

Segregation of replicative DNA polymerases during S phase – DNA polymerase ϵ , but not DNA polymerases α/δ are associated with lamins throughout S phase in human cells*

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Background: Replicative DNA polymerases δ and ϵ are believed to synthesize lagging and leading strands, respectively.

Results: Human DNA polymerases α/δ and ϵ segregate during S phase and DNA polymerase ϵ alone remains bound to lamins.

Conclusion: DNA polymerases δ and ϵ act partly independent.

Significance: Human cell DNA replication differs significantly from prokaryotic replication where DNA polymerase complex catalyzes simultaneous synthesis of both strands.

SUMMARY

DNA Polymerases (Pols) α , δ and ϵ replicate the bulk of chromosomal DNA in eukaryotic cells, Pol ϵ being the main

leading strand and Pol δ the lagging strand DNA polymerase. By applying chromatin immunoprecipitation (ChIP) and quantitative PCR we found that at G1/S arrest, all three DNA polymerases were enriched with DNA containing the early firing lamin B2 origin of replication and, 2 hours after release from the block, with DNA containing the origin at the upstream promoter region of the *MCM4* gene. Pols α , δ and ϵ were released from these origins upon firing. All three DNA polymerases, Mcm3 and Cdc45, but not Orc2, still formed complexes in late S phase. Reciprocal ChIP of the three DNA polymerases revealed that at G1/S arrest and early in S phase, Pols α , δ and ϵ were associated with the same nucleoprotein complexes, whereas in late S phase Pol

ϵ and Pol α/δ were largely associated with distinct complexes. At G1/S arrest, the replicative DNA polymerases were associated with lamins, but in late S phase only Pol ϵ , not Pol α/δ , remained associated with lamins. Consistently, Pol ϵ , but not Pol δ , was found in nuclear matrix fraction throughout the cell cycle. Therefore, Pol ϵ and Pol α/δ seem to pursue their functions at least in part independently in late S phase, either by physical uncoupling of lagging strand maturation from the fork progression, or by recruitment of Pol δ , but not Pol ϵ , to post-replicative processes such as translesion synthesis or post-replicative repair.

Three DNA polymerases (Pols) α , δ and ϵ replicate the bulk of the eukaryotic genome (for reviews see 1-3). Pol α is unique among DNA polymerases by having an intrinsic primase. It is therefore able to start DNA synthesis *de novo* (reviewed in 4-5). The primase acts as a DNA-dependent RNA polymerase synthesizing an RNA primer of about 10 bases long which is then extended by the DNA polymerase activity of Pol α complex to about 30 bases. For duplication of simian virus 40 (SV40) DNA, a classic model system for eukaryotic DNA replication, replication factor C (RFC) is specifically bound to these primers and expels Pol α . RFC then loads the ring-shaped proliferating cell nuclear antigen (PCNA) to form a sliding clamp around the double stranded DNA at the primer end, and recruits Pol δ which synthesizes both leading strand DNA and Okazaki fragments of the lagging strand, the latter being then processed to a continuous strand (for review see 6). Besides Pols α and δ , a third large DNA polymerase, Pol ϵ was found to be essential for yeast *Sacharomyces cerevisiae* (7), and it was found to be involved in synthesis of chromosomal DNA in human cells (8-10). It is also required for efficient DNA synthesis in *Xenopus* egg extracts (11). It has been recently found that *S. cerevisiae* Pol δ and ϵ harboring mutations that confer specific

mutation patterns to the enzymes, sign their mutational signatures to lagging and leading strand, respectively (2,12-13). Based on this evidence and on former work (for review see 14) it is safe to conclude that Pol δ is a main player in synthesis of lagging strand DNA whereas Pol ϵ is predominantly involved in the synthesis of the leading strand DNA.

However, there is also evidence according to which the division of labor between Pol δ and ϵ may be more complex than a simple splitting between lagging and leading strands, respectively. The deletion of the domain containing polymerase and proofreading exonuclease motifs from *S. cerevisiae* causes growth and replication defects but the deletion is not lethal (15-16), indicating that in this case, like in SV40 DNA replication, Pol δ is able to synthesize both strands. Furthermore, when the proofreading activity of Pol δ is mutationally inactivated, the mutation rate is significantly higher than in cells having analogous mutation in Pol ϵ (17-18). Amino acid substitutions in the polymerase domain of Pol δ also seem to generate a higher increase in the mutation rates and cause more severe growth defects than analogous amino acid substitutions in Pol ϵ (19). Further evidence conflicting with the current model comes from studies of human cells. We previously found that (i) a neutralizing antibody against Pol ϵ inhibits DNA synthesis in permeabilized nuclei more efficiently in the early S phase than in the late S phase whereas the contrary is true for antibodies against Pol δ , and that (ii) trapping of Pol ϵ to nascent DNA remained nearly constant throughout the S phase, whereas Pol δ was three to four times more intensely crosslinked to nascent DNA in late compared to early S phase, and that (iii) the chromatin-bound fraction of Pol δ , unlike Pol ϵ , increased in the late S phase (20). These results suggest that the contribution of Pol δ to DNA synthesis increases towards the late S phase while that of Pol ϵ either decreases or remains constant. In contrast, Fuss and Linn (21) proposed that Pol ϵ acts in the replication of heterochromatin during late S phase based

