Evidence of finely tuned expression of DNA polymerase β in vivo using transgenic mice

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Abstract DNA polymerase (Pol) is an error-prone repair DNA polymerase that has been shown to create genetic instability and tumorigenesis when overexpressed by only 2-fold in cells, suggesting that a rigorous regulation of its expression may be essential in vivo. To address this question, we have generated mice which express a transgene (Tg) bearing the *Pol* cDNA under the control of the ubiquitous promoter of the mouse H-2K gene from the major histocompatibility complex. These mice express the Tg only in thymus, an organ which normally contains the most abundant endogenous *Pol* mRNA and protein, supporting the idea of a tight regulation of Pol in vivo. Furthermore, we found no tumor incidence, suggesting that the single Pol overexpression event is not sufficient to initiate tumorigenesis in vivo.

Keywords: DNA replication; DNA polymerase β; Transgenic mice; Gene expression

1. Introduction

There are numerous processes that determine the fidelity of DNA replication in human cells. Several of these involve the accurate and processive behavior of the replicative DNA polymerases δ and ε which perform DNA synthesis of the six billion nucleotides that constitute the human genome. The past several years have seen dramatic progress in our understanding of the world of eukaryotic DNA polymerases. Human cells are now known to contain at least 14 different DNA template-dependent DNA polymerases [1], which could be categorized into two distinct families on the basis of their infidelity in replicating DNA. Emerging information suggests that connections between DNA polymerase function or expression and cancer may occur [2]. This may be the case for the mis-regulation of DNA polymerase β (Pol β), one of the structurally simplest of the known mammalian DNA polymerases, which is believed to function primarily in the repair of damaged bases in normal somatic cells [3]. It is a monomeric protein of 335 amino acids (39 kDa) that lacks exonuclease activities, resulting in inaccuracy of DNA synthesis [4]. In a normal cellular context, Pol β is expressed at a constant low level and its role is believed to be restricted to repair synthesis in the base exision repair (BER) and single strand break repair (SSBR) pathways participating in maintenance of genome stability [5]. The highly coordinated nature of these repair pathways suggests that both loss and overexpression of genes involved in these mechanisms may affect their overall efficiencies. Indeed, this is the case for Pol β: treatment of Pol β null mouse embryonic fibroblasts with DNA alkylating and oxidizing agents leads to increased cytotoxicity, gene mutations and chromosomal aberrations when compared to isogenic wild-type (WT) cell lines [3,6]; conversely, by using transfected mammalian cells and an overexpression vector, we have demonstrated that only a 2-fold overexpression of ectopic Pol β as compared to the endogenous Pol β resulted in an elevated mutation rate, chromosome instability, and accelerated tumorigenesis [7,8]. Excess of Pol β has been found in some human tumors: at the RNA level, *Pol β* is overexpressed in many cancer cells [9]; high levels of Pol β have also been detected at the protein level in ovarian tumors [10] as well as prostate, breast or colon cancer tissues, compared to adjacent normal tissues [11]; furthermore, Pol β level and activity are increased in chronic myelogenous leukemia patients (unpublished data) and melanomas [12]. Using cellular and molecular models, we previously demonstrated that excess Pol β can substitute for the replicative DNA polymerases during error-free DNA transactions such as repair [10], replication [13], or recombination (submitted data) pathways and can render

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Abbreviations: Pol β, DNA polymerase β; RT-PCR, reverse transcription and PCR; WT, wild-type; Tg, transgene; BER, base exision repair; SSBR, single strand break repair

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these processes inaccurate. All these observations led us to propose that a rigorous regulation of the error-prone DNA polymerases is essential for normal cell survival and that their overexpression may be relevant only in cancer cells. In order to address these questions, we report here the generation of mice overexpressing transgenes (Tgs) bearing the *Pol β* Sh-ble cDNA/Sh-ble cDNA fusion genes under the control of two ubiquitously active regulatory sequences, the promoter of the Cytomegalovirus (CMV) [14] that is active during development, and the promoter of the mouse H-2K gene (H-2K) from the major histocompatibility complex, which drives expression after birth [15].

