5354–5361 *Nucleic Acids Research, 2005, Vol. 33, No. 16*
doi:10.1093/nar/gki845

Phosphorylation of human DNA polymerase λ by the cyclin-dependent kinase Cdk2/cyclin A complex is modulated by its association with proliferating cell nuclear antigen

Isabelle Frouin, Magali Toueille, Elena Ferrari, Igor Shevelev and Ulrich Hübscher*

Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Received August 16, 2005; Revised and Accepted August 31, 2005

ABSTRACT

DNA polymerase (Pol) λ is a member of the Pol X family and possesses four different enzymatic activities, being DNA polymerase, terminal transferase, deoxyribose phosphate lyase and polynucleotide synthetase, all localized in its C-terminal region. On the basis of its biochemical properties, Pol λ has been implicated in various DNA repair pathways, such as abasic site translesion DNA synthesis, base excision repair and non-homologous end joining of double strand breaks. However, its role *in vivo* has not yet been elucidated. In addition, Pol λ has been shown to interact with the replication clamp proliferating cell nuclear antigen (PCNA) *in vitro* and *in vivo*. In this work, we searched by affinity chromatography for novel partners and we identified the cyclin-dependent kinase Cdk2 as novel partner of Pol λ. Pol λ is phosphorylated *in vitro* by several Cdk/cyclin complexes, including Cdk2/cyclin A, in its proline-serine-rich domain. While the polymerase activity of Pol λ was not affected by Cdk2/cyclin A phosphorylation, phosphorylation of Pol λ was decreased by its interaction with PCNA. Finally, Pol λ is also phosphorylated *in vivo* in human cells and this phosphorylation is modulated during the cell cycle.

INTRODUCTION

Cell cycle progression is regulated by a family of cyclin-dependent kinases (Cdks) which phosphorylate and activate proteins that execute events critical to cell cycle progression. For activity, Cdks require association with a cyclin and phosphorylation by a Cdk activating kinase (CAK) at a conserved threonine residue (1). Each phase of the cell cycle is characterized by the expression of different Cdk/cyclin complexes that phosphorylate and regulate downstream substrates. In vertebrates, Cdk4/6/cyclin D complexes are active throughout the G1 phase, Cdk2/cyclin E at the G1/S boundary, Cdk2/cyclin A during S phase and Cdk1/cyclin A and Cdk1/cyclin B during the G2/M transition. Studies using knockout mice (2–4) revealed that neither Cdk2 nor cyclin E is essential for mitotic cell division. Cyclin E seems to be important for the control of endoreplication and for quiescent cells which re-enter in cell cycle. Furthermore, Cdk2 is essential in the regulation of the meiotic cell cycle, suggesting a novel tissue-specific function for cyclins and Cdks.

Human DNA polymerase (Pol) λ belongs to the Pol X family based on sequence homology with Pol β, Pol μ and terminal deoxynucleotidyl transferase (5). Pol λ contains a nuclear localization signal (residues 1–35), a BRCA1 C-terminal domain (BRCT, residue 36–132), a proline-serine-rich region (residues 133–243) and a Pol β-like core region (residues 244–575). Pol λ has been well characterized *in vitro* and it possesses four different enzymatic activities: DNA polymerase, terminal transferase, deoxyribose phosphate lyase and polynucleotide synthetase, all localized in its C-terminal region (6–8). On the basis of its biochemical properties, Pol λ has been proposed to be involved in various DNA repair pathways, such as abasic site translesion DNA synthesis (9,10), base excision repair (11,12), repair of oxidative DNA damage (13) and non-homologous end joining of double strand breaks (DSBs) (14,15). In addition, Pol λ has been shown to interact with the replication clamp proliferating cell nuclear antigen (PCNA) (9,16) and with the DNA repair protein ligase IV/XRCC4 (17). Mapping of Pol λ interaction with PCNA identified a helix–hairpin–helix motif localized in its Pol β-like core region (16). The major sites of interaction between PCNA and many of its partners are the interdomain

*To whom correspondence should be addressed. Tel: + 41 16 35 54 72/71; Fax: +41 16 35 68 40; Email: hubscher@vetbio.unizh.ch

© The Author 2005. Published by Oxford University Press. All rights reserved.

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oxfordjournals.org

connecting loop (ID-loop) (amino acids 121–132) and the facing hydrophobic pocket (amino acids 42–46) of PCNA (18,19). We have recently shown that residues 43–45 of the hydrophobic pocket are also essential for the interaction with Pol λ (16). In addition, residues 125–128 of the ID-loop also play an important role in stabilizing this interaction (16). Furthermore, interaction of Pol λ with PCNA has also been demonstrated *in vivo* (16,20). On the other hand, the *in vivo* role of Pol λ is so far poorly understood. To better understand the Pol λ function, we searched by affinity chromatography for novel partners. We identified the cyclin-dependent kinase Cdk2 as novel partner of Pol λ. Pol λ is a substrate for phosphorylation by several Cdk/cyclin complexes *in vitro* and its proline-serine-rich domain is the target of phosphorylation by Cdk2/cyclin A. Moreover, the phosphorylation of Pol λ by Cdk2/cyclin A is decreased upon interaction of Pol λ with PCNA. Finally, we demonstrated that Pol λ is phosphorylated *in vivo* and this phosphorylation is regulated during the cell cycle.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (3000 mCi/mmol) and unlabeled ATP were purchased from Amersham Biosciences, DNA oligonucleotides from Microsynth Gmbh (Balgach, Switzerland). All other reagents were from Merck, Fluka or Sigma.

Enzymes and proteins

Recombinant human wild-type Pol λ was expressed and purified as described by Ramadan *et al*. (7). Human recombinant PCNA was purified as previously described. The SHV43 mutant of human PCNA was generated, expressed in *Escherichia coli* and purified as described previously (21). The pGEX-3X plasmids expressing human wild-type Cdk2 GST fusion protein was kindly provided by C. Bonne-Andréa. The purified Cdk2/cyclin E, Cdk2/cyclin A, Cdk1/cyclin A complexes (22) were gifts from H. P. Nasheuer (Jena, Germany). Histone H1 was purchased from Roche.

