Role of Base and Nucleotide Excision Repair Pathways in Processing of Clustered DNA Lesions Induced by Ionising Radiation

A thesis submitted for the degree of Doctor of Philosophy

by

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Abstract

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Ionising radiation (IR) induces a wide spectrum of lesions in DNA, including double- and single-strand breaks, abasic (AP) sites and a variety of base lesions. IR-induced damage to DNA can range from simple, isolated lesions to clustered DNA damage in which multiple lesions are formed, usually within a single helical turn of the DNA. Individual lesions within a cluster are recognised by repair enzymes of the base excision repair (BER) pathway, however, clustered DNA damage may be recognised as a bulky lesion and be processed by nucleotide excision repair (NER). Additionally, the presence of other closely spaced lesions may affect the rate and fidelity of DNA repair and, in doing so, may contribute to the harmful effects of ionising radiation. The aim of this study is to gain further understanding of the repairability of clustered DNA damage and the effects of multiple lesions on cellular repair systems. 7, 8-dihydro-8-oxoguanine (8-oxoG), thymine glycol (Tg), AP sites and single-strand breaks (SSB), some of the most frequently formed IR-induced DNA lesions, were employed in synthetic oligonucleotides to model various types of clustered lesions and their repairability was studied using purified base excision repair enzymes and cell extracts. It was revealed that BER is the major repair system involved in the processing of clustered DNA lesions, and that some clustered lesions are repaired with decreased efficiency. Both the composition of lesions in a cluster and the positioning of the various lesions determine their repairability by base excision repair enzymes.

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Some of the results of this thesis have been published in the following papers:

1 Budworth, H., Dianova, I.I., Podust, V.N. and Dianov, G.L. Repair of clustered DNA lesions. Sequence-specific inhibition of long-patch base excision repair by 8-oxoguanine. (2002) *JBC* **277**, 21300-21305.

2 Budworth, H. and Dianov, G.L. Mode of inhibition of short-patch base excision repair by thymine glycol within clustered DNA lesions. (2003) *JBC* **278**, 9378-9381.

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Publishers.

Abbreviations

A – adenine

AP site – apurinic/apyrimidinic (abasic) site

APE1 – AP endonuclease 1

ATP – adenosine triphosphate

BER – base excision repair

bp – base pair

C – cytosine

DHU- dihydrouracil

DNA – deoxyribonucleic acid

DTT - dithiothreitol

EDTA – ethylene diamine tetra-acetic acid

εC - ethenocytosine

FEN1 – flap endonuclease 1

G – guanine

Hx - hypoxanthine

IPTG – isopropyl-β-D-thiogalactopyranoside

IR – ionising radiation

NAD^+ - nicotinamide adenine dinucleotide (oxidised form)

NER – nucleotide excision repair

OHMeU – 5-hydroxymethyluracil

PARP-1 – poly (ADP-ribose) polymerase 1

PBS – phosphate-buffered saline

PCNA – proliferating cell nuclear antigen

PCV – packed cell volume

PMSF - phenylmethylsulfonylfluoride

Pol β – DNA polymerase β

Pol δ – DNA polymerase δ

Pol η - DNA polymerase η

RNA – ribonucleic acid

ROS – reactive oxygen species

RPA – replication protein A

SSB – single strand break

T – thymine

Tg – thymine glycol

UDG – uracil DNA glycosylase

XRCC1 – X-ray cross complementing factor 1

5-OHU – 5-hydroxyuracil

8-oxoG - 7, 8-dihydro-8-oxoguanine

Chapter 1 Introduction

1.1 *Ionising Radiation: Sources and Everyday Doses*

Living organisms are constantly exposed to ionising radiation from a variety of sources, both natural and man-made. Natural background radiation comes from three main sources; cosmic radiation, radiation from terrestrial sources and radioactivity in the body. In addition to the natural sources of background radiation, many artificial sources of radiation have been introduced over the past century. These artificial sources now add a significant contribution to the total radiation exposure of the general population [1]. The average annual dose in the UK is 2.6 millisieverts (mSv), in a population of 59 million [2]. Half of this average is from indoor radon exposure, which is the result of the decay of primordial radionuclides such as uranium-238. Individual doses from radon can reach a few hundred millisieverts in areas with high uranium concentrations in the underlying rocks [2]. Medical applications of radiation account for the largest man-made contribution to the overall total (Fig. 1a). Typical doses include; 0.01 mSv from a dental X-ray, 1.5 mSv from a lumbar spine X-ray, 8 mSv from a chest computed tomography (CT) scan and 0.1 to 18 mSv during nuclear medicine procedures, such as barium enema examinations [3]. Fallout from atomic weapons testing, occupational exposures and exposures resulting from radioactive waste discharge contribute less than 1% of the average annual dose [2].

The detrimental health effects of ionising radiation have been known for many decades. Undesired deleterious effects of ionising radiation exposure in man are categorised into

two categories. The first one comprises conditions resulting from massive cell killing, which require a high dose for their manifestation. The second category includes those effects originating from mutational changes in the cellular DNA. These may eventually lead to development of radiation-induced cancer [4].

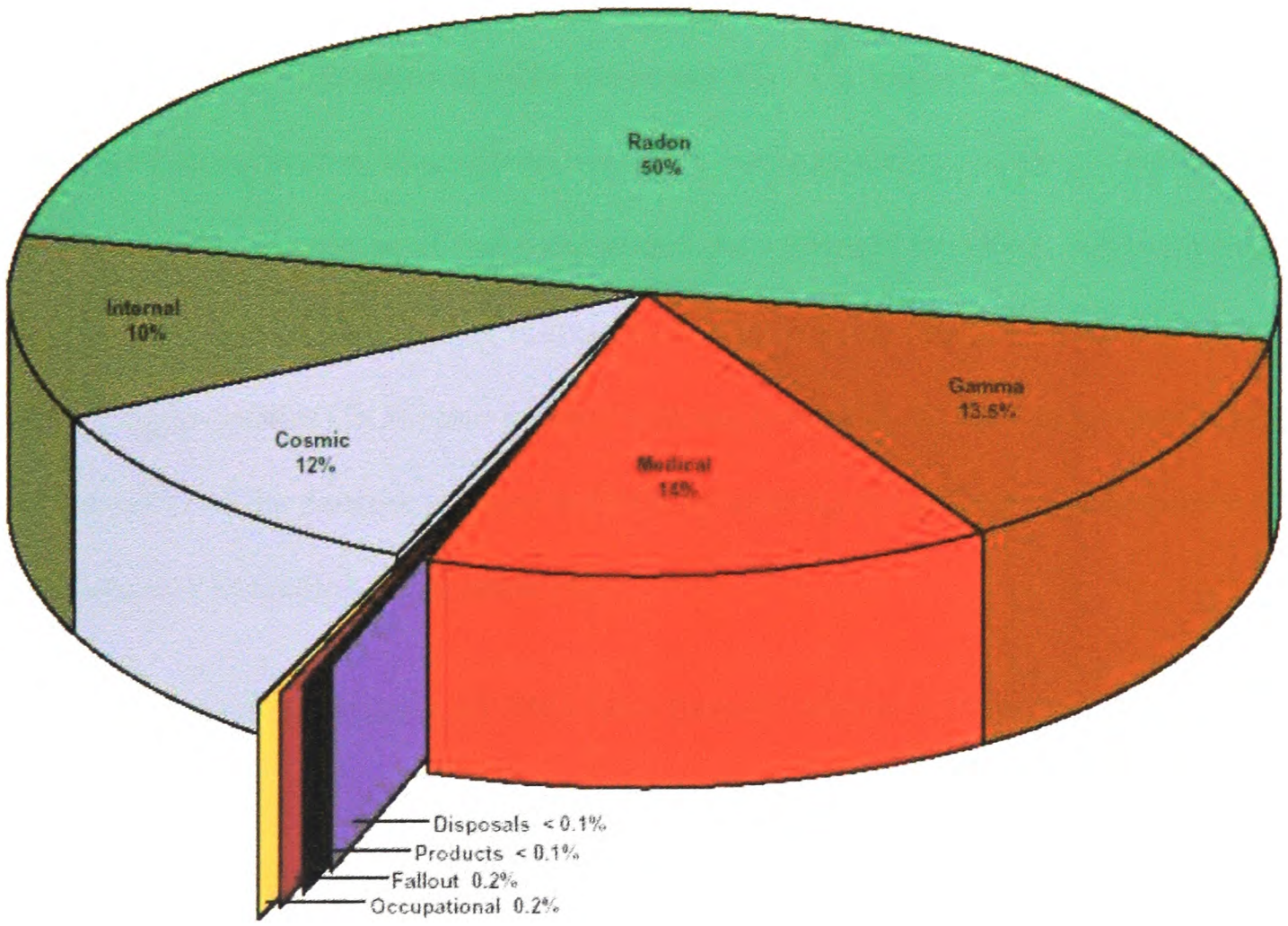


Fig. 1a Average annual radiation dose. (Source: National Radiological Protection Board [2])

1.2 *Ionising Radiation as a Source of Oxidative DNA Damage*

The damaging effects of ionising radiation come from the packages of high energy that are released from radioactive material. The damage to DNA from ionising radiation in mammalian cells is generally considered to arise from two sources; the direct effect and the indirect effect. Direct effect damage originates from energy deposited in the DNA molecule itself, while indirect effect damage arises following attack on the DNA by species produced by ionisations in other molecules [5]. The major source of the indirect effect is the free radicals produced from water. Ionising radiation energy is deposited in the cell and causes ionisation of water molecules that surround the DNA. Ionised water molecules can react with each other to form hydroxyl radicals, one of a number of reactive oxygen species (ROS) that interact with the DNA [5]. Studies have shown that more than 80% of the damage induced in DNA by ionising radiation is a consequence of ROS formed by the radiolysis of water [6].

The effects of ionising radiation on DNA have been the subject of many studies [5, 7-9]. Over the past 35 years radiation chemists, using nucleoside and nucleotide derivatives of DNA, have identified more than 100 DNA radiolysis products [10, 11]. A significant number of these are stable, thus when present in the DNA molecule they could contribute to the biological consequences of radiation damage. Base lesions, abasic (AP) sites, sugar damage and single- and double-strand breaks are the major lesions formed in DNA as a result of exposure to ionising radiation [5]. More than 20 oxidatively damaged purines and pyrimidines have been detected in DNA [12-14]. Such lesions are commonly found as a result of ionising radiation exposure, and are also formed spontaneously

through endogenous oxidative damage. There are a number of major sources of endogenous oxidative damage.

The use of oxygen by aerobic organisms is accompanied by the formation of reactive by-products, free radical forms of oxygen such as those formed as a result of exposure to ionising radiation. Humans consume oxygen during mitochondrial aerobic respiration to generate essential energy, in the form of adenosine triphosphate (ATP), through a series of molecular reduction reactions. During this metabolic process, ROS are produced as by-products. ROS are also formed during other normal metabolic processes such as lipid peroxidation and single-carbon metabolism [15, 16]. There are also a number of additional extracellular sources, including ROS generated by macrophages in the inflammatory response, exposure to alcohol, cigarette smoke, chlorinated drinking water, heavy metals and ultraviolet (UV) radiation [17-20].

A major site of oxidative radical attack in DNA is at the 8 position of guanine to produce 8-oxoguanine (8-oxoG) (Fig 1b) [21]. 8-oxoguanine is one of the most frequently formed base lesions in DNA as a result of oxidative damage [22, 23]. It is formed by the reaction of singlet oxygen or hydroxyl radical either with guanine in DNA or with dGTP. It can be formed spontaneously in the genome of aerobic organisms during normal metabolic processes and also as a result of ionising radiation. 8-oxoguanine is a premutagenic base lesion in DNA as it can base pair with both cytosine (C) and adenine (A) [24]. Plasmid vectors containing a single lesion were used to establish the mutational frequency and specificity of 8-oxoguanine in prokaryotes and eukaryotes [25, 26]. The predominant

mutations were G:C-T:A transversions, targeted to the site of the lesion. This mutational specificity is consistent with the results of primer extension studies which showed that polymerases involved primarily in DNA replication (mammalian polymerases α and δ and *E.coli* pol III) selectively incorporate A opposite template 8-oxoguanine, whereas polymerases involved in repair (mammalian polymerase β and *E.coli* pol I) selectively incorporate C [24]. In contrast to unmodified nucleotides, the 8-oxoguanine lesion intrinsically prefers a *syn* conformation of its glycosylic bond (instead of the *anti* conformation seen in B-form DNA) to avoid a clash between the deoxyribose sugar and the aberrant 8-oxo group on the base [27]. It is in the *syn* conformation that 8-oxoguanine in DNA can form a Hoogsteen base pair with adenine in the *anti*-alignment [28] (Fig. 1c). 8-oxoguanine can also be found in the *anti* conformation in DNA and pairs with cytosine in a Watson-Crick base pair (Fig. 1c). Both 8-oxoguanine:adenine and 8-oxoguanine:cytosine base pairs are relatively non-distorting in duplex DNA [29, 30].

Another frequently formed oxidative base lesion in DNA is thymine glycol (Tg) (Fig. 1d). Approximately 10-20% of the damage to DNA induced by ionising radiation is the result of thymine base oxidation and fragmentation [31]. Thymine glycol is a modified pyrimidine lesion which retains the intact pyrimidine ring, however, unlike the pyrimidine, is non-planar [32]. Thymine glycol is not accomodated into the normal DNA stacking, a thymine glycol residue in DNA appears to be extrahelical because it is not in the central stack of the DNA, due to its loss of planarity [31]. Nuclear magnetic resonance (NMR) results on thymine glycol-containing DNA indicate that the presence of

thymine glycol induces a significant and highly localised alteration in the structure of the DNA [31]. It is likely that this structural perturbation affects the recognition of the DNA by proteins and may also affect the packaging of DNA in the replication complex, in nucleosomes, in viruses and in other contexts. Indeed, the presence of thymine glycol in DNA can have profound consequences on DNA replication, as it is a block to replicative DNA polymerases. Thymine glycol can effectively terminate DNA replication and transcription, is cytotoxic [33, 34], and has been shown to be lethal *in vivo* in *E.coli* [35]. Evidence demonstrates that thymine glycol stops replication either one residue before or at the site of damage, and it has been shown that dA is the residue placed opposite thymine glycol by DNA polymerases [36, 37]. However, the presence of a pyrimidine 5' to the damaged thymine seems to enhance the probability of reading through a thymine glycol site in replication more so than a 5' purine, indicating a sequence-dependent effect [38]. The base 3' to a thymine glycol is apparently also important in determining the extent of the block to replication [39]. Further studies also show that in particular sequence contexts thymine glycol was more readily bypassed than in other sequence contexts, suggesting that the surrounding DNA sequence influences the presentation of the lesion to the DNA polymerase [40].

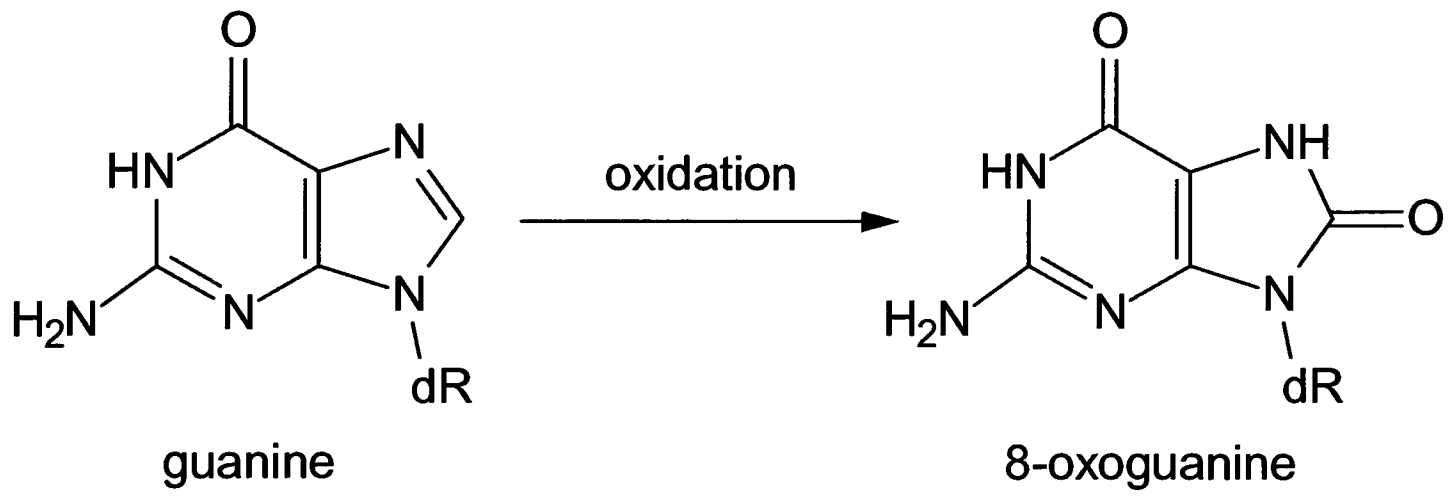
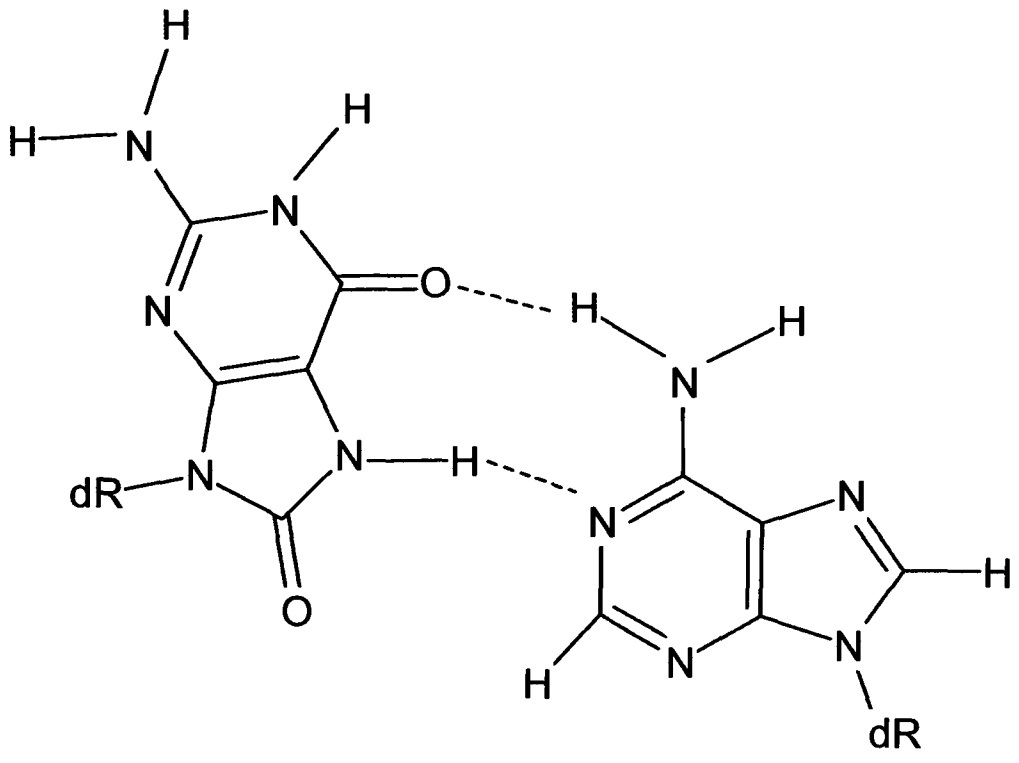
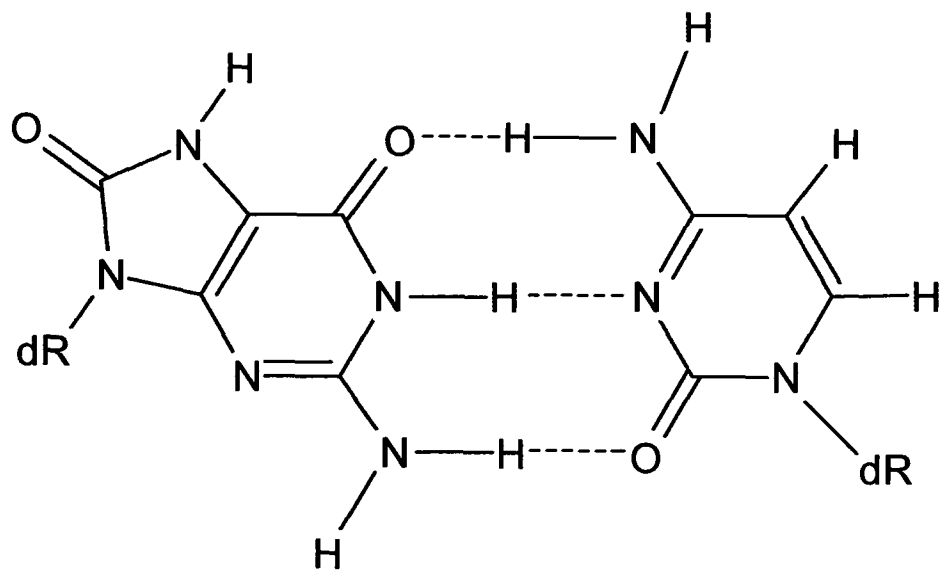


Fig. 1b Structures of guanine and 8-oxoguanine.



8-oxodG (*syn*)-**dA** (*anti*)



8-oxodG (*anti*)-**dC** (*anti*)

Fig. 1c Base pairing properties of 8-oxoguanine.

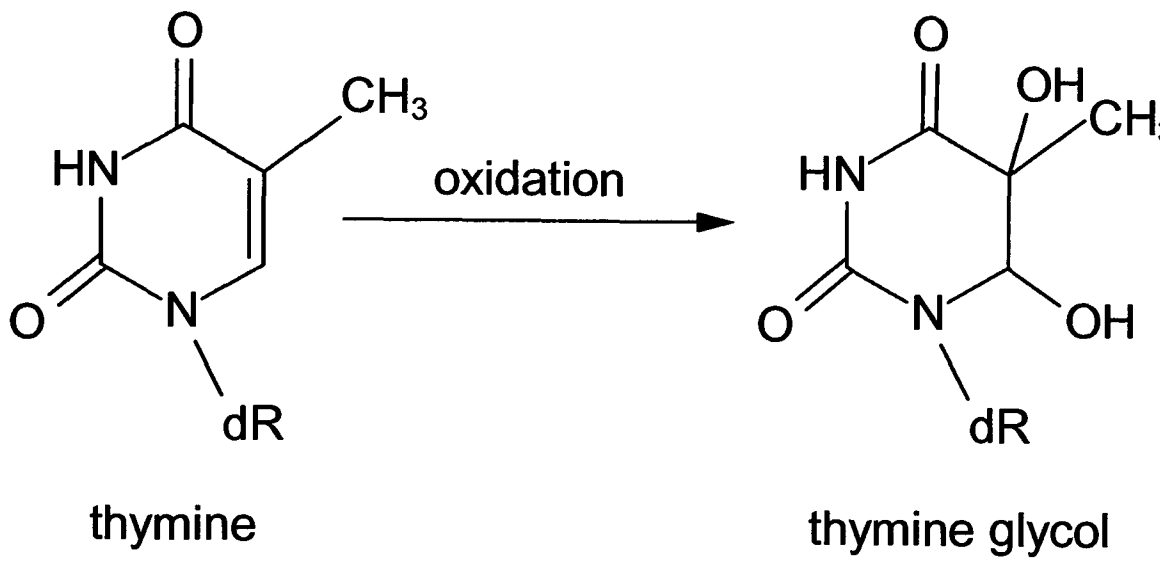


Fig. 1d Structures of thymine and thymine glycol.

Due to the instability of DNA and its sensitivity to damaging agents, cells have evolved sophisticated repair systems to ensure maintenance of genome fidelity. Repair of single oxidative base lesions, formed either spontaneously or through exposure to ionising radiation, is routinely performed by base excision repair (BER) [41], a multiprotein repair pathway with a broad substrate specificity that is determined by the lesion-specific DNA glycosylases. DNA glycosylases initiate BER by recognising damaged or abnormal bases and cleaving the glycosylic bond linking the base to the sugar phosphate backbone [42, 43].

1.3 ***Base Excision Repair***

The background level of endogenous DNA damage per cell (steady state level) is estimated to be between approximately 2200 – 100,000 lesions per cell [41, 44]. The principal endogenous, or so-called spontaneous, DNA lesions are caused by deamination of cytosine to uracil (or methyl-cytosine to thymine), loss of purines to yield abasic sites, and strand breaks [41]. These are in addition to oxidative base lesions caused as a result of exposure to ROS. Base excision repair pathways have been conserved throughout evolution and are present in all forms of life from bacteria to higher mammals, which highlights their importance in maintenance of genome fidelity. There are two pathways for base excision repair, which involve different subsets of proteins and result in replacement of one (short-patch pathway) or more (long-patch pathway) nucleotides [45-48]. Both pathways are initiated by a DNA glycosylase, which removes a damaged base through cleavage of the C1'-*N*-glycosylic bond leaving an AP site [41, 49] (Fig 1e). The

known DNA glycosylases effectively cover repair of the major classes of DNA lesions caused by hydrolysis, oxidation and non-enzymatic methylation [41] (Table 1). Glycosylases can be broadly divided into two different mechanistic subclasses: the monofunctional or "pure" glycosylases, including uracil-DNA glycosylase (UDG), and the bifunctional glycosylases, including the oxidative damage processing enzymes OGG1, which removes oxidized purines, and NTH1, which removes oxidized pyrimidines, which have an associated AP lyase activity that cleaves the phosphodiester backbone 3' to the abasic site. In the former case, the base is displaced by nucleophilic attack at the anomeric centre by an activated water molecule whereas in the latter, an amino group on the enzyme acts as the nucleophile and forms a transient Schiff base intermediate. This Schiff base is primed to undergo a β-elimination reaction resulting in cleavage of the phosphodiester backbone, generating a 5'-phosphate and a 3'-α,β-unsaturated aldehyde. Alternatively, the Schiff base can undergo hydrolysis to leave the phosphodiester backbone intact [50]. The blocking unsaturated aldehyde residue can then be excised by an AP endonuclease (APE1) [51] although the 3'-phosphodiesterase activity of APE1 is significantly (100 fold) weaker than its AP endonuclease activity [52]. The product of sequential AP lyase and 3'-phosphodiesterase processing of an AP site is a one-nucleotide gap with flanking 3'-OH and 5'-phosphate termini.

The degree to which the glycosylase and lyase activities of bifunctional glycosylases are coupled depends on both the particular enzyme and reaction conditions. It has been shown that during repair of oxidative base lesions, in the presence of APE1, the glycosylase activity is stimulated thus bypassing the lyase activity [53, 54]. Subsequent

studies have shown that single-nucleotide base excision repair in mammalian cell extracts is dependent on the deoxyribosephosphate (dRP) lyase activity of DNA polymerase β (Pol β), rather than that of the glycosylase [55]. These data suggest that the AP lyase activity of bifunctional DNA glycosylases has a minor physiological significance and may play a role in a minor subpathway of base excision repair only.

Glycosylase	***Bifunctional***	***Substrates***	***References***
UNG	No	U UNG1-mitochondrial UNG2-nuclear	[56-58]
SMUG1	No	U, OHMeU	[59, 60]
TDG	No	U:G, T:G, εC	[61, 62]
MBD4	No	U:G, T:G in CpG sites	[63, 64]
OGG1	Yes	8oxoG:C, FapyA, FapyG	[65-67]
MYH	No	A:8oxoG	[68, 69]
NTH1	Yes	Oxidised and fragmented pyrimidines (e.g. Tg, DHU), Fapy	[70-72]
AAG (MPG)	No	3-meA, 7-meG, εA, εG, Hx	[73-75]
NEIL1 (NEH1)	Yes	FapyA, FapyG, DHU, Tg, 8-oxoG (low)	[76-78]
NEIL2 (NEH2)	Yes	Cytosine oxidation products (5-OHU, DHU)	[79]

Table 1 Mammalian DNA glycosylases.

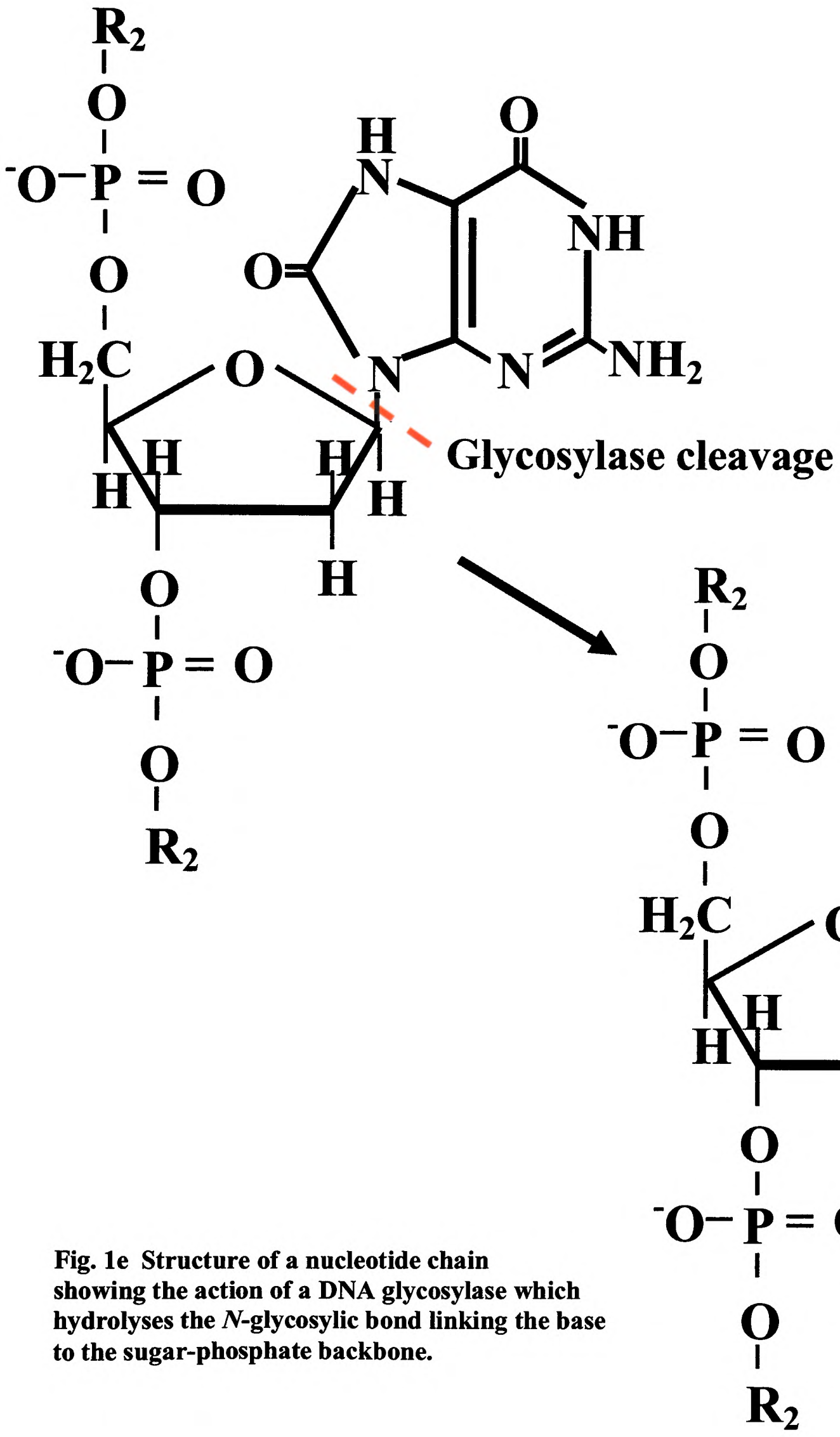


Fig. 1e Structure of a nucleotide chain showing the action of a DNA glycosylase which hydrolyses the *N*-glycosylic bond linking the base to the sugar-phosphate backbone.

Following the action of glycosylases, the resulting AP sites are recognised and cleaved by an AP endonuclease. The major AP endonuclease in mammalian cells is APE1, also known as Ref1 or HAP1, which comprises more than 95% of cellular AP site incision activity [80]. APE1 cleaves phosphodiester bonds hydrolytically in a magnesium (Mg^{2+})-dependent manner, to leave a 3'-hydroxyl group and a 5'-dRP group flanking the nucleotide gap.

The major pathway of base excision repair, short-patch BER, uses Pol β for repair of nuclear DNA, which adds the first nucleotide to the 3'-end of the incised AP site. Pol β, a member of the X family of DNA polymerases, is the lowest molecular mass DNA polymerase, a single polypeptide of 39kDa comprising 335 amino acid residues. Its structure is highly conserved in mammals [81, 82], consisting of two domains connected by a protease-sensitive hinge region. The 8-kDa N-terminal domain carries dRPase activity (to remove the 5'-deoxyribosephosphate from an abasic site generated by AP endonuclease) and template binding functions, whereas the 31-kDa domain carries the DNA polymerase activity [83, 84]. The crystal structure of the rat Pol β enzyme revealed a typical polymerase fold, a thumb, palm and fingers, due to its resemblance to the human hand [85-87]. However, in contrast to other polymerases, Pol β lacks intrinsic accessory activities that may contribute to polymerase fidelity, such as 3'- or 5'-exonuclease, endonuclease, dNMP turnover and pyrophosphorolysis [81, 88-92]. Evidence for the involvement of Pol β in BER came from genetic studies of Pol β-deleted cells, which

exhibited increased sensitivity to monofunctional DNA-alkylating agents and were found to be defective in uracil-initiated base excision repair [91].

Early studies showed that Pol β is non-processive on single-stranded DNA templates, prefers short-gapped DNA substrates, and is capable of filling gaps to completion [93, 94]. Further studies revealed that Pol β fills short gaps (2-6nt) by a processive mechanism that requires a PO_4 group at the 5' margin of the gap [95]. Binding of Pol β to these short-gapped substrates is also strongly enhanced by the presence of a 5' PO_4 [96]. The structural basis for this substrate preference is that the 8kDa single-stranded DNA binding domain of Pol β binds to the 5' termini of the DNA downstream of the gap [84]. It was also demonstrated that the nucleotide insertion fidelity of Pol β is higher on 5'-phosphorylated single-nucleotide gapped DNA compared with other DNA substrates, suggesting that a primary function of Pol β is to fill such gaps which occur as base excision repair intermediates [97].

Normally, the base excision repair reaction continues through the short-patch repair pathway [98, 99] where Pol β removes the 5'-sugar phosphate residue, through β-elimination, to create a nick containing a 3'- hydroxyl and a 5'-phosphate end which can then be sealed by a DNA ligase to complete the repair (Fig. 1f). In the case of short-patch base excision repair, the ligase responsible for sealing the nick has been proposed to be DNA ligase III which exists in a complex with X-ray cross complementing repair protein 1 (XRCC1) [100].

1.3.1 *Long-patch Base Excision Repair*

Both oxidised and reduced AP sites are refractory to β-elimination and repair of such sites must occur by an alternative mechanism [101, 102]. Repair of such lesions proceeds through the long-patch base excision repair pathway [46, 48], in which between 2 and 8 nucleotides are incorporated into the repair gap displacing the dRP as part of an oligonucleotide "flap". This flap is then recognised and removed by a flap endonuclease, FEN1. Human FEN1 exhibits both a 5'-3' exonucleolytic activity and a structure-specific endonucleolytic activity. FEN1 functions in long-patch BER as an endonuclease that cleaves the flap substrate that is generated following strand-displacement synthesis [48]. FEN1 has a flexible loop structure through which the flap has been proposed to thread. Crystal structure studies of FEN1 have indicated that this loop can accommodate single-stranded DNA, but is not large enough for double-stranded DNA; this information prompted suggestions that the flap threads through the hole during tracking [103]. Cleavage of the flap is followed by ligation by DNA ligase I which is able to complete repair by rejoining the free DNA ends [104] (Fig. 1f). Reconstitution of the long-patch BER pathway *in vitro* demonstrated that both DNA polymerase β and DNA polymerase δ (Pol δ) are able to support repair synthesis [48, 102, 105, 106]. Evidence suggests that base excision repair is always initiated by Pol β, which adds the first nucleotide into the repair gap, further synthesis during long-patch BER may be supported by either Pol β or Pol δ [102]. Long-patch base excision repair was also found to be proliferating cell nuclear antigen (PCNA)-dependent [46, 47]. PCNA is a processivity factor for Pol δ [107] and also interacts with and stimulates FEN1 [48, 108] and DNA ligase I [109].

1.3.2 *Role of PARP-1 in Base Excision Repair*

The possibility that the enzyme poly(ADP-ribose)polymerase (PARP-1) is an active participant in BER *in vivo* has been extensively investigated in recent years but remains controversial. PARP-1 is an abundant nuclear protein with a high affinity for single- and double-strand DNA breaks. PARP-1-knockout cells are extremely sensitive to alkylating agents, suggesting the involvement of PARP-1 in base excision repair [110, 111]. PARP-1 binds to single-strand breaks and subsequently catalyses the modification of nuclear proteins with polymers of ADP-ribose which are synthesised from NAD^+. Strand break repair exhibits a dependence on NAD^+ which has been attributed to the activity of PARP-1 [112, 113]. One of the major targets of poly(ADP-ribosyl)ation is PARP-1 itself. PARP-1 binds to strand breaks as a homodimer and after automodification, resulting in accumulation of negative charge on the enzyme, it dissociates from the DNA. Recycling of PARP-1 is provided by PaR-glycohydrolase which catalyses degradation of ADP-ribosyl polymers attached to PARP-1 [114]. A model was proposed whereby PARP-1 plays a passive role in BER, temporarily binding to nicked DNA repair intermediates to protect them from degradation by the cellular milieu [113]. Recent studies of repair by cell extracts from PARP-1-deficient mouse fibroblasts would seem to support this theory. PARP-1 deficient cell extracts showed similar ability to repair X-irradiated or alkylating agent-treated plasmid DNA compared to wild-type cell extracts [115]. Similarly, studies carried out using DNA substrates containing a site-specifically located normal AP site or reduced AP site (long-patch repair substrate) showed that repair in PARP-1 deficient extracts was more efficient than in extracts from the wild-type

[116]. In both studies titration of PARP-1 into the PARP-1-deficient extracts inhibited rather than stimulated repair [115, 116]. However, an alternative theory that PARP-1 plays an active role in DNA damage processing has also been suggested. Experiments with purified recombinant enzymes demonstrated stimulation of Pol β by PARP-1, although only in the absence of NAD^+ [117]. It has also been reported that extracts from PARP-1-deficient mouse fibroblasts show decreased long-patch synthesis in the repair of a DNA substrate containing an abasic site and it was therefore concluded that PARP-1 is required for long-patch BER [118]. It is clear that further work is required to understand the role of PARP-1 in BER.