on the observation that in immunofluorescence microscopy, the enzyme is neighbouring PCNA foci and sites of DNA synthesis in early S phase but co-localises with these sites in late S phase. Our previous study also suggested that ultrastructural localization of the Pols δ and ϵ were essentially distinct although minor colocalization was also detected (20). Depletion of the activity of Pol δ or ϵ in higher eukaryotes causes distinct defects for genome duplication (10,22), arguing for different contributions to DNA replication. All these observations were not expected if Pols ϵ and δ are part of the same replication fork complex, acting on the leading and the lagging strands, respectively, in a similar manner as Pol III in *Escherichia coli* cells (for review see 23). They could still be explained if (i) Pol δ is involved in delayed maturation of accumulating Okazaki fragments or both strands independent of Pol ϵ , (ii) Pol δ , but not Pol ϵ is increasingly involved in post-replicative processes such as DNA translesion synthesis or post-replicative DNA recombination, or if other than in yeast, (iii) the share of labor of Pols δ and ϵ has changed from yeast to human, i.e. human Pol ϵ acts more at early origins of replication and Pol δ more at late origins of replication on both strands. To address these questions we applied chromatin immunoprecipitation (ChIP) techniques and quantitative PCR to study association and release of the three replicative DNA polymerases, Pol α , δ and ϵ , with DNA from two origins of replication, the lamin B2 (*LB2*) gene origin and the upstream promoter region (UPR) of *MCM4* gene, firing at 0h and 2 hours, respectively, after S phase entry, and to study co-existence of the three replicative DNA polymerases, origin recognition complex (ORC) subunit Orc2, a component of the Mcm-helicase complex Mcm3, and cell division control protein Cdc45 in crosslinked nucleoprotein complexes during S phase. We also studied the presence of lamins A/C in these complexes. The results reveal that Pols α , δ and ϵ are loaded to and released from both

origins of replication during S phase. In G1/S arrested cells Pols α , δ and ϵ are present in highly purified nucleoprotein complexes containing 200-1000 base pair long DNA fragments and they are associated with lamins. In late S phase Pols α and δ are segregated from Pol ϵ and lamins A/C whereas Pol ϵ remains associated with lamins. Based on this study and other studies that have been previously published we propose a model according to which Pol ϵ bound to nuclear matrix synthesizes the leading strand and Pol δ synthesizes the lagging strand, but the latter partly trails behind to process still immature lagging strand DNA, and possibly also leading strand DNA, or fulfills post-replicative tasks such as DNA translesion synthesis after Pol ϵ has essentially completed its job.

EXPERIMENTAL PROCEDURES

Antibodies. Primary antibodies used are listed in Table 1. Rabbit polyclonal K31 antibody against human Pol δ catalytic subunit was raised against a polypeptide corresponding to amino acids 108-276, and rabbit polyclonal K32 and K33 antibodies against human Pol δ catalytic subunit were raised against peptides corresponding to amino acids 297-542. The antigens were expressed as GST fusion proteins and purified by preparative SDS-PAGE after thrombin cleavage of the GST as described (9). Secondary antibodies for Western blotting were either alkaline phosphatase conjugated or horseradish peroxidase conjugated (Jackson Immunoresearch or Chemicon).

Cell cultivation, synchronization and cell cycle analysis. HeLa CCL2 monolayer cells (American Type Culture Collection ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (Invitrogen, Paisley, UK) at 37 °C in a 5% carbon dioxide atmosphere. Cells were synchronized by arresting them at G1/S phase with a double thymidine block (28) and then either used as such (0 h) or released from the block to proceed in S phase by replacing the medium containing excess thymidine with

medium without addition of thymidine for the indicated times. Human T98G glioblastoma cells (ATCC CRL 1690) were cultured in Eagle's minimum essential medium (Sigma) supplemented with Earle's salts, 10 % fetal bovine serum, L-glutamine, non essential amino acids, and antibiotics at 37°C in a 5 % carbon dioxide atmosphere. T98G cells were starved in medium containing 0.5 % serum for 6 days followed by addition of conditioned, complete medium. The quality of the synchrony was analysed by flow cytometry of propidium iodine-stained cells (29) using a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany) or a Cell Lab Quanta SC flow cytometer (Beckman Coulter).