DNA substrate

The sequence of the d73mer is : 5′GATCGGGAGGGTAGGAATATTGAGGATGAAGGGTTGAGTTGAGTGGAGATAGTGGAGGGTAGTATGGTGGATA3′. The sequence complementary to the 17mer primer is underlined.

Cell culture

HeLa cells were grown as monolayers in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 10 μg/ml antibiotics (penicillin and streptomycin) at 37°C in a humidified atmosphere containing 5% CO2. Synchronization of HeLa cells at the G1/S border was obtained by growing cells in 2 mM thymidine as described by Stein (23). After thymidine removal, cells were washed twice with PBS, harvested immediately (G1/S border) or were further cultured in complete medium for 3 h (S phase), 6 h (late S phase), 9 h (G2 phase) or 12 h (G2/M phase). Synchronization in M phase was obtained by growing cells in nocodazole (40 ng/ml) for 20 h. After nocodazole removal, cells were washed twice with phosphate-buffered saline (PBS), harvested immediately (M phase) or were further cultured in complete medium for 4 h (G1 phase). Cells were subsequently harvested and washed three times with PBS and were pelleted by low-speed centrifugation (250 *g*, 5 min, 4°C), and the cell pellets stored at −80°C until further use.

Preparations of cell extracts for DNA polymerase λ western blots

HeLa cells were washed twice with cold PBS. Then, 500 μl of Laemmli buffer without β-mercaptoethanol were added to lyse the cells. The lysed cells were scraped off the dish and the DNA was shared by using a syringe. β-mercaptoethanol (1.25%) and bromophenol blue (0.005%) were added and the samples were incubated for 5 min at 95°C.

Affinity chromatography

His-Pol λ or BSA were covalently bound to a HiTrap NHS-activated Sepharose High performance column (Amersham Biosciences) according to the manufacturer's instructions. HeLa cells were lysed for 15 min in a high salt buffer [100 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20% glycerol, 0.5% NP-40, 10 mM glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml bestatin] and centrifuged for 10 min at 10 000 *g* at 4°C, and the supernatant was kept as the total extract. The total extract was diluted 1:4 with dilution buffer (100 mM HEPES, pH 7.5, 1 mM DTT, 2 mM PMSF, 20% glycerol, 10 mM glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml bestatin). Aliquots containing 40 mg of the diluted extract were loaded in parallel onto either a BSA (control) or a Pol λ column. The columns were washed with 2 column volumes with a buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 1 mM DTT and 0.05% NP-40. Subsequently, the bound proteins were eluted with the above mentioned buffer containing, respectively, 150, 350 and 750 mM NaCl. Fractions (20 μl) were analyzed by western blot by using different antibodies.

Antibodies

Antibodies against Cdk2 (sc-748) and PCNA (clone PC10) were purchased from Santa Cruz Biotechnology and α-tubulin (T-6199) from Sigma.

Immunoprecipitation experiments

Total extracts from HeLa cells were prepared as described above. An aliquot of 1.5 mg of total extract was diluted 1:4 in buffer A (10 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$, 0.5% NP-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml bestatin) to yield a final concentration of 100 mM NaCl and were then incubated with 2 μg of antibodies against Cdk2 or 2 μg of rabbit IgG (Vector Laboratories) as a negative control for 3 h at 4°C. Then, 25 μl of protein G-Sepharose (Amersham Biosciences) coated with BSA were added for 1 h at 4°C. The samples were centrifuged for 30 min at 13 000 *g* at 4°C and the pellets washed three times for 10 min at 4°C with a buffer containing 50 mM Tris–HCl, pH 8, 50 mM NaCl, 75 mM KCl, 0.1% NP-40, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml

bestatin. After immunoprecipitation the proteins were heated 5 min at 95°C in Laemmli buffer and analyzed by SDS–PAGE and western blot.

Pull-down assay

Pull-down experiments were performed by incubating 750 ng of GST-Cdk2 with His-tagged Pol λ bound to Ni^{2+}-NTA beads or with Ni^{2+}-NTA beads alone as a control, for 2 h at 4°C in 40 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% (v/v) NP-40, 5 mM imidazole, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml bestatin. After washing four times with the same buffer, the beads were heated for 5 min at 95°C in Laemmli buffer and the co-precipitated proteins analyzed by western blot using the corresponding antibodies.

Enzymatic assays

Kinase assay. Phosphorylation was carried out in a final volume of 15 μl containing kinase buffer [50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 μM [γ-^{32}P]ATP (3000 mCi/mmol)], 66.6 μM ATP, in the presence of substrate (Histone H1 or His-Pol λ) and 40 ng of purified Cdk/cyclin complexes. Reactions were carried out for 20 min at 37°C, and the proteins separated on a 10% SDS–PAGE. The gel was stained by Coomassie brilliant blue, dried and exposed to an X-ray film. The incorporation of [γ-^{32}P]ATP in Pol λ polypeptide was determined by cutting out the band corresponding to Pol λ after Coomassie staining, and by measuring the radioactivity by liquid scintillation counting. Background was determined by cutting out the band corresponding to Pol λ incubated in the reaction mixture in the absence of Cdk/cyclin complex and subtracted from the obtained measurements. Pmols of incorporated radioactivity were calculated according to the specific activity of the [γ-^{32}P]ATP used in the assay.

DNA polymerase assay

Product analysis by His-Pol λ in the linear kinetic region was performed using a sequencing gel. The reaction mixture included in a final volume of 10 μl: 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MnCl_2$ and 10 μM of each unlabeled dNTPs, 20 fmol [^{32}P]5′ end-labeled 17mer primer annealed to the d73mer template (see above). Reactions were incubated for 15 min at 37°C, stopped by addition of sequencing gel loading buffer [95% (v/v) formamide, 20 mM EDTA, pH 8.0] and heated for 5 min at 95°C. The reaction products were resolved on a 10% polyacrylamide, 7 M urea gel. The gels were dried and exposed to an X-ray film.