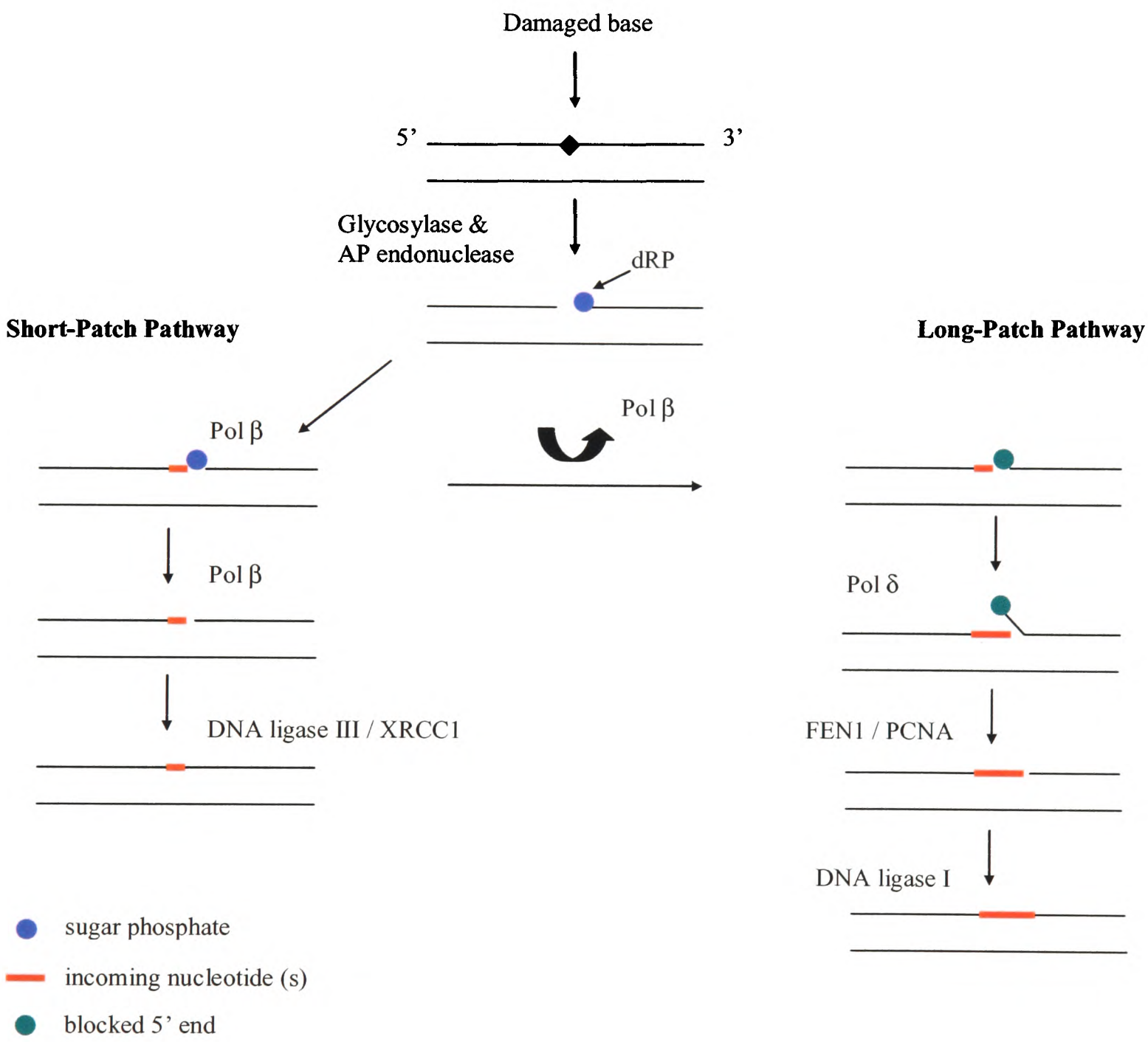


Fig. 1f Base excision repair pathways. (Adapted from Podlutsky et al., 2001 [102])

1.3.3 *Co-ordination of Base Excision Repair*

Base excision repair proceeds via a highly co-ordinated series of concerted reactions [119, 120]. It has been demonstrated that the glycosylase remains associated with the AP site following removal of the damaged base, protecting the AP site from degradation [121]. The glycosylase is subsequently displaced by the next enzyme in the pathway, APE1, which achieves this displacement through having a greater affinity for AP sites by making extensive contacts with the DNA and inducing significant kinking of the helical axis [122]. APE1 hydrolyses the phosphodiester backbone 5' to the AP site, leaving a 3'-OH and a 5'-dRP [123, 124]. Pol β recognises the nicked AP site and adds a single nucleotide by displacing the 5'-terminal sugar phosphate. The 5'-dRP residue must be removed for single-nucleotide patch repair to proceed to completion. In mammals Pol β performs this task through its dRP lyase activity [83]. In practice, Pol β has been demonstrated to have an affinity for the complex between APE1 and an uncleaved AP site and is recruited to the damage site as a ternary complex [125]. Following backbone cleavage Pol β retains affinity for the DNA and is then able to both remove the dRP and introduce a single nucleotide in a concerted fashion. Since Pol β also has an affinity for both DNA ligase I [90, 126] and DNA ligase III (mediated by XRCC1) [100, 127] it is therefore possible for the final step of repair, i.e. ligation of the nick, to be coupled to the rest of the repair process. Indeed, when short-patch BER was reconstituted with purified mammalian proteins both DNA ligase III and DNA ligase I were able to accomplish repair [55, 128].

1.3.4 *Role of Specialised DNA Polymerases in Base Excision Repair*

When lesions are formed in DNA, if they are not repaired prior to replication the DNA polymerase must synthesise across the lesion and the lesion could either block the DNA polymerase and thus be potentially cytotoxic, or it could be bypassed by a DNA polymerase and be potentially mutagenic depending on its ability to mispair. In some cases this translesion synthesis is carried out by the replicative DNA polymerase, but in other cases translesion synthesis seems to be conducted by specialised DNA polymerases, probably depending on the nature of the lesions. Highly mutagenic lesions do not block DNA synthesis and can form base pairs with incorrect nucleotides. Of the modified pyrimidine lesions retaining the intact pyrimidine ring that have been studied to date, only thymine glycol is a strong blocking lesion to DNA polymerases *in vitro* [33] and can be cytotoxic if unrepaired *in vivo* [35]. In contrast, 8-oxoguanine is assumed to be one of the most potent premutagenic lesions in DNA, as it is readily bypassed by polymerases and directs both C and A incorporation during DNA synthesis [24].

Recently, a family of DNA polymerases, the UmuC / Y family of DNA polymerases, involved in translesion synthesis (TLS) has been identified in prokaryotes and eukaryotes, including humans [129-131]. It has been suggested that these specialised DNA polymerases act to minimise the cell death resulting from replication blockage. These polymerases act individually, or in concert, to complete replication of damaged DNA and differ in their specificity for different DNA substrates. Some can insert bases opposite non-coding lesions, whereas others extend DNA from a misincorporated base

[132]. In contrast to the replicative polymerases that generally have a high fidelity of 10^{-5}-10^{-7}, most of these damage-specific polymerases have a low fidelity of ~10^{-2} [133].

Amongst these TLS polymerases are human Pol ι, Pol κ, Pol λ and Pol η, which are suspected to play a role in DNA lesion bypass during replication. Human Pol ι is encoded by the *RAD30B* gene and shares sequence similarity to Pol η [134]. Human Pol ι is a distributive enzyme that is highly error-prone when replicating undamaged DNA [132, 135, 136] and has two unique properties that differentiate it from other known DNA polymerases; (i) violation of the Watson-Crick base pairing rule opposite template T with preferential incorporation of G rather than A [132, 135, 136], and (ii) the T stop that frequently aborts DNA synthesis opposite a template T [135]. Expression of human *POLI* is ubiquitous but elevated in certain tissues, namely testis, heart and pancreas [134].

The human *POLI* gene that encodes the 80kDa Pol ι protein contains a helix-hairpin-helix motif [92, 134] similar to that found in the NH_2-terminal domain of Pol β, which catalyses excision of a 5'-dRP group from DNA during base excision repair [83, 137, 138]. It has recently been reported that Pol ι has intrinsic dRP lyase activity and removes a dRP group at 30-50% of the rate catalysed by Pol β [139, 140]. As with Pol β the reaction proceeds via β-elimination involving an active site residue containing a primary amine [139]. It was subsequently shown that Pol ι can substitute for Pol β in base excision repair reactions *in vitro* [139]. However, Pol ι cannot fully complement all of the cellular functions of Pol β *in vivo* as Pol β-deficient mice are inviable [141, 142], which suggests that Pol ι may only participate in a specialised form of BER.

Human Pol κ is encoded by the *POLK* (*DINB1*) gene. It is a template-directed DNA polymerase with limited processivity and fidelity [143] and lacks detectable 3'-5' proofreading exonuclease activity [144]. Human Pol κ has been shown to be able to incorporate either a C or an A opposite 8-oxoguanine in DNA, consistent with the miscoding property of the lesion, with the preferential insertion of A [145]. Pol κ has also been shown to be able to bypass thymine glycol in DNA and, in contrast to 8-oxoguanine bypass, inserts the correct base, adenine, opposite thymine glycol in preference to the other three bases [146].

According to recent studies [147, 148], Pol β is known to have another family polymerase, DNA polymerase λ (Pol λ). Pol λ is a recently identified eukaryotic DNA polymerase that is homologous to Pol β, sharing 32% identity at the protein level [147]. Pol λ is predicted to have a Pol β-like core formed by two domains, a 31kDa polymerase domain and an 8kDa domain [147]. However, unlike Pol β, Pol λ contains additional N-terminal domains including a BRCT domain that could mediate protein-protein interactions [148-151]. A truncated form of Pol λ, with the N-terminal BRCT motif deleted has been found to retain DNA polymerase activity [152]. Northern blot analysis revealed that the expression of Pol λ was almost the same as that of Pol β [147]. However, it does appear to be predominantly expressed in the testis which suggests that it may play a role in meiotic recombination. As with Pol β, Pol λ has intrinsic dRP lyase activity and removes dRP residues at approx. 25% of the rate catalysed by Pol β, and in a similar manner [153]. *In vitro* experiments indicate that Pol λ can substitute for Pol β

during base excision repair [153]. However, as stated earlier, neither Pol λ nor Pol ι can fully complement all of the cellular functions of Pol β *in vivo* as Pol β-deficient mice are inviable [141, 142].

Human Pol η is encoded by the yeast *RAD30* counterpart, *POLH* (*XPV*). Mutations of *POLH* cause the variant form of xeroderma pigmentosum (XP-V) [154, 155], and XP-V individuals suffer from a high incidence of sunlight-induced skin cancers. Pol η efficiently replicates DNA containing a *cis-syn* thymine-thymine (T-T) dimer by inserting two adenines across from the dimer [156]. The activity of purified human Pol η was also examined on several other types of DNA lesions and was found to bypass 8-oxoguanine [157]. It was further shown that yeast and human Pol η replicate DNA containing 8-oxoguanine efficiently and accurately by inserting a cytosine across from the lesion and by proficiently extending from this base pair [158]. Pol η also has a relatively high efficiency of thymine glycol bypass [159], as it can insert a nucleotide opposite to thymine glycol as efficiently as opposite to an undamaged sequence. Although Pol η misincorporated incorrect nucleotides, especially dGMP, opposite thymine glycol it preferentially elongated from A:Tg pairs and hence the majority (~99%) of translesion synthesis past thymine glycol by Pol η was accurate [159].

Although it is clear that translesion polymerases play an important role during replication, their role in base excision repair remains unclear.

1.4 *Nucleotide Excision Repair*

Nucleotide excision repair (NER) has also been implicated in the removal of minor base lesions, induced by alkylating and oxidising agents - including 8-oxoguanine, which are generally not helix-distorting [88, 160]. Thymine glycol, however, does induce a significant and localised structural change to the DNA double helix that may be recognised by NER [31], and has been shown to be a substrate for this repair pathway [88, 160]. Base excision repair is considered to be the main pathway involved in the repair of such lesions, however, NER may act as a back-up system [99, 161].

NER is the most flexible of all DNA repair mechanisms. It is a highly versatile and sophisticated DNA damage removal pathway that counteracts the deleterious effects of a multitude of DNA lesions, including major types of damage induced by environmental sources. Within the divergent spectrum of NER lesions, significant distortion of the DNA helix by bulky base adducts appears to be a common denominator [162, 163]. The most relevant lesions subject to NER are cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs), two major kinds of injury produced by the shortwave UV component of sunlight [88]. In addition, numerous bulky chemical adducts are eliminated by this process. These include large polycyclic aromatic hydrocarbon adducts (induced by compounds in cigarette smoke amongst other things) and the particularly distorting interstrand crosslinks, induced by chemotherapeutic agents, such as cisplatin, and also by certain cellular metabolites [164-166].

The mechanism of nucleotide excision repair is highly conserved between species and involves recognition of DNA damage followed by DNA unwinding, which provides discrimination of the damaged strand from the non-damaged DNA strand, cleavage of the strand containing the damage (usually on both the 5' and 3' sides of the lesion), removal of an oligonucleotide containing the damage, resynthesis of a repair patch to fill the gap and ligation to the contiguous strand at the end of the gap [167, 168] (Fig 1g). There are two subpathways of NER; global genome nucleotide excision repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER). GG-NER is responsible for repair of lesions over the entire genome, whilst TC-NER is responsible for repair of transcription-blocking lesions present in transcribed DNA strands. Both processes are essentially the same, except for the initial damage recognition step. During GG-NER, damage recognition is performed by XPC/HHR23B, which is the only known NER factor that is dispensable for TC-NER [169]. During TC-NER, the stalled RNA polymerase II complex acts as the damage recognition signal and attracts the other NER factors [170].

In prokaryotes the damage is removed in a 12-13nt-long oligomer [171], whereas in eukaryotes the damage is excised in a 27-29nt-long fragment due to precisely positioned cleavages 5nt on the 3' side of the photoproduct and 24nt on the 5' side [172, 173] (Fig. 1g). Excision in *E.coli* is accomplished by 3 proteins designated UvrA, UvrB and UvrC [174]. In contrast, in humans 16 polypeptides including 7 XP proteins, the trimeric replication protein A (RPA), proliferating cell nuclear antigen (PCNA) and the multi-subunit (7-10) general transcription factor TFIIH, which includes XPB and XPD, are required for the dual incisions [175]. The resulting gap is filled in by the combined

actions of DNA polymerase δ or ε, PCNA, single-strand binding protein (RPA) and DNA ligase [176]. Xeroderma pigmentosum (XP) is an hereditary disease clinically characterised by extreme sensitivity to sunlight, a predisposition to skin cancer and other dermatological changes in exposed areas, and progressive neurological abnormalities. The underlying cause of solar sensitivity is an inability to repair UV-induced DNA damage [88, 177]. XP can be caused by mutations in any one of the 7 genes, *XPA* to *XPG* [177] as these genes are essential for nucleotide excision repair, the primary enzyme system for removal of bulky DNA lesions such as those caused by exposure to UV in sunlight [88, 176, 178].

As base excision repair is considered to be the main pathway for the repair of oxidative base lesions *in vivo*, mouse models deficient in base excision repair proteins have been generated to study the effects of such knockouts. Homozygous *ogg1*$^{-/-}$ null mice have been shown to be viable, but accumulate abnormal levels of 8-oxoguanine in their genomes [179]. Despite this increase in potentially miscoding DNA lesions, OGG1-deficient mice exhibit only a moderately, but significantly, elevated spontaneous mutation rate in non-proliferative tissues. These mice do not develop malignancies, and show no marked pathological changes. It was further shown that extracts of *ogg1* null mouse tissues cannot excise the 8-oxoguanine lesion, but there is significant slow removal *in vivo* from proliferating cells. These findings suggest that in the absence of the DNA glycosylase an alternative repair pathway functions to minimise the effects of an increased load of 8-oxoguanine in the genome and maintain a low endogenous mutation frequency [179]. Similarly, homozygous mNth1 mutant mice showed no detectable

phenotypical abnormality and Tg produced in the mutant mouse DNA disappeared with time, though more slowly than in the wild-type mouse. At least two other DNA glycosylase activities against Tg were found in extracts from the mutant mouse [76]. In addition to redundancy between the substrate specificities of the DNA glycosylases, oxidative damage has also been shown to be a substrate for TC-NER [180]. Evidence suggests that removal of 8-oxoguanine in a wild-type mouse embryonic fibroblast cell line is faster in the transcribed strand (TS) than in the non-transcribed strand (NTS) indicating TC-NER of 8-oxoguanine in mouse cells [180]. Mice deficient in proteins that act at subsequent steps in the base excision repair pathway, such as APE1, Pol β and XRCC1, die during embryogenesis which suggests that these proteins play a critical role in development [141, 181, 182].

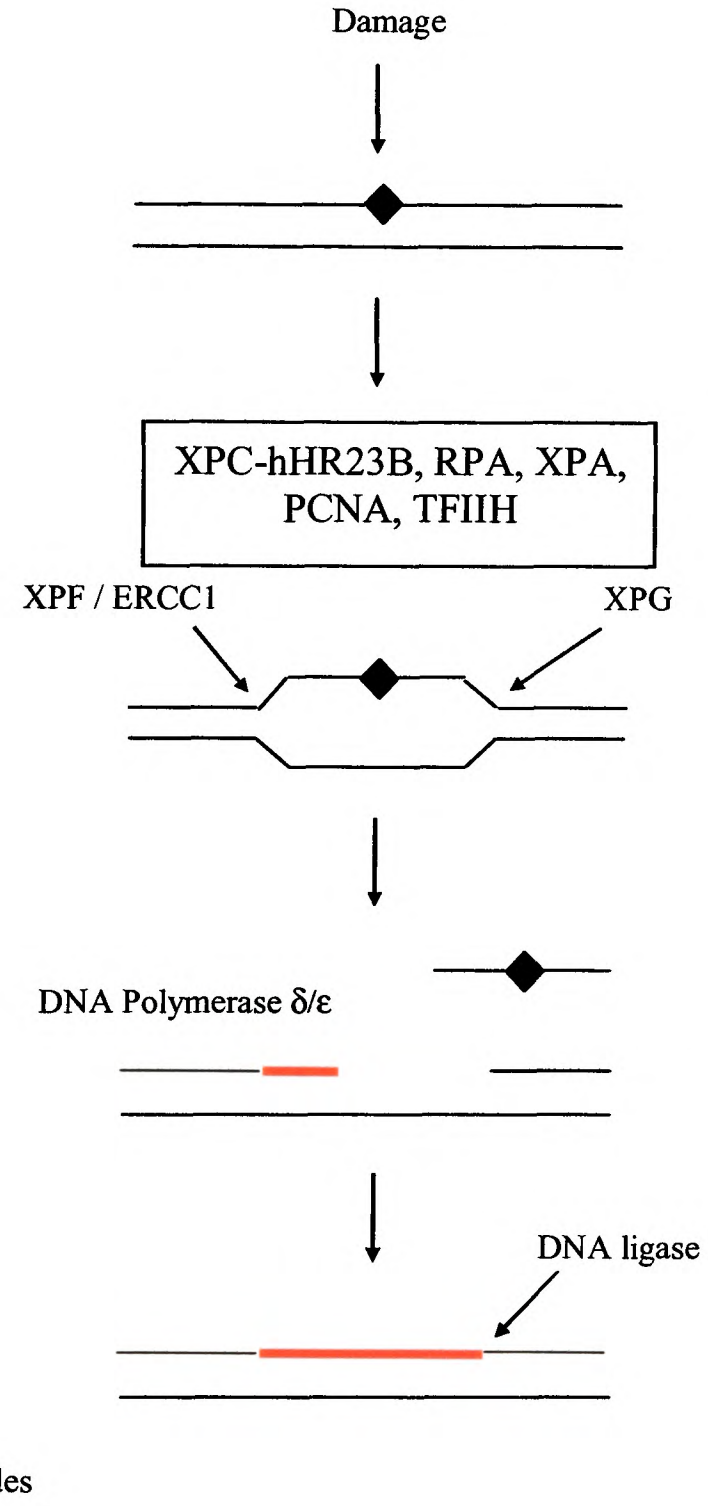


Fig. 1g Nucleotide excision repair pathway.

1.5 *Clustered DNA Lesions*

Despite the existence of DNA repair pathways, ionising radiation is still harmful to cells even in relatively low doses. It was therefore suggested that the distinction between ionising radiation and other DNA damaging agents lies in the types of DNA damage produced. In addition to producing "simple" individual lesions / adducts, ionising radiation and certain chemotherapeutic drugs, such as bleomycin [183, 184], have been shown to generate "complex" multiply damaged sites where several moieties in a local region of the DNA (approx. 10 bp / 1-2 helical turns) are damaged [183, 185-187]. Modelling of radiation track structures has provided strong evidence for radiation-induced damage clusters [188]. Energy from low linear energy transfer (LET) ionising radiation (eg. X-rays and γ-rays) is deposited in the water surrounding the DNA molecule such that between 2 and 5 radical pairs are generated within a radius of 1-4 nm [188]. As a result, multiple single lesions, including oxidised purine or pyrimidine bases, AP sites and single-strand breaks can be formed on one or both strands of the DNA from the same radiation energy deposition event. Indeed, it has been shown that approx. 15% of oxidised pyrimidines, oxidised purines and abasic sites are in damage clusters [189]. Such damage clusters are proposed to be of heightened biological significance due to the greater challenge they present repair systems.

In fact, it has been shown that single-damage lesions induced by OH radicals are not the reason for the high efficiency with which DNA damage induced by ionising radiation kills cells [5]. It is believed that the efficiency of ionising radiation in killing cells, while producing relatively low levels of DNA damage, lies in its ability to produce damage in

which more than one moiety in a localised region of the DNA is damaged [5, 184]. Singly damaged sites produced in cellular DNA by ionising radiation are readily and accurately repaired after biologically significant radiation doses. This might be expected since they are the same lesions as those produced by endogenous oxidation. Post-irradiation repair of isolated single base lesions is efficiently carried out by BER, with a strand break as an intermediate. Accordingly, it is most probable that individual lesions within a multiply damaged site, in particular the base lesions and single-strand breaks, are recognised by repair enzymes of the BER pathway. Simultaneous processing of opposing lesions may lead to the formation of double-strand breaks. In humans, double-strand breaks, in non-proliferating cells, can be repaired by non-homologous end-joining [88, 190, 191]. Although free double-strand breaks are repaired readily in mammalian cells, it is nevertheless desirable to avoid their formation to reduce the frequency of possible misjoining, which might result in translocation mutagenesis. Indeed, double-strand breaks have been shown to play a central role in the cytotoxicity induced by ionising radiation [5].

Therefore, the question remains as to how clustered lesions will be repaired. Individually such lesions are substrates for BER, but will the presence of vicinal lesions affect this process? Various studies have looked at the effect of opposing clustered lesions on the initial steps of base excision repair, due to the potential for formation of double-strand breaks [192, 193]. Studies have shown that bacterial base excision repair enzymes, such as Fpg and Endonucleases III and VIII, can efficiently remove one of two closely opposed base lesions and produce a nick close to the remaining lesion on the opposite

strand [194]. The removal of the remaining base lesion is thereby inhibited. This inhibition is thought to be important in reducing the possibility of generating free double-strand breaks by these repair enzymes. It has also been shown that the Ku 70/80 complex, a human double-stranded DNA binding protein, can bind to a nick opposite dihydrouracil (DHU) leading to a significant inhibition of the human Endonuclease III (hNTH) activity. This increased inhibition of NTH activity by Ku will help to reduce the possibility of generating double-strand breaks during the repair of closely opposed lesions. Furthermore, even if a double-strand break is formed, Ku is involved in non-homologous end-joining and can bind to the ends of a newly generated double-strand break and tether the two ends together as a protein-DNA complex, preventing the possibility of mis-joining [195]. Recently, the *E.coli* DNA-binding protein, HU, has also been shown to inhibit the activity of *E.coli* Endonuclease III when a nick is present in close proximity and opposite to a base lesion [196]. However, it has been shown that double-strand breaks may still be formed during the processing of some opposing lesions, including clustered 8-oxoguanine/abasic site lesions in certain orientations [197]. Interestingly, *E.coli* triple mutant cells (*fpg nth nei*) lacking the major DNA glycosylases, exhibit increased survival toward ionising radiation as compared with either the single mutants or the wild type cells [198]. The increased survival in the triple mutant could be due to a decrease in the ability of the mutant cell to process base lesions within a cluster, thus leading to a decrease in the formation of double-strand breaks. However, the increased survival might be accompanied by an increase in mutation frequency, possibly as a result of mutagenic bypass of the unrepaired base lesions.

1.6 *Biological Consequences of Clustered DNA Lesions*

Many damaged nucleic acids produced by reactive oxygen species, including DNA lesions and damaged DNA precursors, have mutagenic potentials. The damage in DNA, formed either directly or by the incorporation of a damaged DNA precursor, is removed by cellular DNA repair proteins. The repair efficiency of a DNA lesion is one of the determining factors of the mutation frequency in living cells.

Densely ionising radiations interact with DNA to cause heavily clustered sites of damage that are difficult to repair correctly. Repair of base lesions within a heavily clustered damage site is likely to occur relatively slowly and could lead to the formation of more double-strand breaks. As already noted, a delay in repair could result in an increase in the mutation frequency of the base lesion due to its persistence. Indeed, Malyarchuk et. al. recently showed that an 8-oxoguanine situated closely opposed to a second 8-oxoguanine increased the mutation frequency in bacteria by approx. 2-fold compared to that of a single 8-oxoguanine alone [199].

1.7 *Objectives*

DNA damage caused by reactive oxygen species and ionising radiation results in increased genomic instability and has been implicated in several diseases, such as cancer, cardiovascular disease, neurodegenerative disorders and also in aging [200, 201]. In particular, clustered DNA lesions induced by ionising radiation, which can include oxidised purines, pyrimidines, abasic sites or single-strand breaks on the same [202] or opposing DNA strands [189, 203], may be a specific problem for cellular repair systems.

However, little is known about the repairability of clustered lesions in DNA. The aims of this study were to elucidate the roles of different repair pathways in processing clustered DNA lesions.

Two major questions were addressed in this study;

1. Which repair system is involved in the processing of clustered DNA lesions formed on a single strand of DNA?
2. What is the effect of the complexity and the composition of lesions within a cluster on the efficiency and fidelity of repair processes?

To address these questions the base lesions 8-oxoguanine and thymine glycol were employed in various clusters, on both a single DNA strand and opposing strands, and their repair monitored. The effects of various lesion clusters on repair pathways were studied using synthetically generated damage-containing DNA substrates and purified repair proteins and cell extracts.

Chapter 2 Materials and Methods

2.1 *Materials*

Synthetic oligodeoxyribonucleotides, purified by high performance liquid chromatography, were obtained from MWG-Biotech and Sigma Genosys. All concentrations of DNA are given as molar concentration of molecules. [α-^{32}P]dATP and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Perkin-Elmer Life Science Products. Recombinant human Pol β was overexpressed and purified as described previously [204], human APE1 and uracil DNA glycosylase (UDG) with 84 amino acids deleted from the amino terminus were purified as described previously [205, 206] and FEN1 was purified as described previously [207]. DNA ligase I was a gift from A. Tomkinson, mouse OGG1 was a gift from D. Zharkov, Pol η was a gift from F. Hanaoka and Pol δ was a gift from V. Podust. A Bio Rad Molecular Imager FX Pro Plus phosphorimager was used for scanning and quantitation of gels along with Quantity One software.

2.2 *Gel Purification of Oligonucleotides*

Oligonucleotides were purified on a polyacrylamide gel (containing 20% acrylamide in 1x TBE (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0) in 1x TBE. 100 μg of oligonucleotide and 100 μl of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromphenol blue and 0.02% xylene cyanole) was loaded onto a pre-warmed gel and run at 15 W until the dye reached the bottom, approx. 1.5 hrs. Strips of gel from either side of the band were stained in TBE containing ~1 μg/ml of EtBr (ethidium

bromide) for 10-15 mins. The bands of DNA were located under exposure to UV and the strips re-aligned with the gel to determine the position of the DNA. Gel containing the DNA was cut out and incubated at 37°C overnight in water. DNA-containing solution was recovered through an 0.2 μm Spin-X microcentrifuge filter (Sigma), and concentrated in a Centricon-10 ultrafiltration unit (Millipore, Bedford MA) with the buffer exchanged to TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration was measured using a UV spectrophotometer (Beckman) at 260 nm. One optical unit of oligonucleotide solution, in a 1 cm cuvette, was estimated as 33 μg of DNA per ml.

2.3 *Radiolabelling of DNA*

Oligonucleotides were labelled at the 5'-end with T4 polynucleotide kinase and [γ-^{32}P]ATP; 1 μg of DNA was incubated with 20 μCi (6 pmol) (unless otherwise stated) of [γ-^{32}P]ATP and 10 units of T4 polynucleotide kinase (Boehringer) for 1 hr. at 37°C in a buffer supplied by the manufacturer. Unincorporated labelled nucleotides were removed on a Sephadex G-25 spin column (Amersham). 3'-end labelling of oligonucleotide duplexes with TA protruding ends was performed using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP; 400 ng (~40 pmol) oligonucleotide duplex was incubated with 20 μCi of [α-^{32}P]dATP, 0.4 mM TTP and 2 units of the Klenow fragment of DNA polymerase I (Boehringer) in BER buffer (40 mM Hepes-KOH (pH 7.8), 5 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM EDTA) at 37°C for 30 mins. Unincorporated labelled nucleotides were removed on a Sephadex G-25 spin column.

2.4 *Preparation of Closed-circular Tandem Lesion Plasmid Substrate*

Oligonucleotide

5' GATCCCCATATACCGCGGCCGGCCGATCAAGCTTATTGGG 3' (1 μg, 30 pmol) (where G is 8-oxoguanine) was 5'-end labelled with 100 μCi (33 pmol) of [γ-^{32}P]ATP and used for construction of a substrate containing two 8-oxoguanine lesions in circular closed double-stranded DNA. Substrate was constructed by priming 30 μg of single-stranded M13Insert DNA with a 2.5-fold molar excess of the phosphorylated oligonucleotide. The 250 μl reaction mixture containing 50 mM Tris-HCl (pH 7.9), 15 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.02 mg/ml bovine serum albumin (BSA), 100 μM ATP, 500 μM dATP, dCTP, dGTP and TTP, 40 units of T4 DNA polymerase, 35 units of T4 DNA ligase and 100 μg of T4 gene 32 protein (all enzymes from Roche) was incubated for 4 hrs. at 37°C. Closed circular DNA was isolated by CsCl/EtBr density gradient centrifugation; DNA-containing sample was mixed with 8.33 g of CsCl and 560 μl of EtBr (10 mg/ml). The mixture was centrifuged at 55K rpm for 16 hrs. at 20°C, in a Vti-65 rotor in an Optima L-100 XP Ultracentrifuge (Beckman Coulter). The gradient was fractionated and the DNA-containing fractions pooled. The DNA was extracted with butanol and desalted and concentrated by centrifugation in a Centricon-10 ultrafiltration unit (Millipore, Bedford MA). DNA substrates were stored at -20°C in TE.

2.5 ***Cells and Extracts***

HeLa cells, 5 g, were purchased from Paragon, Baltimore, USA. Mouse embryonic fibroblasts derived from normal and PARP-1 knockout mice were kindly provided by Dr. G. de Murcia. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and 2 mM glutamine. Whole-cell extracts were prepared by the method of Tanaka; plates were washed twice with 5 ml of 2x phosphate-buffered saline (PBS) (without calcium and magnesium). Cells were then scraped off the plates in 10 ml of cold PBS and the plates washed with a further 5 ml of PBS. Cells were spun down at 1300 rpm, at 4°C for 25 mins in an Eppendorf centrifuge 5804R. The liquid was decanted off and the pellet resuspended in 10 ml of cold PBS. The cells were spun down again at 1300 rpm, at 4°C for a further 15 mins. Cells were then resuspended in 1 ml of PBS, transferred to an Eppendorf tube and spun down at 1500 rpm for 5 mins. A packed cell volume (PCV) was measured and the cells resuspended in 1 PCV of buffer (10 mM Tris-HCl, pH 7.8 and 200 mM KCl, with 1 mM of DTT, 1μg/ml of each of chymostatin, pepstatin and leupeptin and 1 mM phenylmethylsulfonylfluoride (PMSF) added). 2 PCV of buffer (10 mM Tris-HCl, pH 7.8, 600 mM KCl, 2 mM EDTA, 40% glycerol and 0.2% Nonidet P-40) was added and the cell suspension rocked at 4°C for 2 hrs. and then centrifuged at 40,000 rpm for 20 mins. The supernatant was recovered and dialysed overnight against buffer containing 25 mM Hepes-KOH, pH 7.9, 2 mM DTT, 12 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol and 0.1 M KCl. Extracts were aliquoted and stored at –80°C.

2.6 *Repair of the Closed-circular DNA Substrate*

Standard 50 µl repair reactions contained 100 ng of ^{32}P-labelled 8-oxoguanine-containing DNA (~0.5 pmol), BER buffer, 4 mM ATP, 50 mM phosphocreatine (di-Tris salt, Sigma), 2.5 µg creatine phosphokinase (type I, Sigma), 0.25 mM NAD^+, 8.5% glycerol (Fluka), 10 µg BSA, 40 µM dGTP, dCTP, dATP and TTP, 400 ng pUC19 carrier DNA and 100 µg cell extract. Reactions were carried out for the indicated times at 37°C. Reactions were stopped by the addition of 2 µl EDTA (0.5 M), 2 µl SDS (10%) and 2 µl Proteinase K (5 mg/ml, BDH) and incubated for a further 30 mins. at 37°C. After the reaction, plasmid DNA was purified from the reaction mixture by phenol-chloroform extraction; addition of an equal volume of phenol-chloroform isoamyl alcohol (25:24:1, Sigma) and centrifugation for 2 mins. at 16,000 g in an Eppendorf centrifuge 5415D. 50 µl of the aqueous phase was collected and spun through G-25 columns equilibrated with TE. Samples were evaporated in a Speedvac at 45°C (Eppendorf). The pellet was dissolved in water and treated with 20 units of the indicated restriction endonuclease for 1 hr. at 37°C in a buffer supplied by the manufacturer. The reaction was stopped by addition of 10 µl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel (containing 10% acrylamide and 8 M urea in 1x TBE) in 1x TBE. The amount of radioactivity was quantified on a phosphorimager.

2.7 ***Effect of Vicinal Base Lesions on Incorporation of Nucleotides into a Repair Gap by DNA Polymerase β***

Preparation of DNA substrates:

Oligonucleotide

5' GATCCCCATATACCGCGGCCUGCCGATCAAGCTTATTGGG 3' (1 μg, 81 pmol) (where G is 8-oxoguanine and U is uracil) was 5'-end labelled with 20 μCi (6 pmol) of [γ-^{32}P]ATP. Duplex oligonucleotide substrate was prepared by mixing equimolar solution of 5'-labelled oligonucleotide and complementary oligonucleotide in TE-100 mM KCl and incubation at 90°C for 5 mins., followed by slow cooling to 25°C. Reactions were reconstituted in a reaction mixture (10 μl) that contained BER buffer, 2 mM ATP, 2 mg/ml BSA, 20 μM each of dATP, dGTP, dCTP and TTP, and a ^{32}P-labelled oligonucleotide substrate (5-10 ng, 0.5-1 pmol). Where indicated, substrates (10 pmol) were pre-incubated with UDG (9.4 pmol) and APE1 (5.7 pmol) in BER buffer at 37°C for 20 mins. Alternatively, reactions were initiated by addition of UDG (~625 fmol), APE1 (~300 fmol), Pol β (~25 fmol, or amount indicated) and PARP-1 (~800 fmol, or amount indicated) and NAD^+ (0.2 mM, if indicated). After incubation for 20 mins., or the indicated times, at 37°C, reactions were stopped by addition of 10 μl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel (containing 20% acrylamide and 8 M urea in 1x TBE) in 1x TBE.

2.8 ***Excision of 8-oxoguanine, by OGG1, in the Presence of a Vicinal Strand Break***

Preparation of DNA substrates:

Oligonucleotide 5' ATATACCGCGGCCGGCCGATCAAGCTTATT 3' or

5' GATCCCCATATACCGCGGCCUGCCGATCAAGCTTATTGGG 3' or

5' GATCCCCATATACCGCGUCCGGCCGATCAAGCTTATTGGG 3'

(1 μg, 81 pmol) (where G is 8-oxoguanine and U is uracil) was annealed with complementary oligonucleotide by mixing equimolar solution of both oligonucleotides in TE-100 mM KCl and incubation at 90°C for 5 mins., followed by slow cooling to 25°C. Substrates were labelled at the 5'- (1 and 2 above) or 3'-end (3 above) with 20 μCi (6 pmol) of [γ-^{32}P]ATP or [α-^{32}P]dATP. Single-strand break-containing substrate was prepared by incubation of the uracil-containing substrates (10 pmol) with UDG (9.4 pmol) and APE1 (5.7 pmol) in BER buffer at 37°C for 20 mins.

The reaction was reconstituted in a reaction mixture (10 μl) that contained BER buffer, a ^{32}P-labelled oligonucleotide substrate (5-10 ng, 0.5-1 pmol), OGG1 (128 fmol) and APE1 (570 fmol) and was incubated for the indicated time. After incubation the reaction was stopped by addition of 10 μl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.9 *OGG1 Electrophoretic Mobility Shift Assay*

Substrates were prepared as for section 2.8. The binding reaction was performed in a reaction mixture (10 µl) that contained 25 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 300 µg/ml BSA, 12% glycerol, 0.1% Triton X-100, 3 mM $MgCl_2$, a ^{32}P-labelled oligonucleotide substrate (~8 fmol) and the indicated amount of OGG1. The reaction was incubated on ice for 30 mins. After incubation the reaction was stopped by addition of 2.5 µl of gel loading buffer (30% glycerol, 0.25% xylene cyanol and 0.25% bromphenol blue). The reaction products were separated by electrophoresis in a 12% non-denaturing polyacrylamide gel (containing 12% acrylamide in 0.5x TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, and 1 mM EDTA, pH 8.0) in 0.5x TBE at 5 W at 4°C.

2.10 *Osmium Tetroxide Modification of Thymine to Thymine Glycol*

The 5,6 double bond of thymidine is a target for oxidation by osmium tetroxide, forming a cyclic osmate ester of the *cis*-5,6-dihydro-5,6-glycol (thymine glycol) [208]. Oxidation of the 5,6 double bond of thymidine generates two chiral centres at C5 and C6. The cis-5R,6S form of thymine glycol is generated as the predominant product along with the other diastereomer, the cis-5S,6R form [208]. A reaction mixture (100 µl) containing the thymine-containing oligonucleotide (50 µg), 30 mM OsO_4 and 2 µl of pyridine was incubated at 37°C for 30 mins. and then spun through a Sephadex G-25 spin column. The presence of thymine glycol was confirmed by sensitivity of the modified oligonucleotide to piperidine and by cleavage of the thymine glycol-containing oligonucleotide duplex with the NTH protein.

2.11 ***FEN1 Endonuclease Activity on Modified Flap Strands***

Preparation of DNA substrates:

Oligonucleotide

5' GATCCCCATATACCGCGGCCGGCCGATCAAGCTTATTGGG 3' or

5' GGTTGGAGGAGGAAGAAGAAAGGAAA 3' (1 μg, 81 pmol or 122 pmol) (where G is 8-oxoguanine and T is thymine glycol) was 5'-end labelled with 20 μCi (6 pmol) of [γ-^{32}P]-ATP and used for construction of a substrate containing 3 annealed oligonucleotides to create a 5' flap. Oligonucleotides were annealed by mixing equimolar solution of all oligonucleotides in TE-100 mM KCl and incubation at 90°C for 5 mins., followed by slow cooling to 25°C.

