Localisation of human DNA polymerase κ to replication foci

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Accepted 5 September 2002
Journal of Cell Science 115, 4413-4418 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00162

Summary

The replication of the undamaged genomic DNA requires error-free DNA polymerases δ and ε as part of a protein complex that acts continuously along the double helix. In contrast, when the genomic structure is perturbed, DNA replication needs to function more flexibly to bypass DNA distortions. It has been proposed that the newly discovered error prone DNA polymerases play a role in the replication of irregular structure. Here we report that one of them, the human Polκ, is mostly localised uniformly in the nucleus of undamaged cells, but could be also concentrated in PCNA-containing replication foci. Following treatment with anti-replicative agents, the proportion of foci-containing cells was increased. These data suggest that Polκ may function as part of the replication machinery itself and could be recruited when replicative complexes are stalled. Mutagenesis experiments also indicated that Polκ involvement may affect the accuracy of DNA replication. The results are discussed within the context of the oncogenic process since Polκ has been found as overexpressed in some cancers.

Key words: Polκ, DNA replication, DNA mutagenesis

Introduction

When the conformation of the double helix is regular and unbroken, replicative DNA polymerases Polδ and Polε copy DNA in an accurate way. In contrast, when distortions, adducts or breaks disturb the DNA structure, specialised DNA polymerases including Polζ, Polη, Polι and Polκ, take part in the replication repair of DNA damage that otherwise would not be tolerated (Friedberg et al., 2000; Radman, 1999). This translesion replication of DNA lesions may be more or less accurate, depending on the DNA polymerase involved and the type of damage bypassed. However, in most cases the translesion process mediated by these specialised DNA polymerases results in mutagenesis.

A current working hypothesis suggests that when replicative complexes are stopped at DNA lesions these error-prone DNA polymerases interfere with the replication machinery to facilitate lesion bypass by acting sequentially, perhaps by replacing for the replicative polymerases. For instance Pol η, which is active in the accurate bypass of UV lesions (Johnson et al., 1999), associates with PCNA foci in UV irradiated cells (Kannouche et al., 2001) and interacts with the proliferating cell nuclear antigen (PCNA) (Haracska et al., 2001a), a ring-shaped protein forming a clamp at primer-template junctions of the replication machinery (Kornberg and Baker, 1992). Polι, which incorporates nucleotides opposite abasic sites or the 3′T of the (6,4) T-T photoproduct (Johnson et al., 2000b), also binds the replication machinery via an interaction with PCNA (Haracska et al., 2001b).

The recently discovered (Johnson et al., 2000a) human DNA polymerase κ, (Polκ, previously named Polθ), is a homologue of the *Escherichia coli* DinB (Pol IV) protein, a SOS protein involved in untargeted UV-induced mutagenesis of bacteriophage λ (Brotcorne-Lannoya and Maenhaut-Michel, 1986) and whose activity is stimulated in vitro by PCNA (Haracska et al., 2002). In vitro, Polκ can bypass certain DNA lesions including 1,N^6-ethenodeoxyadenosine or acetylaminofluorene-derived DNA adducts by generating base substitutions and deletions (Gerlach et al., 1999; Levine et al., 2001; Ohashi et al., 2000b; Suzuki et al., 2001), 8-oxo-7,8-dihydrodeoxyguanosine by incorporating dAMP opposite the lesion (Zhang et al., 2000) and (-)-trans-anti-benzo[a]pyrene-N^2-dG in an error-free manner (Zhang et al., 2000). In contrast, Polκ is unable to perform translesion synthesis opposite either a cisplatin adduct (Ohashi et al., 2000b), a *cis-syn* TT dimer or a TT (6-4) photoproduct (Johnson et al., 2000a; Ohashi et al., 2000b; Zhang et al., 2000), although it may extend from a G opposite the 3′T of a TT dimer (Washington et al., 2002). Moreover Polκ was shown to be unable (Johnson et al., 2000a) or inefficient (Ohashi et al., 2000b; Suzuki et al., 2001) in bypassing abasic sites. Here we examine whether Polκ can associate with the replication machinery by determining its cellular localisation in the presence or absence of antireplicative agents. We have used PCNA as a cellular marker in order to identify replisome-containing foci. Our data indicate that Polκ is present at replication forks in some human MRC5 fibroblasts cells in the absence of blocking lesions as well as in most MRC5 cells when replication forks are stalled. These findings thus suggest that Polκ is involved in the replication machinery of untreated cells and, as already shown for Polη and Polι, could be recruited into replisomes at replication arrests.