2. Materials and methods

2.1. Construction of Tg expression vectors

The transgenic vectors were prepared on a pCDNA II backbone (Invitrogen), at the unique *Kpn*I site with a fragment containing the H-2K promoter, the β globin intron, and the β globin poly(A) sequence signal, to give plasmid pC-H-2K. The cDNA of rat *Pol β*, fused in frame with the bacterial gene conferring resistance to zeocin [16], was PCR-amplified from the pUTPolβ plasmid using the primers CCCTGCGCATGGCACTCGTGGAA and TTTAGCGCTCAGTCCTGCTCCTCGGCCA to create a *Eco*47III–*Fsp*I fragment that was inserted into the pC-H-2K plasmid, opened with *Bam*HI and then treated with Klenow fragment polymerase. The *Kpn*I fragment was used as Tg for the microinjections of one-cell stage mouse embryos (Fig. 1).

2.2. Generation of transgenic mice

The 3.8 Kb-long CMV and the 5.2 Kb-long *Kpn*I (H-2K) fragments containing the *Pol β* cDNA Tgs were micro-injected into fertilized (CBA × C57Bl/6) × (CBA × C57Bl/6) oocytes to obtain Tg founders, which were crossed with (CBA × C57Bl/6) WT mice and then backcrossed to WT mice or intercrossed to derive Tg lines.

2.3. Southern blot

Genomic DNA was digested by *Eco*RV and products were electrophoretically separated in a 0.8 % agarose gel. DNA present in the gel was depurinated in HCl 0.25 N solution and denaturated in 0.4 N NaOH solution before being transferred on nylon HYBOND N+ membrane. Membrane was prehybridized overnight at 42 °C in Hybrisol solution (Oncor) and hybridized in the same buffer with ^{32}P labelled Zeo (Sh) probe overnight at 42 °C. The membrane was washed twice with SSPE 2×, 0.1 % SDS for 15 min at room temperature and once with SSPE 0.1×/SDS, 0.1 % for 15 min. Detection was achieved by autoradiography.

2.4. PCRs

PCR was achieved on tail genomic DNA using primers specific for the Sh sequence (Zeo 5′: GCCAAGTTGACCAGTGCCGTT; Zeo 3′: CGGAAGTTCGTGGACACGACCT).

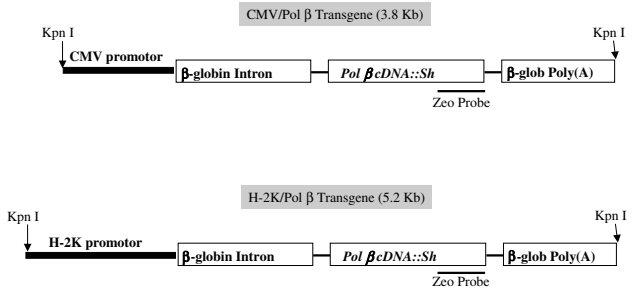

Fig. 1. Diagram of the CMV-Pol β::Sh and the H-2K-Pol β::Sh Tgs. The transgenic vectors were prepared as indicated in Section 2. The *Kpn*I fragments were used as Tgs for the microinjections of one-cell stage mouse embryos.

2.5. RT-PCRs

Total RNA preparation: 30 mg of freshly excised tissue was ground in RLT buffer in the presence of resin using a pestle adapted for Eppendorf tubes (Geno Technology). After centrifugation, RNA extraction was performed with Rneasy Protect kit (Qiagen). Quality of RNA was confirmed by analysis with Agilent Technologies 2100 Bioanalyzer – Bio Sizing (Version A.02.05 S1243). Reverse transcription and PCR (RT-PCR) were processed in one tube using the Access RT-PCR kit (Promega). One microgram of total RNA was reverse transcribed and the PCR amplification was achieved with the specific Zeo 5′ and Zeo 3′ primers.

2.6. Northern blot

Total RNA was prepared as described above. For the Northern blot analysis, 30 μg of total RNA was denatured 10 min at 65 °C in denaturation buffer. RNA samples were electrophoretically fractionated in a 2% gel containing MOPS 1× and formaldehyde 0.5× and blotted to a N+ membrane in SSC 10× buffer. Membrane was prehybridized overnight at 42 °C in Hybrisol solution (Oncor) and hybridized in the same buffer with ^{32}P labelled Zeo probe overnight at 42 °C. The membrane was washed once with SSC 0.5× for 15 min. at room temperature and twice with SSC 0.1×/SDS 0.1% for 15 min at 55 °C. Detection was achieved by phosphoImager storm system analysis (Molecular Dynamics).