The reaction was reconstituted in a reaction mixture (10 μl) that contained 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 100 μg/ml BSA, and a ^{32}P-labelled oligonucleotide triplex substrate (5-10 ng, 0.5-1 pmol). The reaction was initiated by addition of FEN1 at the amount indicated in the figure legends. After incubation for the indicated time at 37°C the reaction was stopped by addition of 10 μl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.12 ***Preparation of DNA Substrates for BER in the Presence of Opposing 8-oxoguanine Lesions***

Oligonucleotide 5' AATAAGCTTGATCGGCCGGUCGCGGTATAT 3' or 5' AATAAGCTTGATCGGCCGUCCGCGGTATAT 3' (1 μg, 109 pmol) (where U is uracil) was 5'-end labelled with 20 μCi (6 pmol) of [γ-^{32}P]ATP. Duplex oligonucleotide substrate was prepared by mixing equimolar solution of 5'-labelled oligonucleotide and complementary oligonucleotide, containing guanine or 8-oxoguanine at the indicated position (either directly opposite to or one nucleotide downstream of the uracil residue), in TE-100 mM KCl and incubation at 90°C for 5 mins., followed by slow cooling to 25° C. Prior to assembly of the BER reaction, the DNA substrate (500 ng, 50 pmol) was pretreated with UDG (200 ng, 6.25 pmol) in UDG buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, and 70 mM KCl) to generate an abasic site. The reaction mixture was incubated at 37°C for 1 hr. Due to the instability of the AP-containing DNA, the substrates were prepared just before performing the reactions. When indicated, the resulting AP site was reduced by addition of sodium borohydride to 0.1 M and, after incubation on ice for 10 mins. the reaction buffer was exchanged to TE by filtering through a Sephadex G-25 spin column. To generate substrate containing pre-incised AP sites, the AP-containing substrate was pretreated with APE1 (128 fmol) in BER buffer for 10 mins. at 37°C.

2.13 *DNA Polymerase Assays*

The reactions (10 µl) contained BER buffer, 2 mM ATP, 2 mg/ml BSA, 20 µM each of dATP, dGTP, dCTP and TTP, and a ^{32}P-labelled oligonucleotide substrate (5-10 ng, 0.5-1 pmol). The reactions were initiated by addition of Pol β/Pol δ/Pol η at the amount indicated in the figure legends. Reactions with Pol δ also included proliferating cell nuclear antigen (PCNA) (860 fmol). After incubation for the indicated time at 37°C (20 mins. if not indicated) the reaction was stopped by addition of 10 µl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.14 *Incorporation Opposite to 8-oxoguanine by DNA Polymerase β*

Preparation of DNA substrates:

Oligonucleotide 5' AATAAGCTTGATCGGCCGGUCGCGGTATAT 3' (1 µg, 81 pmol) (where U is uracil) was annealed to its complementary strand containing guanine or 8-oxoguanine directly opposite to the uracil residue. The equimolar solution of both oligonucleotides in TE-100 mM KCl was incubated at 90°C for 5 mins., and the solution was allowed to cool slowly to 25° C. Prior to assembly of the incorporation reaction, the duplex oligonucleotide substrate (100 ng, 10 pmol) was pretreated with UDG (200 ng, 6.25 pmol) in UDG buffer to generate an AP site. The reaction mixture was incubated at 37°C for 1 hr.

The reaction was reconstituted in a reaction mixture (10 µl) that contained BER buffer, 2 mM ATP, 2 mg/ml BSA, 20 µM of labelled dATP/unlabelled dCTP mix or labelled dCTP/unlabelled dATP mix and the unlabelled duplex oligonucleotide substrate (5-10 ng, 0.5-1 pmol). The reaction was initiated by addition of Pol β at the amount indicated in the figure legends. After incubation for 20 mins. at 37°C the reaction was stopped by addition of 10 µl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.15 ***Reconstituted BER Reaction in the Presence of an Opposing 8-oxoguanine Lesion***

Substrates were prepared as for the DNA polymerase assays. The BER reaction was reconstituted in a reaction mixture (10 µl) that contained BER buffer, 2 mM ATP, 20 µM each of the indicated dNTPs, 2 mg/ml BSA and ^{32}P-labelled oligonucleotide substrate (10 ng, 1 pmol). The reactions were initiated by addition of purified APE1 (60 fmol), Pol β (70 fmol), and DNA ligase I (80 fmol). After incubation at 37°C for the times indicated in the figure legends, the reactions were stopped by addition of 10 µl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.16 ***DNA Repair Synthesis and FEN1 Cleavage Reaction***

Preparation of DNA substrates:

Oligonucleotide 5' AATAAGCTTGATCGGCCGUCCGCGGTAT 3' (1 μg, 116 pmol) (where U is uracil) was annealed to the complementary 30-mer containing two extra nucleotides (TA) at the 5'-end and labelled by filling protruding ends using *E. coli* DNA polymerase Klenow fragment in the presence of [α-^{32}P]dATP (6 pmol) and cold TTP (0.4 mM). Prior to the assembly of the reaction, the oligonucleotide substrate (100 ng, 10 pmol) was pre-treated with UDG (200 ng, 6.25 pmol) in UDG buffer and incubated at 37°C for 1 hr. The resulting AP site was reduced by the addition of sodium borohydride to 0.1 M and, after incubation on ice for 10 mins., the reaction buffer was exchanged to TE by filtration through a Sephadex G-25 spin column.

The reaction was reconstituted in a reaction mixture (10 μl) that contained BER buffer, 2 mM ATP, 2 mg/ml BSA, 20 μM each of dATP, dGTP, dCTP and TTP, and a [^{32}P] 3'-labelled oligonucleotide substrate (5-10 ng, 0.5-1 pmol). The reaction was initiated by addition of purified APE1 (100 fmol), Pol β (25 fmol) and FEN1 (60 fmol) at the amount indicated in the figure legends. After incubation for the indicated time at 37°C the reaction was stopped by addition of 10 μl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.17 ***Preparation of DNA Substrates for BER in the Presence of Opposing Thymine Glycol Lesions***

Oligonucleotide

5' TTCTCTCCTTCCCTTCCTCTCTUCCCTTTCTCCTCTCCTCTCTTC 3' (1 μg, 75 pmol) (where U is uracil) was 5'-end labelled with 20 μCi (6 pmol) of [γ-^{32}P]ATP. To prepare the oligonucleotide duplex, labelled oligonucleotide containing a uracil residue was annealed to its complementary strand containing thymine or thymine glycol directly opposite to the uracil residue. The equimolar solution of both oligonucleotides in TE-100 mM KCl was incubated at 90°C for 5 mins., and the solution was allowed to cool slowly to 25° C. Prior to assembly of the excision reaction, the DNA substrates (500 ng, 50 pmol) were pretreated with UDG (200 ng, 6.25 pmol) in UDG buffer. The reaction mixture was incubated at 37°C for 1 hr. To generate substrate containing pre-incised AP sites, the AP-containing substrate (1 pmol) was pretreated with APE1 (0.3 pmol) in BER buffer for 10 mins. at 37°C.

2.18 ***DNA Polymerase β Synthesis Opposite to Thymine Glycol***

The reaction was carried out in a reaction mixture (10 μl) that contained BER buffer, 2 mM ATP, 2 mg/ml BSA, 20 μM each of dATP, dGTP, dCTP and TTP, and a [^{32}P]-labelled oligonucleotide substrate (5-10 ng, 0.5-1 pmol). The reaction was initiated by addition of Pol β, at the amount indicated in the figure legends. After incubation for the indicated time at 37°C the reaction was stopped by addition of 10 μl of gel loading buffer.

Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.19 *dRP Lyase Activity of DNA Polymerase β in the Presence of an Opposing Thymine Glycol Lesion*

Preparation of DNA substrates:

5' TTCTCTCCTTCCCTTCCTCTCTUCCCTTTCTCCTCTCCTCTCTTC 3' (1 μg, 75 pmol) (where U is uracil) was annealed to the complementary oligonucleotide (containing thymine or thymine glycol opposite to the uracil) containing two extra nucleotides (GA) at the 5'-end and labelled by filling protruding ends using *E. coli* DNA polymerase Klenow fragment in the presence of [α-^{32}P]dCTP (6 pmol) and cold TTP (0.4 mM). This was measured under the same conditions as described for the DNA synthesis reaction. The indicated amounts of Pol β were added to reaction mixtures containing 1 pmol of 3'-end labelled substrate duplex with preincised AP site and, after incubation for 20 mins. at 37°C, abasic sites were reduced by addition of 2 μl of 0.5 M $NaBH_4$ and incubation on ice for 10 mins. Reactions were stopped by addition of 10 μl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.20 ***DNA Ligase III Activity in the Presence of an Opposing Thymine Glycol***

Preparation of DNA substrates:

Oligonucleotide 5' TTCTCTCCTTCCCTTCCTCTCTA 3' (1 μg, 147 pmol) was 5'-end labelled with 20 μCi (6 pmol) of [γ-^{32}P]ATP. The labelled oligonucleotide was used for construction of a substrate containing 3 annealed oligonucleotides to create a single-strand break opposite to thymine or thymine glycol. Oligonucleotides were annealed by mixing equimolar solution of all oligonucleotides in TE-100 mM KCl and incubation at 90°C for 5 mins., followed by slow cooling to 25°C.

The indicated amounts of DNA ligase III were added to reaction mixtures containing 1 pmol of substrate duplex under the same conditions as described for the DNA synthesis reaction. Reactions were incubated for 10 mins. at 37°C. Reactions were stopped by addition of 10 μl of gel loading buffer. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.21 ***BER in the Presence of an Opposing Thymine Glycol with Whole Cell Extract***

The reactions were carried out in a reaction mixture (10 μl) that contained BER buffer, 2 mM ATP, 0.4 mg/ml BSA, 20 μM each of the indicated dNTPs, and ^{32}P-labelled oligonucleotide substrate, as before (1 pmol). The reactions were initiated by addition of whole cell extract (5 μg) and incubated for the indicated time at 37°C. Reactions were stopped by addition of 10 μl of 0.2 M EDTA and 20 μl of chloroform-isoamyl alcohol

(24:1) mixture. After centrifugation for 2 mins. at 16,000 g in an Eppendorf centrifuge 5415D, 10 µl of the aqueous phase was collected and 10 µl of gel loading buffer was added. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel in 1x TBE.

2.22 *Protein Purification of DNA Polymerase β*

Purification of hexahistidine fusion protein of the wild type polymerase β was performed as follows: All buffers used in the purification procedures contained the following protease inhibitors: 1µg/ml Apotinin and 0.1µg/ml each of Pepstatin A, Chymotrypsin, Antipain and Leupeptin (Sigma). Vectors were transformed into $CaCl_2$ competent *E. coli* λDE3 lysogen strain BL21 (DE3) (Novagen) and late log phase cultures (cultures were grown at 37°C to optical density (at 600 nm) of 0.6) (200 ml) were induced to produce polymerase β with 2mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 hrs. at 30°C. Cells were harvested and resuspended in 4 ml buffer A (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole), lysed by sonication and then centrifuged at 5000 rpm at 4°C for 20 mins. in a Beckman Avanti-J25 centrifuge to remove debris. The cleared lysate was then added to 0.5 ml of histidine binding resin, Ni-NTA (Qiagen, Valencia CA), pre-equilibrated with buffer A and mixed gently for 1 hr. at 4°C. The mixture was loaded on a gravity flow column and the resin was washed with buffer B (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole), protein was eluted with buffer C (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 250 mM imidazole) and 0.5 ml fractions were collected.

Fractions were dialysed against buffer containing 50 mM Hepes-KOH (pH 7.8), 1 mM EDTA, 50 mM KCl and 10% glycerol and stored at -80°C. Final protein concentrations were determined using the Bradford assay; in which the amount of protein present in the sample is determined using a simple colorimetric reaction and the results compared with those obtained from standard amounts of protein. Samples were run on a 10% Tris-glycine gel (Invitrogen) in 10x TGS (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) running buffer and analysed for purity.

All experiments were repeated at least 3-5 times and representative gels are shown.

Lists of all oligonucleotide sequences and buffers used are included in the appendix.

Chapter 3

Repair of Tandem Lesions

3.1 *Introduction*

Clustered lesions in DNA may have several degrees of complexity and have been predicted to be problematic for the cell's repair machinery [5, 184]. Tandem base damage, in which two or more lesions are formed within a few bases on the same strand of DNA, has been shown to be readily formed by ionising radiation [209-214] and also by photosensitisation reactions [215]. Clustered lesions on a single-strand of DNA could be substrates for either base- or nucleotide excision repair pathways. Distortion of the DNA helix may be recognised as a substrate for NER proteins and the damage removed as part of an oligomer. However, each lesion within a cluster is likely to be a substrate for BER. Short-patch BER may, however, be affected by the presence of vicinal lesions on the same strand as this could affect access of BER enzymes to each lesion or could affect recognition of the lesions.

To address the question of how tandem lesions are repaired, and the role of BER and NER in processing of such lesions, a closed-circular DNA substrate, containing two 8-oxoguanine residues at defined sites, was constructed (Fig 3a) and repair of the two lesions by whole cell extracts was monitored. The extracts used had previously been shown to be NER proficient (G. Dianov, as in [216]). NER in mammalian cells results in the excision of DNA fragments, of 27- 29 nucleotides, containing the damage and resynthesis of the DNA. Due to the position of the two 8-oxoguanine lesions and the ^{32}P

label, such fragments would be visible on an electrophoresis gel after repair. As no such fragments were observed on the gels in the repair assays, it was therefore assumed that nucleotide excision repair pathways were not responsible for repair of these tandem lesions. It was found that each lesion is repaired independently by base excision repair enzymes, however, we observed an asymmetric effect on the rate of repair of each lesion. It was shown that a lesion situated 3' to another lesion on the same DNA strand exhibits a delay in repair after removal of the base and incision of the abasic site. A lesion situated 5' to another lesion on the same strand showed no such delay and was repaired efficiently and at the same rate as a single 8-oxoguanine. We speculate that in such cases one lesion may persist as a single-strand break and could go through to replication. Such complex lesions, therefore, may be responsible for some of the harmful effects of ionising radiation.

3.2 ***Results***

3.2.1 ***Substrate Characterisation***

The substrate DNA was designed such that the two 8-oxoguanine residues and an upstream ^{32}P label were located within a 59-mer *Hin*d III restriction fragment (Fig 3a). The two 8-oxoguanine residues were positioned within two *Hae* III recognition sequences, effectively blocking *Hae* III cleavage at these sites until the lesions have been repaired. However, it should be noted that repair and subsequent *Hae* III cleavage at the lesion situated closest to the label would prevent monitoring of repair at the second downstream lesion. The 8-oxoguanine lesion furthest upstream (which will be referred to as lesion 1 from hereon in) was also positioned within a *Btg* I recognition sequence. The

downstream lesion (which will be referred to as lesion 2 from hereon in) was also positioned within a *Hpa* II recognition sequence. These individual restriction endonuclease recognition sites were intended to allow monitoring of the repair of each lesion individually.

The substrate was carefully characterised prior to use in experiments: treatment of the substrate with *Hin*d III generated a 59-mer labelled fragment alone. Treatment of unrepaired substrate with *Hin*d III and the other restriction endonucleases resulted predominantly in a 59-mer labelled fragment only, indicating that the 8-oxoguanine lesions prevented cleavage at these sites, although some cleavage by *Btg* I (6.1%) and *Hpa* II (24.9%) was observed suggesting that these restriction endonucleases are not completely inhibited by the presence of an 8-oxoguanine in the restriction site (Fig. 3b). As a result of this we were not able to use *Hpa* II to monitor repair of lesion 2. The presence of lesion 1 was verified by digestion of the repaired substrate, after incubation with whole cell extracts, with *Hin*d III and *Btg* I which generated a 43-mer fragment (Fig. 3j). The presence of the two 8-oxoguanine residues was also verified by double digestion of the repaired substrate with both *Hin*d III and *Hae* III generating both 48-mer and 52-mer fragments (Fig. 3e).

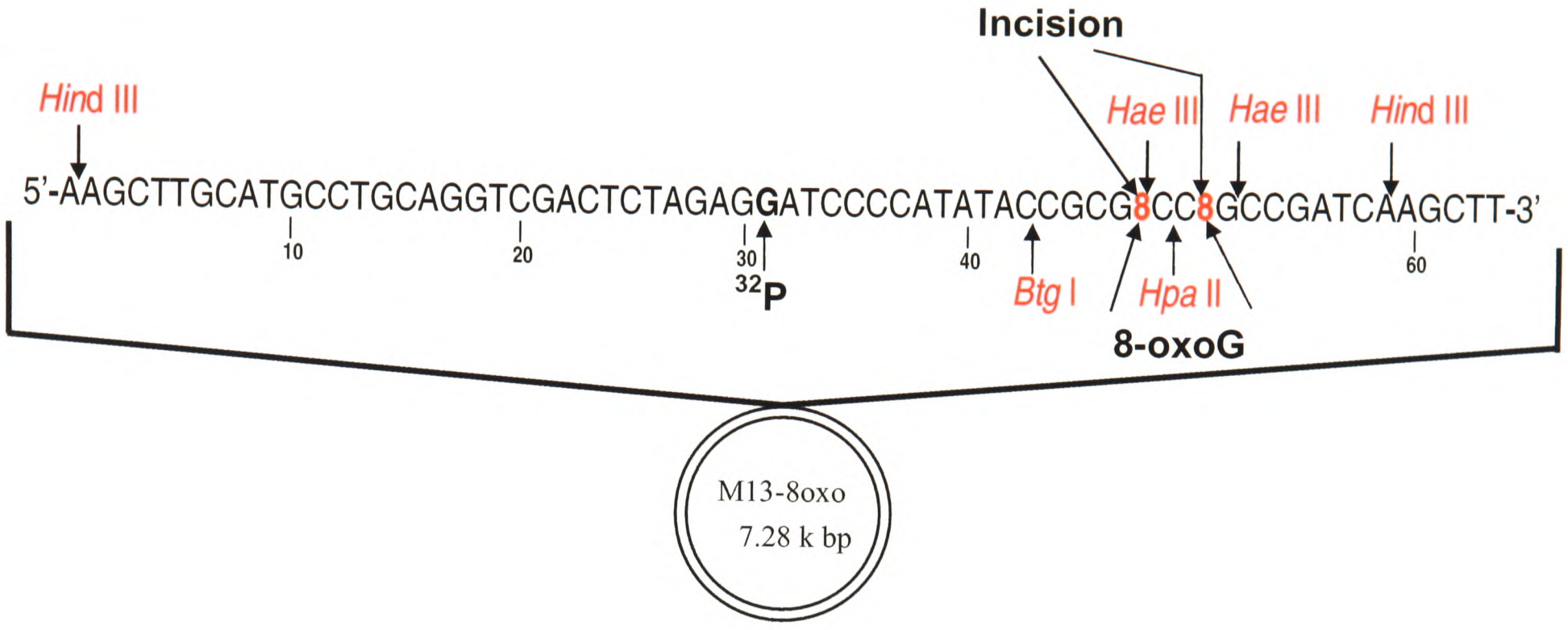


Fig. 3a Closed-circular DNA substrate. Schematic representation of the closed-circular M13 DNA substrate containing the sequence of interest. Two 8-oxoguanine residues separated by two nucleotides are situated downstream from a ^{32}P label within a *Hin*d III restriction fragment. Each 8-oxoguanine residue is positioned within a *Hae* III restriction site effectively blocking cleavage by *Hae* III at these sites until the lesions have been repaired. Due to the location of the ^{32}P label repair of the lesion closest to the label and subsequent cleavage by *Hae* III prevents analysis of the downstream lesion. The lesion closest to the label, which will be referred to as lesion 1, is positioned within a *Btg* I site and prevents cleavage by *Btg* I until the lesion has been repaired. The lesion downstream of this, which will be referred to as lesion 2, is positioned within a *Hpa* II site and partially prevents cleavage by *Hpa* II until the lesion has been repaired.

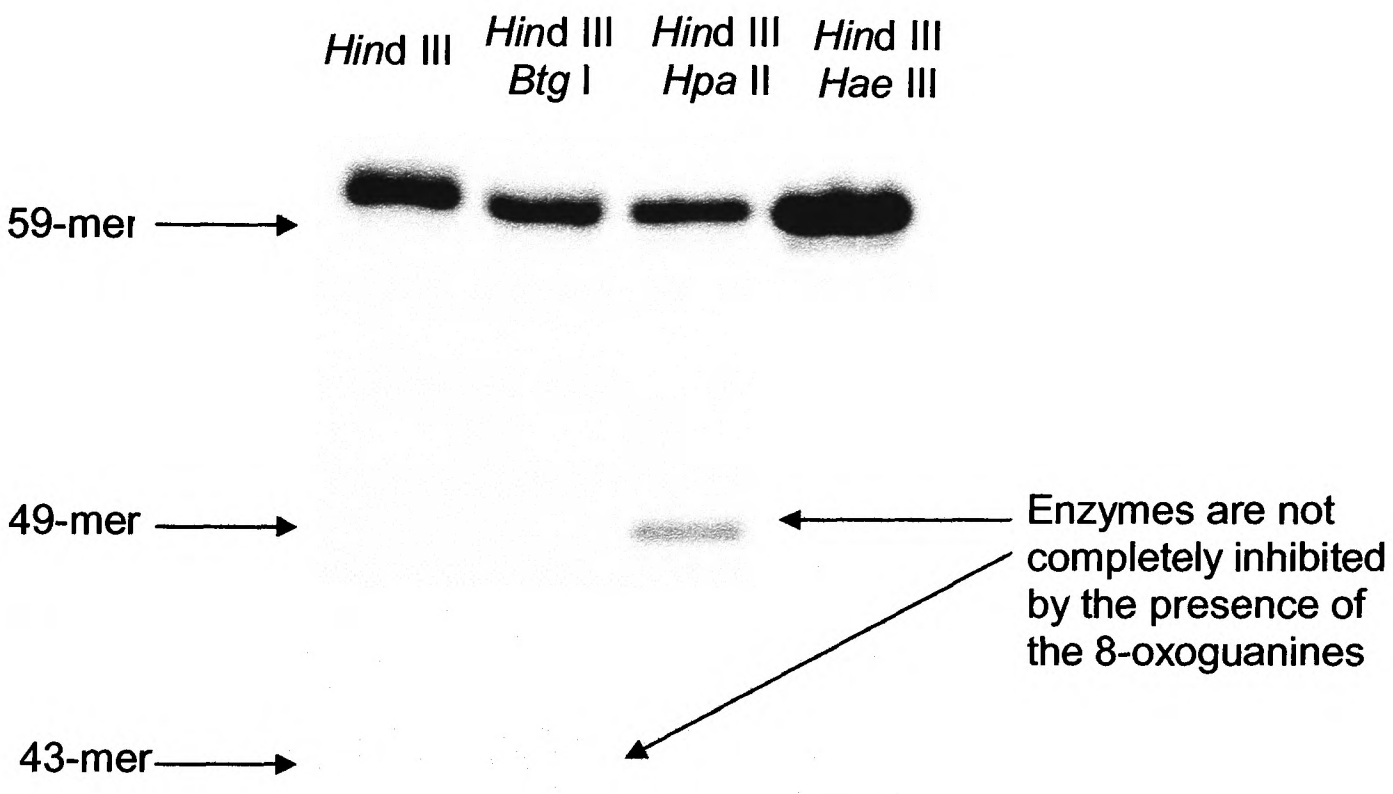


Fig. 3b Characterisation of the substrate. Untreated substrate was incubated with the indicated restriction endonucleases for 1 hr. at 37°C. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

3.2.2 *Nucleotide Excision Repair is not Responsible for Repair of Tandem 8-oxoguanine Lesions*

Nucleotide excision repair has been shown to be able to act on base lesions, including 8-oxoguanine, which in general do not cause distortion to the DNA double helix [88, 160]. The basis for this recognition is unclear, as is the coordination of the two repair pathways, NER and BER, in the repair of such base lesions. As previously noted, base excision repair is considered to be the main pathway involved in the repair of such lesions, however, NER may act as a back-up system [99, 161]. It was therefore interesting to test whether tandem 8-oxoguanine lesions in DNA would act as substrates for nucleotide excision repair enzymes. To investigate the role of nucleotide excision repair in the processing of tandem 8-oxoguanine residues, the closed circular DNA substrate was incubated with whole cell extract for the indicated time, and then subjected to restriction analysis. In mammalian cells nucleotide excision repair excises the damaged DNA in a 27-29nt-long fragment due to precisely positioned cleavages 5nt on the 3' side of the lesion and 24nt on the 5' side [172, 173]. Therefore, recognition of either single 8-oxoguanine or both lesions would result in excision of a fragment containing both 8-oxoguanine residues and the ^{32}P label (Fig. 3c). No such fragments were observed after incubation of the substrate with whole cell extract (Fig. 3d). It was therefore concluded that nucleotide excision repair is not involved in repair of tandem 8-oxoguanine lesions.

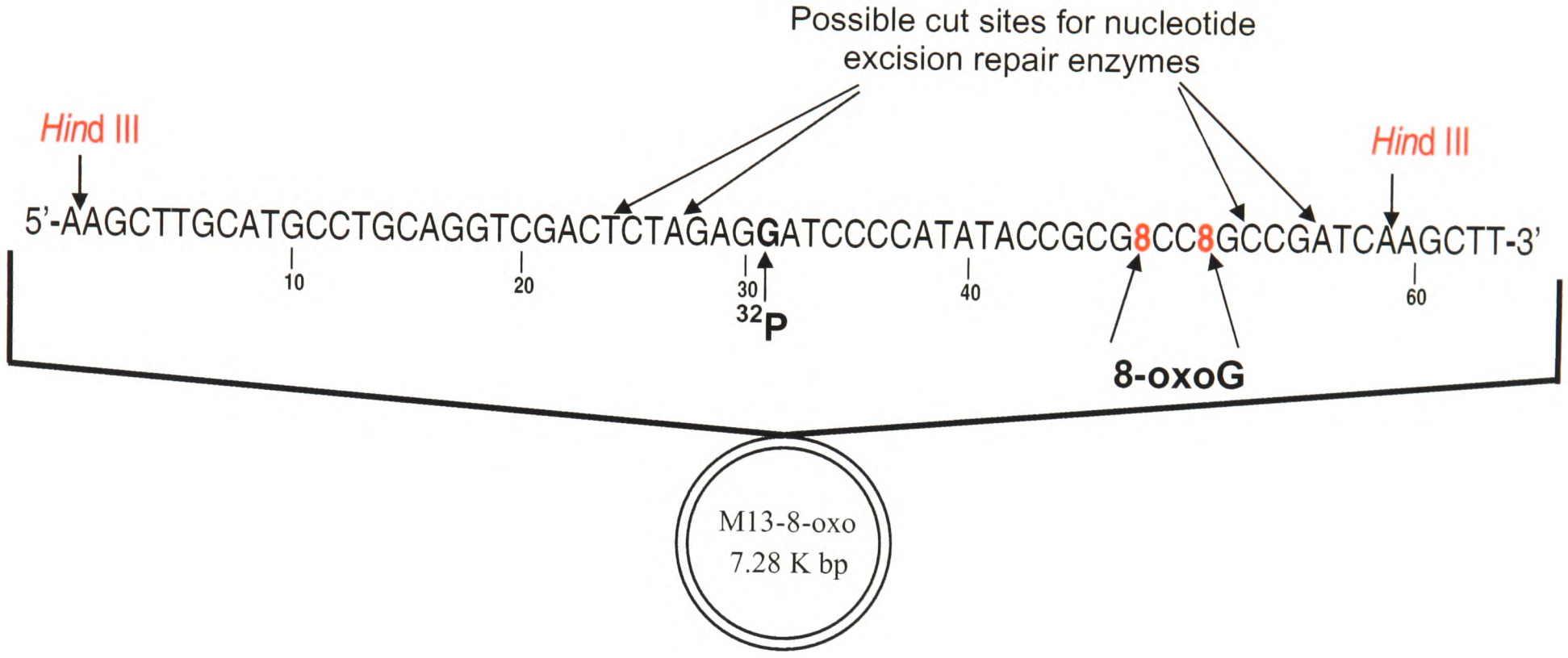


Fig. 3c Excision of DNA by Nucleotide Excision Repair. Position of possible nucleotide excision repair cleavage sites are marked. Excision of the damaged DNA by NER would result in a fragment containing both 8-oxoguanine lesions and the ^{32}P label.

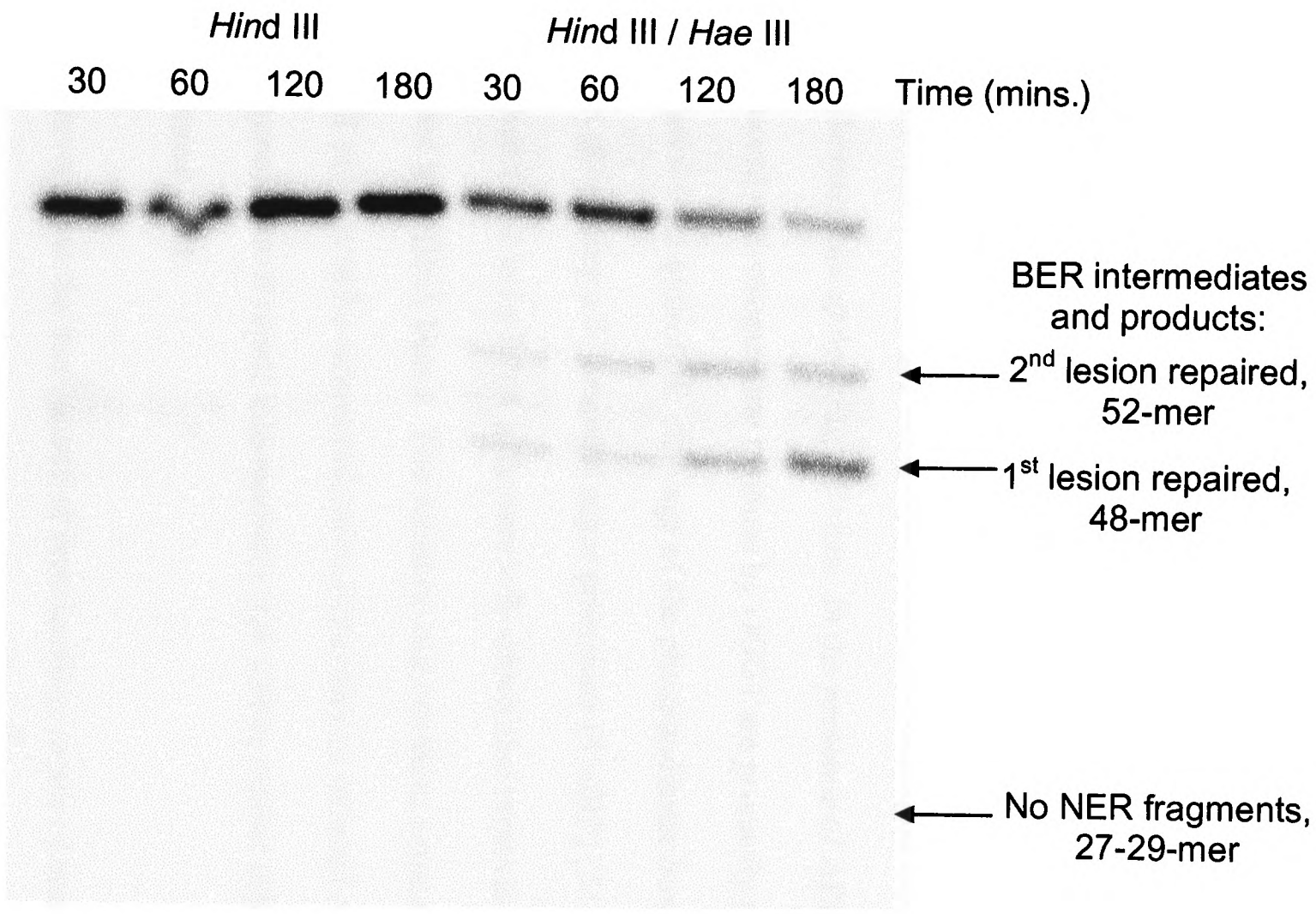


Fig. 3d Base excision repair is the major repair system involved in repair of tandem lesions in human whole cell extract. 0.5 pmol of the labelled closed-circular substrate containing the two 8-oxoguanine residues was incubated for the indicated times at 37°C with 100 μg of HeLa whole cell extract in the presence of 0.25 mM NAD^+ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

3.2.3 *Base Excision Repair of the Tandem Lesions by Whole Cell Extracts*

Having characterised the tandem 8-oxoguanine-containing substrate, and tested for the role of nucleotide excision repair in processing these lesions, we then tested whether the presence of a vicinal lesion in the same DNA strand would affect repair of an 8-oxoguanine residue by base excision repair. Single-standing 8-oxoguanine residues in DNA are substrates for base excision repair. In mammalian cells, BER of 8-oxoguanine is initiated by the action of the OGG1 DNA *N*-glycosylase, which catalyses the hydrolysis of the *N*-glycosyl bond linking the damaged base to the sugar phosphate backbone; thus generating AP sites. In the presence of APE1, AP sites resulting from the removal of 8-oxoguanine residues by OGG1 are primarily processed by APE1, which catalyses the hydrolytic cleavage of the phosphodiester bond immediately 5' to the AP site, generating a 5'-terminal sugar phosphate (5'-dRP) [54, 55, 217]. The resulting gap is then filled in by DNA polymerase β, which also removes the 5'-dRP and the ends sealed by a DNA ligase. The substrate used in these experiments allows every step of the BER process to be monitored, since deficiency or reduced rate of any step will result in accumulation of repair intermediates that will be visible following *Hind* III cleavage of repaired DNA.

To investigate the mechanism of base excision repair of tandem lesions the substrate DNA was incubated with human whole cell extract for the indicated times, and then subjected to cleavage with *Hind* III (to visualise repair intermediates) or *Hind* III and *Hae* III (to visualise repaired DNA). The incision intermediate for lesion 2 (Fig. 3e, *50-mer*) is clearly visible, whereas there appears to be much less incision intermediate for

lesion 1 (Fig. 3e, *47-mer*) and only repair product can be seen (Fig. 3e, *48-mer*). However, at the later time points (120 and 180 mins.), repair and subsequent cleavage by *Hae* III of the first lesion prevents analysis of the second lesion as lesion 1 is situated closest to the label. From this data it was concluded that, although initiation of repair of individual 8-oxoguanine lesions within a tandem is random, further processing appears to exhibit an asymmetry.

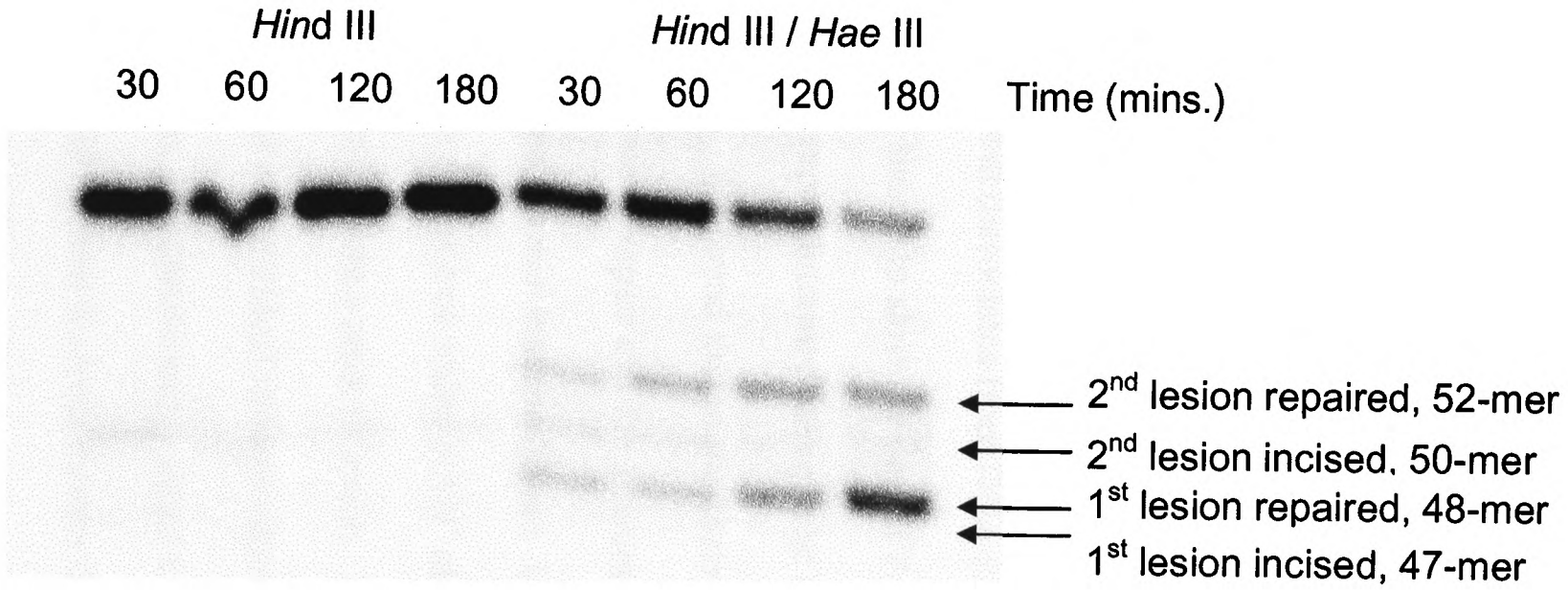


Fig. 3e Kinetics of base excision repair of tandem lesions by human whole cell extract. 0.5 pmol of the labelled closed-circular substrate containing the two 8-oxoguanine residues was incubated for the indicated times at 37°C with 100 μg of HeLa whole cell extract in the presence of 0.25 mM NAD^+ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

3.2.4 *Repair of the Tandem Lesions by Whole Cell Extracts Without the Addition of NAD^+*

To further investigate the asymmetric repair of lesions within a tandem, repair reactions were performed under conditions which allowed clearer visualisation of repair intermediates. The enzyme poly(ADP-ribose) polymerase (PARP-1) has a high affinity for the single strand break intermediates of BER [113, 116]. In the presence of NAD^+, this affinity is transient since binding to nicked DNA triggers an auto-modification reaction in which PARP-1 accumulates negatively charged polymers of poly(ADP)-ribose, and as a result dissociates from DNA [112]. Omitting NAD^+ from repair reactions has been shown to slow the rate of PARP-1 turnover and, accordingly in the absence of NAD^+, repair intermediates can be visualised as they remain bound by PARP-1 for a longer time. We found that in the absence of added NAD^+ there is a pronounced asymmetry in the rate of repair and the amount of incision intermediates for each lesion (Fig. 3fI, *47-mer and 50-mer intermediates*). Quantitative analysis of repair intermediates in the absence of added NAD^+ allows us to follow the dynamics of repair of the individual lesions within a tandem lesion. In agreement with previous data (Fig. 3e) we found that repair of individual lesions within a tandem lesion was randomly initiated, as at 30 and 60 mins. an approximately equal total amount of incised intermediates and repair products could be seen for both lesions (Fig. 3fI, *47- and 48-mer, and 50-mer* and Fig. 3fII). However, following incision, repair of lesion 2 appears to be uncoordinated as further accumulation and persistence of the incision product (Fig. 3fI, *50-mer*) is observed (Fig. 3fII). Repair of lesion 1, however, does not appear to be affected by the presence of a downstream lesion as repair is tightly coupled and the incision product is

rapidly converted to repaired product (Fig. 3fI). Quantitative analysis, using Quantity One software, of three independent experiments confirmed these conclusions (Fig. 3fII).