Phosphatase treatment

HeLa cells were grown as previously described, washed once with ice-cold PBS, scraped off the dish and collected in ice-cold PBS. The cells were spinned down, the packed cell volume (PCV) was estimated and the cells were resuspended in 1 PCV of buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml bestatin]. After incubation on ice for 15 min the samples were centrifuged for 10 min at 10 000 *g* at 4°C, and the supernatants kept as cell lysates. The phosphatase assay was carried out in a volume of 20 μl containing 25 μg of cell lysate, the λ-phosphatase mixture (50 mM Tris–HCL, pH 7.5, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35 and 2 mM $MnCl_2$) and 400 U of λ-phosphatase (New England Biolabs). Incubation was at 30°C for 45 min. Two negative controls were added one without λ-phosphatase and the other with λ-phosphatase in the presence of the phosphatase inhibitors EDTA (5 mM) and Na_3VO_4 (1 mM). Then, 2× Laemmli buffer was added, the samples were heated at 90°C for 2 min and loaded on a 10% SDS–PAGE gel. The shift of the phospho-band was analyzed by western blot by using the corresponding antibodies.

RESULTS

DNA polymerase λ interacts with Cdk2 *in vitro* and *in vivo*

First, we searched for novel partners of Pol λ by affinity chromatography. His-Pol λ or BSA were covalently bound on HiTrap NHS-activated Sepharose HP columns. Total extracts of HeLa cells were prepared as described in Materials and Methods and loaded in parallel onto the two affinity columns. Bound proteins were eluted successively by 150, 350 and 750 mM NaCl. The purification was carried out essentially by following the PCNA elution, a protein known to interact with Pol λ *in vitro* (9) and *in vivo* (16). The elution profiles of BSA and Pol λ are shown in Figure 1A. Proteins binding unspecifically were eluted in peak 1 at low NaCl concentrations from the Pol λ and the BSA columns. Two additional peaks were exclusively present in the Pol λ column elution. They contained proteins eluted at higher NaCl concentration, suggesting that they interact specifically with Pol λ. The eluted fractions were tested by western blot by using different antibodies. We first confirmed that, as expected, PCNA specifically bound to the Pol λ column (data not shown). Next, we found that the cyclin-dependent kinase Cdk2 was eluted in peak 2 (fractions 24–28) from the Pol λ column (Figure 1B), whereas it bound only unspecifically to the BSA column (peak 1). We further confirmed this interaction *in vitro* by a His pull-down experiment. For this, GST-Cdk2 was incubated with His-Pol λ bound to Ni-beads or with Ni-beads alone as a negative control (Figure 1C). Western blot analysis against Cdk2 showed a specific co-precipitation of Pol λ with Cdk2, suggesting a direct interaction of these two proteins. Finally, the interaction was tested *in vivo* by immunoprecipitation. Anti-Cdk2 or control IgG bound to protein G-Sepharose was incubated with HeLa cells total extracts. After immunoprecipitation, western blot against Pol λ showed that Pol λ was co-immunoprecipitated with Cdk2 only when the anti-Cdk2 antibody was used (Figure 1D). In summary, these results indicate that Cdk2 can physically interact with Pol λ *in vitro* and *in vivo*.

DNA polymerase λ is phosphorylated by the Cdk2/cyclin E, Cdk2/cyclin A and Cdk1/cyclin A complexes *in vitro*

Next, we searched for potential Cdk phosphorylation sites in the amino acid sequence of Pol λ. We found three serines (amino acids 167, 177 and 230, respectively) which could be part of consensus sites for Cdk phosphorylation. They are all located in the proline-serine-rich domain of Pol λ. These sites are specific to Pol λ, since they do not occur in the sequence of any of the other Pol X family members