Upregulation of Polκ has been recently found in lung tumours in comparison with adjacent nontumorous tissues (O-Wang et al., 2001), and has been observed by Serial Analysis of Gene Expression (SAGE) (Velculescu et al., 1995) in human ovarian and prostate cancers (http://www2.ncbi.nlm.nih.gov/SAGE/, Cancer Genome Anatomy Project from NCBI), indicative of a role in tumor development. In view of the potential presence of Polκ at replication forks, we investigated in the present study the role of the enzyme in mutagenesis. Our findings are discussed with reference to the mutagenic impact of high levels of hPolκ on cell proliferation and carcinogenesis processes.

Materials and Methods

Construction of eGFP-polκ protein

Polκ cDNA was amplified by PCR using the pHSE2 DNA (provided by H. Ohmori, Kyoto, Japan) as a template and 5′-cccaagctTG-GATAGCACAAAGGAGAA-3′ and 5′-gcgggatccTTACTTAAAA-AATATATCAA-3′ oligonucleotides as primers. The PCR product was digested using *Hin*dIII and *Bam*HI, and was inserted into the restricted peGFP-C2 vector (Clontech) to produce peGFP-polκ. DNA sequencing confirmed the correct construction.

Cell lines and DNA transfection

MRC5 (ATCC) and XP12ROSV40 (provided by B. Salles, France) cells were grown in MEM alpha (Gibco) medium supplemented with 10% foetal calf serum, penicillin and streptomycin. Tranfection of plasmids was performed using Lipofectamin Plus reagent (Gibco).

Irradiation and drug treatment

Cells in PBS were irradiated using a 254 nm UV lamp. For drug treatment, cisplatin was diluted in complete medium to a final concentration of 30 μM and added onto the transfected cells for 1 hour. After 1 hour the cells were washed with PBS and incubated in complete medium. For hydroxyurea treatment, the cells were incubated for 12 hours with complete medium containing 1.5 mM hydroxyurea, washed with PBS and incubated in drug free complete medium for 8 hours.

Immunofluorescence microscopy

For the localisation of eGFP or eGFP-polκ, cells were grown on coverslips, washed in PBS and fixed in 4% formaldehyde for 30 minutes at 4°C, then rinsed three times in PBS and mounted in Mowiol. To visualise the eGFP-polκ and PCNA proteins at the same time, cells were fixed in 4% formaldehyde for 30 minutes at 4°C and rinsed three times in PBS. Permeabilisation was then performed by incubating the cells for 10 minutes at –20°C in the presence of cold methanol. They were then washed before treatment with 0.25% Triton X-100 for 5 minutes at room temperature. After washing three times with PBS, the cells were blocked for 10 minutes at room temperature in FCS 1% in PBS and incubated for 1 hour with primary antibody against PCNA diluted 1:200 with FCS 1% in PBS (PC10 monoclonal anti-PCNA, Dako). Finally they were washed three times over a 3 minutes period in FCS 1% in PBS, incubated for 30 minutes with TRITC conjugated goat anti-mouse IgG (Sigma), and washed three times more in PBS before mounting. More than 1000 fluorescent cells were examined in each experiment.

Cell cycle analysis

Cells were grown to 80% confluency and stained with propidium iodide. DNA content was evaluated using a FACScan flow cytometer (Beckson Dickinson). Histograms were analysed using ModFit cell cycle analysis software.

Selection of Polκ-overexpressing MRC5 cells

The Tet-Off gene expression system (Clontech) was used to obtain regulated, high-level overexpression of Polκ. Human *polκ* cDNA was PCR-amplified from the pHSE2 plasmid using *Pfu* polymerase and the primers, 5′-CCggatccTCAGATAAGTTTATA-3′ and 5′-CCatcgatATGATAAAATGTTCA-3′. The PCR product was then digested using *Bam*HI and *Cla*I before insertion into the *Bam*HI-*Cla*I sites of the pTRE2 (Clontech), which carries the Tet operator sequence and the CMV minimal promoter, to obtain the pTRE2-polκ plasmid. MRC5 cells were transfected according to the manufacturer's protocol (Lipofectamin, Gibco) with the pTet-Off vector which carries the regulatory protein TetR/HSV-VP16. A stable transfectant clone displaying the highest level of Doxycyclin (Dox)-dependent induction was screened out by transient transfections of more than forty clones using an inducible pTRE2-luc vector carrying the luciferase gene. The selected clone was then stably transfected with pTRE2-polκ plasmid to select for Polκ-overexpressing MRC5 cells.