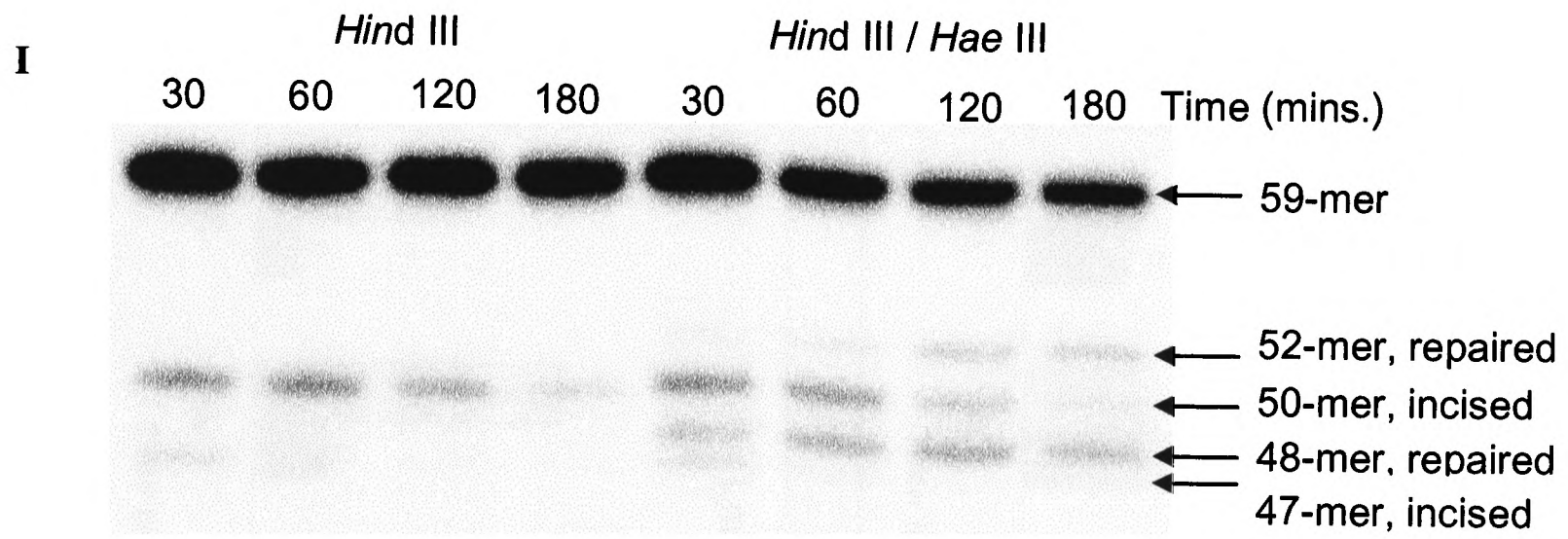
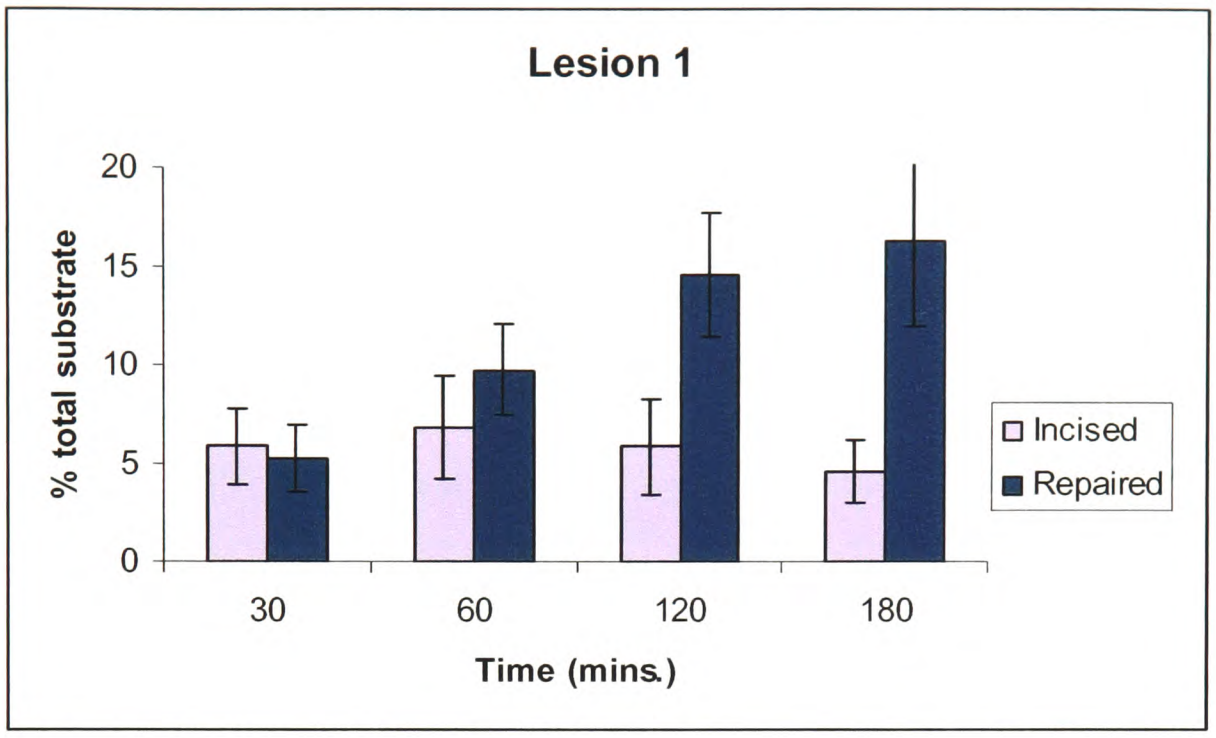


Fig. 3f Repair of the two lesions by whole cell extract without the addition of NAD^+. (I) 0.5 pmol of the labelled closed-circular substrate containing the two 8-oxoguanine residues was incubated for the indicated times at 37°C with 100 μg of HeLa whole cell extract without the addition of NAD^+ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

II



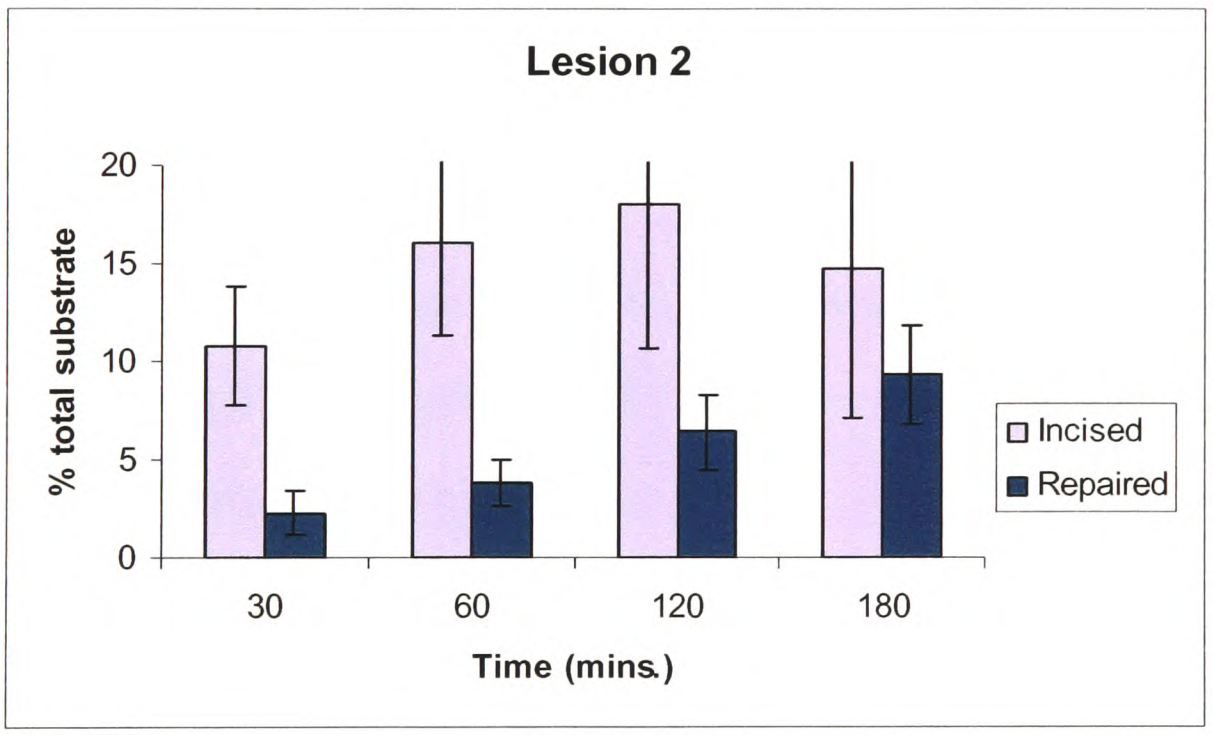


Fig. 3f (II) Graphical analysis of three independent experiments. The amount of incised and repaired product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

3.2.5 *Repair of the Tandem Lesions in PARP-1$^+$ and PARP-1$^-$ Cell Extracts*

From the previous data, it was observed that the repair intermediates of lesion 2 accumulated at the stage of repair in which the protein PARP-1 may be involved, and that the accumulation of intermediates was affected by NAD^+. Therefore, the role of PARP-1 in repair of tandem lesions was investigated. Although non-essential, PARP-1 has been shown to intervene in the course of BER in living cells [218, 219]. Evidence for the involvement of PARP-1 in BER was provided by the fact that PARP-1 null mice are hypersensitive to ionising radiation and alkylating agents [110, 220, 221]. Moreover, the physical interaction of PARP-1 with Pol β and XRCC1 also points to its role in BER [222, 223]. Repair of the two 8-oxoguanine lesions was compared in PARP-1$^+$ and PARP-1$^-$ cell extracts to determine whether PARP-1 played a role in the asymmetrical pattern of repair that had been observed for the two lesions.

We found that repair of both lesions was significantly faster in PARP-1$^-$ extracts, as shown previously [116], suggesting that PARP-1 interferes with base excision repair (Compare Fig. 3g to 3h). No repair intermediate products were observed with the PARP-1$^-$ extracts (Fig. 3h) and both lesions appeared to be repaired at similar rates (Fig. 3h, *48-mer and 52-mer*) at the early time points (5 and 10 mins.), although with time lesion 1 repair product will predominate in *Hin*d III / *Hae* III digests due to being closest to the label (Fig. 3h, *right panel, 48-mer, 20 and 30 mins.*). This suggests that PARP-1 may play a role in the observed delay in repair of lesion 2 in whole cell extracts. In fact, it has previously been shown that PARP-1 binds only weakly to BER intermediates when single nucleotide BER proceeds normally to completion. In contrast, when single

nucleotide BER is stalled by a block in the excision step, PARP-1 binds strongly to the BER intermediate, along with APE 1, Pol β and FEN1 [224], presumably to protect the intermediate from the cellular milieu.

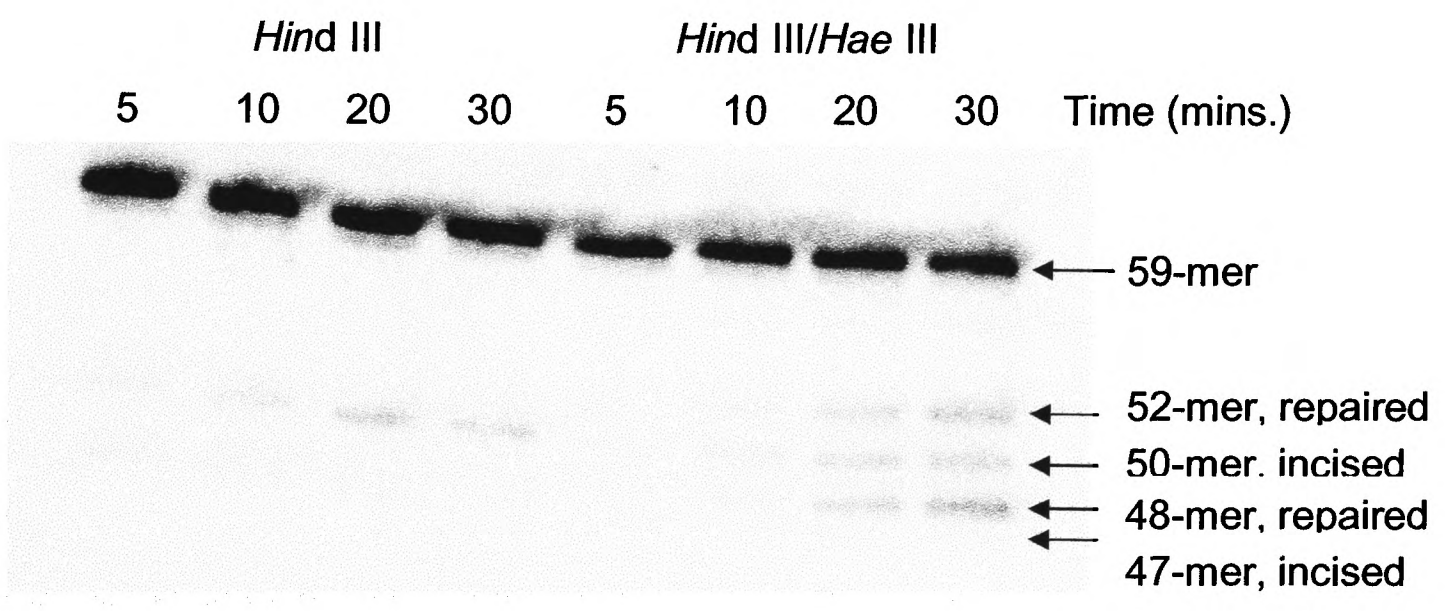


Fig. 3g Repair of the two 8-oxoguanine lesions by PARP-1$^+$ cell extract. 0.5 pmol of the labelled closed-circular substrate containing the two 8-oxoguanine residues was incubated for the indicated times at 37°C with 100 μg of PARP-1$^+$ cell extract in the presence of 0.25 mM NAD$^+$ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

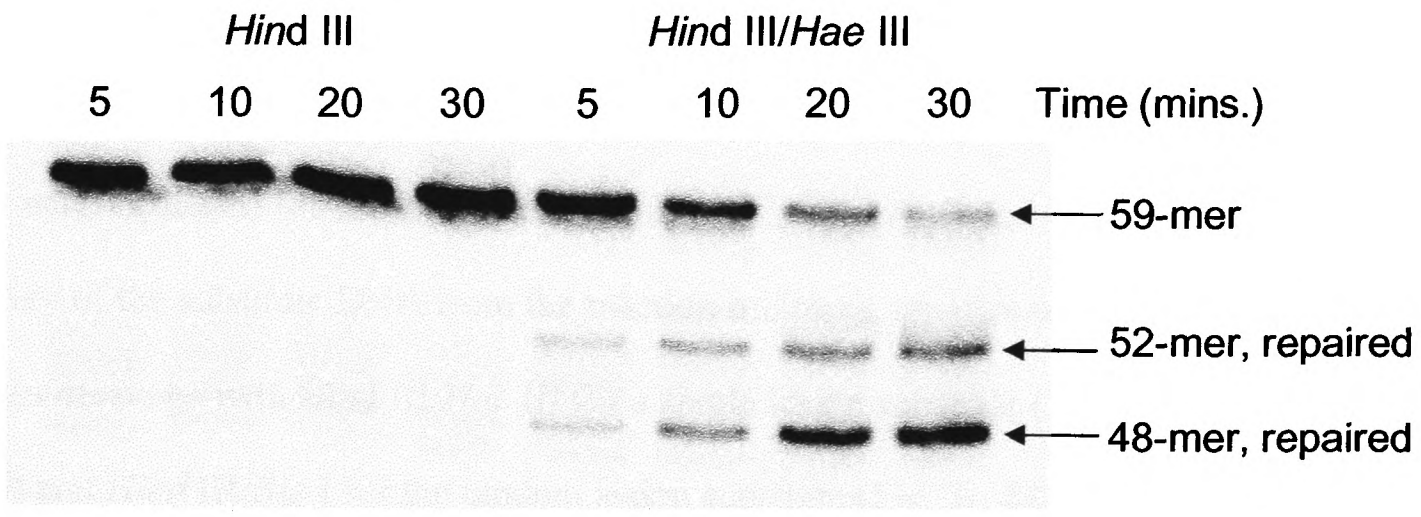


Fig. 3h Repair of the two 8-oxoguanine lesions by PARP-1$^-$ cell extract. 0.5 pmol of the labelled closed-circular substrate containing the two 8-oxoguanine residues was incubated for the indicated times at 37°C with 100 μg of PARP-1$^-$ cell extract in the presence of 0.25 mM NAD^+ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

3.2.6 *Comparative Repair Rates of a Single 8-oxoguanine Lesion and an 8-oxoguanine Within a Tandem Lesion*

To compare the effect of a neighbouring lesion on the repair of individual lesions we compared the rates of repair of lesion 1 within a tandem lesion with the rate of repair of a substrate containing only a single 8-oxoguanine lesion, occupying the same position as lesion 1 in the tandem lesion substrate. Note, the rate of repair of lesion 2 could not be analysed due to the incomplete block of *Hpa* II cleavage by 8-oxoguanine (Fig. 3b). Substrates were incubated for the indicated times at 37°C with whole cell extract and, after recovery of the substrate DNA from the reaction mixtures, repair was monitored by simultaneous cleavage with *Hin*d III/*Hae* III for a single lesion substrate (Fig. 3i, *48-mer, right panel*) and *Hind* III/*Btg* I for the tandem lesion substrate (Fig. 3j, *43-mer, right panel*). It was demonstrated that lesion 1 was repaired as fast as a single-standing lesion, suggesting that there is little or no effect of the second lesion when it is located 3' downstream to the lesion under repair (Fig. 3k). It should be noted, however, that the repair rate of a single lesion in the same position as lesion 2 in the tandem lesion substrate was not tested, therefore sequence-dependent effects cannot be ruled out.

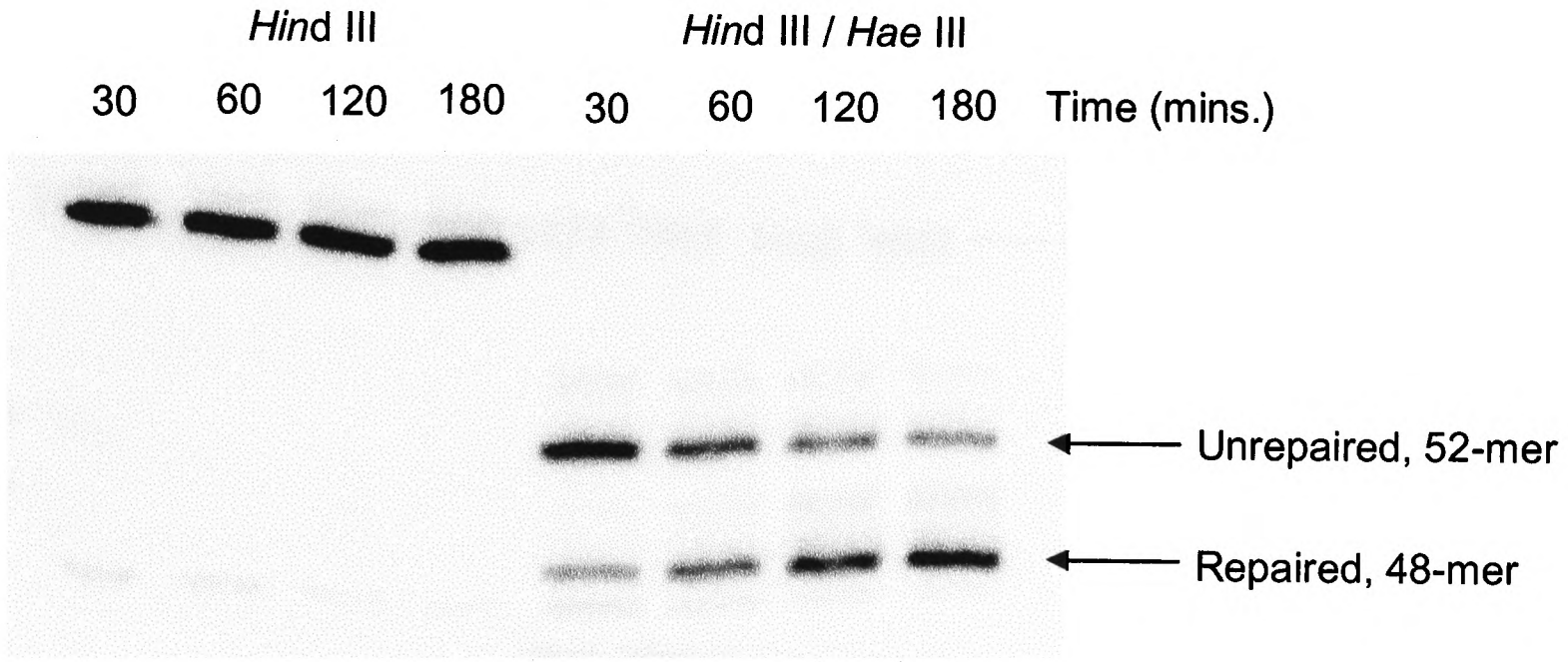


Fig. 3i Repair of a single 8-oxoguanine lesion by whole cell extract. 0.5 pmol of a labelled closed-circular substrate containing a single 8-oxoguanine residue, corresponding to lesion 1 of the double lesion substrate, was incubated for the indicated times at 37°C with 100 μg of HeLa whole cell extract in the presence of 0.25 mM NAD^+ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

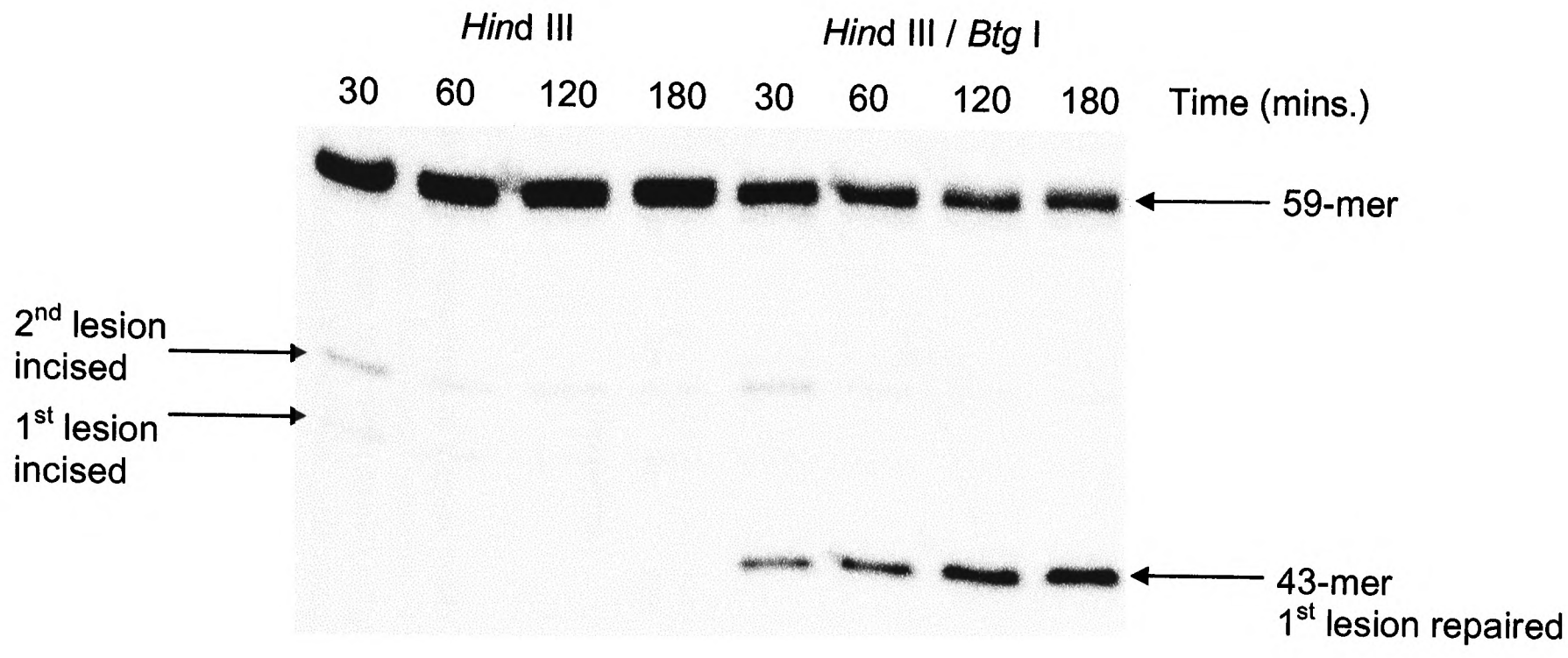


Fig. 3j Repair of lesion 1 within the tandem by whole cell extract. 0.5 pmol of the labelled closed-circular substrate containing the two 8-oxoguanine residues was incubated for the indicated times at 37°C with 100 μg of HeLa whole cell extract in the presence of 0.25 mM NAD^+ in conditions described under Materials and Methods. After incubation with cell extract, substrate DNA was purified from the reaction mixture and subjected to cleavage with the indicated restriction enzymes at 37°C for 1 hr. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

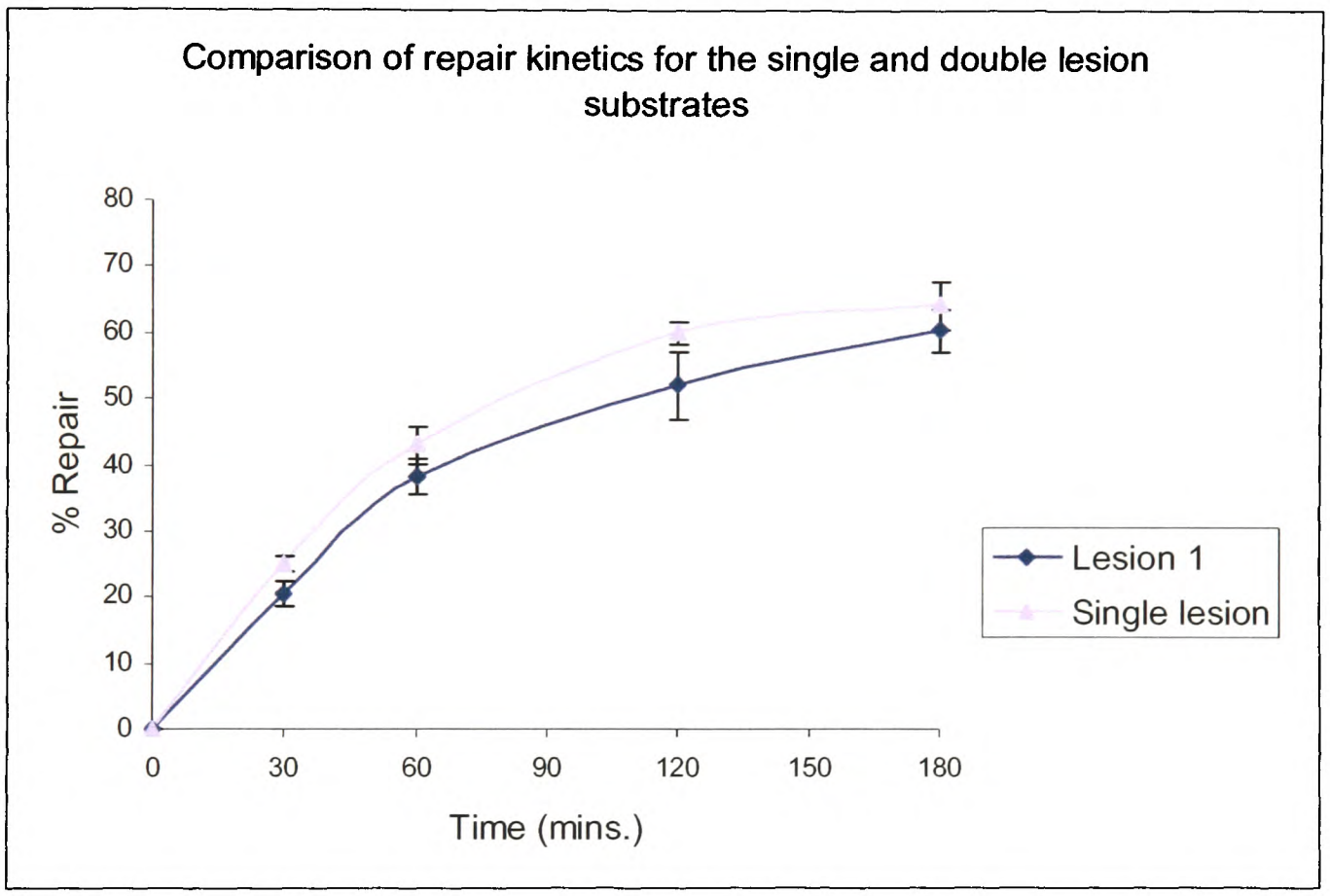


Fig. 3k Graphical comparison of repair rates of a single lesion and lesion 1 with the tandem lesion substrate. Graphical analysis of three independent experiments. The amount of repaired product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

3.2.7 ***Repair Synthesis by DNA Polymerase β***

As can be seen from the previous results, repair of lesion 2 appears to be uncoupled and is stalled after excision of the damaged base and incision of the resulting AP site by AP endonuclease. Due to the design of the assay, incorporation of the first nucleotide into the gap by DNA polymerase β would be visible on the gel after *Hin*d III restriction enzyme digestion as a 51-mer. However, the absence of a 51-mer (Fig. 3e) might suggest that insertion of a nucleotide into the gap by Pol β is delayed in the presence of a second lesion 5' to the gap, since only the APE1 incision product is observed (Fig. 3e, *50-mer*). This could be a reduced ability of the polymerase to recognise or be able to bind to the damage site. Alternatively, this could be due to the interference of other proteins at the gap, which may prevent access for the polymerase. To test this, an oligonucleotide substrate identical in sequence to that in the closed-circular substrate (Fig. 31 I), but with an uracil residue in the place of lesion 2, was used with purified Pol β to determine whether Pol β is able to incorporate a nucleotide into the gap in the presence of another lesion 5' to the damage site. Pre-incubation of the 40-mer 5'-labelled oligonucleotide duplex (Fig. 31 I), containing 8-oxoguanine two nucleotides 5' to an uracil residue, with UDG and APE1 creates a substrate with an incised AP site two nucleotides 3' to an 8-oxoguanine residue, this mimics the repair intermediate that appears to be stalled during repair by whole cell extracts. Further incubation with Pol β allows the effect of an upstream 8-oxoguanine on gap filling by Pol β to be monitored. The result clearly shows that the presence of 8-oxoguanine 5' to the incised AP site does not affect the ability of Pol β to incorporate a nucleotide into the gap, as 21-mer products (indicating insertion of

a nucleotide into the gap) are observed with both a guanine-containing (*left panel*) and 8-oxoguanine-containing substrate (*right panel*) at even the lowest concentration of Pol β (Fig. 31 II). Graphical analysis even suggests a slight stimulation of Pol β insertion by the presence of a 5' 8-oxoguanine lesion (Fig. 31 III). This suggests that other proteins may be responsible for the observed stalling of repair in whole cell extract.

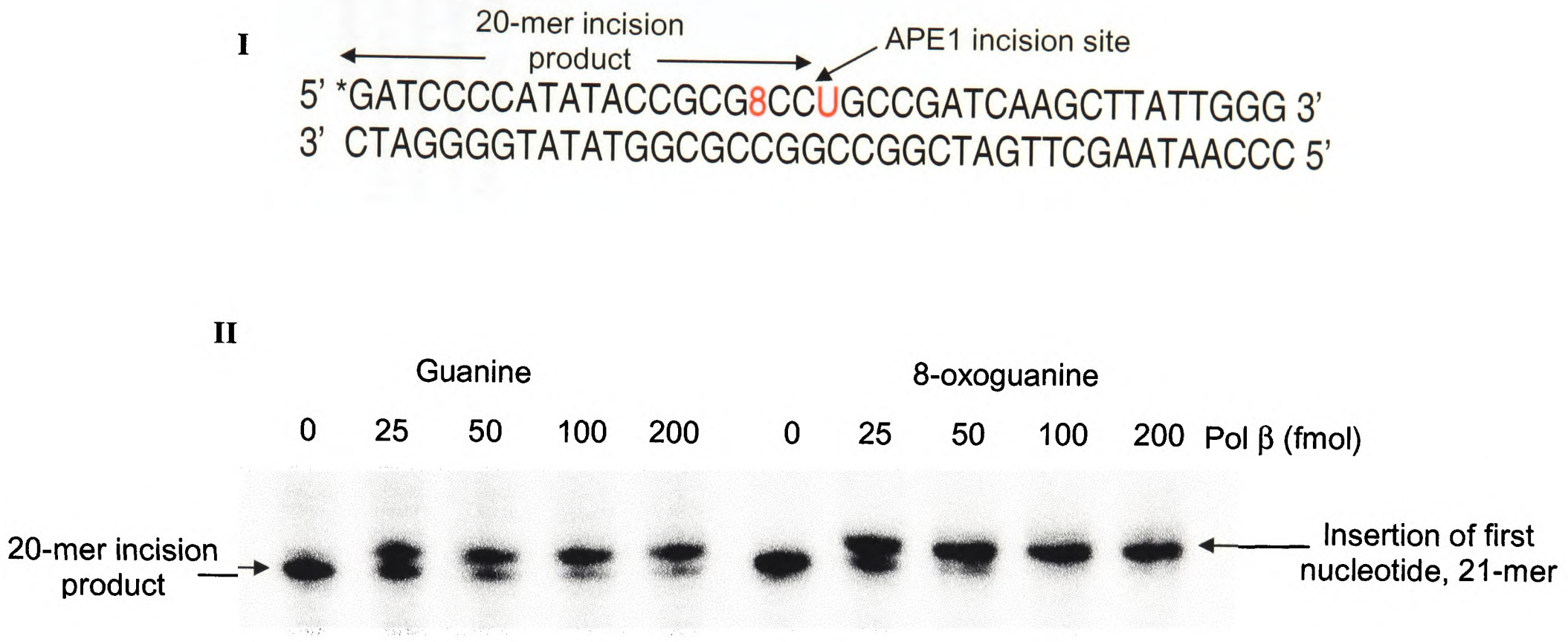


Fig. 31 Insertion of nucleotides into a gap by Pol β. **(I)** Schematic representation of the oligonucleotide substrate **(II)** 10 pmol of the ^{32}P-5'-labelled (*) substrate containing 8-oxoguanine two nucleotides 5' to an uracil residue was pre-incubated with 9.4 pmol of UDG and 5.7 pmol of APE1 for 20 mins. at 37°C. Substrates were further incubated for 20 mins. at 37°C with the indicated amount of Pol β in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

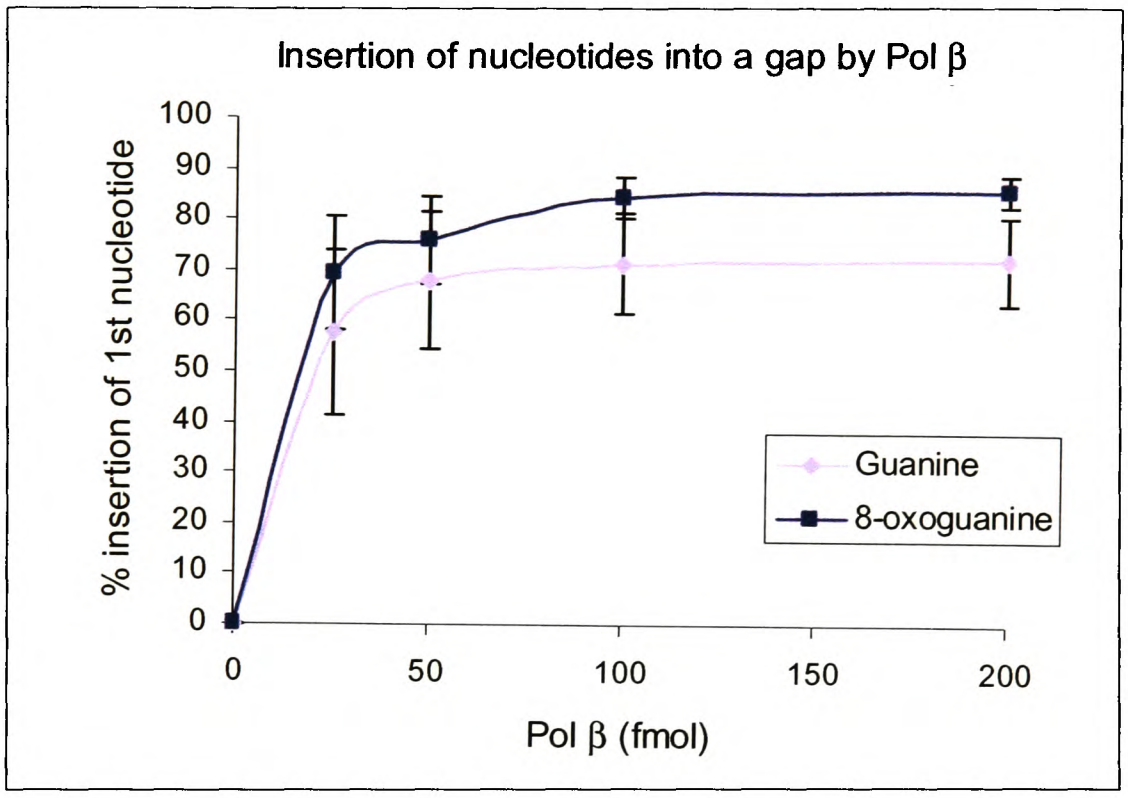


Fig. 3l III Graphical comparison of insertion of nucleotides into a gap by Pol β in the presence or absence of an upstream 8-oxoguanine. Graphical analysis of three independent experiments. The % of insertion was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

3.2.8 *Effect of PARP-1 on Gap Filling by DNA Polymerase β*

The difference in the rate of repair of each lesion in PARP-1$^+$ extracts was much greater than in PARP-1$^-$ extracts (Figs. 3g and 3h). PARP-1 is known to have a high affinity for the single-strand break intermediates of BER [113] and has also been shown to interact with Pol β [118]. We found that in a reconstituted system, that does not contain PARP-1, Pol β is able to incorporate a nucleotide into a gap in the presence of an upstream 8-oxoguanine lesion. Therefore, we tested whether the presence of PARP-1 in the reaction would affect the insertion of nucleotides by Pol β. Using the same conditions as in the assay for insertion of nucleotides by Pol β, the 5'-labelled substrate was pre-incubated with UDG and APE 1 (to create a repair gap) and then Pol β and PARP-1 were added. In the absence of NAD^+, the turnover of PARP-1 is reduced and therefore, as expected, increasing concentrations of PARP-1 inhibited nucleotide insertion by Pol β on both the guanine-containing substrate and the substrate containing 8-oxoguanine (Fig. 3m, *left and right panel respectively, less 21-mer insertion product*). It was also interesting, therefore, to test whether the presence of a second nearby damage had an effect on PARP-1 turnover on a single-nucleotide gap, in the presence of NAD^+. Kinetics of incorporation of the first nucleotide, by Pol β, in the presence of PARP-1 and NAD^+ were monitored and it was found that in the presence of NAD^+, incision of the AP site (Fig. 3n, *20-mer*) was followed by insertion of the first nucleotide (Fig. 3n, *21-mer*). An 8-oxoguanine upstream of the AP site did not affect the polymerase reaction (Fig. 3nI and 3nII). It can therefore be concluded that PARP-1 does not affect incorporation of nucleotides into a repair gap situated 3' of an 8-oxoguanine lesion.