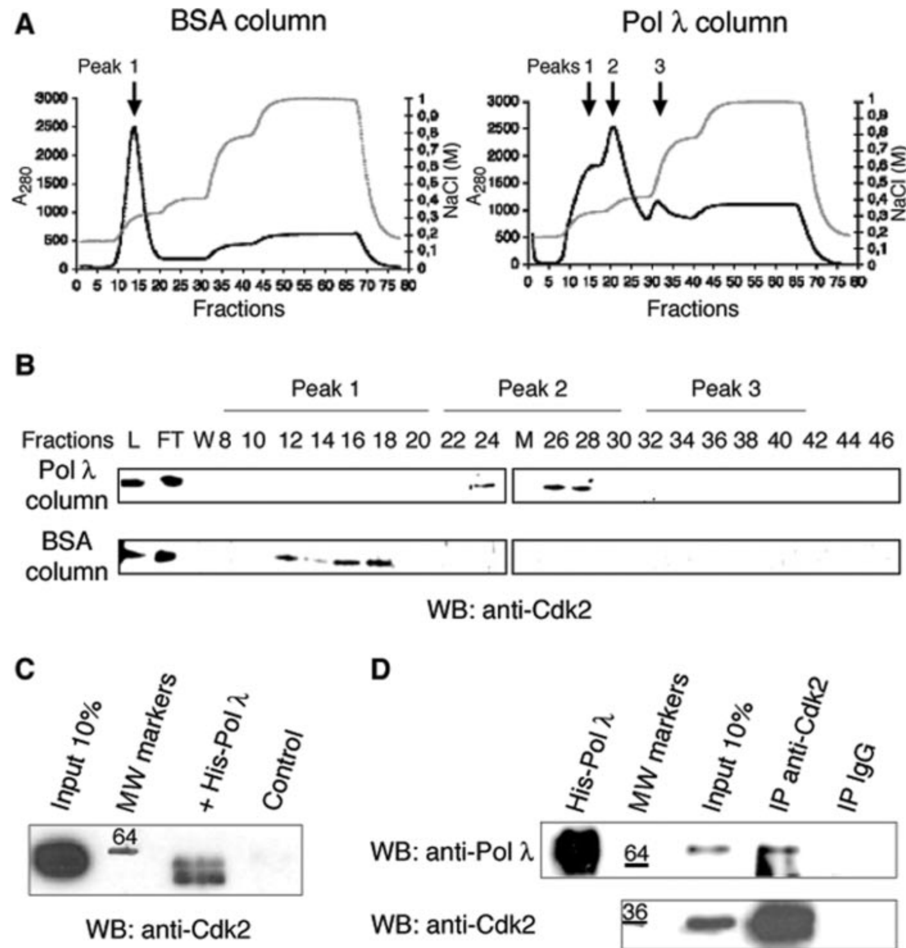

Figure 1. DNA polymerase λ interacts with Cdk2 *in vitro* and *in vivo*. (**A**) Elution profile of BSA and Pol λ affinity columns. Aliquots containing 40 mg of HeLa cells total extract were each loaded in parallel onto a BSA and Pol λ affinity column, respectively. Bound proteins were eluted successively by 150, 350 and 750 mM NaCl. (**B**) Aliquots containing 20 µl elution fractions from the BSA and Pol λ columns were loaded on a SDS–PAGE gel and a western blot against Cdk2 was performed. (**C**) His-Pol λ interacts with GST-Cdk2 *in vitro*. Pull-down experiments were performed by incubating 750 ng of GST-Cdk2 with His-tagged Pol λ bound to Ni^{2+}-NTA beads or with Ni^{2+}-NTA beads alone (Control), for 2 h at 4°C. After washing four times with the same buffer, the beads were heated for 5 min at 95°C in Laemmli buffer and the co-precipitated proteins were analyzed by western blot. The 'Input 10%' lane shows 10% of the total amount of GST-CDK2 used in the pull-down. (**D**) Pol λ and Cdk2 interact *in vivo*. Aliquots containing 1.5 mg of total HeLa cell extract were incubated with 2 µg of antibody against Cdk2 or 2 µg of rabbit IgG as a negative control for 3 h at 4°C. Then, 25 µl of protein G-Sepharose were added for 1 h at 4°C. Immunoprecipitated proteins were heated for 5 min at 95°C and analyzed by SDS–PAGE and western blot. The 'Input 10%' lane shows the signal obtained when 10% of the amount of cell extract used in the immunoprecipitation were loaded on the gel.

(Figure 2A). In order to verify whether Pol λ could be a substrate for Cdk-dependent phosphorylation, kinase assays with different Cdk/cyclin complexes were performed in the presence of recombinant Pol λ and [γ-^{32}P]ATP. As shown in Figure 2B, a [^{32}P]labeled band of the expected molecular weight for Pol λ appeared after incubation with the Cdk2/cyclin E, Cdk2/cyclin A and Cdk1/cyclin A complexes. Histone H1 was used as a positive control. The identity of the phosphorylated band with Pol λ was confirmed by SDS–PAGE and immunoblot analysis (data not shown, and Figures 1D and 3B, left panel). In summary, these results suggest that Pol λ is a target for phosphorylation by these three Cdk/cyclin complexes *in vitro*.

The proline-serine-rich domain of DNA polymerase λ is the target of phosphorylation by Cdk2/cyclin A

In order to map the phosphorylation domains of Pol λ, kinase assays were performed by using deletion mutants of Pol λ (illustrated in Figure 3A). Figure 3B (left panel) shows a Coomassie staining of the samples after the phosphorylation reaction separated by SDS–PAGE. The same gel was then exposed to an X-ray film to reveal the phosphorylated products. As shown in Figure 3B (right panel), Pol λ, deleted for the BRCT domain, was still phosphorylated by Cdk2/cyclin A, whereas the mutant missing both the BRCT and proline-serine-rich domain was not. This result supported our findings that the three potential sites of phosphorylation are located in the proline-serine-rich domain (see Figure 2A) and suggested that this domain of Pol λ is the target for Cdk2/cyclin A.

Phosphorylation of DNA polymerase λ does not affect its polymerase activity and is regulated by its interaction with PCNA

Furthermore, the effect of Pol λ phosphorylation on its polymerase activity was tested. For this, His-Pol λ was

Figure 2. DNA polymerase λ is phosphorylated by Cdk2/cyclin E, Cdk2/cyclin A and Cdk1/cyclin A complexes *in vitro*. (**A**) Multiple amino acid alignment of the human Pol X family and potential Cdk phosphorylation sites in Pol λ. (**B**) Pol λ is a substrate of Cdk2/cyclin E, Cdk2/cyclin A and Cdk1/cyclin A. Aliquots containing 500 ng of Pol λ or 100 ng Histone H1 were incubated in a kinase buffer in the presence of 40 ng of different Cdk/cyclin complexes for 20 min at 37°C. Proteins were then separated on a 10% SDS–PAGE, the gel stained by Coomassie brilliant blue, dried and exposed to an X-ray film.

Figure 3. The proline-serine-rich domain of DNA polymerase λ is the target of phosphorylation by Cdk2/cyclin A. (**A**) Schematic domain organization of Pol λ wt and deletion mutants. (**B**) Kinase assay by Cdk2/Cyclin A. Aliquots containing 500 ng of Pol λ wt and deletion mutants or 100 ng Histone H1 were incubated in a kinase buffer containing 40 ng of Cdk2/Cyclin A complex for 20 min at 37°C. Proteins were then separated on a 10% SDS–PAGE, the gel stained by Coomassie brilliant blue (left panel), dried and exposed to an X-ray film (right panel).

phosphorylated *in vitro* by Cdk2/cyclin A in the presence of ATP. Preliminary experiments showed that the degree of Pol λ phosphorylation was between 56 and 84% considering that it can be phosphorylated on one to three sites (see Materials and Methods). This suggested that any effect due to phosphorylation of Pol λ could be visualized by the sensitive method of product analysis. As negative controls, His-Pol λ was either incubated in the presence of Cdk2/cyclin A without ATP, or in the presence of ATP without Cdk2/cyclin A. The Pol λ polymerase activity was then tested on a 5′-labeled d17:d73mer primer-template and the elongation products were visualized on a polyacrylamide denaturating gel. DNA synthesis by Pol λ was stimulated when it was incubated with ATP, either alone (lanes 7–9) or in combination with Cdk2/cyclin A (Figure 4A, lanes 1–3), compared with the reactions where ATP was omitted (lanes 4–6, lanes 10–12). Stimulation was greater when Pol λ was incubated in the presence of ATP alone (lanes 7–9), suggesting that the observed stimulation was rather due to the presence of ATP than to phosphorylation. This effect could be explained by the fact that Pol λ, similarly to Pol μ (24), is able to incorporate ribonucleotides opposite a DNA template (K. Ramadan and U. Hübscher, unpublished data). In this case, ATP could be used as a nucleotide substrate by Pol λ, thus explaining the increased product elongation observed upon addition of ATP (Figure 4A, lanes 7–9). Moreover, the slight decrease in the effect of ATP in the presence of Cdk2/cyclin A may be due to its consumption occurring upon Pol λ phosphorylation (Figure 4A, lanes 1–3). From these results, we concluded that phosphorylation of Pol λ by Cdk2/cyclin A does not directly affect its polymerase activity. In addition, by testing the effect of phosphorylation on Pol λ terminal deoxynucleotidyl transferase activity, we showed that phosphorylation did not affect this activity (data not shown).