Quantitative RT-PCR analysis

RNA was extracted from cells using TriReagent (GibcoBRL). 120 ng RNA were incubated with 0.2 mM dNTPs, 1 mM $MgSO_4$, 0.25 u AMV reverse transcriptase, 0.25 u Tfl ADN polymerase and specific primers for *polκ* and *hprt*. The RT-PCR reaction was performed at 48°C for 45 minutes, 94°C for 2 minutes and then at 94°C for 30 seconds, 52°C for 1 minute, and 68°C for 45 seconds for 30 cycles. After the first 94°C incubation, [α^{32}P]dGTP was added and 12 μl aliquots were removed after 10, 20 and 30 cycles. Gel electrophoresis was then carried out in 2% agarose. The gel was dried and scanned in a phosphoimager (Storm 840, Molecular Dynamics).

Mutagenesis experiments

Experiments were carried out as described (Canitrot et al., 1998) and mutant frequencies were corrected for plating efficiency.

Results

Polκ is localised in the nucleus and can be concentrated in nuclear spots

In order to study the intracellular localisation of Polκ, we designed a peGFP-C2/polκ vector coding for a fusion protein comprising the enhanced green fluorescent protein (eGFP) in frame with the amino-terminus of Polκ. Immortalised human MRC5 pulmonary fibroblasts were transfected either with this vector or the control eGFP-expressing empty vector peGFP before analysis by confocal fluorescence microscopy. The eGFP protein expressed by the control vector was present in both the nucleoplasm and cytoplasm of all the transfected cells (Fig. 1Aa) whereas the eGFP-Polκ fused protein was distributed homogeneously in the nucleoplasm only in 80% of the transfected cells (Fig. 1Ab). In the remaining 20% cells of the cell population, corresponding to cells in S-phase (Fig. 1C), eGFP-Polκ was concentrated in fluorescent nuclear spots (Fig. 1Ac) which colocalised with the replisome marker PCNA (Fig. 1B). These data suggest that Polκ-containing spots are associated with DNA replication forks.