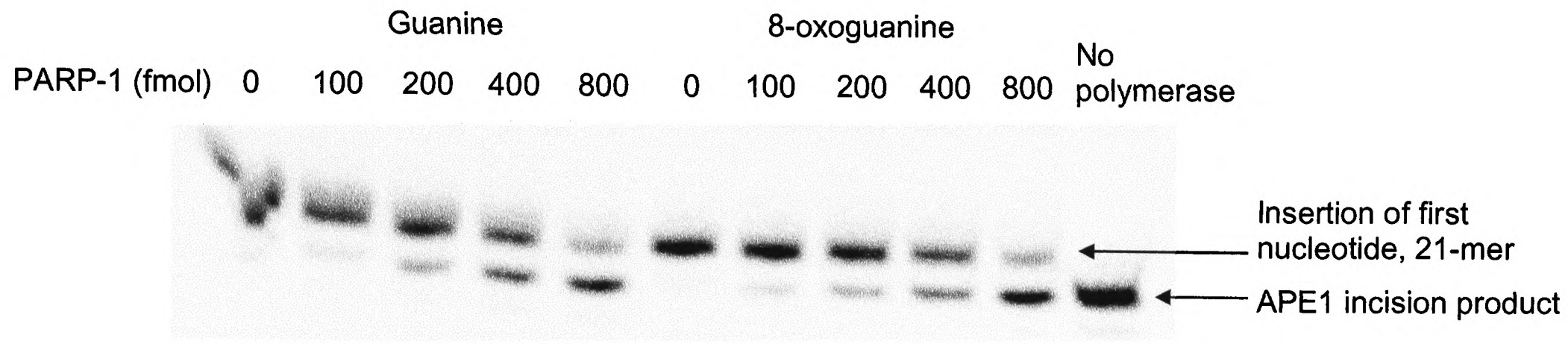


Fig. 3m Effect of PARP-1 on insertion of nucleotides by Pol β. 10 pmol of the ^{32}P-5'-labelled (*) substrate used in the previous assay (see Fig. 31 I), containing an 8-oxoguanine residue two nucleotides 5' to an uracil residue, was pre-incubated with 9.4 pmol of UDG and 5.7 pmol of APE1 for 20 mins. at 37°C. Substrates were further incubated for 20 mins. at 37°C with ~25 fmol of Pol β and the indicated amount of PARP-1 in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

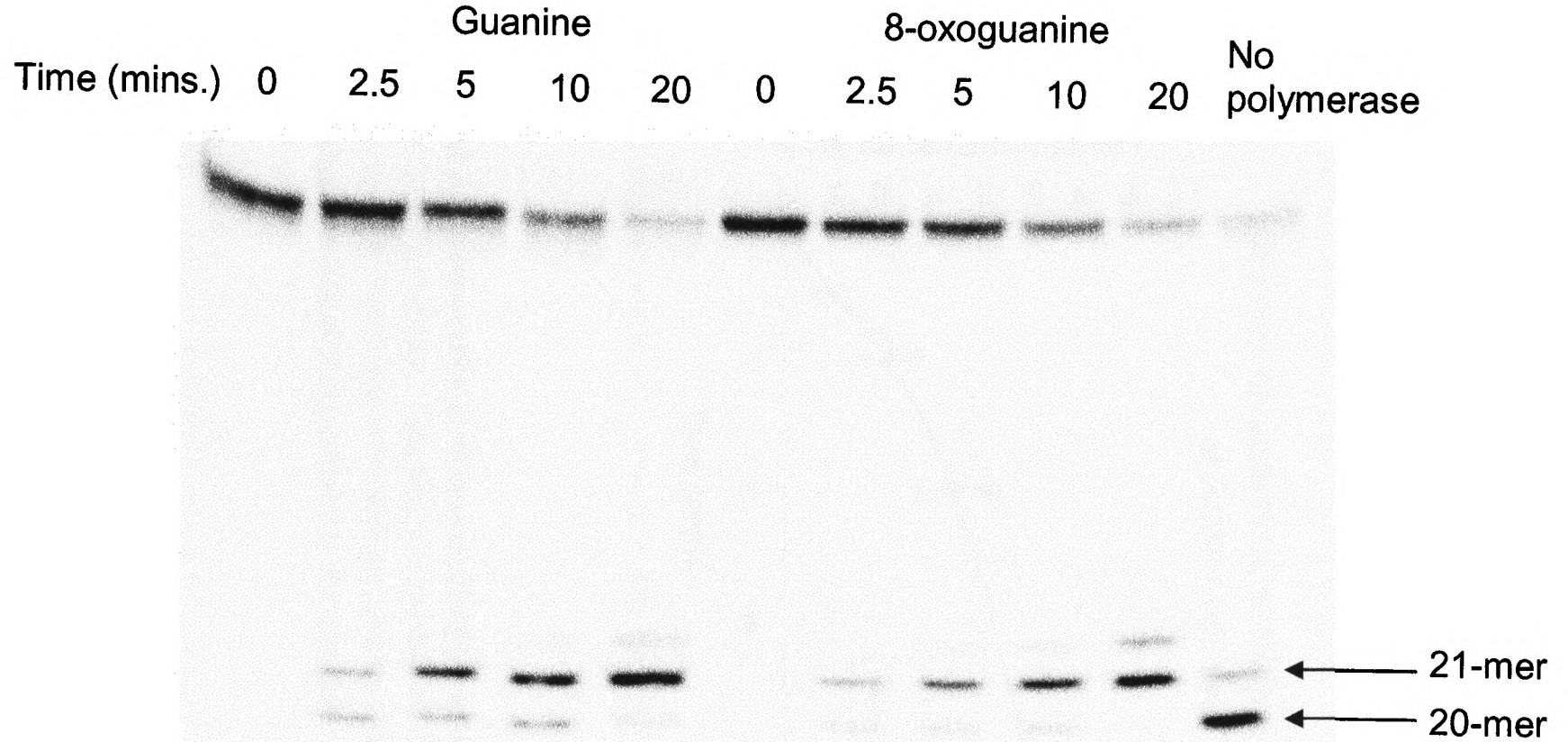


Fig. 3n Kinetics of nucleotide insertion into a gap by Pol β in the presence of PARP-1 and NAD^+. (I) 1 pmol of the ^{32}P-5'-labelled (*) substrate used in the previous assay (see Fig. 31 I), containing an 8-oxoguanine residue two nucleotides 5' to an uracil residue, was incubated for the indicated times at 37°C with ~625 fmol of UDG, ~300 fmol of APE1, ~25 fmol of Pol β and ~800 fmol of PARP-1 and 0.2 mM NAD^+ in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

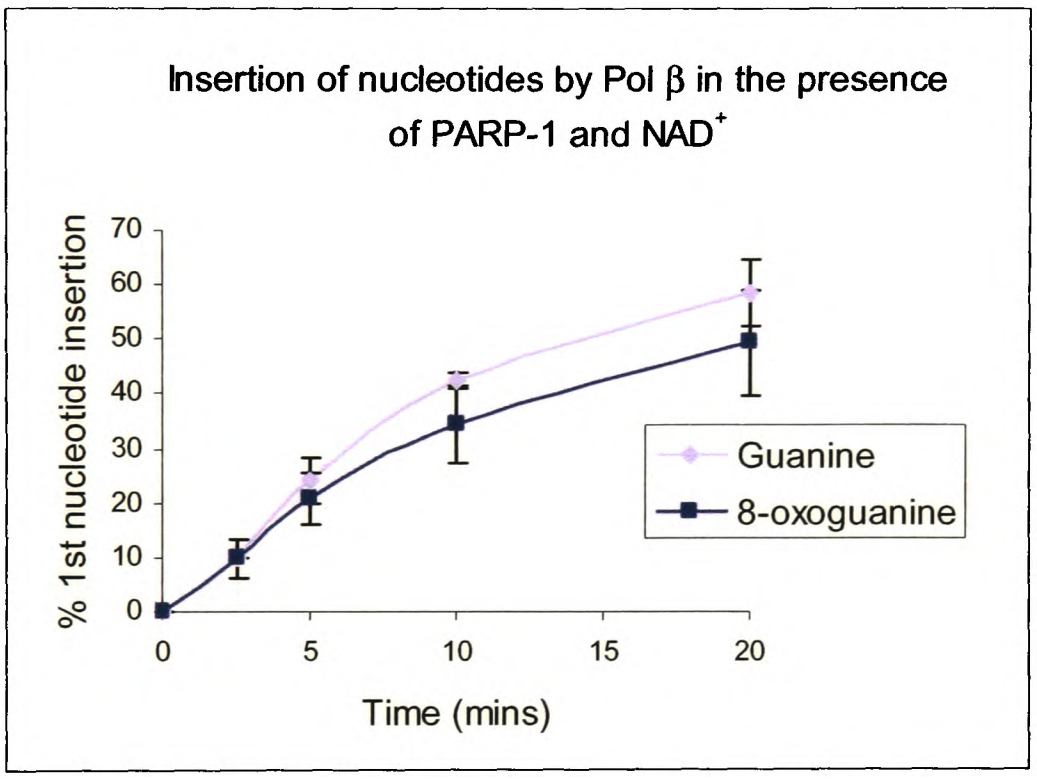


Fig. 3n (II) Graphical comparison of nucleotide insertion into a gap and a gap with a second upstream 8-oxoguanine. Graphical analysis of three independent experiments. The amount of nucleotide insertion was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

3.2.9 ***Reduced Incision by OGG1 of an 8-oxoguanine 3' of an Incised AP Site***

Since we did not find any inhibitory effect of a 5' 8-oxoguanine on the efficiency of the BER enzymes involved in processing of a 3' AP site, we tested the hypothesis that processing of this 5' 8-oxoguanine itself (lesion 1) might be the major reason for delayed repair of a 3' 8-oxoguanine. It had previously been shown that the presence of a nearby strand break can inhibit certain glycosylases in an asymmetrical manner [225, 226]. In such cases, it might be possible that the glycosylase remains bound to the base lesion unproductively. We therefore decided to test whether this was also the case for OGG1.

To test this model oligonucleotide substrates simulating repair intermediates arising during processing of tandem lesions were used. An oligonucleotide containing a single 8-oxoguanine (Fig. 3oI) and an oligonucleotide containing 8-oxoguanine near the 3' incised AP site were labelled at the 5'-end (Fig. 3qI). An oligonucleotide containing 8-oxoguanine near the 5' incised AP site was 3'-labelled (Fig. 3pI). Oligonucleotides were incubated with OGG1 and APE1. Removal by OGG1 of single-standing 8-oxoguanine or 8-oxoguanine located near the 3' strand break, and subsequent cleavage of AP site by APE1, generates 10- and 17-mer labeled products respectively (Figs. 3oII and 3qII). Since OGG1 has pronounced deoxyribophosphodiesterase activity [227], incubation of the substrate containing 8-oxoguanine near the 5' strand break with OGG1 and APE1 led to the removal of 5'-terminal deoxyribose phosphate residue from the incised AP site (Fig. 3pII, *24-mer product*) as well as to removal of the 8-oxoguanine located near the 5'-terminus and cleavage of the generated AP site (Fig. 3pII, *21-mer product*). The rates of 8-oxoguanine removal for these substrates were quantitated and it was found that while

removal of 8-oxoguanine located near the 5' break was similar to the removal rate of single-standing 8-oxoguanine, there was a four fold reduction in excision when 8-oxoguanine was located near the 3' break. The data from three independent experiments are summarized in Fig. 3r. This result suggests that persistence of 8-oxoguanine near a 3'-terminus may lead to accumulation of the intermediate products containing DNA strand breaks.

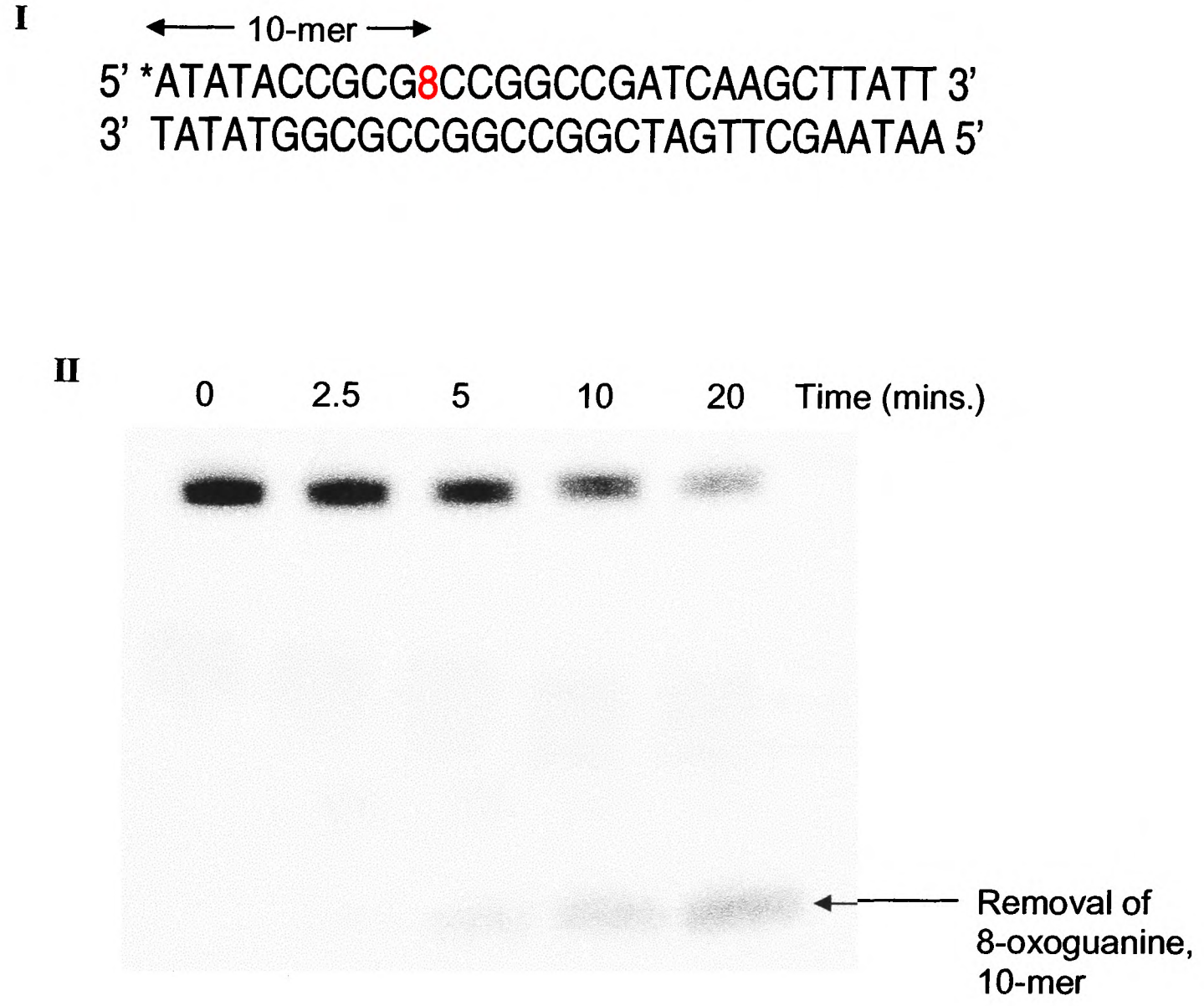


Fig. 3o Excision of 8-oxoguanine by OGG1. (I) Schematic representation of the oligonucleotide substrate **(II)** 1 pmol of the ^{32}P-5'-labelled (*) substrate containing a single 8-oxoguanine residue was incubated for the indicated time at 37°C with 128 fmol of OGG1 and 570 fmol of APE1 in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

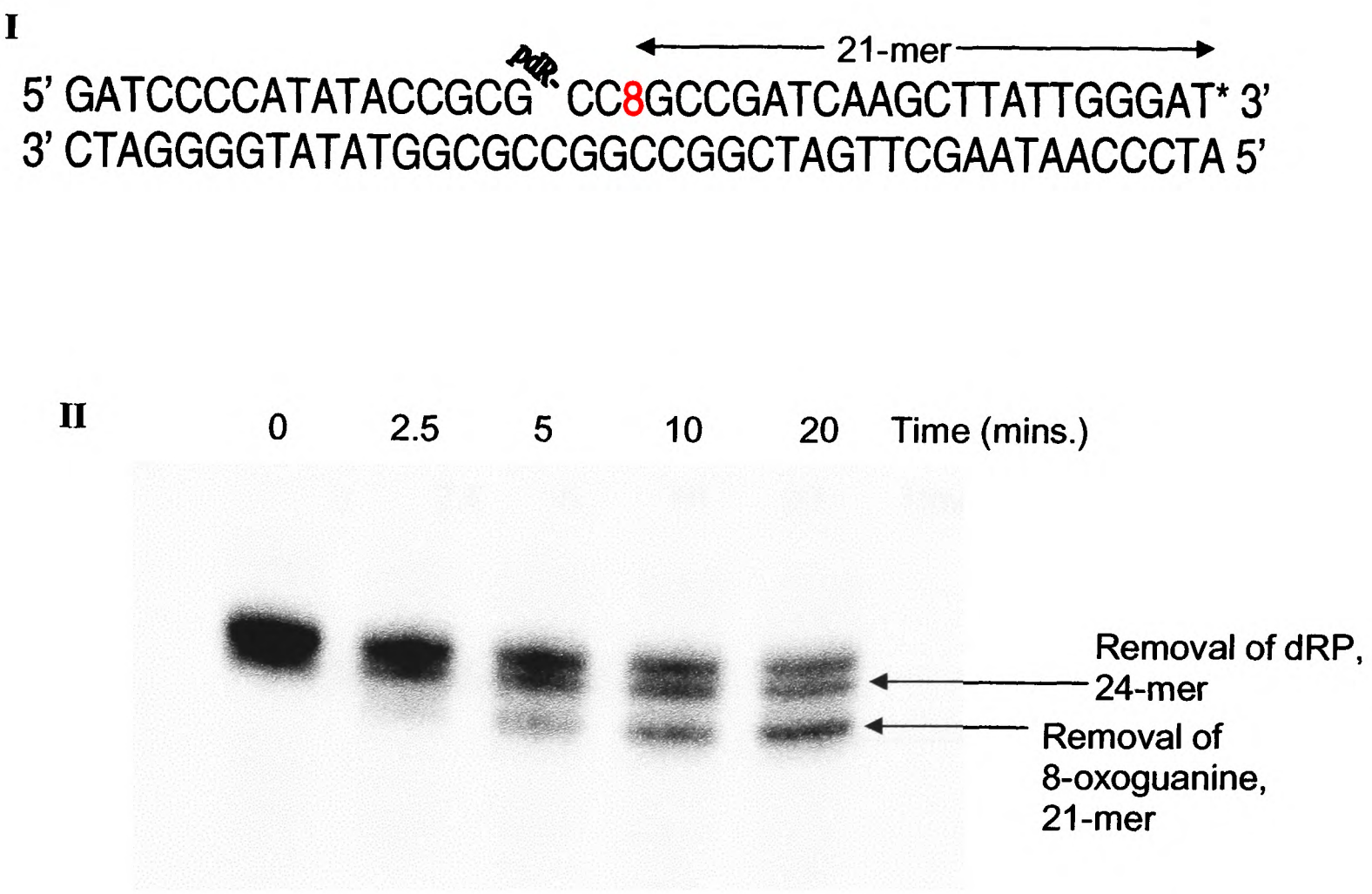
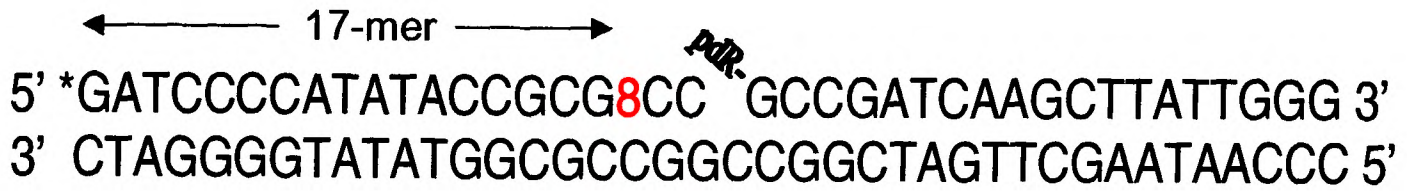


Fig. 3p Excision of 8-oxoG by OGG1 in the presence of a 5'-strand break. (I) Schematic representation of the oligonucleotide substrate **(II)** 1 pmol of the ^{32}P-3'-labelled (*) substrate containing an 8-oxoguanine residue two nucleotides 3' to a strand break was incubated for the indicated time at 37°C with 128 fmol of OGG1 and 570 fmol of APE1 in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

I



II

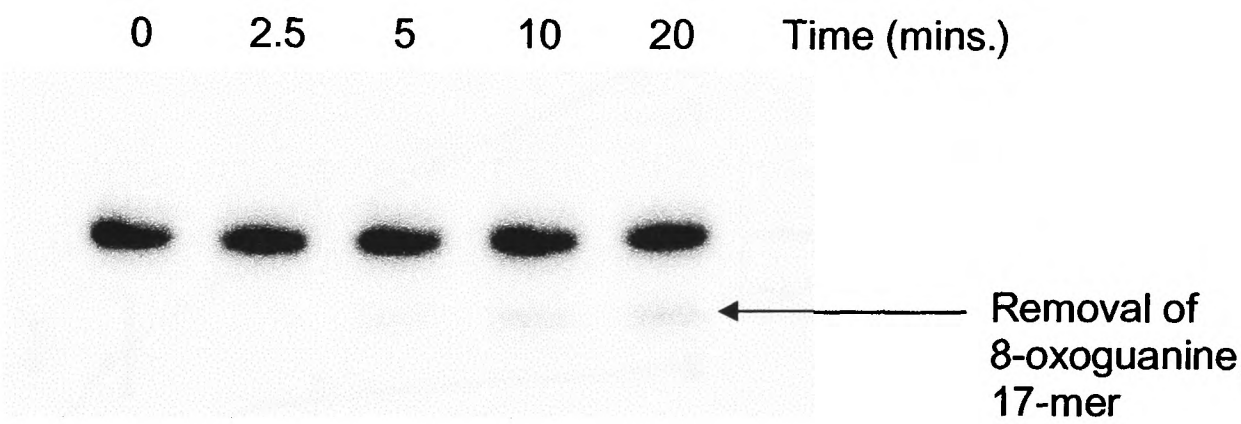


Fig. 3q Excision of 8-oxoguanine by OGG1 in the presence of a 3'-strand break. (I) Schematic representation of the oligonucleotide substrate **(II)** 1 pmol of the ^{32}P-5'-labelled (*) substrate containing an 8-oxoguanine residue two nucleotides 5' to a strand break was incubated for the indicated time at 37°C with 128 fmol of OGG1 and 570 fmol of APE1 in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and, after incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

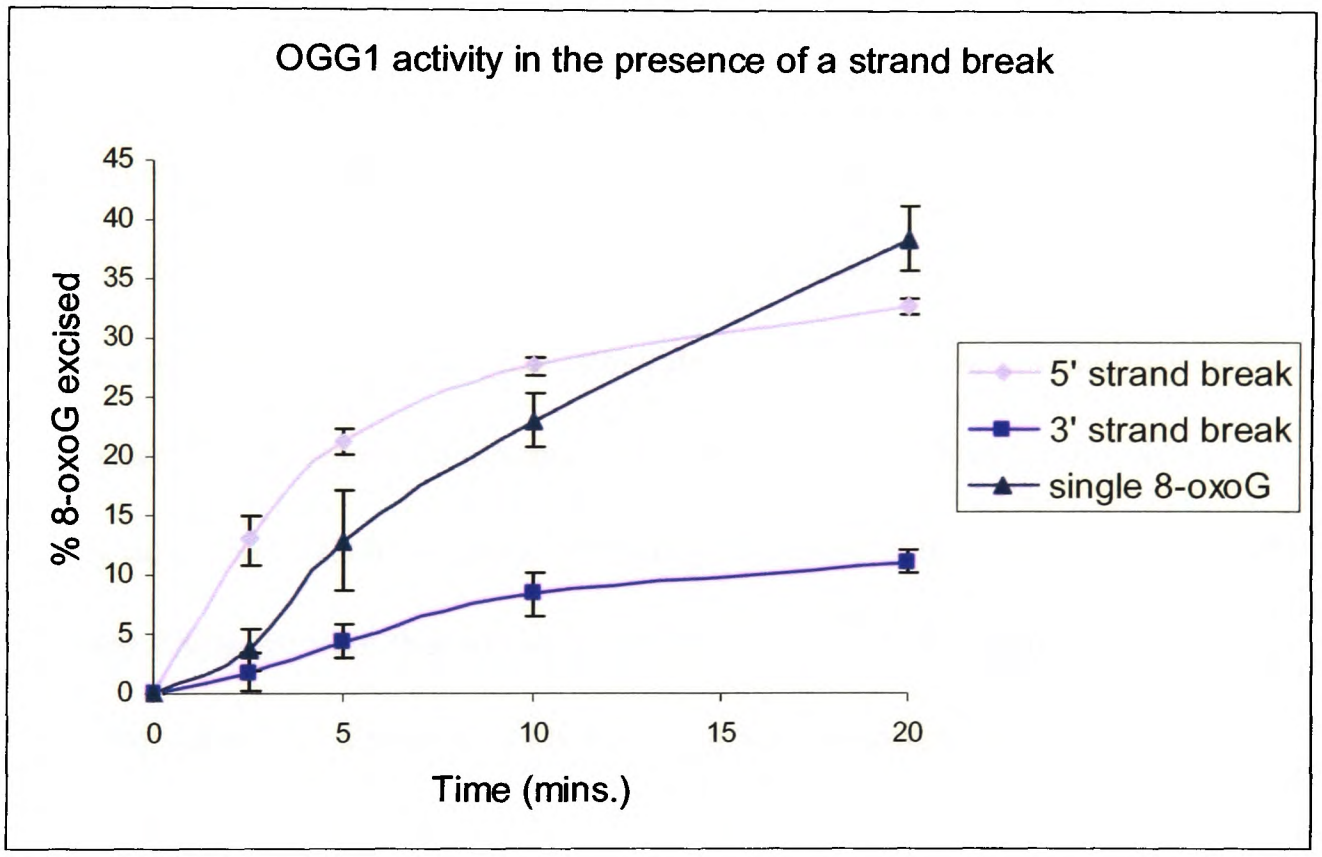


Fig. 3r Graphical comparison of 8-oxoguanine excision by OGG1 in the presence of either a 5'- or 3'-strand break. Graphical analysis of three independent experiments. The amount of cleavage product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

3.2.10 *Reduced Affinity of OGG1 to an 8-oxoguanine Near the 3'-Terminus of an Incised AP Site*

OGG1 shows a 4-fold reduction in reaction rate for excision of an 8-oxoguanine located near a 3'-terminus (Fig. 3r). In order to determine if this inhibition is due to a reduced turnover of OGG1 on such a substrate or to a reduced affinity of the enzyme for the substrate, we incubated the ^{32}P-labelled substrates with OGG1 and analysed the products of the reactions using the electrophoretic mobility shift assay (EMSA). To minimise cleavage of DNA duplexes by OGG1, the binding reactions and gel electrophoresis were performed at 4°C. Figs. 3s and 3t show that incubation of the single- and tandem 8-oxoguanine and incised AP site lesion substrates with increasing concentrations of OGG1 produced increasing amounts of a band with reduced electrophoretic mobility, presumably an OGG1-DNA complex. Binding of OGG1 to the single lesion substrate was significantly more than that to either of the tandem lesion substrates (25-fold) (Compare Fig. 3s to 3t). Binding of OGG1 to an 8-oxoguanine near the 5'-terminus of an AP site was 2-fold more than that to an 8-oxoguanine near the 3'-terminus (Fig. 3t, *compare right panel to left*). However, the reduction in rate of excision by OGG1 on an 8-oxoguanine near a 3'-terminus was 4-fold less than that for an 8-oxoguanine near a 5'-terminus, therefore it could be possible that the presence of an upstream AP site also affects the turnover of OGG1 on an 8-oxoguanine residue. As a result of the reduced excision rate of an 8-oxoguanine near a 3'-terminus by OGG1, the 8-oxoguanine residue may persist longer.

I

5' *ATATACCGCG8CCGGCCGATCAAGCTTATT 3'
3' TATATGGCGCCGGCCGGCTAGTTCGAATAA 5'

II

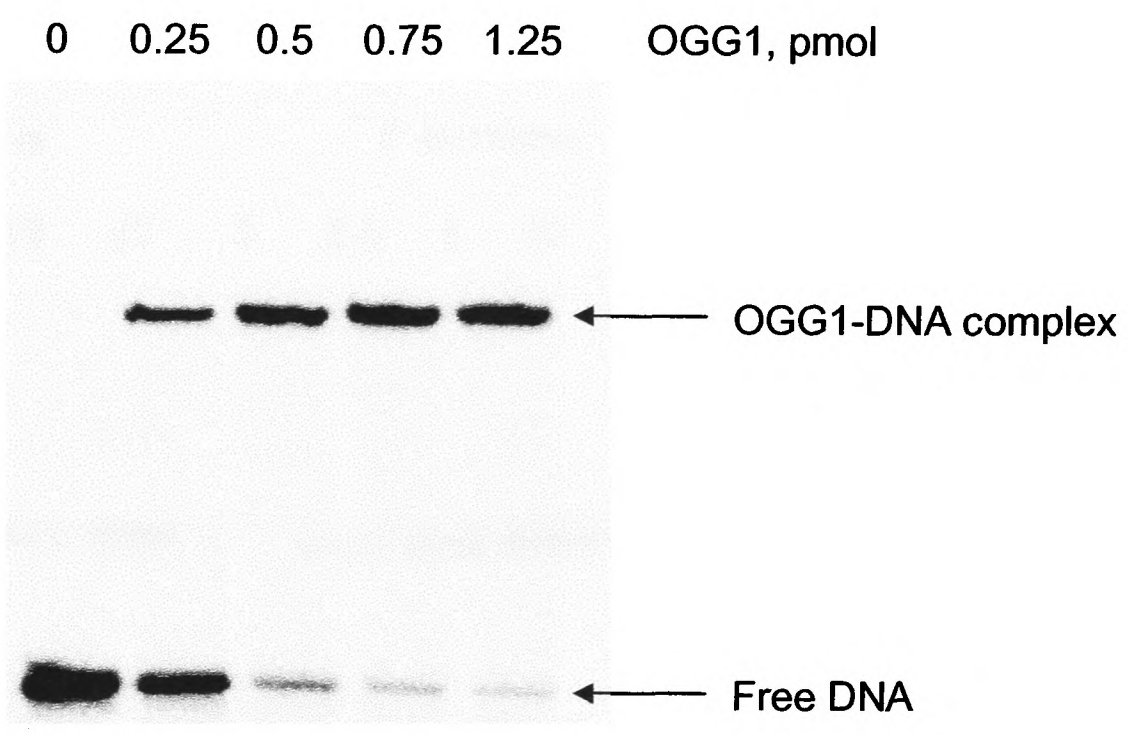


Fig 3s Electrophoretic mobility shift assay (EMSA) of OGG1 protein with a single 8-oxoguanine-containing substrate. (I) Schematic representation of the oligonucleotide substrate **(II)** 8 fmol of the ^{32}P-5'-labelled (*) substrate containing a single 8-oxoguanine residue was incubated with increasing concentrations of OGG1 at 4°C for 30 mins. in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and the reaction products were separated by electrophoresis run at 4°C in a 12% non-denaturing polyacrylamide gel.

I

5' *GATCCCCATATACCGCG8CC PdR GCCGATCAAGCTTATTGGG 3'
3' CTAGGGGTATATGGCGCCGGCCGGCTAGTTCGAATAACCC 5'

5' *GATCCCCATATACCGCG PdR CC8GCCGATCAAGCTTATTGGGAT 3'
3' CTAGGGGTATATGGCGCCGGCCGGCTAGTTCGAATAACCCTA 5'

II

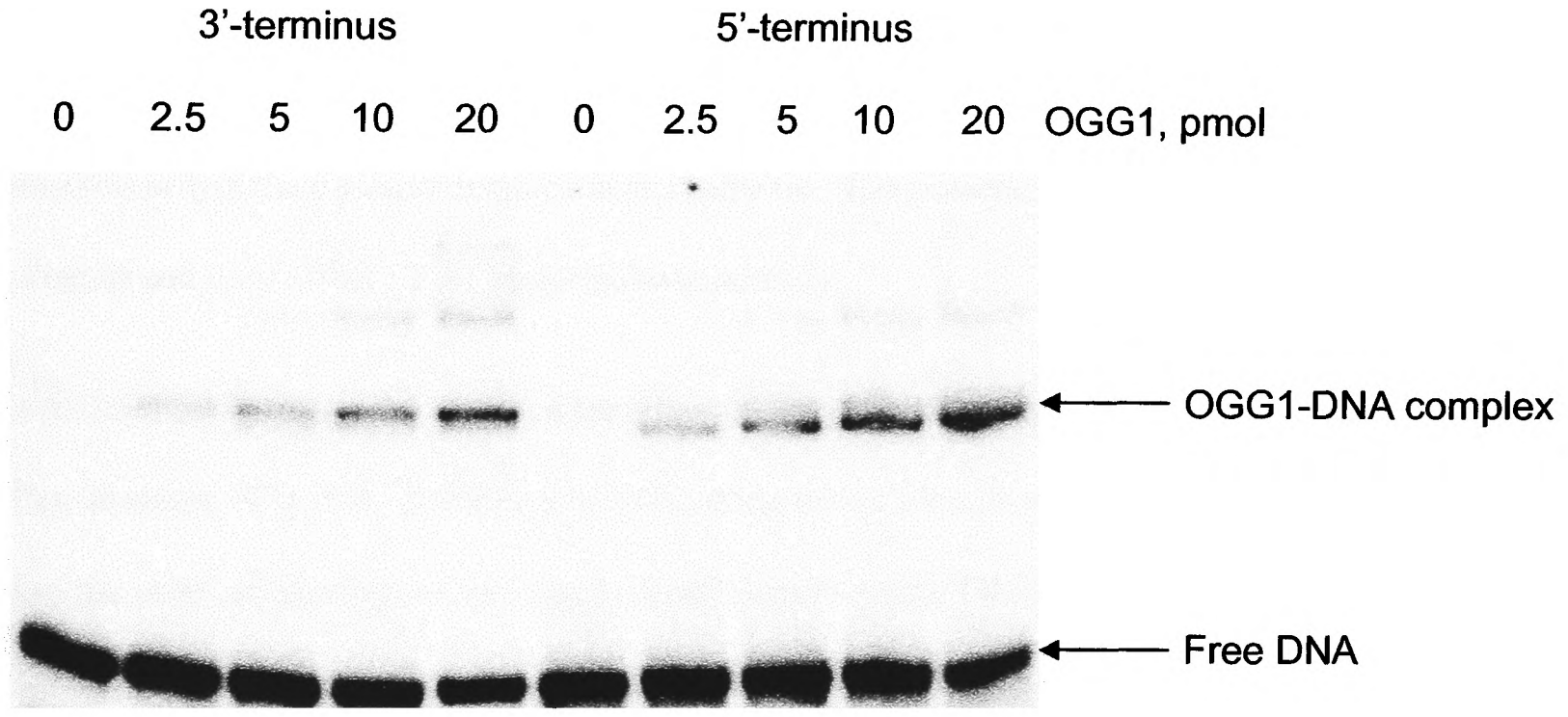


Fig 3t Electrophoretic mobility shift assay (EMSA) of OGG1 protein with tandem lesion substrates. **(I)** Schematic representation of the oligonucleotide substrates **(II)** 8 fmol of the ^{32}P-5'-labelled (*) substrates containing an 8-oxoguanine residue near to an incised AP site was incubated with increasing concentrations of OGG1 at 4°C for 30 mins. in conditions described under Materials and Methods. Reactions were stopped by addition of gel loading buffer and the reaction products were separated by electrophoresis run at 4°C in a 12% non-denaturing polyacrylamide gel.

3.3 *The Effects of Tandem Lesions on Long-patch Base Excision Repair*

Tandem lesions formed on the same strand of DNA appear to be substrates for base excision repair pathways. There are two pathways of base excision repair, that depend on the ability of Pol β to remove the 5'-dRP residue of an abasic site. Modified abasic sites are resistant to β-elimination by Pol β, therefore such sites are repaired through the long-patch base excision repair pathway [48]. Flap endonuclease 1 (FEN1) functions in long-patch BER as an endonuclease that cleaves a flap substrate generated following strand-displacement synthesis [48]. The presence of vicinal lesions on the same strand during long-patch BER could result in the presence of damaged bases in the flap strand that is formed by polymerase strand displacement synthesis. The presence of damaged bases in the flap strand may affect FEN1 endonuclease activity.

The preferred substrate for FEN1 is a template with a primer having an unannealed 5' tail or flap structure (Fig. 3u). It appears to track along the full length of the flap from the 5' end to the point of cleavage at the single-strand/double-strand DNA junction [228]. FEN1 has a flexible loop structure through which the flap has been proposed to thread [229]. Crystal structure studies of FEN1 have indicated that this loop can accommodate single-stranded DNA, but is not large enough for double-stranded DNA; this information prompted suggestions that the flap threads through the hole during tracking [103]. Certain modifications in the flap strand have been shown to inhibit FEN1 activity [103, 230]. We therefore tested whether the presence of base lesions in the flap strand would affect long-patch base excision repair by affecting FEN1 activity. Synthetic oligonucleotide substrates, containing tandem base lesions were generated to test whether

the presence of such lesions in the flap strand would affect FEN1 tracking and cleavage (Fig. 3vI and Fig. 3wI).

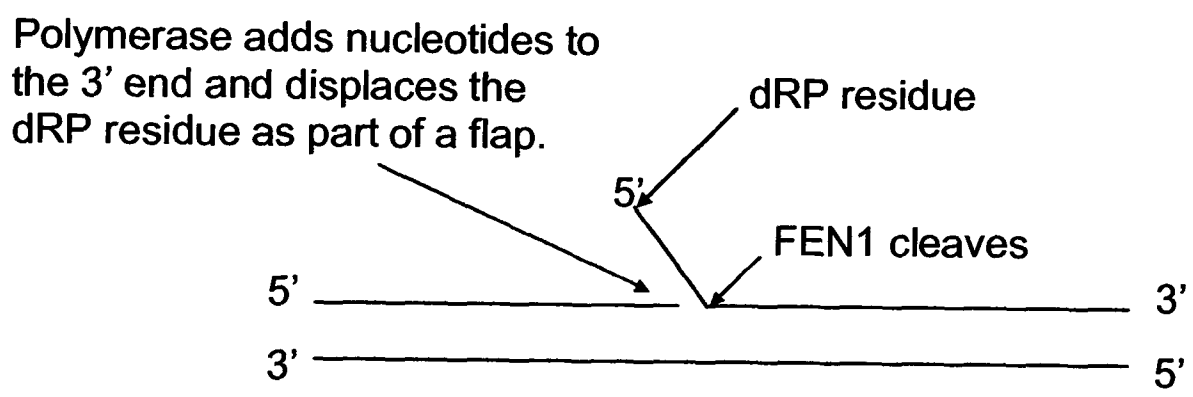


Fig. 3u Schematic representation of a FEN1 substrate.

3.4 *Results*

3.4.1 *The Effect of 8-oxoguanine Lesions in the Flap Strand on FEN1 Flap Endonuclease Activity*

In this study the effect of 8-oxoguanine lesions in a flap strand on FEN1 activity was studied. A triplex oligonucleotide substrate was constructed containing three 8-oxoguanine lesions in a 5'-labelled flap strand. Substrates were incubated with purified human FEN1, and the endonuclease activity on modified and unmodified substrates was determined by the formation of flap cleavage products (Fig. 3vII, *23-mer*). It is shown that the presence of 8-oxoguanine lesions in the flap strand does not affect FEN1 endonuclease activity, as kinetics of accumulation of the 23-mer cleavage product are similar for both the guanine-containing and 8-oxoguanine-containing substrates upon incubation of the substrate with FEN1 (Fig. 3vII, *right and left panel respectively*). Graphical analysis of the data suggests a possible stimulation of FEN1 activity by the presence of the base lesion in the flap strand (Fig. 3vIII).