Since Pol λ and PCNA have been shown to interact *in vivo* and *in vitro* (9,16), and PCNA is known to interact with Cdk/cyclin complexes (19), we next tested the effect of PCNA on

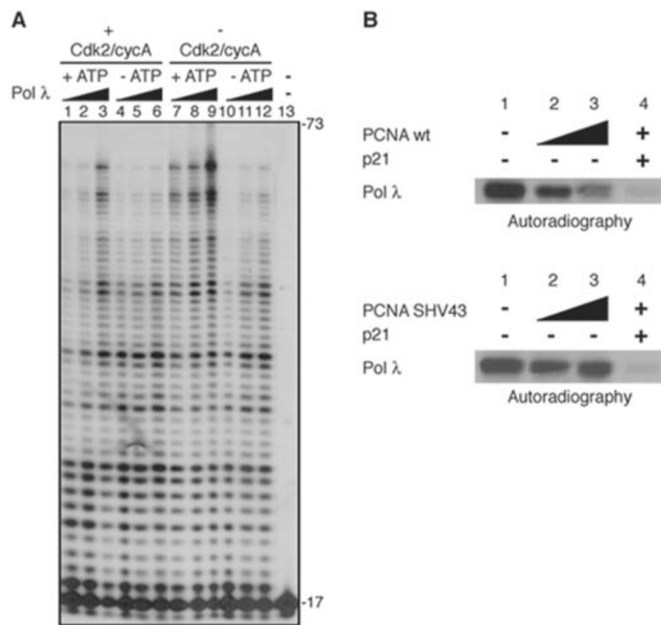

Figure 4. Phosphorylation of DNA polymerase λ does not affect its polymerase activity and is regulated by its interaction with PCNA. (**A**) Product analysis of DNA synthesis by phosphorylated and non-phosphorylated Pol λ (75, 150 and 300 ng) on a d17:d73mer (20 fmol). His-Pol λ was incubated in kinase buffer for 20 min at 37°C with 40 ng Cdk2/cyclin A either with ATP (350 μM) (lanes 1–3, phosphorylated Pol λ) or without ATP (lanes 4–6, non-phosphorylated Pol λ). Two controls were added where His-Pol λ was incubated in kinase buffer without Cdk2/cyclin A but with (lanes 7–9) or without (lanes 10–12) ATP. Positions of the 17mer and 73mer are indicated on the right side of the figure. (**B**) Effect of the interaction between Pol λ and PCNA on phosphorylation of Pol λ by Cdk2/cyclin A. Aliquots containing 500 ng of His-Pol λ were incubated with 0, 500 or 750 ng of PCNA wt (upper panel, lanes 1–3) or the same amount of PCNA SHV43 mutant (lower panel, lanes 1–3) for 5 min on ice followed by a kinase assay by Cdk2/cyclin A. In each experiment, a control was performed in the presence of the p21 inhibitor (750 ng, lane 4). Proteins were then separated on a 10% SDS–PAGE, the gel stained by Coomassie brilliant blue, dried and exposed to an X-ray film.

phosphorylation of Pol λ. Incubation of Pol λ with PCNA during phosphorylation by Cdk2/cyclin A *in vitro* resulted in a decrease in Pol λ phosphorylation (Figure 4B, upper panel). However, when the same experiment was performed by using the SHV43 PCNA mutant (18), no decrease of the Pol λ phosphorylation was observed (Figure 4B, lower panel). In both cases, Pol λ phosphorylation was abolished by addition of the specific Cdk inhibitor p21, confirming that phosphorylation was due to Cdk2/cyclin A. It has been shown that the PCNA mutant SHV43, in which the residues S43, H44 and V45 in the hydrophobic pocket on the C-side of the trimer were changed to A (18), is no more able to interact with Pol λ (16) but can still bind Cdk2 (25). Thus, this result suggested that the physical interaction of PCNA with Pol λ prevented phosphorylation by Cdk2/cyclin A, suggesting a possible regulation of the Pol λ phosphorylation status via its interaction with PCNA.

DNA polymerase λ is phosphorylated during the cell cycle

Having shown that Pol λ can be phosphorylated by Cdk/cyclin complexes *in vitro*, the next step was to test whether Pol λ is