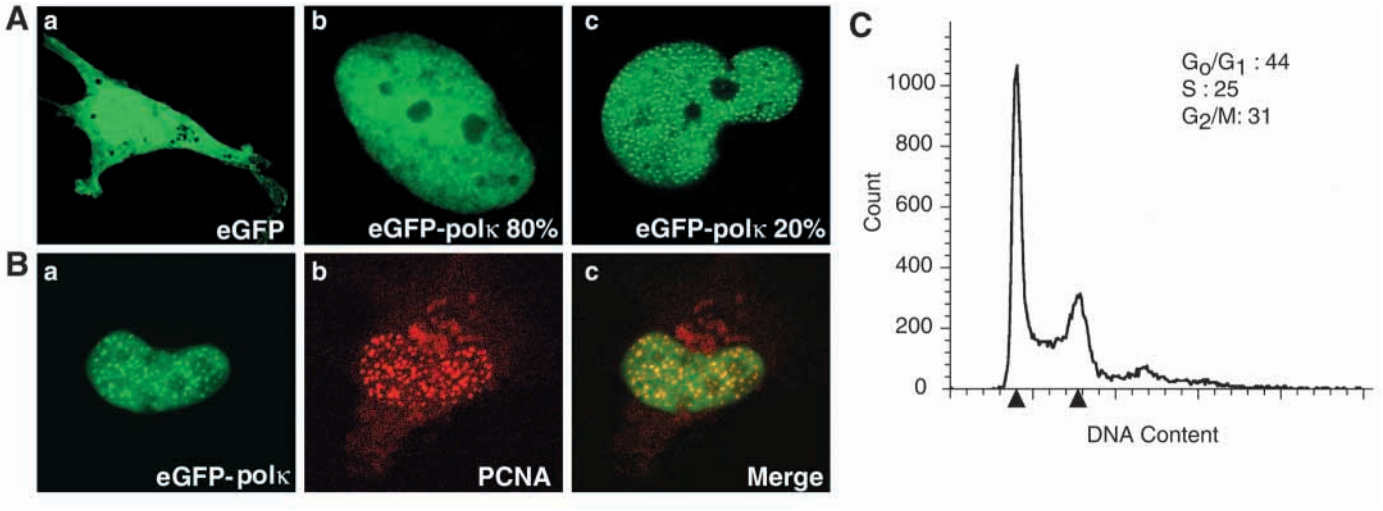

Fig. 1. Localisation of eGFP-polκ in the whole MRC5 cells (A) and colocalisation of eGFP-polκ with PCNA in 20% MRC5 cells corresponding to the cell population in S phase (B). MRC5 cells were transfected with plasmid peGFP-C2 or peGFP-C2/polκ. Cells were then fixed, stained with anti-PCNA mAb, incubated with TRITC-conjugated secondary antibody, and analysed by using confocal microscopy for the localisation of either eGFP or eGFP-polκ fluorescent proteins or PCNA. The localisation of eGFP-polκ (green) and PCNA (red) was observed in the same cell and the colocalisation is indicated by the yellow pattern (Merge). About 1000 fluorescent cells were analysed. DNA content was evaluated by analysing 20,000 collected events by flow cytometry (C).

Antireplicative treatments increase the local concentration of eGFP- Polκ

When the replicative forks were arrested following treatment with hydroxyurea (HU), an agent which halts the biosynthesis of dNTPs from rNTPs, we observed a dramatic increase in the number of cells containing fluorescent Polκ nuclear spots. Twelve hours after treatment, the proportion of cells containing spots increased from 20% for untreated controls to 70% for cells treated with HU. Moreover, these foci colocalised with PCNA in the presence of the antimetabolic drug (Fig. 2Ab,c). When treated cells were rinsed to remove HU we found that the number of cells with eGFP-polκ foci dropped to 10% (Fig. 2Ba) and that 90% of the remaining cells did not display GFP and PCNA foci (Fig. 2Bb,c). Taken together, these results indicate that eGFP-polκ foci are associated with HU-mediated stalled replication forks.

To further demonstrate the relation between replicative arrests and hPolκ-containing foci, the polκ-transfected cells were exposed to UV irradiation (Fig. 3A) or treated with cisplatin (Fig. 3B), both of which one known to induce bulky DNA adducts not bypassed in vitro by Polκ. The proportion of transfected cells containing foci increased following both genotoxic treatments reaching maximal respective values of 75% and 65% six hours after 10 J/m^2 UV irradiation (Fig. 3Aa) or one hour incubation in the presence of cisplatin (Fig. 3Ba). These foci all colocalised with PCNA in both cases (Fig.

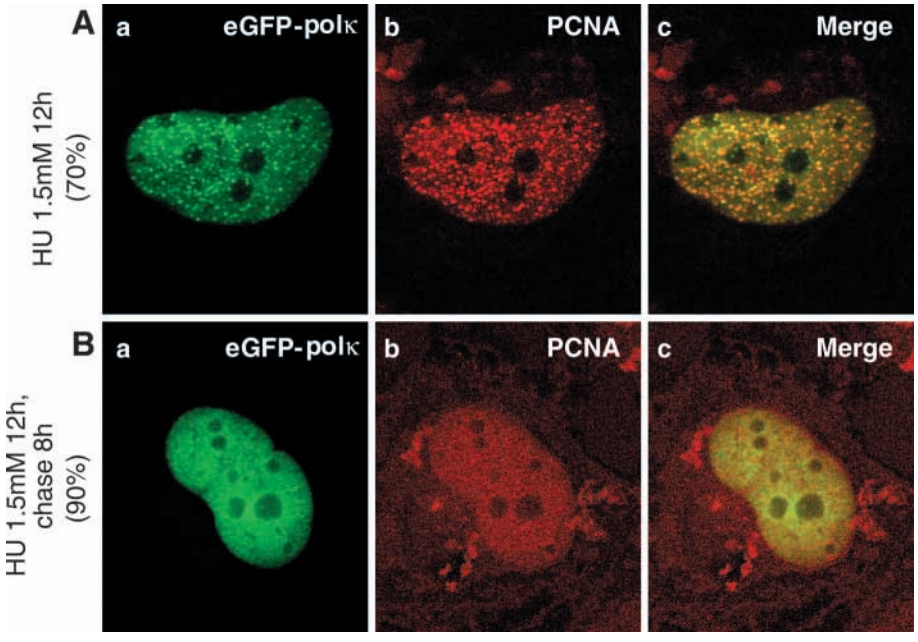

Fig. 2. Association of Polκ with the replication machinery. Cells were transfected with peGFP-C2/polκ and treated for 12 hours with 1.5 mM HU (A,B). HU was maintained (A) or chased by rinsing cells with fresh medium and then incubated for 8 hours (B). Cells were fixed and stained with anti-PCNA mAb followed by TRITC-conjugated secondary antibody. The localisation of eGFP-polκ (green) and PCNA (red) was observed in the same cell and the colocalisation is indicated by the yellow pattern (Merge). About 1000 fluorescent cells were analysed.