2.7. Western blotting

Organs were disrupted and lysed in SDS-containing buffer according to the protocol described previously [11]. For analysis of Pol β, 100 μg of organ protein was electrophoresed in a 12% SDS–PAGE gel and transferred to PVDF membrane (Appligen). Blots were blocked in TBS-T (0.1% Tween) with 5% skimmed milk and incubated with Pol β polyclonal antibody (1/5000) (provided by Dr. Sam Wilson, NIEHS, NC) followed by incubation with horseradish peroxidase conjugated anti rabbit IgG, and revealed using the ECL system (Amersham). Equal loading was determined using monoclonal antibody to actin (1/5000) (Chemicon, Euromedex, France). Quantification was made by PhosphoImager Storm-system analysis (Molecular Dynamics) using Imagequant software.

2.8. Macroscopic examination of the mice

Mice were housed in a room lighted from 7 a.m. to 7 p.m., kept at a temperature of 19–21 °C with a relative humidity of 60 ± 15%. Food and sterile water were provided ad libitum. The mice were housed 5 per cage and kept until natural death. Animal care consisted of weekly cleaning of the cages and bottles, changing sawdust litter (pine, heat treated), daily monitoring of the animals and the use of germ-limited conditions by the keeper (gloves, gown, covered hair). Body weights were recorded monthly. This study was performed in compliance with FELASA recommendations. An animal care committee approved the experiment.

Some of the animals were excluded from the necropsy study when postmortem decomposition was too severe. Thus, the necropsy was performed on 23 mice (16 Polβ mice and 7 control mice). Macroscopic examination was systematically performed. The following organs were removed: lungs, heart, thymus, liver, spleen, pancreas, intestinal tract, lymph nodes, kidneys, adrenal glands, ovaries, uterus, testis, bone marrow, salivary glands, and brain. Some tissue samples were frozen for further analysis (see below) and the remaining tissues were fixed in formalin. After paraffin embedding, 5-μm thick sections were prepared and the slides stained with hematoxylin–eosin.

3. Results and discussion

The design of the CMV-Pol β::Sh Tg and H-2K-Pol β::Sh Tg (Fig. 1) was based on three main considerations: (a) the Pol β-Sh cDNA was placed under the control of either the CMV promoter or that of the murine MHC class I H-2K gene to drive Tg expression in a wide variety of tissues, as shown for other reporters; indeed, when CMV [14] or when 2 Kb of the H-2K upstream regulatory sequences were fused to several reporter genes, such as hGH [15], c-myc [17], or v-jun [18], fusion genes were ubiquitously expressed in adult organs of

transgenic mice, (b) the bacterial *ble Sh* gene, which confers resistance to the broad-spectrum zeocin antibiotic of the phleomycin family, was fused in frame with the NH_2 terminus of the Pol β protein to discriminate between Tg and endogenous *Pol β* expression; and (c) the Pol β-Sh cDNA was linked to the β-globin intron and polyadenylation site to enhance transcription and stabilize mRNA, respectively. Only one CMV Tg line and five independent H-2K Tg lines, named 1, 3a, 3b, 8a, and 8b were derived (Fig. 2A), which have distinct Tg integration patterns as demonstrated by Southern blot analysis (Fig. 2B). The line 3b was eliminated since it was sterile.

For all lines, the heterozygous mice developed to maturity with no obvious abnormalities. These heterozygotes were then bred to homozygosity to increase the potential effect of the deregulated polymerase. Mice homozygous for the Pol β overexpression were obtained at the expected Mendelian frequency. When intercrossed, their offspring were viable and fertile. We first analyzed the Tg mRNA expression by Northern blot in different adult tissues of each Tg line. Expression of Tg was also investigated by Western blot analysis using a polyclonal anti-Pol β antibody. No expression of the CMV Tg was observed, strongly suggesting a deleterious effect of Pol β overexpression during development. In contrast, we observed expression of the Tg when driven by the H-2K promoter in one of the 5 lines, the 8a line, whereas the lines 1, 3a and 8b did not express the Tg. Using a Tg specific probe (Sh), expression of the Tg in the 8a line was observed at the mRNA level exclusively limited to the thymus (Fig. 3). This observation was confirmed with the protein expression analysis by Western blot (Fig. 4), which indicates that the level of Tg expression is several fold above the level of endogenous Pol β. These findings show that, despite a strong promoter, most of the organs do not tolerate high level of the ectopic Pol β, supporting that tight regulation of Pol β occurs in these organs. Since we could not observe Tg expression at RNA and protein level in organs other than thymus, we could speculate that Pol β overexpression is down regulated by transcriptional control or RNA degradation. The thymus is a highly replicative organ where all the replicative and repair machineries are activated; furthermore, the mutagenic impact of high level of Pol β is thwarted in this organ by excess mismatch repair proteins which can eliminate mismatches introduced into DNA by the up-regulated Pol β.