3.5.2 *The Effect of Thymine Glycol Lesions in the Flap Strand on FEN1 Flap Endonuclease Activity*

Thymine glycol, another frequently formed oxidative base damage, induces a significant alteration in the structure of double-stranded DNA and is known to block both DNA and RNA polymerases [33, 34]. We therefore tested whether this more distorting lesion would affect FEN1 activity. A triplex oligonucleotide substrate was constructed containing two thymine glycol lesions in a 5'-labelled flap strand. Substrates were

incubated with FEN1, and the endonuclease activity on modified and unmodified substrates was determined by the formation of flap cleavage products (Fig. 3wII, *6-mer*). As with 8-oxoguanine, the presence of thymine glycol lesions in a flap strand does not affect flap removal by FEN1 protein (Fig. 3wII). Again, graphical analysis of the data suggests a possible stimulation of FEN1 flap cleavage by the lesions (Fig. 3wIII).

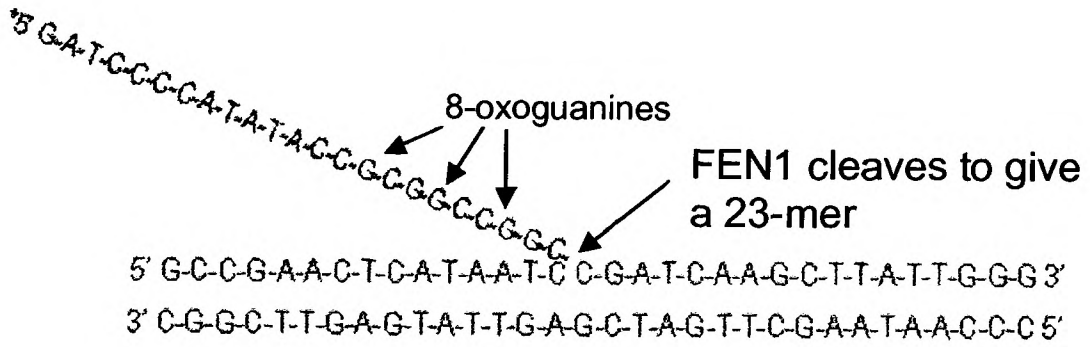


Fig. 3vI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

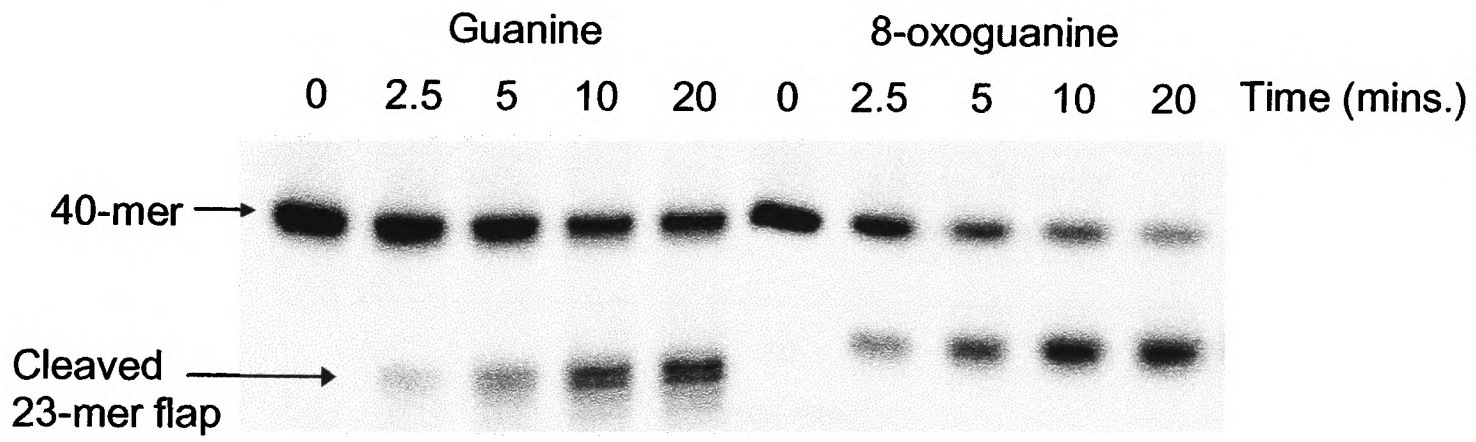


Fig. 3vII FEN1 cleavage of an 8-oxoguanine-containing and guanine-containing flap strand. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (right panel) or guanine (left panel) residues in the flap strand, was incubated for the indicated time with 950 fmol of FEN1. Reactions were stopped by the addition of an equal volume of gel loading buffer and products were analysed by electrophoresis in a 20% denaturing polyacrylamide gel.

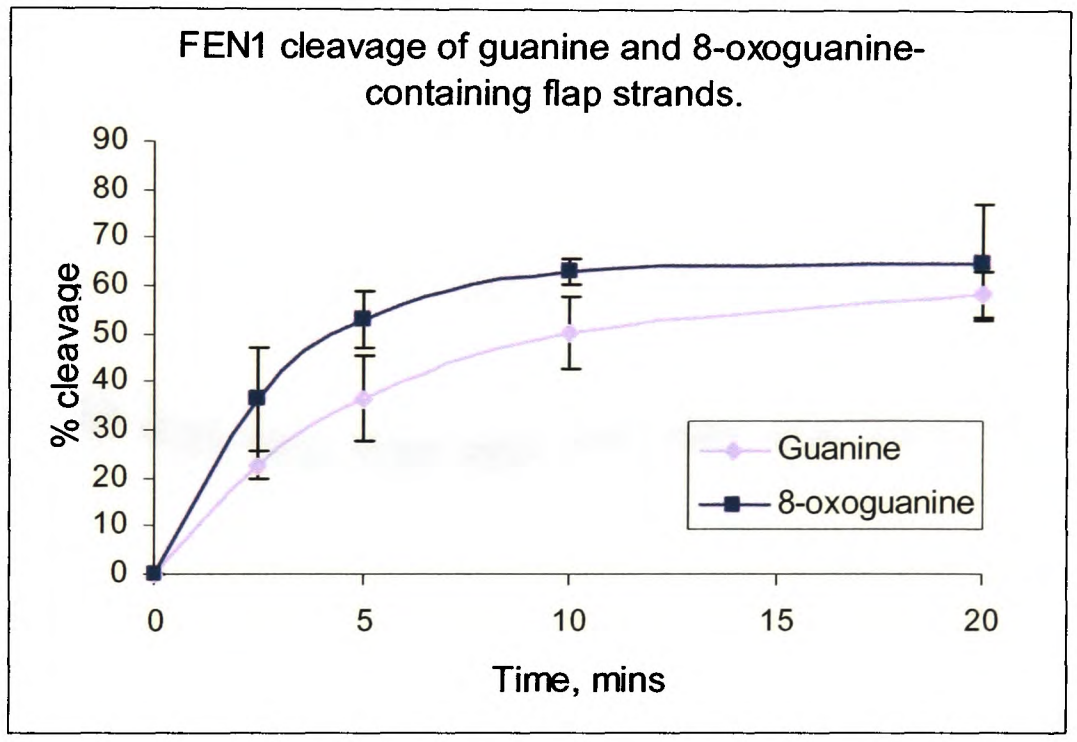


Fig. 3vIII Quantification of the data presented in Fig. 3vII. Graphical analysis of three independent experiments. The amount of cleavage product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

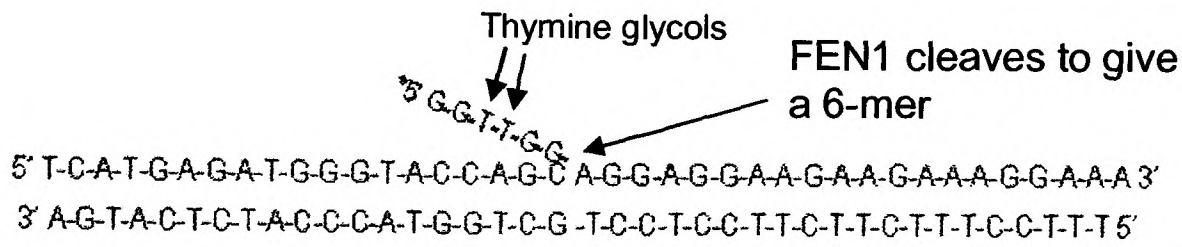


Fig. 3wI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

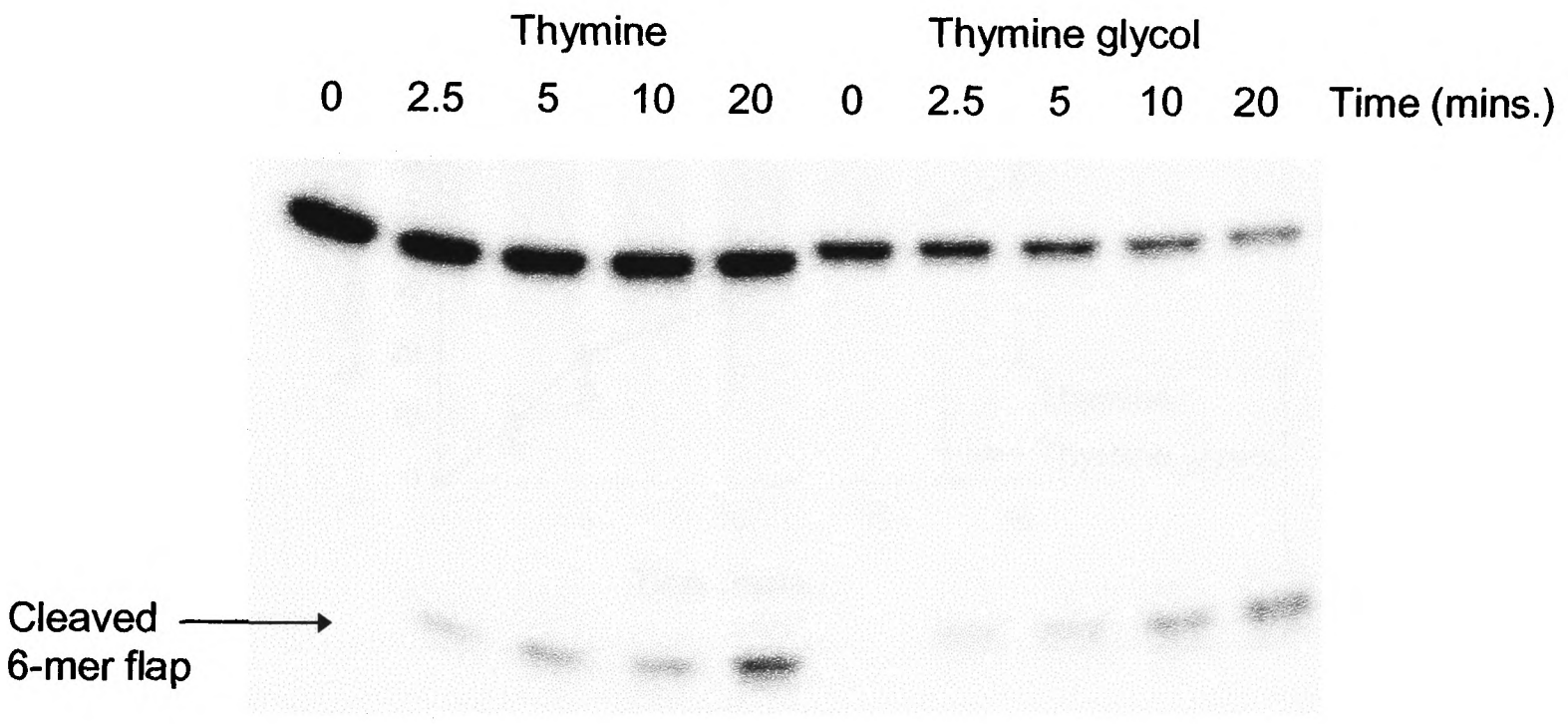


Fig. 3wII FEN1 cleavage of a thymine glycol-containing and a thymine-containing flap strand. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing thymine or thymine glycol residues in the flap strand, was incubated for the indicated time with 48 fmol of FEN1. Reactions were stopped by the addition of an equal volume of gel loading buffer and products were analysed by electrophoresis in a 20% denaturing polyacrylamide gel.

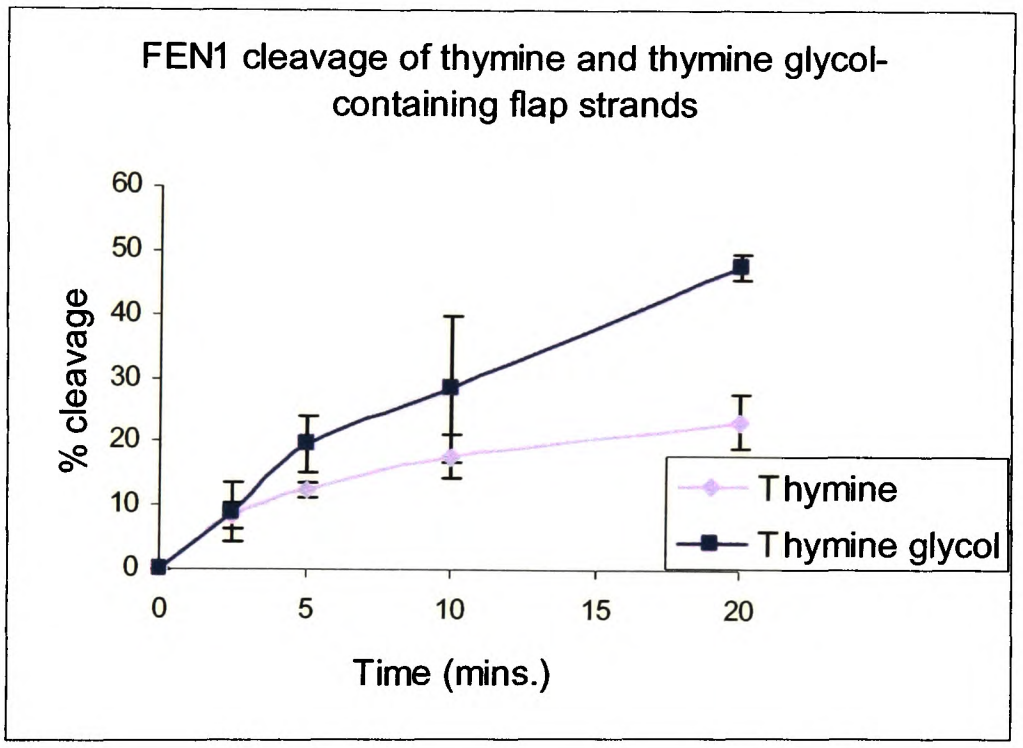


Fig. 3wIII Quantification of the data presented in Fig. 3wII. Graphical analysis of three independent experiments. The amount of cleavage product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

3.5 *Discussion*

The biological effects of ionising radiation are thought to arise from the formation of clustered DNA damage, ie. two or more lesions formed within one helical turn of the DNA, by a single radiation track. Such damage clusters have been suggested to pose problems for DNA repair pathways and as such have been implicated in contributing to the harmful effects of ionising radiation [231]. Repair of opposing clustered lesions has been studied extensively, due to their potential to form DNA double strand breaks (reviewed in [193, 232, 233]). However, the repair of multiple closely-spaced lesions on the same strand of DNA has received much less attention. To the best of our knowledge, only two papers have been published on the repair of tandem lesions [225, 234]. Both papers addressed repair of tandem lesions by purified bacterial and mammalian DNA repair enzymes, however neither of them investigated the repairability of such lesions by human whole cell extracts. To determine the mode of repair of such clustered damages by human whole cell extracts we designed a substrate that allowed us to monitor the repair of two closely-spaced lesions. We find that BER of an individual 8-oxoguanine lesion within a tandem lesion is predominantly initiated independently and proceeds to completion in the case of the 5' lesion in the tandem, whereas repair of the 3' lesion is delayed after incision of the AP site generated during processing. In order to understand the mechanism for such delay we analysed the rate of repair of both lesions in PARP-1^{+} and PARP-1^{-} cell extracts. It was found that repair of both lesions proceeded much faster in PARP-1^{-} cell extracts, as had previously been shown [116], and also that the rate of repair of each lesion was similar for both lesions in PARP-1^{-} extracts, whereas there was

a delay in the rate of repair of lesion 2 in PARP-1$^+$ extracts, suggesting that PARP-1 may play a role in the delay of repair of lesion 2. To further elucidate this we next analysed the rate of DNA synthesis by Pol β in the presence of an upstream 8-oxoguanine, as well as PARP-1 affinity and turnover on such a substrate, and found no effect of a neighbouring 8-oxoguanine lesion on these reactions. Another possibility for the uncoupled repair of lesion 2 was the processing of the individual 8-oxoguanine lesions by OGG1. Since different rates of removal of damaged bases located near strand breaks were previously reported for UDG [226] and human endonuclease III homolog (hNTH) [225], we further proposed that a similar mechanism may apply to OGG1 and that the rate of removal of 8-oxoguanine depends on its orientation towards the neighbouring incised AP site. In agreement with previous reports that show different rates of removal of damaged bases located near strand breaks, we found that a similar mechanism applies to OGG1, and that the rate of removal of 8-oxoguanine depends on its orientation towards a neighbouring incised AP site. To study this, oligonucleotide substrates containing 8-oxoguanine residues near to a strand break were tested, to mimic the situation during repair of the lesions in whole cell extracts, with purified OGG1. Both the excision rates and the binding affinity of OGG1 for the different substrates were analysed. It was demonstrated that 8-oxoguanine located near the 5'-terminus was removed at least four times faster than an 8-oxoguanine residing near the 3'-terminus. We found that OGG1 shows a four-fold reduction in excision rate for an 8-oxoguanine situated near the 3'-terminus and also a two-fold reduction in affinity for the same substrate. Therefore, the different activity of OGG1 towards the two 8-oxoguanines in the tandem lesion is not

responsible for the observed delay in repair of lesion 2. However, as a result of the reduced excision rate of an 8-oxoguanine near a 3'-terminus by OGG1, the 8-oxoguanine residue may persist longer.

In summary, these studies indicate that tandem lesions, formed on the same strand of DNA by ionising radiation, are substrates for base excision repair pathways rather than nucleotide excision repair. The presence of vicinal base lesions on the same strand during short-patch base excision repair has an asymmetric effect on the rate of repair of the individual lesions. A lesion situated 5' to another lesion is repaired efficiently and at the same rate as a single lesion. Whereas a lesion situated 3' to another lesion exhibits a delay in repair after excision of the damage and incision of the AP site by APE1. As already noted, it has previously been shown that PARP-1 binds only weakly to BER intermediates when single nucleotide BER proceeds normally to completion but binds strongly to the BER intermediate when single nucleotide BER is stalled by a block in the excision step [224]. A delay in repair could attract PARP-1 to the repair intermediate which could cause the observed delay in repair of lesion 2. This is in good agreement with the data showing that repair of both lesions occurs at a similar rate in PARP-1$^-$ extracts, with little accumulation of repair intermediates (Fig. 3h). These studies also highlighted the fact that OGG1 displays different activity towards 8-oxoguanine lesions situated close to a strand break, it was shown that 8-oxoguanine located near the 5'-terminus was removed at least four times faster than an 8-oxoguanine residing near the 3'-terminus, which could lead to a persistence of some 8-oxoguanine residues.

During long-patch base excision repair, the region of DNA downstream of the lesion is displaced by polymerase synthesis and is recognised and cleaved by the flap endonuclease FEN1. In the case of clustered lesions present on the same strand of DNA during long-patch BER, strand displacement synthesis would cause these lesions to be displaced as part of the flap strand that is a substrate for FEN1. As FEN1 has previously been shown to be affected by the presence of some modifications in the flap strand we tested whether the base lesions 8-oxoguanine and thymine glycol would affect FEN1 tracking and cleavage. The results show that the presence of these base lesions in a flap strand does not affect FEN1 tracking or cleavage and would therefore not affect this step of long-patch base excision repair. It was also noted that the presence of lesions in the flap strand caused a slight stimulation of FEN1 activity, this could be due to changes in the base stacking around the damages. It should be noted that different concentrations of FEN1 were used in the 8-oxoguanine and thymine glycol assays, due to the different lengths of flap strand in the substrates. This accounts for the difference in the shape of the curves.

Chapter 4

Repair of Opposing Lesions: the Effects of 8-oxoguanine

4.1 *Introduction*

The effects of opposing clustered lesions on DNA glycosylases have been well studied due to their propensity to form double-strand breaks [192-194, 235]. However, relatively little is known about the repair of such lesions. Abasic sites can arise in DNA in close opposition to 8-oxoguanine either during repair of mismatched bases (ie. 8-oxoG:A mismatches) or, more frequently as a consequence of ionising radiation exposure. During repair, different types of clustered lesions, including oxidised bases and/or abasic sites (such as 5-hydroxycytosine opposite 8-oxoguanine or an AP site opposite 8-oxoguanine) will be converted to a lesion containing 8-oxoguanine opposite a single-strand break containing a 5'-sugar phosphate, thus inhibiting excision of the 8-oxoguanine. Depending on the status of the 5'-sugar phosphate (ie. normal or oxidised /reduced) this lesion will be a substrate for either short-patch or long-patch base excision repair. The fidelity and the rate of bypass of 8-oxoguanine by Pol β has been studied before in primer extension reactions [24], however, the ability of Pol β to incorporate opposite to 8-oxoguanine in a single nucleotide repair gap has not been studied. Also, the effects of the presence of 8-oxoguanine in a repair gap on the dRP lyase activity of Pol β and the efficiency of DNA ligase has not been studied. To address these questions, a substrate containing 8-oxoguanine opposite to an uracil residue was constructed. Treatment of the substrate with UDG and APE1 creates a substrate containing an incised AP site directly

opposite to an 8-oxoguanine residue (Fig. 4aI). Repair of this substrate can then be monitored to study the effect of the opposing lesion on the enzymes of the base excision repair pathway.

4.2 *Results*

4.2.1 *8-oxoguanine in a Template Strand does not Affect Either the Efficiency or Fidelity of Short-patch Base Excision Repair*

During short-patch base excision repair DNA polymerase β incorporates a single nucleotide into the repair gap, using the opposite strand as a template, and removes the 5'-sugar phosphate, after which DNA ligase is able to seal the DNA ends. To investigate whether the presence of an 8-oxoguanine residue in the repair gap would affect short-patch BER, a substrate oligonucleotide duplex containing a strand break with a 5'-sugar phosphate moiety opposite to an 8-oxoguanine on the template strand was constructed.

Firstly, the DNA repair synthesis step was examined and it was found that, at the concentrations used, Pol β could efficiently bypass 8-oxoguanine and was able to extend synthesis beyond one nucleotide (Fig. 4aII). In order to examine the specificity of insertion opposite to 8-oxoguanine by Pol β, an assay in which the unlabelled substrate was incubated with equal molar amounts of dCTP and dATP, one of which was labelled, was performed, and incorporation of only dCMP was observed (Fig. 4bII, *left panel*). Moreover, we were able to show an equal efficiency of incorporation opposite to guanine

and 8-oxoguanine (Fig. 4cII). It was thus concluded that 8-oxoguanine situated in the repair gap does not affect either the efficiency or the fidelity of repair synthesis by Pol β.

To investigate whether an opposing 8-oxoguanine affects removal of the 5'-sugar phosphate or the ligation step of repair, short-patch base excision repair was reconstituted with purified APE1, Pol β and DNA ligase I. Incubation of the substrate with APE1 resulted in a 19-mer product generated by the incision of the oligonucleotide at the AP site (Fig. 4dII, *lane* 2). DNA polymerase β and DNA ligase I, when added to the reaction, stimulated the complete repair and restoration of the full length 30-mer product within 20 mins. for both the guanine (Fig. 4dII, *lanes 7-10*) and 8-oxoguanine-containing substrates (Fig. 4dII, *lanes 3-6*). It was therefore concluded that repair of the 5'-nicked AP site opposite to 8-oxoguanine is as effective as repair of the control oligonucleotide duplex containing a 5'-nicked AP site opposite to guanine.

Therefore, repair of a lesion in the presence of an 8-oxoguanine in the opposing strand can proceed in an efficient and error-free manner and such a damage cluster does not pose a threat to short-patch base excision repair. This result is important as such a substrate may be formed after excision of adenine, misincorporated opposite to an 8-oxoguanine during replication, by the Mut Y homologue (MYH) DNA glycosylase [69, 236]. In such a situation, the resulting repair gap could be filled correctly and the 8-oxoguanine could subsequently be repaired by OGG1.

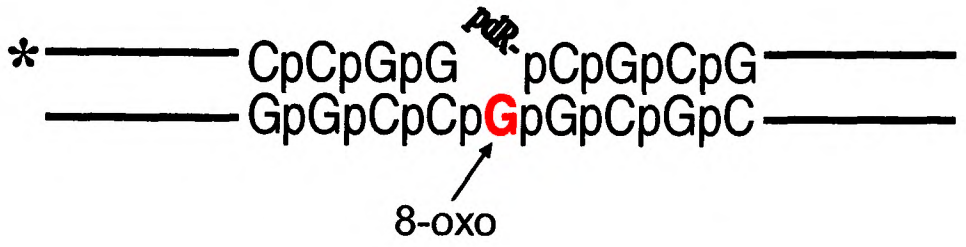


Fig. 4aI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

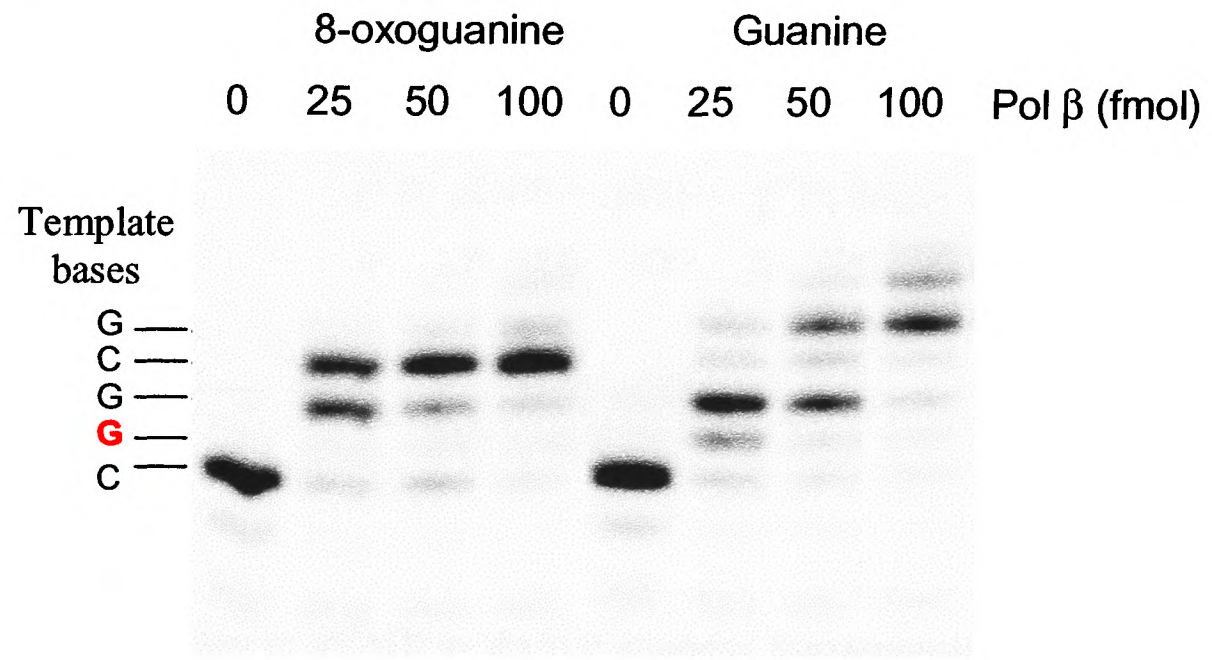


Fig. 4aII Incorporation opposite 8-oxoguanine and guanine by Pol β. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (left panel) or guanine (right panel) opposite to the single-strand break with 5'-sugar phosphate (pdR) was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel. The corresponding template bases are shown.

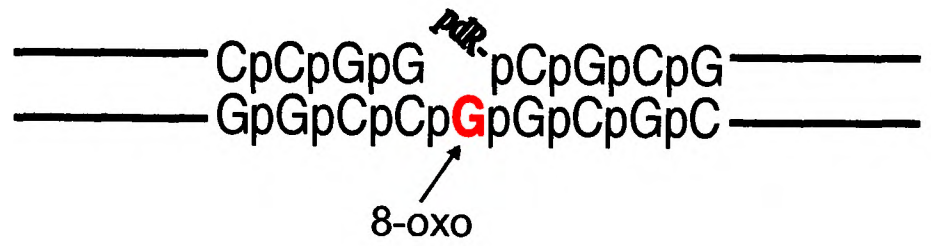


Fig. 4bI Schematic representation of the oligonucleotide substrate.

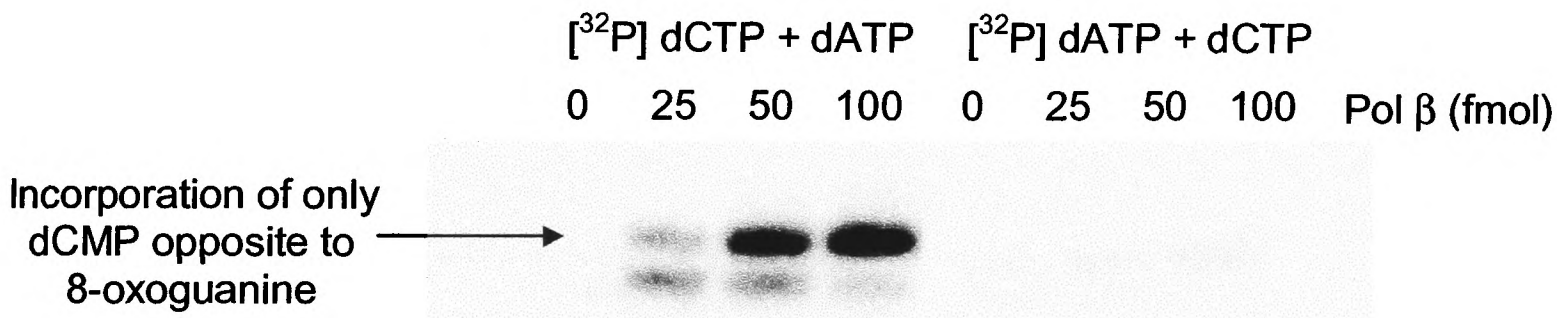


Fig. 4bII Insertion of dCMP or dAMP opposite 8-oxoguanine by Pol β. 1 pmol of the unlabelled substrate oligonucleotide duplex, containing 8-oxoguanine opposite to the single-strand break, was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods, in the presence of equal amounts (20μM each) of dCTP and dATP, of which either dCTP (left panel) or dATP (right panel) was labelled. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.



Fig. 4cI Schematic representation of the oligonucleotide substrate.

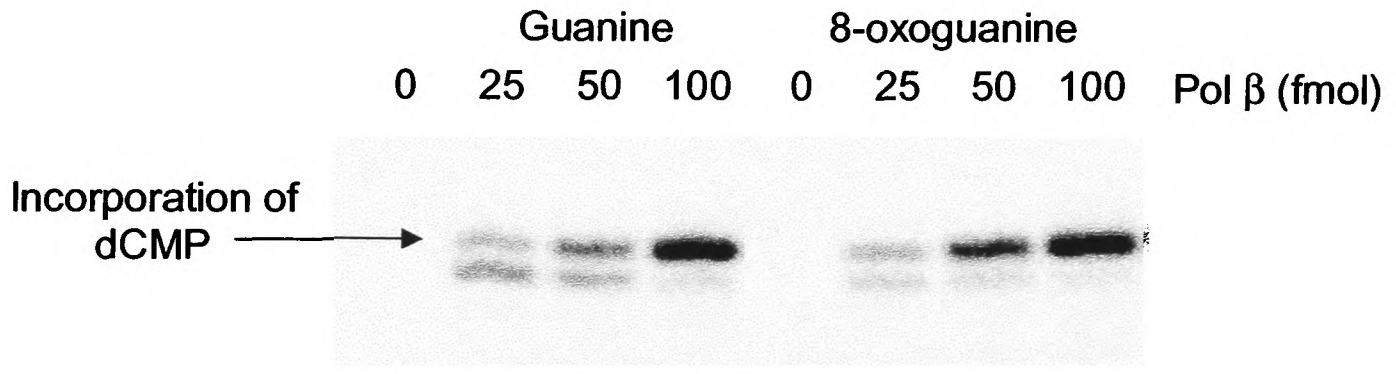


Fig. 4c Insertion of dCMP opposite guanine and 8-oxoguanine by Pol β. 1 pmol of the unlabelled substrate oligonucleotide duplex, containing guanine (left panel) or 8-oxoguanine (right panel) opposite to the single-strand break, was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods, in the presence of 20 μM labelled dCTP. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.



Fig. 4dI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

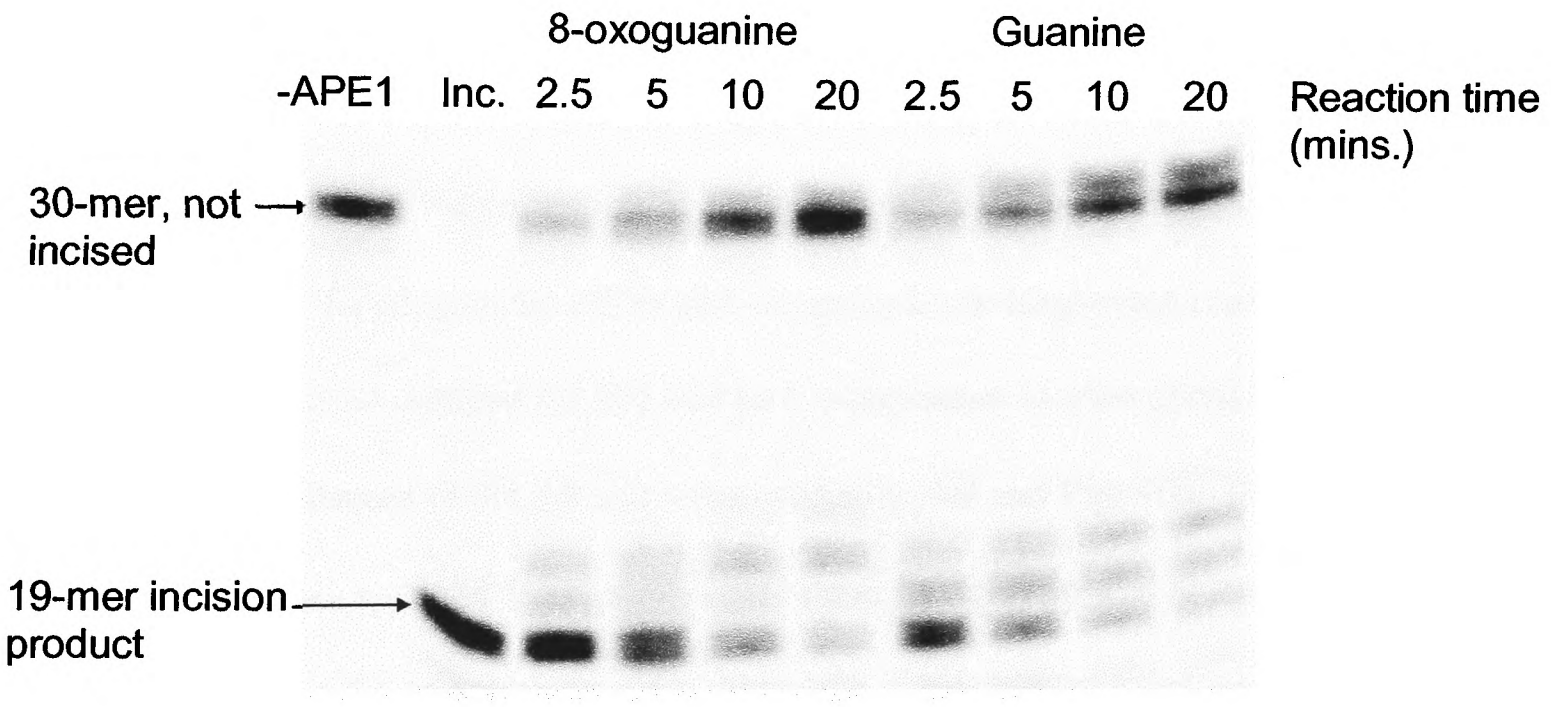


Fig. 4dII Reconstitution of short-patch repair of an AP site opposing 8-oxoguanine with purified proteins. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (left panel) or guanine (right panel) opposite to an abasic site (ϕ), was incubated for the indicated time in buffer containing magnesium, dNTPs, 60 fmol of APE1, 70 fmol of Pol β and 80 fmol of DNA ligase I. Reactions were stopped by the addition of an equal volume of gel loading buffer and, following incubation at 90°C for 3 mins., products were analysed by electrophoresis in a 20% denaturing polyacrylamide gel. Lane 1 contains substrate without APE1 added (-APE1) and lane 2 contains substrate incised (Inc.) with APE1.

4.2.2 *Effect of 8-oxoguanine in a Template Strand on Long-patch DNA Repair Synthesis by DNA Polymerase β*

Long-patch BER can repair regular AP sites and also altered AP sites that are not susceptible to β-elimination, as it relies on phosphodiester bond hydrolysis for excision. Sites with oxidised or reduced sugar groups and those with fragmented bases or sugars can be repaired only by long-patch BER [46]. This versatility may be especially important in the repair of DNA damage caused by ionising radiation, as it has been shown that approx. 80% of the γ-irradiation-induced DNA lesions that can be repaired by BER are resistant to dRP-elimination by DNA polymerase β and require FEN1 for processing [48]. To address the effect of 8-oxoguanine on long-patch repair, substrates containing an incised reduced AP site and an 8-oxoguanine located opposite to it or one nucleotide downstream of the AP site were used (Fig. 4eI and Fig. 4fI). Polymerase β is unable to remove a reduced 5'-sugar phosphate and incorporation of at least 2-4 nucleotides, using the opposing strand as template, is required to accomplish repair of the reduced AP site [83, 237].

No effect of reduction of the 5'-sugar phosphate on polymerase synthesis through an 8-oxoguanine located opposite to the SSB was found (Fig. 4eII); however, the position of 8-oxoguanine in the template strand had a dramatic effect on the efficiency of bypass. When 8-oxoguanine was moved one nucleotide downstream of the SSB, Pol β was able to insert a nucleotide opposite the base preceding 8-oxoguanine but was unable to efficiently bypass the 8-oxoguanine itself (Fig. 4fII, *left panel*). In a control experiment

with a substrate containing guanine instead of 8-oxoguanine Pol β readily generated strand displacement even at the lowest concentration used (Fig. 4fII, *right panel*).