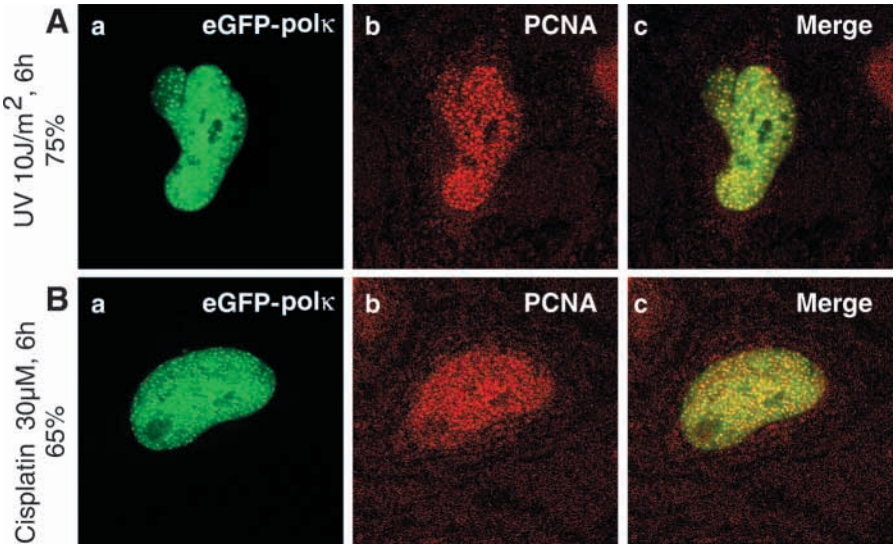

Fig. 3. Formation of eGFP-polκ foci in UV or cisplatin-treated cells. Cells were transfected with peGFP-C2/polκ and UV-irradiated 20 hours later at 10 J/m^2 dose (A) or treated for 1 hour with 30 μM cisplatin (B). Cells were fixed and stained with anti-PCNA mAb followed by TRITC-conjugated secondary antibody. The localisation of eGFP-polκ (green) and PCNA (red) was observed in the same cell and the colocalisation is indicated by the yellow pattern (Merge). About 1000 fluorescent cells were analysed.

3A,Bb,c), further supporting the view that the eGFP-Polκ protein is locally concentrated near PCNA-containing replicative complexes arrested by UV or cisplatin damage.

We then conducted experiments with the XPA-deficient XP12R0 cells, that are unable to excise UV lesions. In such cells, the number of non-coding UV adducts is increased in comparison with control MRC5 cells where many lesions are repaired. We thus observed that the number of foci-containing cells was higher for the XP12R0 cells as compared to the MRC5 cells following UV irradiation (Fig. 4). For example, at 2 J/m^2, we observed a 2-fold increase in the number of foci in XP cells. In the XP12R0 cells, all the eGFP-Polκ foci colocalised with the PCNA spots (data not shown). These data further indicate that hPolκ colocalises near replication forks when the replisomes are stopped and does not seem to be present in complexes controlling repair synthesis following the excision of UV or cisPt adducts.

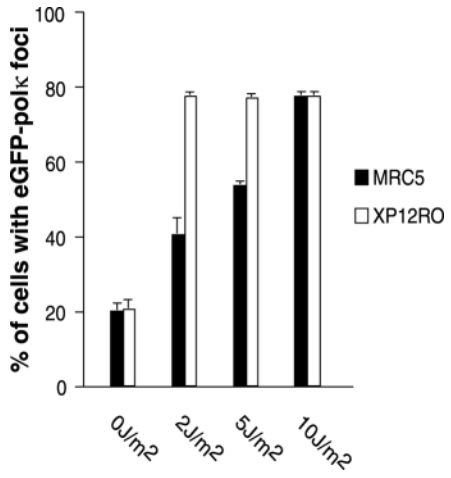

Fig. 4. Relocalization of eGFP-polκ in UV-treated MRC5 and XP12RO cells. Twenty hours after DNA transfection, peGFP-C2/polκ-containing cells were irradiated at 0 J/m^2, 2 J/m^2, 5 J/m^2 or 10 J/m^2 and incubated for 6 hours at 37°C, 5% CO_2. About 1000 fluorescent cells were then analysed as already described.