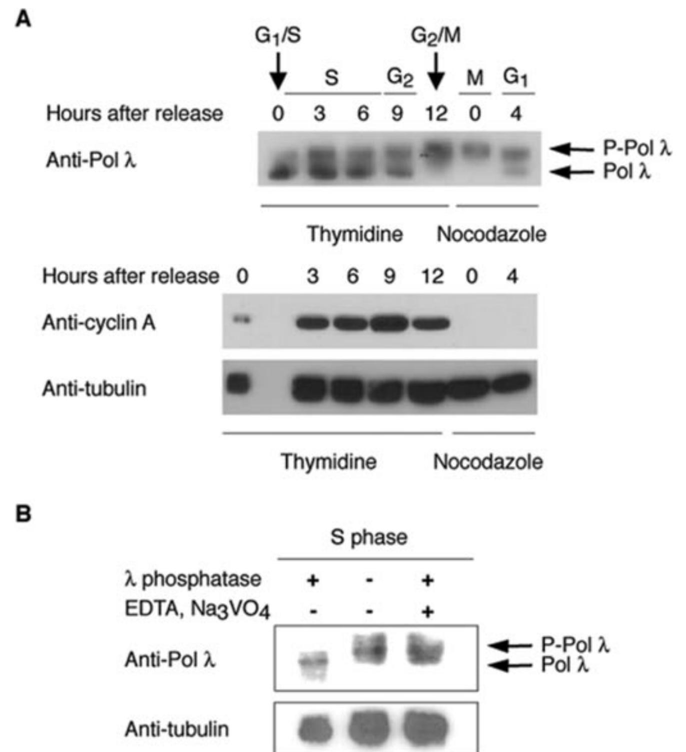

Figure 5. DNA polymerase λ is phosphorylated during the cell cycle. (**A**) Synchronization of HeLa cells at the G1/S border was achieved by double blocks of thymidine (2 mM). After thymidine removal, cells were harvested immediately (G1/S border) or further cultured in complete medium for 3 h (S phase), 6 h (late S phase), 9 h (G2 phase) or 12 h (G2/M phase). Synchronization in M phase was obtained by growing cells in nocodazole 40 ng/ml for 20 h. Cells were harvested immediately after nocodazole removal (M phase) or further cultured in complete medium for 4 h (G1 phase). The cells were lysed in 500 μl Laemmli buffer and 20 μl extract were loaded on a 7.5% SDS–PAGE and analyzed by western blot with antibodies against cyclin A, Pol λ and α-tubulin, respectively. α-tubulin was used as loading control. (**B**) Aliquots containing 25 μg of total extract from HeLa cells synchronized in S phase were treated with or without 400 U of bacteriophage λ-phosphatase for 45 min at 30°C. The reaction was stopped by adding Laemmli buffer and the samples were loaded on a 7.5% SDS–PAGE and analyzed by western blot with antibodies against Pol λ and tubulin.

phosphorylated during the cell cycle. For this, HeLa cells were synchronized at the G1/S transition by double thymidine block, and subsequently released in normal medium. Samples were collected at four different time points: 3 h (corresponding to mid-S phase), 6 h (late S phase), 9 h (G2 phase) and 12 h (G2/M phase). Synchronization in M phase was performed by treatment of the cells with nocodazole. Cells released in normal medium progressed in G1 phase and were collected after 4 h. Synchronization was confirmed on total extracts by western blot using antibodies against cyclin A, a specific marker of S and G2 phases of the cell cycle (Figure 5A, lower panel). A western blot against Pol λ using cell extracts from synchronized cells revealed the presence of two forms of Pol λ, represented by a faster and a slower migrating band, throughout the cell cycle. In S phase, the faster migrating band was more abundant, whereas in late G2 and M phases, only the slower migrating band could be detected (Figure 5A, upper panel). To verify that these two different forms of Pol λ were due to phosphorylation, we treated a total extract of S phase HeLa cells with bacteriophage λ-phosphatase. In

the presence of the phosphatase only one form of Pol λ was visible (Figure 5B, lane 1), corresponding to the faster migrating form, as revealed by comparison with reaction without phosphatase (lane 2) or in the presence of phosphatase inhibitors (lane 3), suggesting that Pol λ is phosphorylated *in vivo* and that its phosphorylation status is modulated during cell cycle.

DISCUSSION

In our efforts to better understand the Pol λ function, we searched for partners by affinity chromatography and we found Cdk2 as a novel interacting partner of Pol λ. Analysis of the amino acid sequence of Pol λ revealed the presence of three potential phosphorylation sites (S167, S177 and S230) for Cdk in its proline-serine-rich domain and we showed that this domain is the target of phosphorylation of Pol λ by the Cdk2/cyclin A complex. This finding is not unexpected, since in other proteins proline-rich domains have been shown to be targets of post-translational modifications and to be involved in the regulation of protein function. Moreover, we found that Pol λ is phosphorylated during the cell cycle and exists in two forms in S phase with a majority of hypophosphorylated form, whereas it is present exclusively in its hyperphosphorylated form from G2 to M phase (Figure 5). The different phosphorylation status of Pol λ could be linked with different Pol λ functions *in vivo*. Interestingly, Pol λ is mainly present in the hypophosphorylated form in the S phase of the cell cycle when PCNA is also abundant. This is in agreement with our findings that the presence of PCNA reduces Pol λ phosphorylation *in vitro* and suggests that this regulation may also happen *in vivo*.

In terms of physiological role, it has been shown that PCNA stimulated translesion synthesis of Pol λ past an abasic site as it did for Pol ι, Pol κ and Pol η but by a different mechanism of interaction. Thus, it is tempting to speculate that the interplay among Cdk2/cyclin A, PCNA and Pol λ, revealed by our study, might act in regulating the 'switch' between the replicative Pol δ an the translesion Pol λ through direct competition for PCNA, yielding to elongation of the nascent DNA strand past abasic sites. This model was supported by the finding that Pol λ interacts with PCNA at the same site as Pol δ and many other partners and that PCNA could promote bypass of abasic sites during DNA replication (9,16). Moreover, we showed that phosphorylation of Pol λ does not influence its polymerase activity, suggesting that phosphorylation of Pol λ rather regulates its interaction with cellular factors (e.g. PCNA) than its intrinsic activity.

In summary, our results allow to hypothesize that regulation of Pol λ phosphorylation by PCNA during the cell cycle may be important in modulating the interaction of Pol λ with other protein partners, or its recruitment to specific sites on chromatin. The effect of Cdk/cyclin phosphorylation on several replication proteins has been studied and seems to be specific for each protein [reviewed in (26)]. For example, phosphorylation has an inhibitory or stimulatory effect on the activity on Pol α depending on which Cdk/cyclin complexes phosphorylate it (27). In other cases, phosphorylation has been shown to regulate interaction between proteins, such as Fen 1 (28) or DNA ligase I (29,30), with PCNA. In addition, phosphorylation of replication protein A (RP-A) by Cdk1/cyclin A and Cdk1/cyclin B in late S phase leads to the dissociation of the RP-A trimer (31).