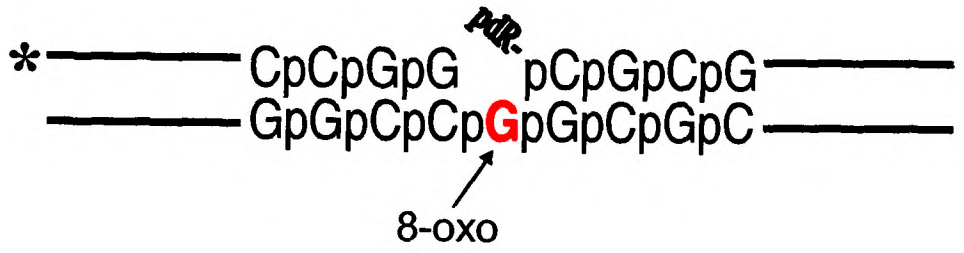


Fig. 4eI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

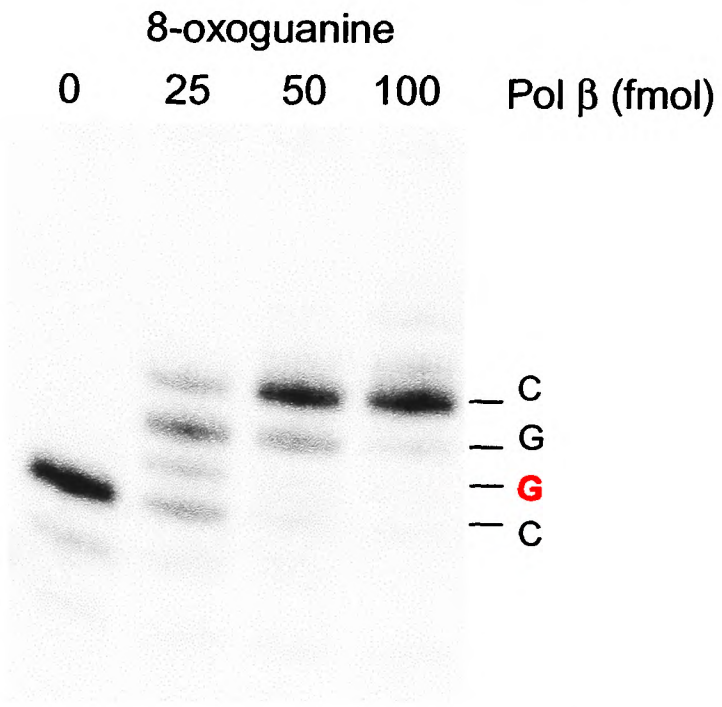


Fig. 4eII Effect of 8-oxoguanine on long-patch repair synthesis by Pol β. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine opposite to a SSB with a reduced 5'-sugar phosphate, was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

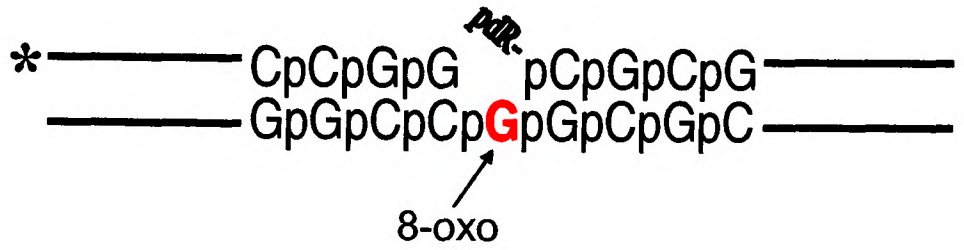


Fig. 4fI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

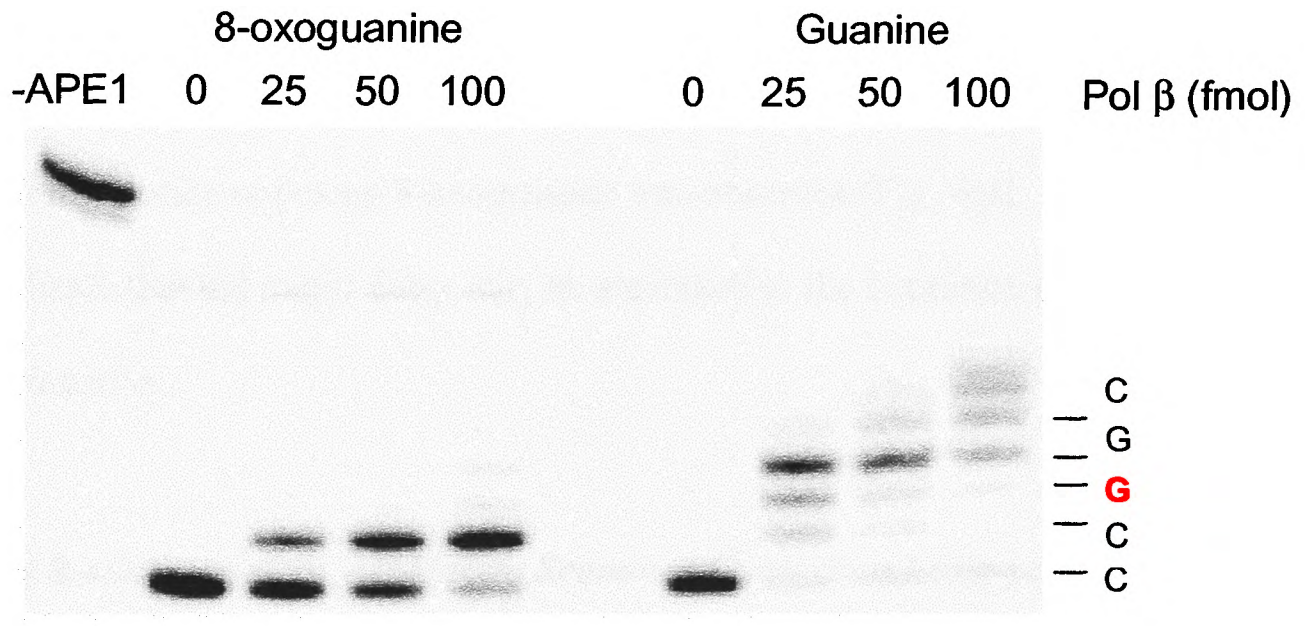


Fig. 4fII Effect of 8-oxoguanine on long-patch repair synthesis by Pol β. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (*left panel*) or guanine (*right panel*) positioned one nucleotide downstream opposite to a SSB with a reduced 5'-sugar phosphate, was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

4.2.3 *Effect of 8-oxoguanine in a Template Strand on Primer Extension by DNA Polymerase β*

The presence of 8-oxoguanine in the template strand may directly affect the polymerisation step itself or may indirectly affect the DNA synthesis reaction by reducing the efficiency of strand displacement by the DNA polymerase. To evaluate the contribution of each of these components to the observed block of repair synthesis, we tested whether 8-oxoguanine in the same sequence would block polymerisation by Pol β in a primer extension reaction. It was found that Pol β readily bypassed 8-oxoguanine (second nucleotide in a template strand) and only a moderate reduction in the rate of addition of the nucleotide opposing 8-oxoguanine was observed (Fig. 4gII, *lane 6*). These data indicate that the major delay may be attributed to the inhibition of the strand displacement reaction.

4.2.4 *Effect of 8-oxoguanine in a Different Sequence Context on Long-patch DNA Repair Synthesis by DNA Polymerase β*

If strand displacement efficiency plays a major role in the inhibitory effect of 8-oxoguanine, then the inhibitory effect should be less pronounced in an AT-rich sequence; due to the existence of only two hydrogen bonds between A:T base pairs, compared with three between G:C base pairs. To test this, a substrate containing 8-oxoguanine in an AT-rich sequence context was constructed (Fig. 4hI). Using this substrate, it was found that although 8-oxoguanine still inhibits the strand displacement reaction (Fig. 4hII, *left panel*), the effect is not as dramatic as that seen with the GC-rich substrate (compare Figs.

4fII and 4hII). The presence of an 8-oxoguanine in the template strand during long-patch repair synthesis will block Pol β, in certain sequence contexts. These results are consistent with published data which have shown the sequence-specificity of 8-oxoguanine repair [238].

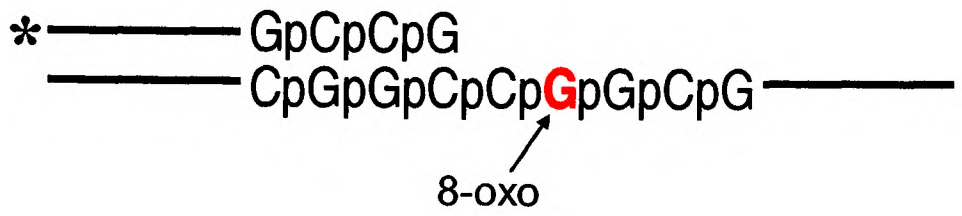


Fig. 4gI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

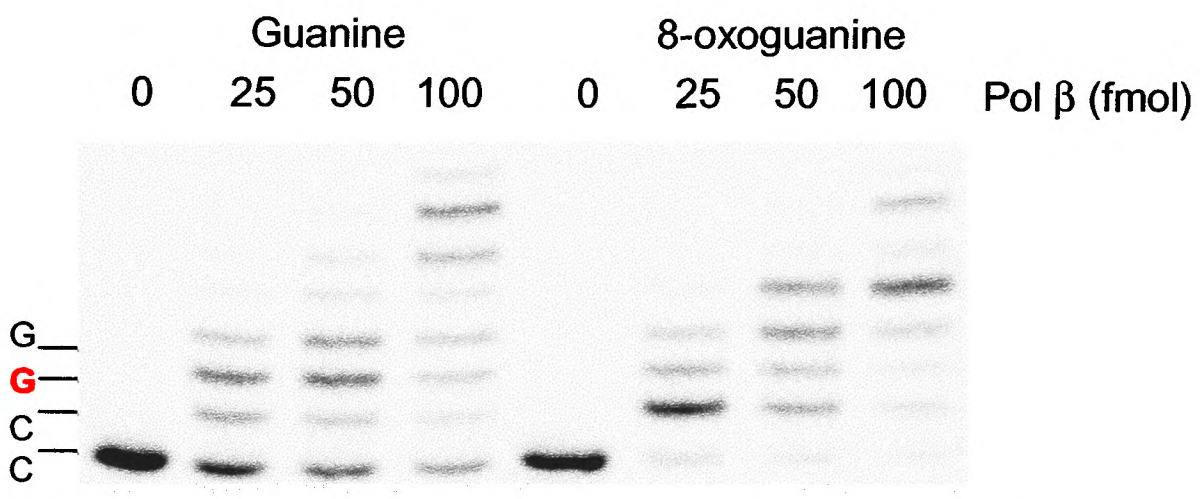


Fig. 4gII Bypass of 8-oxoguanine by Pol β during primer extension. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (right panel) or guanine (left panel) in the template strand, was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

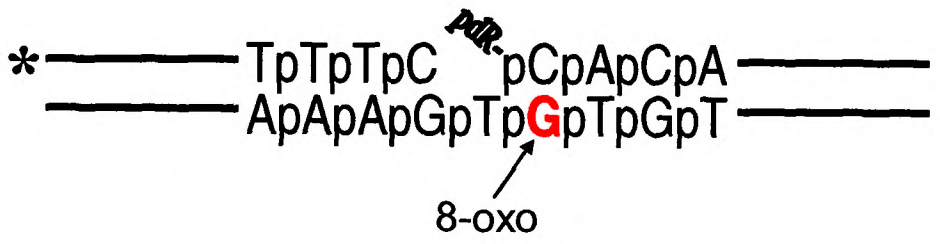


Fig. 4hI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

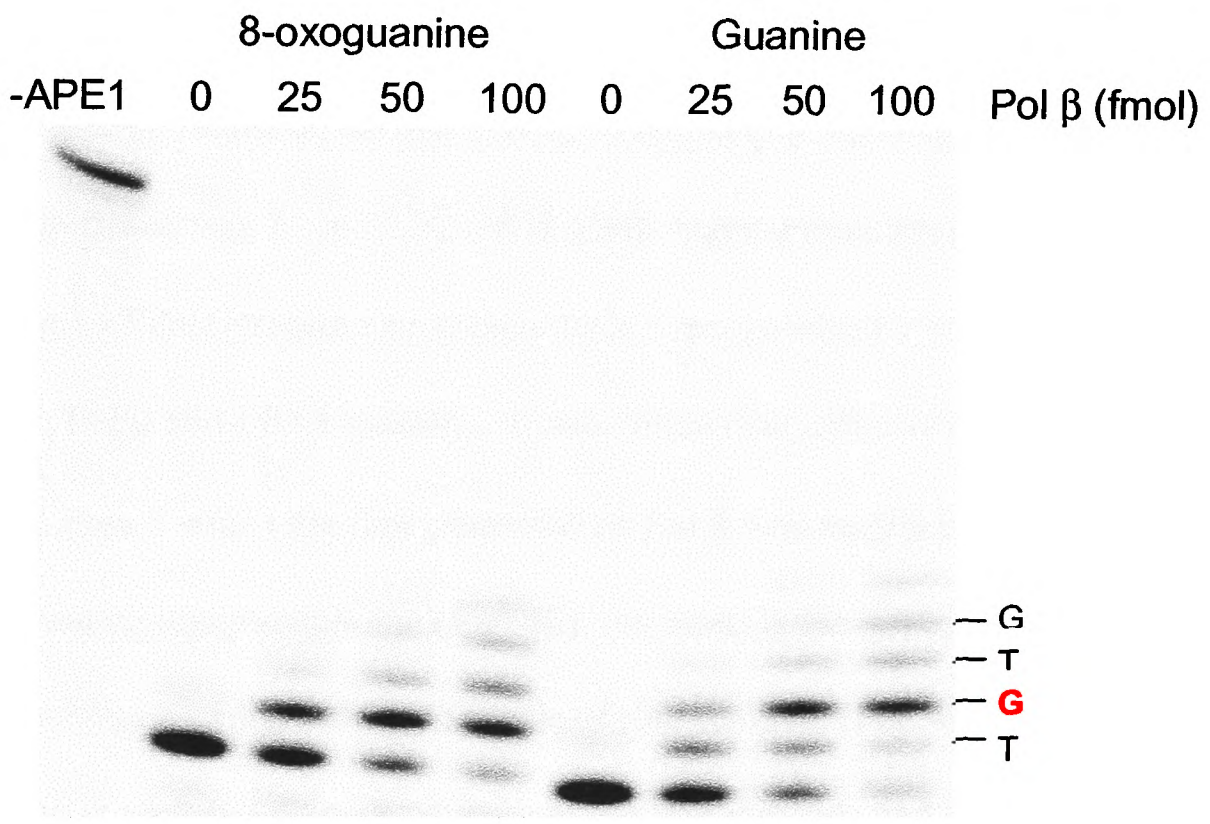


Fig. 4hII Effect of sequence context on 8-oxoguanine bypass during long-patch repair synthesis by Pol β. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (left panel) or guanine (right panel) positioned one nucleotide downstream opposite to a SSB with a reduced 5'-sugar phosphate, was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

4.2.5 *8-oxoguanine in a Template Strand Inhibits Flap Removal by FEN1*

During long-patch base excision repair, the incorporation of several nucleotides into the repair gap leads to the generation of a flap containing 5'-deoxyribose phosphate. The FEN1 protein alone is not able to remove the 5'-deoxyribose phosphate but releases it as part of a small flap oligonucleotide (2-4-mer) [237]. Impaired strand displacement caused by the presence of 8-oxoguanine in a template strand may also affect flap excision and, as a consequence, the entire long-patch BER pathway. To address this question, a substrate containing a reduced AP site and an 8-oxoguanine one nucleotide downstream in the opposite strand was 3'-end labelled (Fig 4iI) and the excision of the Pol β-generated flap by FEN1 protein was measured in a reconstituted system containing human APE1, Pol β and FEN1 proteins. It was found that, after APE1 cleaved the AP site (Fig. 4iII, *time 5 mins.*) the flap generated by Pol β was readily removed from the control substrate containing guanine (Fig. 4iII, *left panel*). However, 8-oxoguanine in the template strand significantly reduced the rate of flap removal (Fig. 4iII, *right panel*). Some cleavage is visible, however, with cleavage products two and three nucleotides shorter than the original APE1 cleavage product. This indicates that some bypass of the 8-oxoguanine by Pol β is occurring. This is in contrast to the previous experiments in which no bypass of the 8-oxoguanine was observed (Fig 4fII). It has been reported that strand displacement synthesis by Pol β is stimulated by the presence of FEN1, which may explain these results [207].

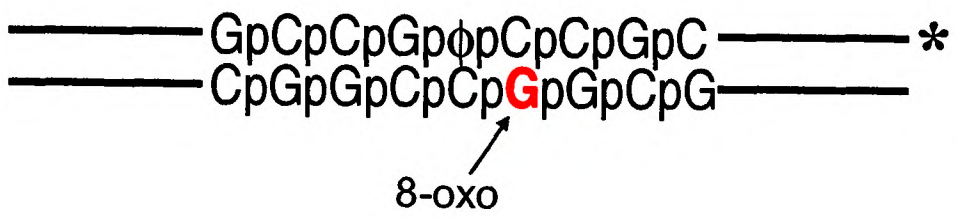


Fig. 4iI Schematic representation of the ^{32}P-3'-labelled (*) oligonucleotide substrate.

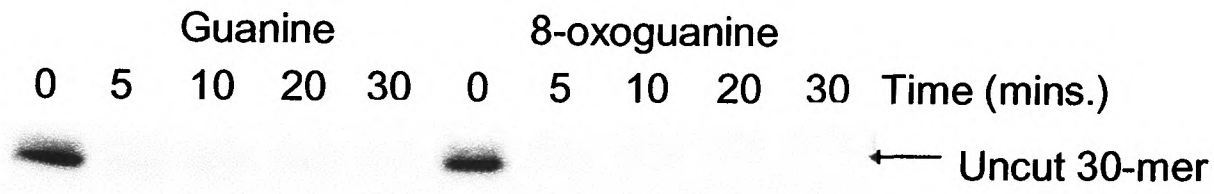




Fig. 4iII Effect of 8-oxoguanine on the FEN1 step of long-patch repair synthesis. 1 pmol of the 3'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (right panel) or guanine (left panel) positioned one nucleotide downstream opposite to the reduced AP site (ϕ) was incubated for the indicated time at 37°C in buffer containing magnesium, dNTPs, 100 fmol of APE1, 25 fmol of Pol β and 60 fmol of FEN1 under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer, and following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

4.2.6 *Effect of 8-oxoguanine in a Template Strand on Long-patch DNA Repair Synthesis by DNA Polymerase δ*

The role of different DNA polymerases in long-patch base excision repair in the cellular context is still unclear. Although it was recently demonstrated that Pol β is responsible for the initiation of both short- and long-patch BER [102], it is still uncertain as to the polymerase responsible for further incorporation during long-patch BER [239]. Evidence shows that long-patch DNA synthesis can be catalysed by Pol β or by Pol δ/ε [46-48, 106]. It was therefore interesting to test whether 8-oxoguanine would also interfere with strand displacement by Pol δ. Using the same oligonucleotide substrates (Fig. 4jI) as used for Pol β (Fig. 4f), strand displacement synthesis by Pol δ was monitored and it was found that the polymerisation reaction was nearly completely blocked after addition of the first nucleotide (Fig. 4jII, *left panel*). Whereas with a control substrate, containing guanine, efficient strand displacement synthesis was observed, resulting in complete strand displacement and accumulation of the 30-mer full length product (Fig. 4jII, *right panel*).

By itself Pol δ is a poorly processive enzyme due to its unstable interaction with the DNA substrate, however, a physical interaction with the processivity factor PCNA leads to a catalytically competent processive holoenzyme [240]. The consequence of such an interaction is a marked increase in the processivity [241]. PCNA enhances processivity both by modestly increasing the rate of single nucleotide incorporation [242] and by dramatically decreasing the dissociation of Pol δ from template-primers [243]. It is

thought that PCNA performs this latter function by acting as a sliding clamp that tethers Pol δ to DNA during replication [244]. It has been reported that PCNA increases Pol δ synthesis past 8-oxoguanine on a primer-template substrate [245]. However, as we show here, an 8-oxoguanine in the template strand during strand displacement synthesis by Pol δ is a significant block to the polymerase even in the presence of PCNA.

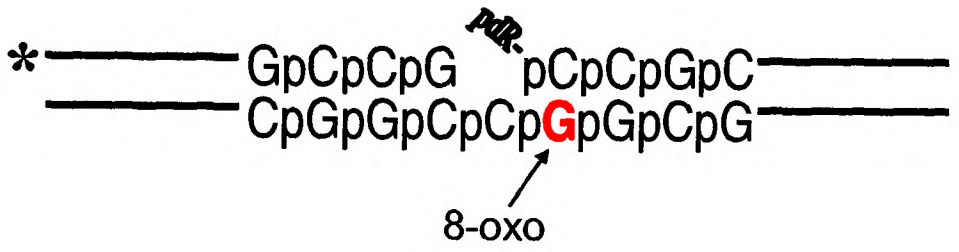


Fig. 4jI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

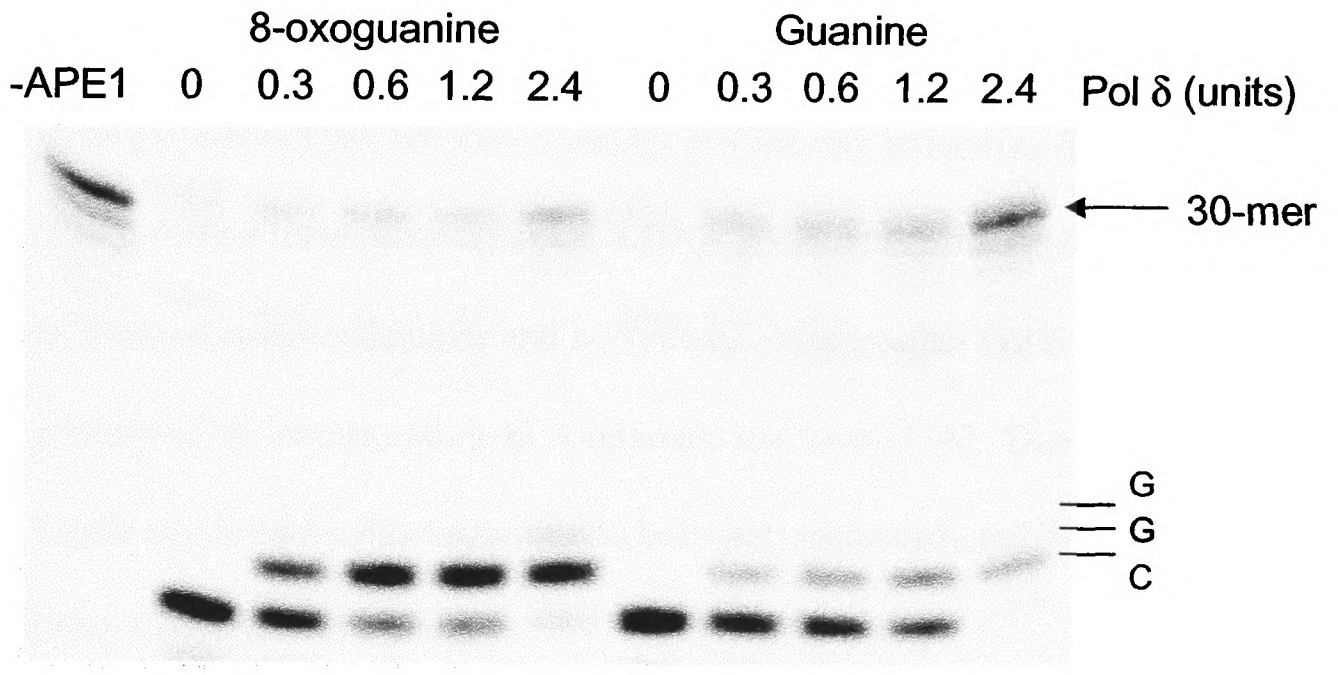


Fig. 4jII Effect of 8-oxoguanine on long-patch repair synthesis by Pol δ. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (left panel) or guanine (right panel) positioned one nucleotide downstream opposite to a SSB with a reduced 5'-sugar phosphate, was incubated for 20 mins. at 37°C with the indicated amount of Pol δ in the presence of 100 ng of PCNA under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer, and following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

4.2.7 *Effect of 8-oxoguanine in a Template Strand on Long-patch DNA Repair Synthesis by DNA Polymerase η*

How else might cells overcome such a block to strand displacement and long-patch repair? Studies have shown that there are low fidelity, distributive polymerases in both prokaryotes and eukaryotes that are involved in translesion DNA synthesis [129-131]. The *XPV* gene encodes Pol η, a DNA polymerase that is specialised for replicating past certain lesions. The activity of purified human Pol η was examined on several types of DNA lesions and was found to bypass 8-oxoguanine [157]. It was also shown that yeast and human Pol η replicate DNA containing 8-oxoguanine efficiently and accurately by inserting a cytosine across from the lesion and by proficiently extending from this base pair [158]. Pol η differs from other replicative DNA polymerases in its ability to replicate past 8-oxoguanine efficiently and accurately. Mammalian Pol δ and Pol α bypass 8-oxoguanine by incorporating an A opposite the lesion [24]. Due to the block of long-patch repair synthesis by 8-oxoguanine to both polymerases β and δ, Pol η was tested for ability to bypass the lesion.

Pol η appears to bypass the 8-oxoguanine lesion efficiently with only a slight stalling before the lesion being observed (Fig. 4kII, *left panel*). It has been reported that Pol η predominantly inserts C opposite to 8-oxoguanine, with occasional incorporation of A. However, evidence suggests that the extension efficiency of Pol η is greatly reduced from 3' mispaired bases [158]. Therefore translesion synthesis across 8-oxoguanine by Pol η would most likely result in an error-free bypass of the lesion. The ability of Pol η to

efficiently synthesise DNA opposite to an 8-oxoguanine lesion either during repair or replication should have an impact on lowering mutagenesis resulting from its misreplication. Indeed, spontaneous mutation rates are elevated in the absence of Pol η in the yeast *ogg1* mutant [158].

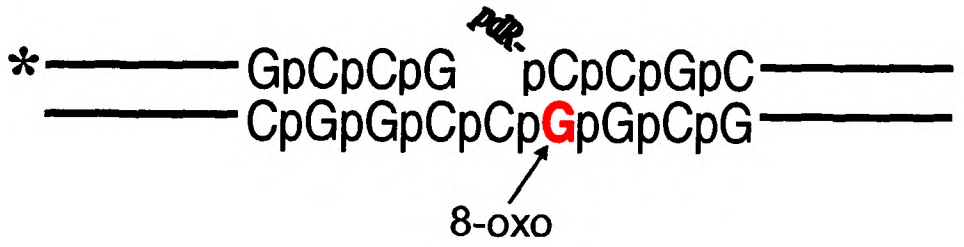


Fig. 4kI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

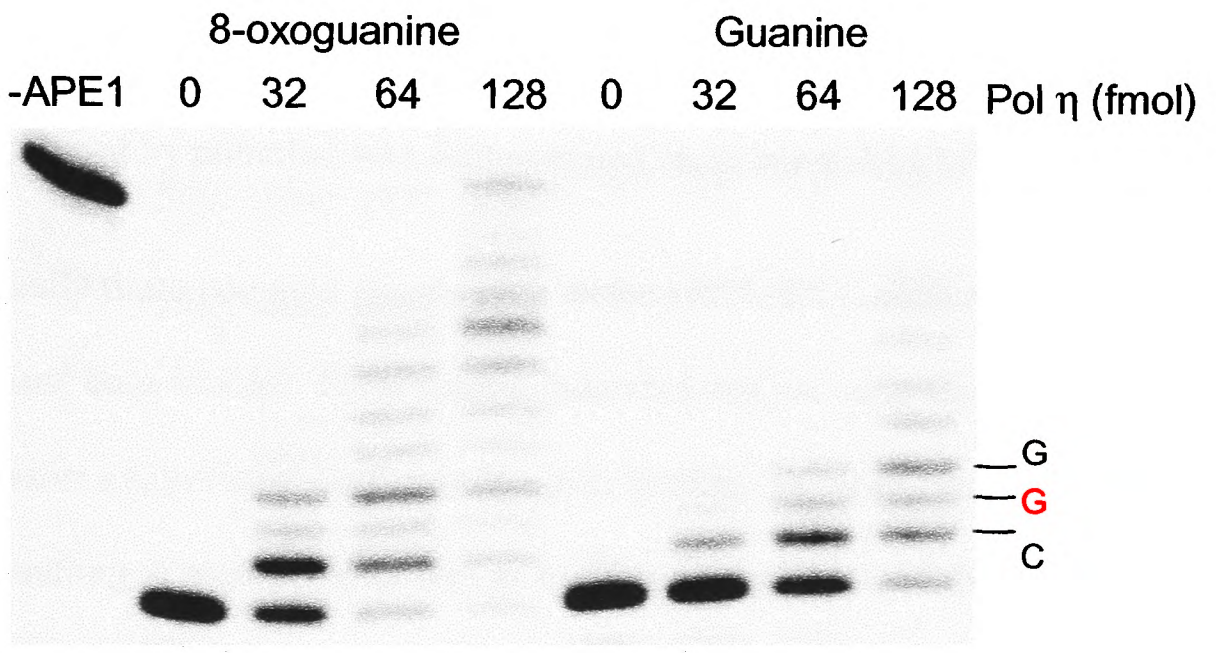


Fig. 4kII Effect of 8-oxoguanine on long-patch repair synthesis by Pol η. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing 8-oxoguanine (left panel) or guanine (right panel) positioned one nucleotide downstream opposite to a SSB with a reduced 5'-sugar phosphate, was incubated for 20 mins. at 37°C with the indicated amount of Pol η under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

4.3 *Discussion*

About 70-80% of radiation induced DNA lesions are the result of reactive oxygen species generated by hydrolysis of water in the vicinity of DNA [5]. Although it was earlier proposed that a high density of hydroxyl radicals induced by irradiation may induce multiple closely spaced DNA lesions [246], until recently these lesions were considered as regular oxidative lesions that can be repaired by BER. The first indication of the unique nature of radiation induced DNA damage emerged from the theoretical studies of radiation-induced track structures [231, 247]. Later on, the existence of complex DNA lesions induced by radiation was demonstrated experimentally [203, 248, 249].

Theoretically these complex lesions may include different combinations of base lesions, or SSBs and base lesions. Early studies indicated that the complexity of clustered DNA lesions poses a significant challenge to repair systems and may cause both infidelity of repair, resulting in mutations, as well as inefficient repair potentially leading to the formation of deletions and chromosome rearrangements [250-252]. In this study the repairability of opposing lesions containing 8-oxoguanine, by different BER pathways, was investigated. It is revealed that the damaging effect of such lesions depends on the sequence in which they occur as well as on the relative positioning of primary lesions within a cluster. Short-patch BER is normally accomplished by removal and replacement of a single-nucleotide [98]. For this repair pathway, the major threat may be the repair of simultaneous modifications of complementary bases. Processing of one of the lesions would generate an AP site or SSB that would block removal of the opposing modified base [253-255] and would lead to the presence of a modified base in the repair gap on the

template strand. To model this scenario the repair of an AP site located opposite to 8-oxoguanine was studied. It was found that 8-oxoguanine in a repair gap does not affect either the fidelity (dCMP is normally incorporated opposite to 8-oxoguanine) or the efficiency of repair (in a reconstituted system the rate of repair of an AP site located opposite to guanine or 8-oxoguanine is similar). Although this is the first data on the tolerance of both the dRP lyase activity by Pol β, and the efficiency of DNA ligase to the presence of 8-oxoguanine in the repair gap, the fidelity and the rate of bypass of 8-oxoguanine by Pol β have been studied before by several groups. It is generally accepted now that 8-oxoguanine bypass depends on the DNA polymerase and the structure of the substrate used [24, 158]. Indeed, Pol β has evolved as a distributive polymerase designed to fill one nucleotide gaps in short-patch BER, and has increased activity and fidelity on single-nucleotide gap substrates [97, 256] which is in good agreement with this data. However, when the fidelity of bypass was measured on substrates with longer gaps, or in primer extension reactions catalysed by Pol β, both the efficiency and fidelity of DNA synthesis were reduced [256].

To accomplish repair of chemically modified AP sites via long-patch BER either Pol β or Pol δ should incorporate at least 2 nucleotides into the repair gap before FEN1 would be able to remove the generated two-nucleotide flap [257]. As is shown here, 8-oxoguanine located one nucleotide downstream from an incised AP site in the template strand strongly inhibits strand-displacement DNA synthesis by both DNA polymerases β and δ, (Fig. 4fII and Fig. 4jII) and as a consequence inhibits flap excision, thus blocking long-

patch BER (Fig. 4iII). This is an unexpected result, because from previous studies it was known that both Pol β and Pol δ [24] can bypass 8-oxoguanine reasonably well in a primer extension reaction. Indeed, reasonable bypass of 8-oxoguanine by Pol β in a primer extension reaction was also observed (Fig. 4gII). However, 8-oxoguanine bypass was significantly reduced under strand-displacement conditions. In support of the "strand displacement model" for the explanation of the bypass block by 8-oxoguanine present in the repair gap, it is demonstrated that the severity of the polymerase block depends on the sequence around 8-oxoguanine: the more G:C base pairs, the more difficult it is for DNA polymerase to unwind DNA and at the same time to bypass 8-oxoguanine. However, the error-prone translesion synthesis polymerase, Pol η, was able to bypass the 8-oxoguanine in the template strand during strand displacement synthesis (Fig. 4kII). It may be possible that tranlesion synthesis polymerases may act to rescue the block seen here by 8-oxoguanine to long-patch base excision repair.

Chapter 5

Repair of Opposing Lesions: the Effects of Thymine Glycol

5.1 *Introduction*

Thymine glycol is formed as a product of exposure to ionising radiation and other oxidative stresses and is a major replication-interfering lesion *in vivo*, as it can terminate DNA replication and transcription, and is cytotoxic [33, 34]. Thymine is an easily oxidised base, and Tg is frequently found as a component of clustered lesions [203, 249]. Studies on incorporation of nucleotides opposite to thymine glycol have shown that there is some incorporation of nucleotides opposite to the lesion, and that insertion of the correct nucleotide, dATP, is favoured [33, 36, 159]. However, little is known about the efficiency of incorporation opposite to thymine glycol by Pol β, and also the effect of thymine glycol on APE1 and DNA ligase III, key enzymes in the major base excision repair pathway. In order to address these questions, a single thymine-containing oligonucleotide was chemically modified with osmium tetroxide to introduce specifically a single thymine glycol lesion. A duplex oligonucleotide substrate containing thymine glycol opposite to an uracil residue was constructed. As previously, treatment of the substrate with UDG and APE1 creates a substrate containing a single-strand break directly opposite to a thymine glycol lesion. Repair of the gap in the presence of a template thymine glycol residue can then be monitored, using purified human BER proteins or human cell extracts, to study the effect of thymine glycol on the enzymes of the base excision repair pathway.

5.2 *Results*

5.2.1 *Thymine Glycol in a Template Strand Affects the Efficiency of DNA Synthesis by DNA Polymerase β*

In human cells, after incision of an AP site by APE1, base excision repair is continued by Pol β, which incorporates a single nucleotide into the repair gap [91, 98, 102]. Using an oligonucleotide duplex substrate, containing an AP site or a preincised AP site with a 5'-sugar phosphate moiety opposite thymine glycol on the template strand (Fig. 5aI), it was first investigated whether thymine glycol located opposite to the damage would affect DNA repair synthesis by Pol β. Surprisingly, it was found that purified human Pol β is able to incorporate the first nucleotide, opposite to thymine glycol, although at a slower rate than on the undamaged template (Fig. 5aII). Incorporation on the thymine glycol-containing substrate by Pol β is reduced by approximately 3-fold compared to the control substrate, containing thymine, with 25 fmoles of Pol β per reaction (Fig. 5aII, *lanes 2 and 6*), however at higher concentrations Pol β was more than 80% efficient.

5.2.2 *Incorporation Opposite to Thymine Glycol*

The important question remains whether the incorporation opposite to thymine glycol is error-free. Using reactions containing only one out of four deoxyribonucleotide triphosphates we next analysed which nucleotide is incorporated and found that incorporation by Pol β was very specific with the dAMP residue exclusively incorporated opposite to thymine glycol (Fig. 5bII, *lane 1*).

These data are in agreement with those for other polymerases tested, such as Pol α, which can incorporate dAMP opposite thymine glycol lesions, however, more than 30% of the Pol α reaction was inhibited before insertion of a nucleotide opposite the lesion [33].

5.2.3 *Removal of the dRP Residue by DNA Polymerase β is Unaffected by Thymine Glycol*

At the next step of short-patch BER Pol β catalyses removal of the 5'-dRP residue. To study the effect of thymine glycol on this reaction a 3'-end labelled substrate was constructed, containing thymine or thymine glycol opposite to the preincised AP site (Fig. 5cI). Removal of the dRP from this substrate will generate an 11-mer labelled fragment, while a dRP-containing fragment will migrate slightly slower on a gel. After reactions all samples were treated with sodium borohydride to stabilise the AP sites and prevent their degradation during electrophoresis. Under these conditions no inhibitory effect of thymine glycol on dRP removal by Pol β was found. In fact, removal of the dRP from thymine glycol-containing substrate was even slightly faster than from control substrates containing thymine (Fig. 5cII).

5.2.4 *Ligation of the Nick is Reduced in the Presence of Thymine Glycol*

During the last step of BER, DNA ligase III seals the DNA ends broken during repair. The ability of thymine glycol to affect DNA ligase III was tested with a substrate simulating the last step of BER: an oligonucleotide duplex containing a single strand break opposite to thymine or thymine glycol (Fig. 5dI). Only a moderate (1.5-fold)

reduction of the ligation rate for a substrate containing thymine glycol compared to the control substrate containing a normal thymine was found (Fig. 5dII, *conversion of the 22-mer to the 45-mer product*). Thus, at certain enzyme concentrations both purified Pol β and DNA ligase III are able to catalyse repair of an AP site opposite thymine glycol.