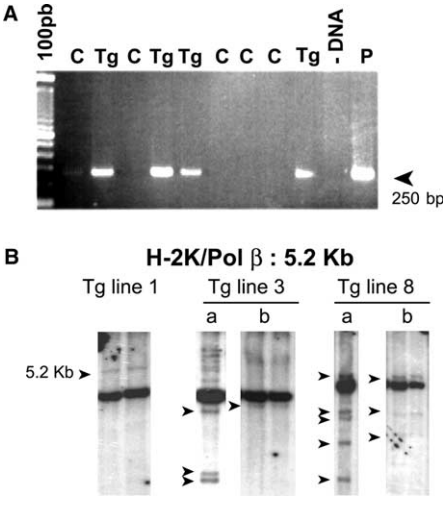

Fig. 2. Generation of transgenic mice. (A) Transgenic founders were identified by PCR on tail genomic DNA using primers specific for the Sh sequence; C: non-transgenic control littermates; Tg: Transgenic animals; P: positive control PCR using the vector bearing the Tg as DNA template. (B) Analysis of the integration of the H-2k/Pol β construct by Southern blot in the Tg lines 1, 3a, 3b, 8a, and 8b. The arrows represent the 3′ flanking sequences.

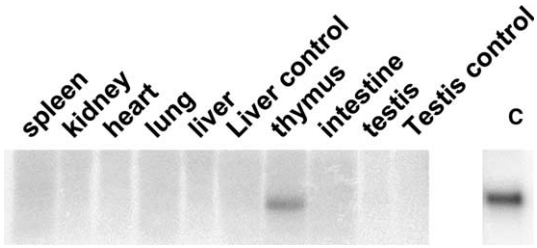

Fig. 3. Analysis of the expression of the Tg mRNA by Northern blot in various tissues of control and the 8a transgenic mice. RNA preparation and Northern blot analysis were performed as indicated in Section 2. C, Control experiment using the CHO::Pol β cell line transfected by the pUTPolβ plasmid, overexpressing Pol β by 4-fold [8].

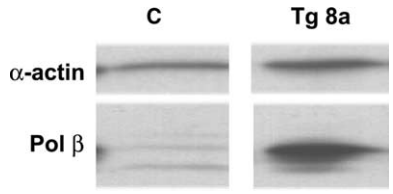

Fig. 4. Immunoblotting analysis of Pol β level in thymus from control and the 8a Tg mice. Expression of Pol β in the thymus of Control (C) and Tg8a lines was analyzed by using Pol β polyclonal antibodies that were provided by Dr. S. Wilson, NIEHS, NC, USA. α-Actin served as a loading control.

In order to examine the survival of H-2K/Pol β 8a animals that overexpressed Pol β, we maintained 300 WT, 300 heterozygotes, and 69 homozygous animals in identical conditions and recorded the time when they became moribund or the time of natural death. None of the three classes of mice died during the first 6 months of life. Between 6 and 9 months after birth (mean 7-month-old), twenty five (36%) of the homozygous animals died prematurely quite suddenly without any premortem symptoms, equally among males and females. Such a mortality was never observed for WT and heterozygous animals. These results suggest that Tg Pol β expression in thymus or in other organs even if undetected (less than 2-fold overexpression) could be responsible for shortened life span. Because Pol β is the major BER polymerase, a tightly coordinated mechanism for repair of DNA base damage via alkylation and oxidation as well as base losses, imbalance of its expression may cause an overall functional deficiency of the elimination of the numerous endogenous oxidative DNA damage events that have accumulated during the first months of life of the mice. This could explain the deleterious impact of excess Pol β in vivo that we observed in this study. Moreover, we found that homozygous Tg mice showed a distinctive phenotype with thinness and frequent spastic crisis resembling epilepsy. At 6 months of age, homozygous female mice weighed between 20 and 30 g (mean at 24–25 g) and homo-

zygous males between 20 and 38 g (mean at 30 g), significantly less than 6-month-old-WT or heterozygous animals (average at 30 g for the females and 35 g for the males) ($P < 0.01$).