The interaction between Cdk2 and Pol λ also fits with the proposed role of Pol λ in non-homologous end joining (14,15). In fact, Cdk2 has been recently shown to regulate DNA DSB repair when associated to cyclin A1. Cyclin A1 is a second A-type cyclin abundantly expressed in testis (32,33). Since Pol λ is also known to be abundant in testis (34) one could propose a role of Pol λ in DSB repair in germ cells. Additional observations of Cdk2 localization at the telomeric ends of chromosome from leptotene to diplotene stage of meiosis (35) could support a role of Pol λ by its terminal transferase activity (7) in germ cells. In addition, our data are supported by the recent findings that Pol λ is selectively involved in NHEJ processing DNA with complementary overhangs, which occur upon DNA damage, whereas Pol μ seems to be mainly involved in NHEJ of DNA with non-complementary ends (36). In this context, phosphorylation of Pol λ may be one of the factors influencing selectively the recruitment of Pol λ on places where its activity is needed.

In conclusion, we showed for the first time that Pol λ is post-translationally modified in human cells, and that this modification is regulated during the cell cycle, opening a way for a better understanding of the Pol λ function *in vivo*.

ACKNOWLEDGEMENTS

The authors thank L. Blanco for providing the plasmid expressing the recombinant human wild-type Pol λ, C. Bonne-Andréa for providing the pGEX-3X plasmid expressing GST-Cdk2 and H.-P. Nasheuer for providing purified Cdk2/cyclin E, Cdk2/cyclin A, and Cdk1/cyclin A complexes and G. Maga and M. Stucki for their precious advises. This work was supported by the Swiss National Science Foundation (grant 31. 61 361. 00) to I.F., M.T. and U.H.; the EU (project No QLK3-CT-2002-02071 REBIOTECH) to U.H. and I.F., the Kanton of Zürich to I.F., I.S. and U.H. and a FEBS fellowship to M.T. Funding to pay the Open Access publication charges for this article was provided by University of Zürich-Irchel.

Conflict of interest statement. None declared.

REFERENCES

1. Morgan,D.O. (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu. Rev. Cell Dev. Biol.*, **13**, 261–291.
2. Geng,Y., Yu,Q., Sicinska,E., Das,M., Schneider,J.E., Bhattacharya,S., Rideout,W.M., Bronson,R.T., Gardner,H. and Sicinski,P. (2003) Cyclin E ablation in the mouse. *Cell*, **114**, 431–443.
3. Aleem,E., Berthet,C. and Kaldis,P. (2004) Cdk2 as a master of S phase entry: fact or fake? *Cell Cycle*, **3**, 35–37.
4. Ortega,S., Prieto,I., Odajima,J., Martin,A., Dubus,P., Sotillo,R., Barbero,J.L., Malumbres,M. and Barbacid,M. (2003) Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nature Genet.*, **35**, 25–31.
5. Hubscher,U., Maga,G. and Spadari,S. (2002) Eukaryotic DNA polymerases. *Annu. Rev. Biochem.*, **71**, 133–163.
6. Garcia-Diaz,M., Bebenek,K., Kunkel,T.A. and Blanco,L. (2001) Identification of an intrinsic 5′-deoxyribose-5-phosphate lyase activity in human DNA polymerase lambda: a possible role in base excision repair. *J. Biol. Chem.*, **276**, 34659–34663.