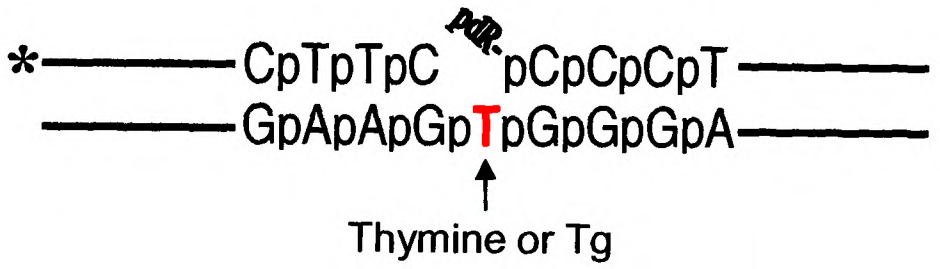


Fig. 5aI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

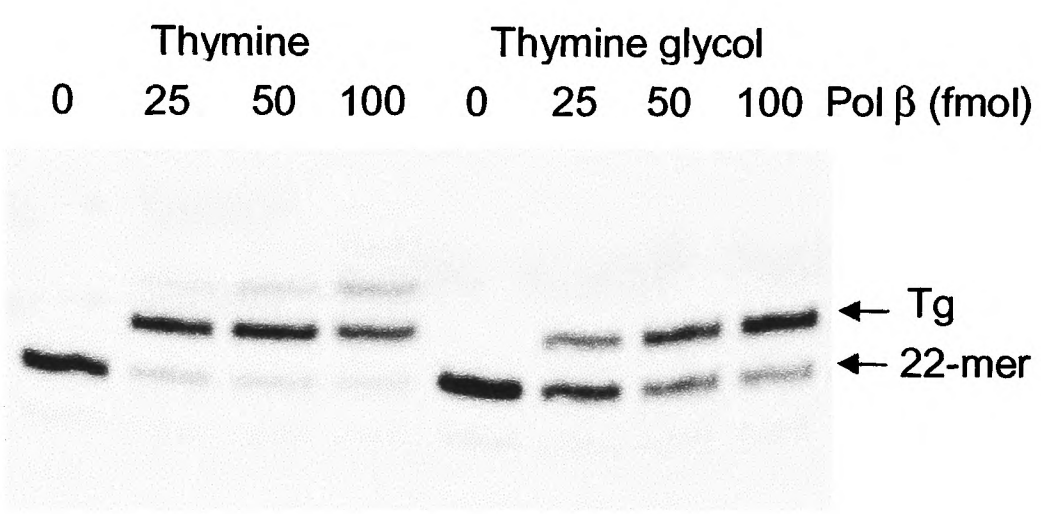


Fig. 5aII Incorporation opposite thymine and thymine glycol by Pol β. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing thymine (left panel) or thymine glycol (right panel) opposite to the pre-incised AP site was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

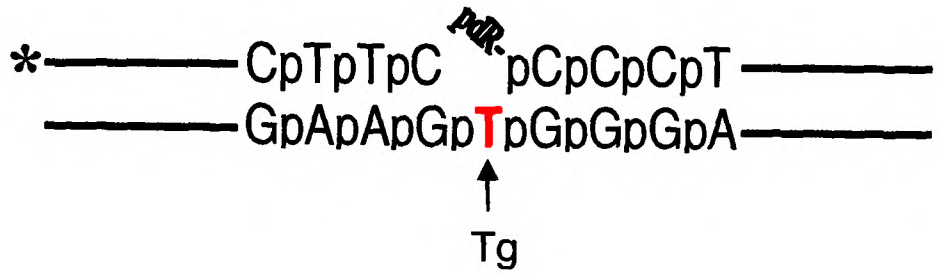


Fig. 5bI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

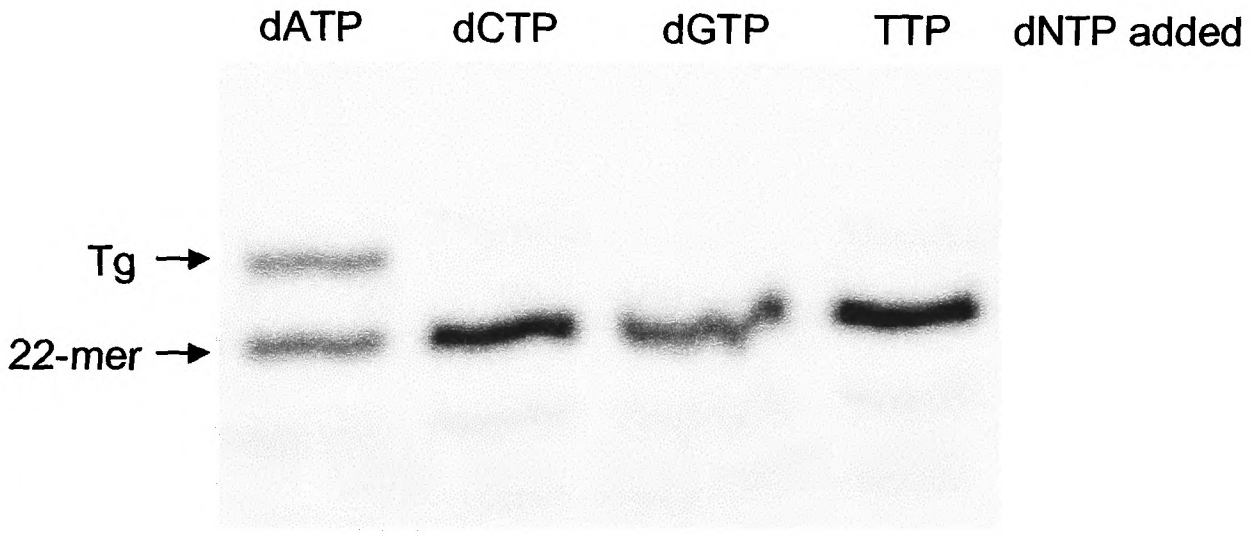


Fig. 5bII Specificity of incorporation opposite to thymine glycol by purified Pol β. 1 pmol of the 5'-end labelled substrate oligonucleotide duplex, containing thymine glycol opposite to the pre-incised AP site was incubated for 20 mins. at 37°C with 25 fmol of Pol β under the conditions described in Materials and Methods in the presence of either dATP, dCTP, dGTP or TTP (20 μM). After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

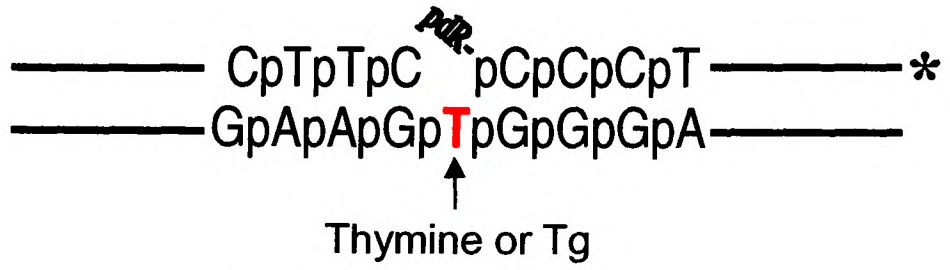


Fig. 5cI Schematic representation of the ^{32}P-3'-labelled (*) oligonucleotide substrate.

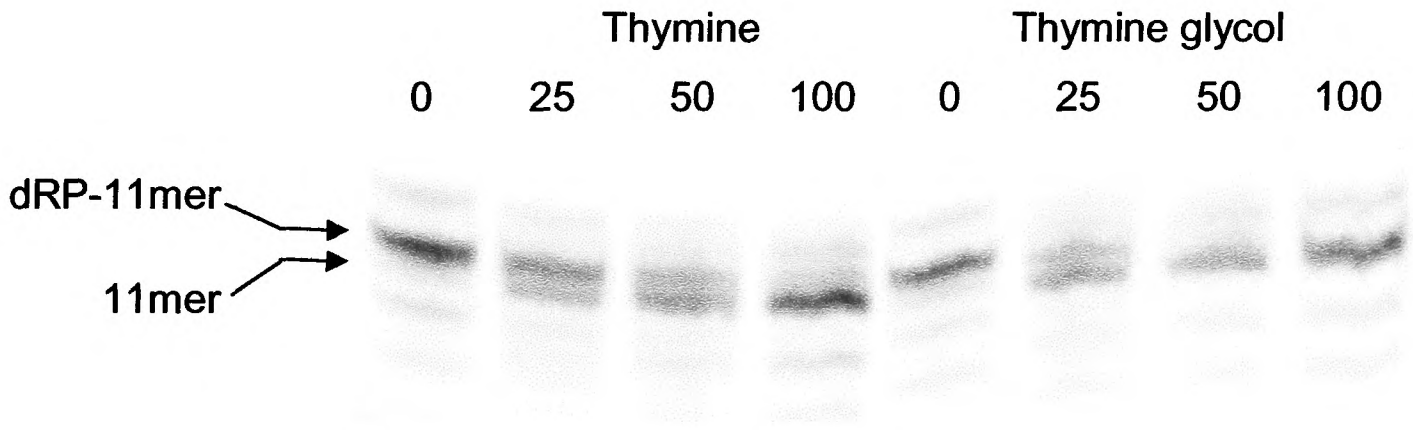


Fig. 5cII Effect of thymine glycol on removal of the dRP residue by Pol β. 1 pmol of the 3'-end labelled (*) substrate oligonucleotide duplex, containing thymine (left panel) or thymine glycol (right panel) opposite to the pre-incised AP site was incubated for 20 mins. at 37°C with the indicated amount of Pol β under the conditions described in Materials and Methods. After incubation, reactions were further incubated for 10 mins. on ice with 0.1 M $NaBH_4$. Reactions were stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

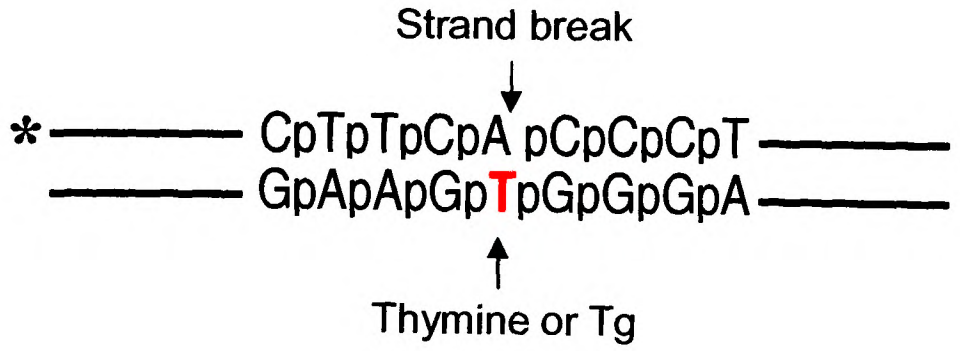


Fig. 5dI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

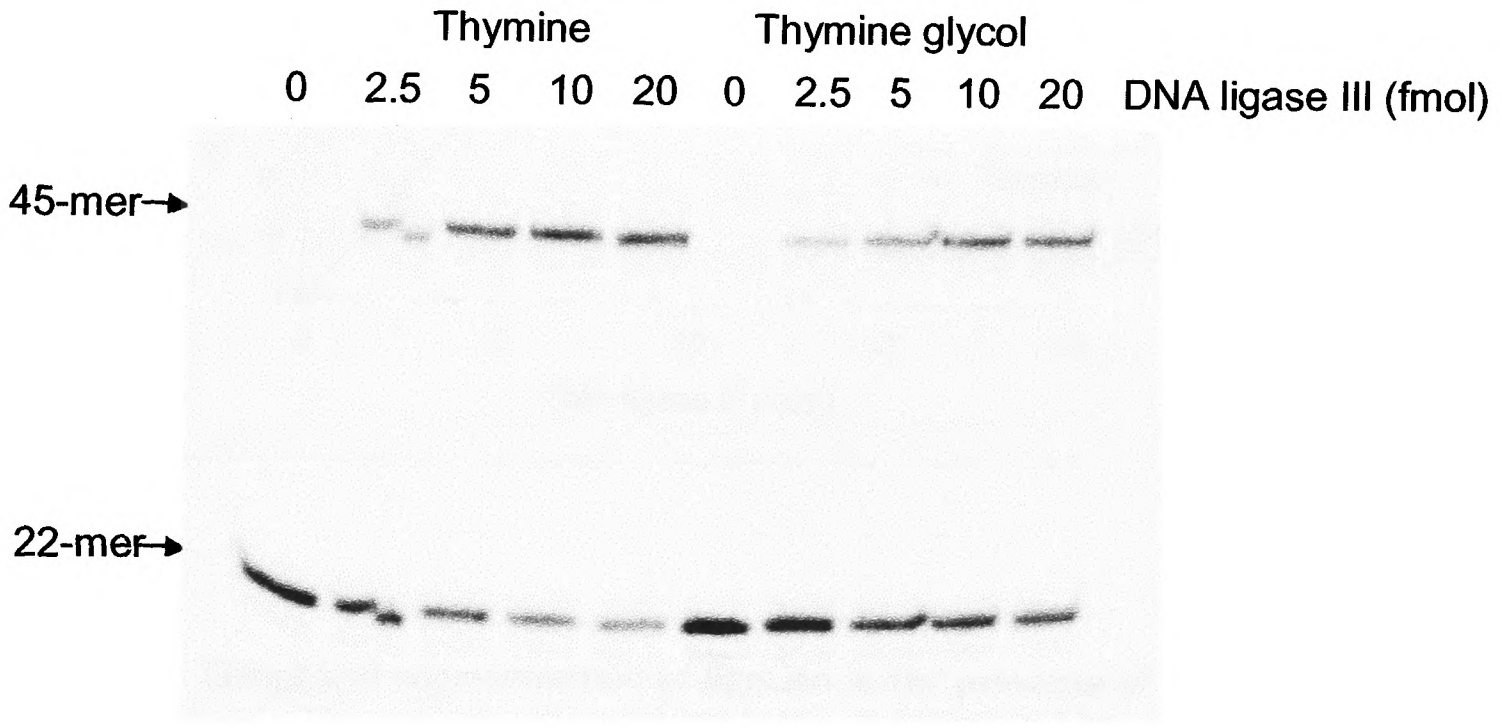


Fig. 5dII Effect of thymine glycol on DNA ligase III. 1 pmol of the 5'-end labelled substrate oligonucleotide triplex, containing thymine (left panel) or thymine glycol (right panel) opposite to the single-strand break was incubated for 10 mins. at 37°C with the indicated amount of DNA ligase III under the conditions described in Materials and Methods. After incubation, the reaction was stopped by the addition of gel loading buffer and, following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

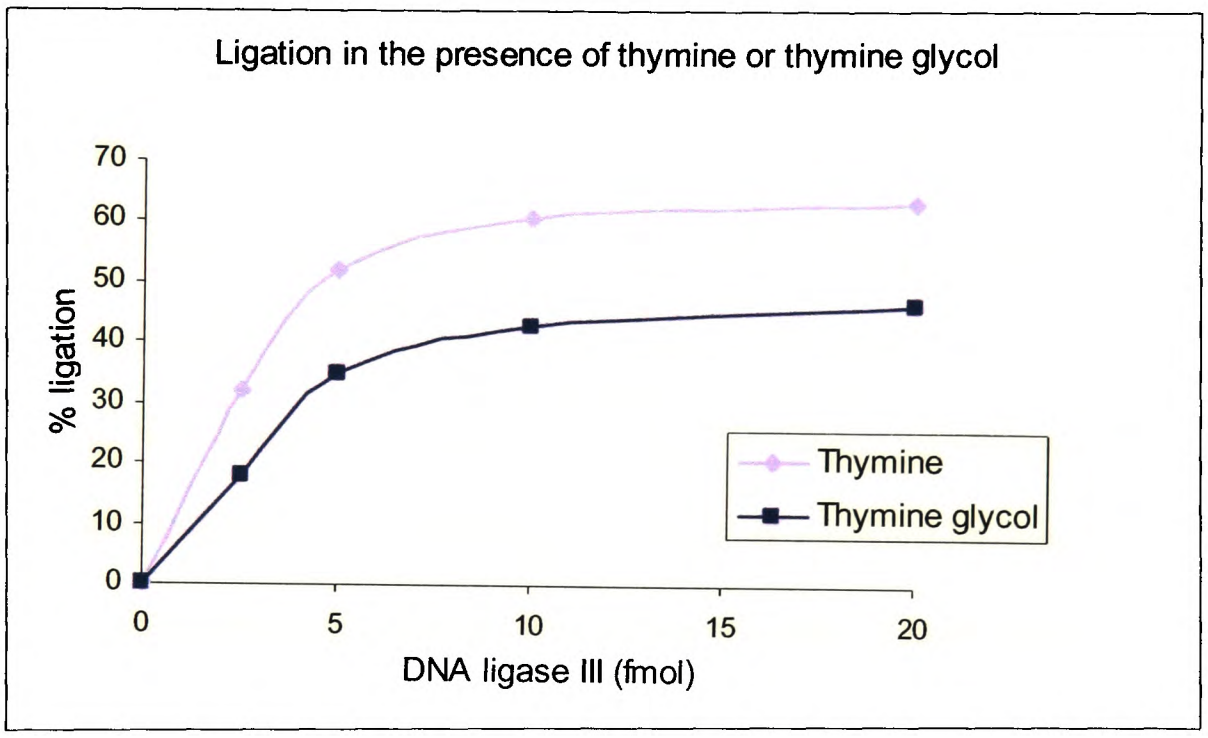


Fig. 5dIII Graphical representation of ligation in the presence of thymine or thymine glycol. The amount of ligated product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis.

5.2.5 ***Repair in the Presence of Thymine Glycol in Whole Cell Extracts***

An important question, however, is whether the concentration of Pol β and DNA ligase III in whole cell extracts is high enough to support efficient repair of clustered lesions containing thymine glycol. To address this question repair of the oligonucleotide duplexes, containing an AP site located opposite to thymine glycol or opposite to thymine, by human whole cell extracts was compared. Thymine glycol located opposite to the AP site did not affect cleavage of the AP site by human AP endonuclease. When incubated with human whole cell extract, the AP site-containing substrate was cleaved immediately, generating the 22-mer 5'-labelled incision product even at the attempted '0' time point (Fig. 5eII, *lane 1*). Further repair of the AP site was monitored as restoration of the full-length 45-mer labelled product (Fig. 5eII). It was found that approx. 20% of the thymine glycol-containing substrate was repaired within 20 mins. incubation with cell extract in comparison to 50-60 % for the thymine-containing substrate (Fig. 5eII).

It can thus be concluded that, although thymine glycol does not completely block repair and such lesions are to some degree repairable by BER, the inhibitory effect of thymine glycol may cause substantial delay in the repair of clustered lesions by BER enzymes.

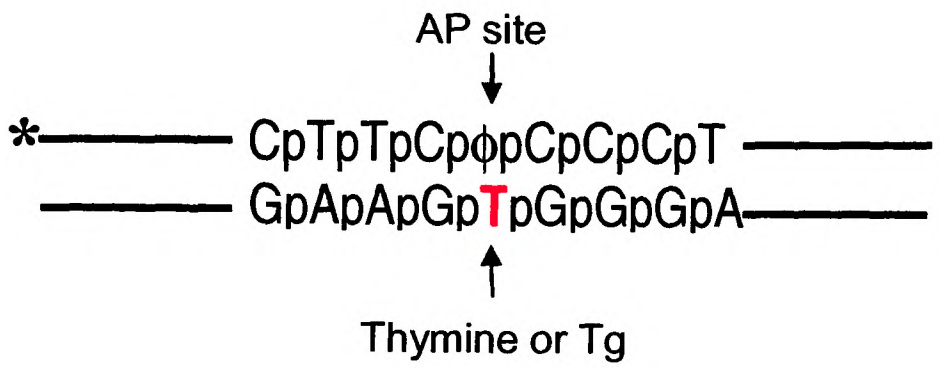


Fig. 5eI Schematic representation of the ^{32}P-5'-labelled (*) oligonucleotide substrate.

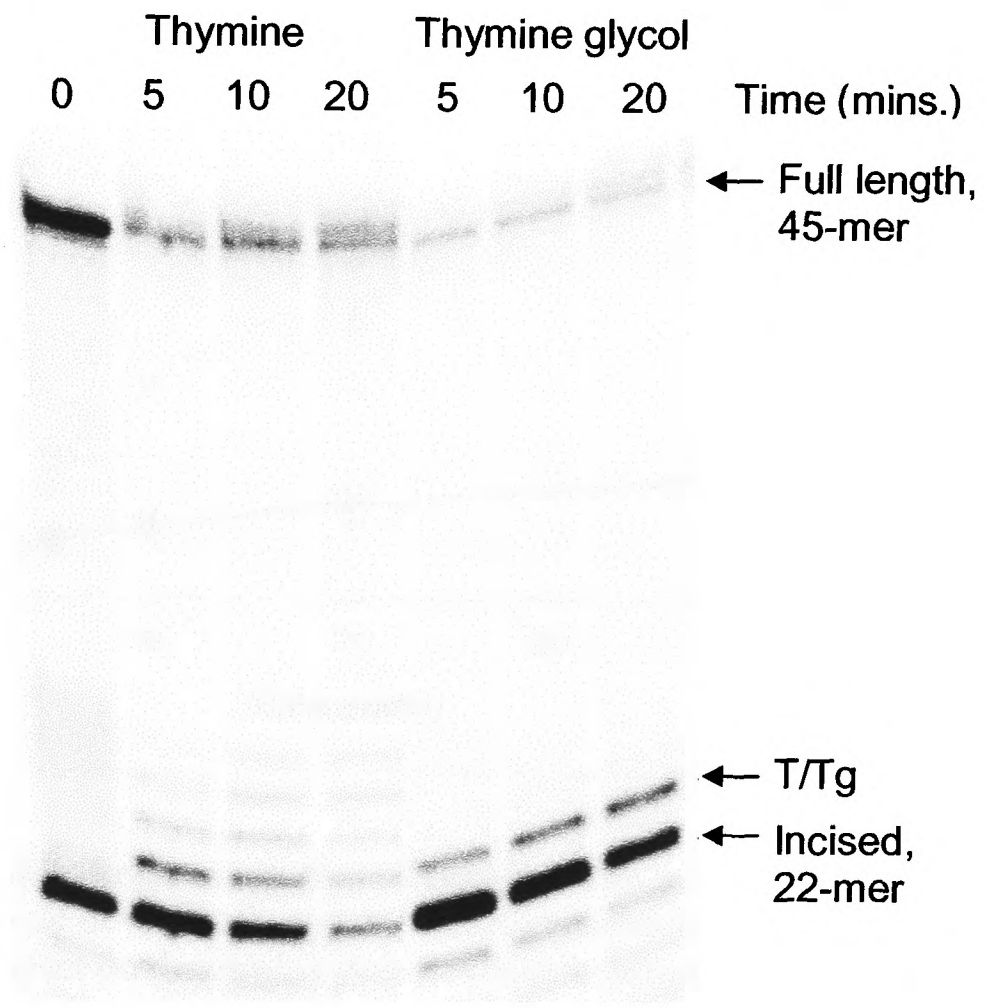


Fig. 5eII Short-patch base excision repair in whole cell extract. 1 pmol of the 5'-end labelled oligonucleotide duplex containing thymine (left panel) or thymine glycol (right panel) opposite to the AP site was incubated for the indicated time at 37°C with 5 μg of HeLa whole cell extract under the conditions described in Materials and Methods. Reactions were stopped by the addition of EDTA and a chloroform/isoamyl alcohol mixture. After centrifugation for 2 mins. at 16,000 x *g*, 10 μl of the aqueous phase was collected and 10 μl of gel loading buffer was added. Following incubation at 90°C for 3 mins., the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

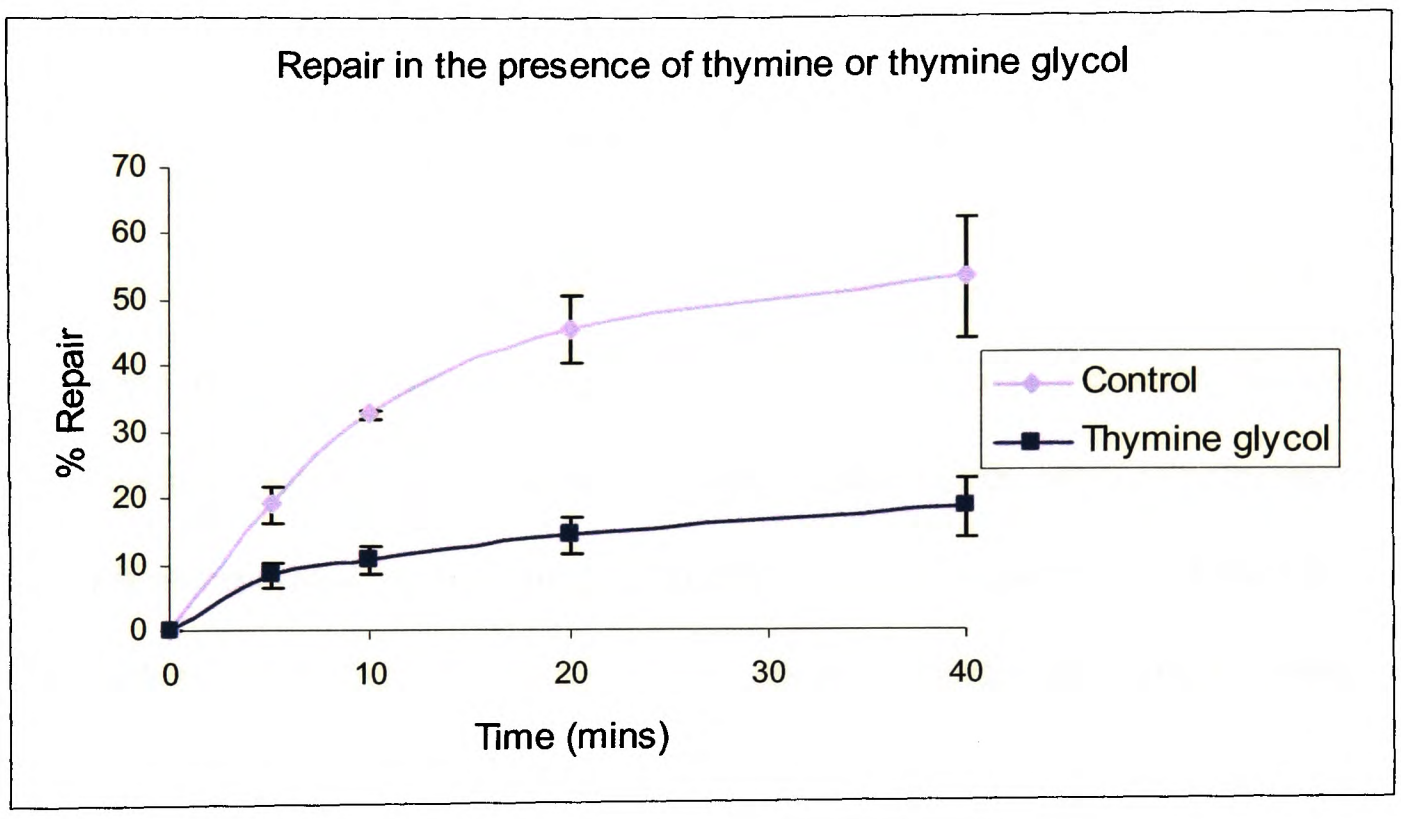


Fig. 5eIII Graphical representation of repair in the presence of thymine or thymine glycol. The amount of repaired product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using phosphorimager analysis. Analysis of three independent experiments.

5.3 *Discussion*

In this chapter the repair of clustered lesions containing thymine glycol was addressed. During short-patch BER, when a single damaged nucleotide is excised by repair enzymes, the major threat may be a simultaneous damage of the complementary base, and the presence of thymine glycol in the repair gap may have a major impact on the quality and the rate of repair. Nevertheless, as is demonstrated in this study, not all BER reactions are affected to the same extent by such damages. Incision of an AP site by APE1 as well as removal of the 5'-sugar phosphate by the dRP lyase activity of Pol β were not affected at all and thymine glycol had only a moderate (1.5-fold) inhibitory effect on the ligation reaction. However, thymine glycol in a repair gap significantly affected the incorporation of nucleotides by Pol β. Although the correct nucleotide (dAMP) is incorporated, addition of the first nucleotide was significantly slowed by the thymine glycol and further incorporation was completely blocked in repair reactions reconstituted with purified Pol β (Fig. 5aII), as well as in cell extract (Fig. 5eII, *right panel*). This suggests that long-patch base excision repair, which requires incorporation of at least 2 nucleotides, would not be efficient in repair of such lesions.

Thymine glycol, when present on the DNA template strand blocks progression of replication by the major replicative DNA polymerases α and δ, although a limited incorporation opposite the lesion is observed [34, 146, 159]. It was also found here that Pol β was able to catalyse limited incorporation opposite to thymine glycol and that thymine glycol does not change the specificity of incorporation (Fig. 5aII and Fig. 5bII).

In agreement with these findings early studies indicated that thymine glycol forms a reasonably stable base pair with adenine and that the DNA sequence immediate to thymine glycol does not affect the specificity of incorporation by Klenow fragment of DNA polymerase I or by DNA polymerase α [34, 159]. Theoretically, the two major known isomeric forms of thymine glycol (5S and 5R) [258] may differently affect human DNA polymerases. In this study, thymine glycol was generated by direct oxidation of the single thymine in the template DNA using osmium tetroxide. This procedure generates 85% of the 5R isomer [259], suggesting that the reduced rate of incorporation of dAMP opposite thymine glycol observed in these experiments was mainly due to the effect of this isomer. In support of this conclusion kinetic analyses performed by Hanaoka's group revealed that Pol α incorporates dAMP opposite 5R-thymine glycol about 16-fold less efficiently than on undamaged template [159]. It can be concluded from these studies that thymine glycol, present within a clustered damage site, will pose problems for the base excision repair pathway which could lead to persistence of lesions in the DNA.

Chapter 6

Discussion

Previous studies have shown that ionising radiation can introduce clusters of damages within a few base pairs in DNA [185, 186, 188, 209-214, 231, 247, 249], and that the increased lethality of ionising radiation compared to other oxidising agents may be explained by the decreased ability of the damage cluster to be repaired [260].

It had previously been shown that some oxidative base lesions, including 8-oxoguanine, may be processed by nucleotide excision repair [88, 99, 160, 161]. Base excision repair is considered to be the main pathway responsible for the repair of such lesions, it was therefore hypothesised that nucleotide excision repair may act as a back-up [99, 161]. However, the roles of these repair pathways in repair of tandem base lesions, formed on the same strand of DNA, had never been studied. A single 8-oxoguanine in duplex DNA has been shown to be relatively non-distorting [29, 30], however, the presence of tandem 8-oxoguanine lesions on a single strand of DNA may cause a structural distortion to the DNA double helix. Therefore, we tested the relative roles of both BER and NER pathways in the repair of tandem 8-oxoguanine lesions. It was found that tandem 8-oxoguanine lesions on the same strand of DNA are not recognised by enzymes of the nucleotide excision repair pathway. Lesions formed within a few base pairs on the same strand of DNA appear to be repaired independently by base excision repair. However, the repair of such lesions displays asymmetry at a certain stage in the repair pathway. Repair of a base damage with another damage 3' of it is repaired accurately and

efficiently and at the same rate as a singly damaged site. Whereas a base damage with another damage 5' of it displays uncoupled repair and repair is delayed after incision of the AP site following removal of the damage by a DNA glycosylase. Such a delay may lead to the persistence of a single-strand break intermediate, which, if not repaired before replication of the DNA, could block replication and lead to the formation of a more harmful double-strand break.

In addition, repair of tandem lesions by the long-patch base excision repair pathway was also investigated. Previous studies on the long-patch repair enzyme FEN1 had shown that certain modifications to the substrate flap strand hindered FEN1 tracking and cleavage activities [103, 230, 261]. The presence of oxidative base lesions, such as 8-oxoguanine and thymine glycol, in the flap strand during long-patch BER had not been tested. We therefore tested such substrates with purified human FEN1 and found that long-patch BER can proceed unhindered by the presence of 8-oxoguanine and thymine glycol lesions in the displaced flap strand. Indeed, this mode of repair would appear to be a more efficient method of repairing tandem lesions as several lesions may be removed as part of the displaced oligonucleotide flap.

Another type of clustered damage formed as a result of exposure to ionising radiation is one in which lesions are formed on both strands of the DNA within a few base pairs. Repair of such lesions could lead to the formation of more double-strand breaks either through unfavourable endonuclease action at base lesions on complementary DNA strands or through interaction of base lesions with the replication machinery [233]. A

variety of studies have looked at the effect of opposing lesions on enzymes of the base excision repair pathway due to the potential for forming a DNA double-strand break [193, 253-255, 262-265]. It has been shown that in the case of closely opposing base lesions or a base lesion and an AP site, the glycosylase and AP endonuclease activities are not inhibited by the presence of an opposing base lesion, but are inhibited by the presence of an opposing single strand break [253-255, 262, 263]. Also, *in vitro* studies have indicated that if base lesions are situated less than 3bp apart on opposing strands, repair of one lesion is inhibited until repair of the lesion in the opposite strand is completed [266, 267]. Therefore, during repair, such damaged sites will be converted to a lesion opposite to a single strand break. Little is known about the effect of a directly opposing base lesion on repair of a single-strand break.

Therefore, in order to address this question, the effects of two base lesions, 8-oxoguanine and thymine glycol, on repair of a single-strand break by both the short- and long-patch pathways of base excision repair were investigated. 8-oxoguanine and thymine glycol are the major oxidative lesions induced by indirect effects of ionising radiation, caused by generation of reactive oxygen species [22, 23, 31]. Individually, these lesions are efficiently repaired by BER [99, 161]. We were able to demonstrate that the presence of 8-oxoguanine in a repair gap does not affect repair of the break by short-patch BER, with the exclusive incorporation of cytosine opposite to the 8-oxoguanine. This result is significant as such a substrate would result from MYH excision of mis-incorporated adenine opposite to 8-oxoguanine. The ability to correctly repair such a gap would allow further repair of the 8-oxoguanine to occur and would avoid the formation of mutations.

Structural studies of Pol β with a template 8-oxoguanine lesion have indicated that the 5'-phosphodiester linkage of the modified base plays a critical role in determining the fidelity of incorporation by repositioning itself in the polymerase active site [268].

In contrast, thymine glycol is a much more harmful lesion when located within a cluster. The presence of thymine glycol directly opposite to a single-strand break causes a substantial delay in short-patch BER of the break. The presence of thymine glycol in the repair gap slows base excision repair by delaying nucleotide insertion opposite to the damaged base and also by moderately affecting the ligation of the nick. This data suggests that clustered lesions containing thymine glycol are repaired slower than a single-standing lesion of a similar type and may be partially responsible for the deleterious effects of ionising radiation. However, it was demonstrated that Pol β can again insert the correct nucleotide opposite to the damaged template. These data suggest that Pol β acts specifically during base excision repair to incorporate the correct nucleotide into the repair gap, even in the presence of a damaged template. The increased activity and fidelity of Pol β on single nucleotide gapped DNA has been shown previously [97], however, the ability of Pol β to correctly incorporate nucleotides opposite to modified bases during repair has been much less well studied.

An 8-oxoguanine in the template strand during long-patch BER, however, was shown here to be able to block repair at the strand displacement synthesis step, in a sequence-dependent manner. Long-patch base excision repair DNA synthesis has been shown to be supported by both Pol β and Pol δ [46, 48, 106]. It was subsequently demonstrated

that Pol β is the polymerase responsible for initiation of long-patch BER by inserting the first nucleotide [102]. In this study an 8-oxoguanine lesion present in the template strand during long-patch BER was found to block strand displacment synthesis by both Pol β and Pol δ, in a sequence-dependent manner. *In vivo*, this block may be overcome by the presence of accessory proteins, such as the WRN helicase that has been shown to stimulate strand displacment synthesis by Pol β by facilitating unwinding of the DNA substrate [269]. *In vitro*, the translesion DNA polymerase, Pol η, was able to bypass the lesion, and may play a role in bypass of 8-oxoguanine lesions *in vivo*, as spontaneous mutation rates have been shown to be elevated in the absence of Pol η in the yeast *ogg1* mutant [158].

In conclusion, as is demonstrated here, the repairability of clustered lesions significantly depends on the relative location of the primary DNA lesions as well as on the sequence context of the damaged DNA. Certain lesion combinations within a cluster that will block lesion processing by DNA repair enzymes may result in genetic instability and reduced cell viability. Indeed, various studies have reported elevated mutation rates as a result of clustered lesions [199, 252, 270]. Delays in repair may lead to gapped DNA being exposed for a longer time to the cellular milieu. Delays in processing of repair intermediates may cause a significant increase in genomic instability and affect cellular resistance to ionising radiation [271, 272].

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Appendices

List of Oligonucleotide Sequences

(G is 8-oxoguanine, T is thymine glycol and U is uracil)

Chapter 3:

Tandem Lesions

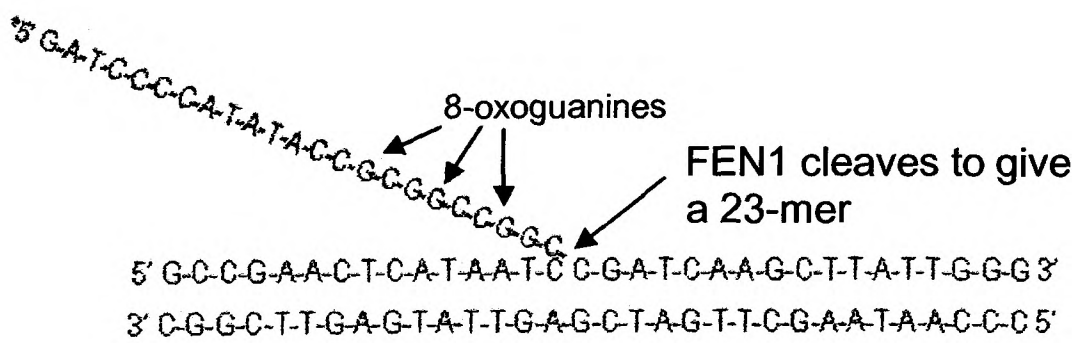
```
*5'GATCCCCATATACCGCGGCCGGCCGATCAAGCTTATTGGG 3'
 3'CTAGGGGTATATGGCGCCGGCCGGCTAGTTCGAATAACCC 5'

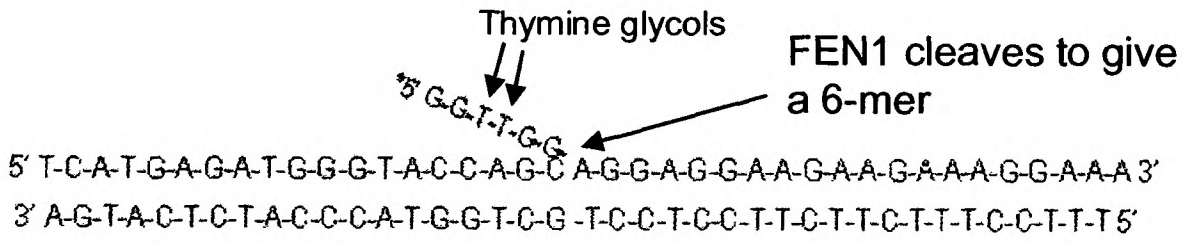
*5'GATCCCCATATACCGCGGCCUGCCGATCAAGCTTATTGGG 3'
 3'CTAGGGGTATATGGCGCCGGCCGGCTAGTTCGAATAACCC 5'

5'GATCCCCATATACCGCGUCCGGCCGATCAAGCTTATTGGG *3'
3'CTAGGGGTATATGGCGCCGGCCGGCTAGTTCGAATAACCC 5'
```

```
*5'ATATACCGCGGCCGGCCGATCAAGCTTATT 3'
 3'TATATGGCGCCGGCCGGCTAGTTCGAATAA 5'
```

Effect of base lesions in the flap strand on FEN1





Chapter 4:

*5′AATAAGCTTGATCGGCCGGUCGCGGTATAT 3′

3′TTATTCGAACTAGCCGGCCGGCGCCATATA 5′

*5′AATAAGCTTGATCGGCCGUCCGCGGTATAT 3′

3′TTATTCGAACTAGCCGGCCGGCGCCATATA 5′

*5′GGATAACAATTTCUCACAGGAAACAGCTAT 3′

3′CCTATTGTTAAAGTGTGTCCTTTGTCGATA 5′

Chapter 5:

*5′TTCTCTCCTTCCCTTCCTCTCTUCCCTTTCTCCTCTCCTCTCTTC 3′

3′AAGAGAGGAAGGGAAGGAGAGATGGGAAAGAGGAGAGGAGAGAAG

DNA ligase assay



*5′TTCTCTCCTTCCCTTCCTCTCTACCCTTTCTCCTCTCCTCTCTTCCT 3′

3′AAGAGAGGAAGGGAAGGAGAGATGGGAAAGAGGAGAGGAGAGAAGGA 5′

List of Buffers

1x TBE: 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0

Gel loading buffer: 95% formamide, 20 mM EDTA, 0.02% bromphenol blue, 0.02% xylene cyanole

TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

BER buffer: 40 mM Hepes-KOH (pH 7.8), 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA

UDG buffer: 10 mM Hepes (pH 7.9), 1 mM EDTA, 70 mM KCl