triplicate wells for each experimental point) in growth medium hygromicin/zeocin free. After 20-24 hours from seeding, cells were exposed to MMS. A different range of concentration of MMS was used to treat wt (0-5 mM MMS) and Pol β null (0-2 mM MMS) transfected and untransfected cells, since they show different alkylating agents sensitivity. High glucose D-MEM complemented with 5% of fetal bovine serum and antibiotics free was used for treatment. After 1 hour from MMS addition cells were washed, fed with complete medium (containing also hygromicin and zeocin) and, 3 days later, harvested and counted by cell counter. Results were expressed as the number of cells in MMS-treated wells relative to cells in control wells (% control growth).
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