Excess hPolκ induces spontaneous mutagenesis

In view of the error-prone characteristics of Polκ in vitro and its potential involvement at replication forks in MRC5 human cells, it is reasonable to suppose that its overexpression might affect the fidelity of DNA replication in these cells. We first selected a single clone stably overexpressing *hPolκ* mRNA by 2.1-fold, as estimated semi-quantitatively by RT-PCR (Fig. 5A). Cells from this selected clone were then incubated in the absence of doxycycline (Dox) in order to maintain high intracellular levels of Polκ. It should be noted that the Polκ levels remained constant during the incubation (data not shown). 6-thioguanine was then added and *hprt* mutants counted a week later. A 7-day and 52-day incubation of Polκ-overexpressing cells led to 4.6- and 16.1-fold respective increases in the number of *hprt* mutants (Fig. 5B), compared to control 8-4 cells incubated in the presence of doxycycline. In an additional control experiment, mutagenesis rates were examined in 8-TRE2 cells that are similar to 8-4 cells but which contain both pTet-Off and an empty pTRE2 plasmid. Fig. 5B shows that these cells are not mutated in the presence or absence of doxycycline as compared with parental MRC5 cells, indicating that ectopic Polκ expression is specific in its impact. The data suggest that excess Polκ induces spontaneous mutagenesis.

Discussion

DNA polymerase κ belongs to a newly identified class of error-prone DNA polymerases whose role in mammalian cells is not well understood. These enzymes share two common features, namely (1) an ability to bypass DNA lesions, either alone or in association with other polymerases, and (2) a propensity to misincorporate nucleotides during the replication of an undamaged DNA tract. We have investigated whether human Polκ could be present in replication complexes during the synthesis of undamaged or damaged DNA in human cells and whether it can affect the accuracy of DNA replication when up regulated.

By analysing the intracellular localisation of a eGFP-Polκ fusion protein in human fibroblasts, we found, in cells probably in S-phase, that the tagged Polκ is present in nuclear foci

A

RT-PCR signal expression ratio			
	10 cycles	20 cycles	30 cycles
Hprt	1.0	1.1	ND
Polκ	2.0	2.1	ND

B

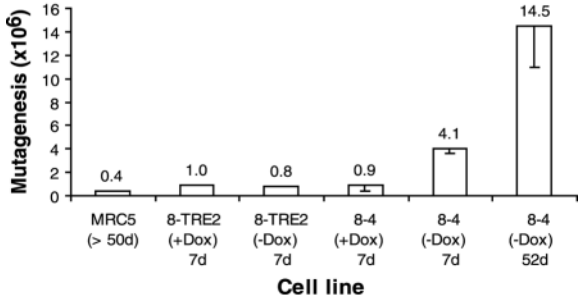

Fig. 5. Chromosomal mutagenesis in Polκ-overexpressing cells. Polκ overexpression was estimated by semi-quantitative RT-PCR (A). A 30-cycle amplification led to a saturating signal (ND, not detectable), whereas a 10- or a 20-cycle procedure allowed measurement of the signal overexpression ratio between RNA extracts from untreated and doxycyclin-treated pTet-Off/pTRE2-polκ-8-4-containing cells. (B) Untransfected MRC5 cells or MRC5 cells containing either pTet-Off/pTRE2 (8-TRE2) or pTet-Off/pTRE2-polκ (8-4) plasmids were grown in the presence or absence of doxycycline and then incubated with 6-thioguanine to select *hprt* mutants. Errors were calculated from three independent experiments.

containing the essential replicative cofactor PCNA. We demonstrated that under conditions in which replication is arrested, the number of cells harbouring eGFP-hPolκ foci increases. These findings suggest that Polκ might be spontaneously part of the replication machinery although we cannot rule out the possibility that only specialised replicative forks, such as those replicating heterochromatin, or secondary DNA structures, require Polκ. They also suggest that Polκ could be recruited when DNA replication is halted by certain lesions.