7. Ramadan,K., Maga,G., Shevelev,I.V., Villani,G., Blanco,L. and Hubscher,U. (2003) Human DNA polymerase lambda possesses terminal deoxyribonucleotidyl transferase activity and can elongate RNA primers: implications for novel functions. *J. Mol. Biol*., **328**, 63–72.
8. Ramadan,K., Shevelev,I.V., Maga,G. and Hubscher,U. (2004) De novo DNA synthesis by human DNA polymerase lambda, DNA polymerase mu and terminal deoxyribonucleotidyl transferase. *J. Mol. Biol*., **339**, 395–404.
9. Maga,G., Villani,G., Ramadan,K., Shevelev,I., Tanguy Le Gac,N., Blanco,L., Blanca,G., Spadari,S. and Hubscher,U. (2002) Human DNA polymerase lambda functionally and physically interacts with proliferating cell nuclear antigen in normal and translesion DNA synthesis. *J. Biol. Chem*., **277**, 48434–48440.
10. Blanca,G., Villani,G., Shevelev,I., Ramadan,K., Spadari,S., Hubscher,U. and Maga,G. (2004) Human DNA polymerases lambda and beta show different efficiencies of translesion DNA synthesis past abasic sites and alternative mechanisms for frameshift generation. *Biochemistry*, **43**, 11605–11615.
11. Lebedeva,N.A., Rechkunova,N.I., Dezhurov,S.V., Khodyreva,S.N., Favre,A., Blanco,L. and Lavrik,O.I. (2005) Comparison of functional properties of mammalian DNA polymerase lambda and DNA polymerase beta in reactions of DNA synthesis related to DNA repair. *Biochim. Biophys. Acta*., **1751**, 150–158.
12. Braithwaite,E.K., Prasad,R., Shock,D.D., Hou,E.W., Beard,W.A. and Wilson,S.H. (2005) DNA polymerase lambda mediates a back-up base excision repair activity in extracts of mouse embryonic fibroblasts. *J. Biol. Chem*., **280**, 18469–18475.
13. Braithwaite,E.K., Kedar,P.S., Lan,L., Polosina,Y.Y., Asagoshi,K., Poltoratsky,V.P., Horton,J.K., Miller,H., Teebor,G.W., Yasui,A. *et al.* (2005) DNA polymerase lambda protects mouse fibroblasts against oxidative DNA damage and is recruited to sites of DNA damage/repair. *J. Biol. Chem*., **280**, 31641–31647.
14. Ma,Y., Lu,H., Tippin,B., Goodman,M.F., Shimazaki,N., Koiwai,O., Hsieh,C.L., Schwarz,K. and Lieber,M.R. (2004) A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol. Cell*, **16**, 701–713.
15. Lee,J.W., Blanco,L., Zhou,T., Garcia-Diaz,M., Bebenek,K., Kunkel,T.A., Wang,Z. and Povirk,L.F. (2004) Implication of DNA polymerase lambda in alignment-based gap filling for nonhomologous DNA end joining in human nuclear extracts. *J. Biol. Chem*., **279**, 805–811.
16. Maga,G., Blanca,G., Shevelev,I., Frouin,I., Ramadan,K., Spadari,S., Villani,G. and Hubscher,U. (2004) The human DNA polymerase lambda interacts with PCNA through a domain important for DNA primer binding and the interaction is inhibited by p21/WAF1/CIP1. *FASEB J*., **18**, 1743–1745.
17. Fan,W. and Wu,X. (2004) DNA polymerase lambda can elongate on DNA substrates mimicking non-homologous end joining and interact with XRCC4-ligase IV complex. *Biochem. Biophys. Res. Commun*., **323**, 1328–1333.
18. Jonsson,Z.O., Hindges,R. and Hubscher,U. (1998) Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. *EMBO J*., **17**, 2412–2425.
19. Maga,G. and Hubscher,U. (2003) Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J. Cell Sci*., **116**, 3051–3060.
20. Shimazaki,N., Yazaki,T., Kubota,T., Sato,A., Nakamura,A., Kurei,S., Toji,S., Tamai,K. and Koiwai,O. (2005) DNA polymerase lambda directly binds to proliferating cell nuclear antigen through its confined C-terminal region. *Genes Cells*, **10**, 705–715.
21. Maga,G., Jonsson,Z.O., Stucki,M., Spadari,S. and Hubscher,U. (1999) Dual mode of interaction of DNA polymerase epsilon with proliferating cell nuclear antigen in primer binding and DNA synthesis. *J. Mol. Biol*., **285**, 259–267.
22. Voitenleitner,C., Fanning,E. and Nasheuer,H.P. (1997) Phosphorylation of DNA polymerase alpha-primase by cyclin A-dependent kinases regulates initiation of DNA replication *in vitro*. *Oncogene*, **14**, 1611–1615.
23. Stein,G.S. and Stein,J.L. (1989) Cell synchronisation. In Baserga,R. (ed.), *Cell Growth and Division.* IRL Press, Oxford, UK, pp. 133–137.
24. Nick McElhinny,S.A. and Ramsden,D.A. (2003) Polymerase mu is a DNA-directed DNA/RNA polymerase. *Mol. Cell. Biol*., **23**, 2309–2315.
25. Koundrioukoff,S., Jonsson,Z.O., Hasan,S., de Jong,R.N., van der Vliet,P.C., Hottiger,M.O. and Hubscher,U. (2000) A direct interaction between proliferating cell nuclear antigen (PCNA) and Cdk2 targets PCNA-interacting proteins for phosphorylation. *J. Biol. Chem*., **275**, 22882–22887.
26. Henneke,G., Koundrioukoff,S. and Hubscher,U. (2003) Multiple roles for kinases in DNA replication. *EMBO Rep*., **4**, 252–256.
27. Voitenleitner,C., Rehfuess,C., Hilmes,M., O'Rear,L., Liao,P.C., Gage,D.A., Ott,R., Nasheuer,H.P. and Fanning,E. (1999) Cell cycle-dependent regulation of human DNA polymerase alpha-primase activity by phosphorylation. *Mol. Cell. Biol*., **19**, 646–656.
28. Henneke,G., Koundrioukoff,S. and Hubscher,U. (2003) Phosphorylation of human Fen1 by cyclin-dependent kinase modulates its role in replication fork regulation. *Oncogene*, **22**, 4301–4313.
29. Ferrari,G., Rossi,R., Arosio,D., Vindigni,A., Biamonti,G. and Montecucco,A. (2003) Cell cycle-dependent phosphorylation of human DNA ligase I at the cyclin-dependent kinase sites. *J. Biol. Chem*., **278**, 37761–37767.
30. Rossi,R., Villa,A., Negri,C., Scovassi,I., Ciarrocchi,G., Biamonti,G. and Montecucco,A. (1999) The replication factory targeting sequence/PCNA-binding site is required in G(1) to control the phosphorylation status of DNA ligase I. *EMBO J*., **18**, 5745–5754.
31. Treuner,K., Findeisen,M., Strausfeld,U. and Knippers,R. (1999) Phosphorylation of replication protein A middle subunit (RPA32) leads to a disassembly of the RPA heterotrimer. *J. Biol. Chem*., **274**, 15556–15561.
32. Diederichs,S., Baumer,N., Ji,P., Metzelder,S.K., Idos,G.E., Cauvet,T., Wang,W., Moller,M., Pierschalski,S., Gromoll,J. *et al*. (2004) Identification of interaction partners and substrates of the cyclin A1-CDK2 complex. *J. Biol. Chem*., **279**, 33727–33741.
33. Muller-Tidow,C., Ji,P., Diederichs,S., Potratz,J., Baumer,N., Kohler,G., Cauvet,T., Choudary,C., van der Meer,T., Chan,W.Y. *et al*. (2004) The cyclin A1-CDK2 complex regulates DNA double-strand break repair. *Mol. Cell. Biol*., **24**, 8917–8928.
34. Garcia-Diaz,M., Dominguez,O., Lopez-Fernandez,L.A., de Lera,L.T., Saniger,M.L., Ruiz,J.F., Parraga,M., Garcia-Ortiz,M.J., Kirchhoff,T., del Mazo,J. *et al*. (2000) DNA polymerase lambda (Pol lambda), a novel eukaryotic DNA polymerase with a potential role in meiosis. *J. Mol. Biol*., **301**, 851–867.
35. Ashley,T., Walpita,D. and de Rooij,D.G. (2001) Localization of two mammalian cyclin dependent kinases during mammalian meiosis. *J. Cell Sci*., **114**, 685–693.
36. Nick McElhinny,S.A., Havener,J.M., Garcia-Diaz,M., Juarez,R., Bebenek,K., Kee,B.L., Blanco,L., Kunkel,T.A. and Ramsden,D.A. (2005) A gradient of template dependence defines distinct biological roles for family x polymerases in nonhomologous end joining. *Mol. Cell*, **19**, 357–366.