We then performed systematically macroscopic examination of 46 mice (23 homozygous animals alive at 6–9 months; 16 prematurely dead homozygous mice and 7 control mice). The following organs were removed: lungs, heart, thymus, liver, spleen, pancreas, intestinal tract, lymph nodes, kidneys, adrenal glands, ovaries, uterus, testis, bone marrow, salivary glands, and brain. When analysis was performed randomly on 14 homozygous animals alive at 6–9 months, enlarged thymus was observed. On the prematurely dead transgenic animals, we found no obvious abnormality that could explain the mortality. Liver and pancreas were histologically normal, brain structure and histology were not modified. There was no significant difference between Tg mice and controls in terms of neuronal and astrocytic cellularity as revealed by the GFAP immunostainings (not shown). The heart, lungs, kidneys, and intestinal tract were normal. Lymphoid organs such as thymus and spleen were histologically normal. Furthermore, macroscopic and microscopic analysis did not reveal any tumoral lesion. This indicates that the unique event, overexpression of Pol β, is not sufficient to induce tumorigenesis in vivo. Mutagenesis, chromosome instability and tumorigenesis impacts of Pol β overexpression have been previously described ex vivo in CHO cells [7,8] where ectopic expression of Pol β induces high frequency of spontaneous mutations, aneuploidy, an abnormal localization of the centrosome-associated γ-tubulin protein during mitosis, a deficient mitotic checkpoint, and promotes tumorigenesis in nude immunodeficient mice. Thus, it was proposed that alteration of Pol β expression in CHO cells appears to induce major genetic changes associated with a malignant phenotype. Some of the molecular bases leading to these genetic instabilities were analyzed and it was found that excess Pol β could compete with the accurate DNA polymerases during DNA transactions such as DNA replication [13] or DNA repair [10], resulting in highly error-prone DNA synthesis. However, the CHO/AA8 cell line used in these sudies was a derivative from the CHO-K1 cell line which has a mutant p53 sequence. Preliminary attempts to overexpress Pol β in a normal p53 background CHO were unsuccessful (unpublished data). Thus, it is possible that tolerance of high level of Pol β in murine cells may require another oncogenic event. Our in vivo data presented here support this hypothesis.

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References

[1] Burgers, P.M. et al. (2001) J. Biol. Chem. 276, 43487–43490.
[2] Kunkel, T.A. (2003) Cancer Cell 3, 105–110.
[3] Sobol, R., Horton, J., Kühn, R., Gu, H., Singhal, R., Prasad, R., Rajewsky, K. and Wilson, S. (1996) Nature 379, 183–186.
[4] Kunkel, T. (1985) J. Biol. Chem. 260, 5787–5796.
[5] Zmudzka, B., Fornace, A., Collins, J. and Wilson, S. (1988) Nucleic Acids Res. 16, 9589–9596.
[6] Ochs, K., Sobol, R.W., Wilson, S.H. and Kaina, B. (1999) Cancer Res. 59, 1544–1551.
[7] Canitrot, Y., Cazaux, C., Frechet, M., Bouayadi, K., Lesca, C., Salles, B. and Hoffmann, J. (1998) Proc. Natl. Acad. Sci. USA 95, 12586–12590.
[8] Bergoglio, V. et al. (2002) Cancer Res. 62, 3511–3514.
[9] Scanlon, K., Kashani-Sabet, M. and Miyachi, H. (1989) Cancer Invest. 7, 581–587.
[10] Canitrot, Y., Hoffmann, J.S., Calsou, P., Hayakawa, H., Salles, B. and Cazaux, C. (2000) FASEB J. 14, 1765–1774.
[11] Srivastava, D., Husain, I., Arteaga, C. and Wilson, S. (1999) Carcinogenesis 20, 1049–1054.
[12] Servant, L., Cazaux, C., Bieth, A., Iwai, S., Hanaoka, F. and Hoffmann, J. (2002) J. Biol. Chem. 277, 50046–50053.
[13] Servant, L., Bieth, A., Hayakawa, H., Cazaux, C. and Hoffmann, J.S. (2002) J. Mol. Biol. 315, 1039–1047.
[14] Schmidt, E.V., Christoph, G., Zeller, R. and Leder, P. (1990) Mol. Cell. Biol. 10, 4406–4411.
[15] Morello, D., Moore, G., Salmon, A.M., Yaniv, M. and Babinet, C. (1986) EMBO J. 5, 1877–1883.
[16] Bouayadi, K., Hoffmann, J., Fons, P., Tiraby, M., Reynes, J. and Cazaux, C. (1997) Cancer Res. 57, 110–116.
[17] Morello, D., Lavenu, A., Pournin, S. and Babinet, C. (1993) Oncogene 8, 1921–1929.
[18] Schuh, A.C., Keating, S.J., Monteclaro, F.S., Vogt, P.K. and Breitman, M.L. (1990) Nature 346, 756–760.