It has been shown that the yeast DNA polymerase Polζ is an efficient extender of base-pair mismatches in undamaged DNA or blocking lesions in damaged DNA (Johnson et al., 2000b). Human Polκ has also been shown to possess similar properties (Washington et al., 2002). It can extend mispaired termini on untreated DNA with high frequency (10^{-1}-10^{-2}) and can also extend from a G base opposite the 3′T of a *cis-syn* T-T dimer (Washington et al., 2002). The role of Polκ in replicative foci may therefore be to extend mispairs or to promote full bypass replication following the action of other DNA polymerases such as Polι or Polη in the case of UV lesions.

PCNA endows polymerase δ with high processivity (Kornberg and Baker, 1992) and seems also to be a Pol ε cofactor late in the S phase (Fuss and Linn, 2002). As has been previously suggested for Polη and Polι (Haracska et al., 2001a; Haracska et al., 2001b), our data are consistent with the possibility that PCNA could target Polκ to replication machinery stalled at some lesion sites, thereby allowing replication to resume. This hypothesis is supported by recent work showing that purified Polκ and PCNA proteins are able to form a complex in vitro, PCNA stimulating the Polκ-mediated DNA synthesis (Haracska et al., 2002).

Polκ makes about nine errors when replicating one kilobase of undamaged DNA in vitro (Ohashi et al., 2000a). We hypothesised that the involvement of an error-prone DNA polymerase in replication may affect accuracy when overexpressed in vivo. Ogi et al. have reported that transient expression of mouse *polκ* cDNA in untreated murine cells results in increased mutagenesis (Ogi et al., 1999). Here we provide evidence that the controlled and stable expression of human Polκ in human cells also induces spontaneous mutagenesis. Previously we have published similar data for another error-prone DNA polymerase, Polβ (Canitrot et al., 1998) and we have also demonstrated that excess Polβ interferes with the replication machinery (Servant et al., 2002). In the light of the data presented here, it is reasonable to assume that excess Polκ might similarly interfere with replisomes and affect their capacity to correctly copy DNA.

The intracellular balance between error-free and error-prone DNA polymerases appears to be of great importance within the context of genetic integrity. Because of its high mutagenic incidence, misregulated expression of an error-prone DNA polymerase may generate variant cells able to rapidly proliferate and reduce therapeutic efficacy. For example, deficiency in Polη, which is involved in the accurate bypass of UV lesions, causes skin cancers because of the inability of the cell to properly copy pyrimidine dimers (Johnson et al., 1999; Masutani et al., 1999). We have shown that Polβ, which is over produced in some human cancer tissues (Srivastava et al., 1999), is also associated with cell proliferation and tumour progression (Louat et al., 2001). In the case of Polκ, which was found to be upregulated in lung tumors relative to physically adjacent non tumorous tissue [(O-Wang et al., 2001), unpublished data from our group], and in human ovarian and prostate tumour cell lines (Cancer Genome Anatomy Project), its error-prone features may also favour cancer progression. It has been suggested that an *x*-fold increase in an in vivo mutation rate could increase cancer incidence by a factor of x^n, where *n* is the number of mutations required to develop a tumour (Yao et al., 1999). The 7-9-fold increase in the number of mutations that we observed here as a result of a limited 2-fold overexpression of Polκ shows how a weak misregulation might exert a significant influence on the progression of cancer.

DNA repair genes are currently considered as tumour suppressors since their deficiency is often related to cancer susceptibility. The root sources of the loss of genomic integrity are however multiple. Alterations in DNA replication genes, which are represented in human cells as DNA repair genes [Online Mendelian Inheritance in Man, OMIM™ (2000) McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/omim)], or misregulation of their protein expression, could also contribute to tumour genomic heterogeneity. Further studies are however required to determine which human cancers might be associated with an upregulation of error-prone DNA polymerases such as Polκ or the expression of hyperactive mutants. An understanding of why error-prone polymerases are

abnormally expressed in some cancer cells, and whether the protein is induced by endogenous and/or exogenous stress will be the next challenge.

We are grateful to H. Ohmori for providing us with the *polκ* cDNA and G. Villani and U. Hübscher for the gift of PCNA protein. We acknowledge C. Cougoule for technical assistance and C. Astarie-Dequeker, J. Middleton and C. Topham for critical reading of the manuscript. This study was supported by the Ligue Nationale Contre le Cancer (Labellisation).

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