Chromatin immunoprecipitation. In vivo crosslinking and subsequent chromatin immunoprecipitations (ChIPs) were performed from double thymidine synchronized, and exponentially growing monolayer HeLa cells as described (30). The steps are essentially (i) *in vivo* crosslinking-treatment of cells with formaldehyde, (ii) homogenization of cells and collection of nuclear material, (iii) isolation of nucleoproteins by CsCl gradient centrifugation, (iv) sonication and digestion of the nucleoproteins with micrococcal nuclease optimized to result in DNA fragments of 200-1000 base pairs, (v) immunoprecipitation with a selected protein antibody, and (vi) division of the immunoprecipitate for Western blotting and DNA extraction. For Western blotting proteins were eluted from crosslinked nucleoproteins, and for DNA extraction proteins were hydrolyzed by proteinase K and DNA was purified by phenol-chloroform extraction and ethanol precipitation. Immunoprecipitation reactions contained 5 µg of K19 and 10 µg of K27 for Pol ε, 2.5 µg each of K30, K31, K32 and K33 for Pol δ, 5 µg of p140 for Pol α, 5 µg of N18 for Lamin A/C, or 5 µg of rabbit IgG as negative control. For colourimetric immunodetection of Pols ε, δ, and α, lamin A/C, Mcm3, Cdc45 and Orc2 a mixture of G1A, H3B and E24C antibodies, a mixture of K30, K31, K32 and

K33 antibodies, p140 antibody, lamin A/C (N18) antibody, Mcm3 (N20) antibody, Cdc45 (3G10) antibody and Orc2 (3B7) antibody were used, respectively.

Quantitative PCR analysis. Quantitative PCR (qPCR) reactions were run with Opticon Monitor program in Chromo 4 Peltier Thermal Cycler (MJ Research). The *LB2* gene origin of replication (31) and the origin located in the UPR of the *MCM4* gene (32) were utilized in this study, and PCR analysis was performed from the areas shown (Fig. 1 and 2). The primers used are presented in Table 2. The quantity of specific DNA in the immunoprecipitate is given as a relative ratio of DNA precipitated over input DNA.

Cell fractionation studies. 100 mm plates of T98G cells were cooled to +4 °C, washed twice with cold TBS (150 mM NaCl, 20 mM Tris-Cl pH 7.5) and twice with hypotonic KM buffer (10 mM NaCl, 1 mM MgCl₂, 2 mM DTT and 10 mM MOPS-NaOH pH 7.0). Cells were then lysed by hypotonic extraction with 1 ml of KM buffer containing 0.5 % Nonidet P-40 for 30 minutes with occasional gentle agitation. The resulting supernatant represented the detergent-soluble fraction (S). The cell remnants were washed twice with KAc buffer (5 mM potassium acetate, 0.5 mM MgCl₂, 2 mM DTT and 30 mM HEPES-KOH pH 7.4) followed by incubation with 1 ml of DNase I solution (150 mM NaCl, 1.5mM CaCl₂, 6mM MgCl₂, complete EDTA-free protease inhibitors, 50 µg/ml RNase A, 50 U/ml DNase I and 40mM Tris-Cl pH 8.0) at room temperature for half an hour. The supernatant containing proteins released by the DNase I digest represented the chromatin-bound fraction (B) and was collected. The remaining cell matrix was washed twice with DNase solution (without DNase I and RNase A) and solubilised by addition of 1 ml of lysis buffer (100 mM NaCl, 0.5 mM MgCl₂, 0.5 mM DTT, 5 mM KCl, 0.5% SDS and 20 mM HEPES-KOH pH 7.7). The insoluble matrix fraction (M) was collected using a cell scraper. Total cell extract (T) was prepared by washing a parallel 100 mm plate twice with cold TBS followed by lysis with lysis buffer and cell scraper. All samples were rapidly

frozen in liquid nitrogen and stored at -70°C . For Western detection proteins were separated by SDS PAGE, transferred onto a PVDF membrane and detected using chemiluminescence reagents (Pierce and BioRad). Antibodies PC10, 3G10, PDG-1E8, and a combination of G1A, H3B and E24C were used for detection of PCNA, Cdc45, Pols δ and ϵ , respectively.

RESULTS

Association of Pol ϵ defines replication timing of the lamin B2 gene origin and the MCM4 gene UPR origin. In the yeast *S. cerevisiae*, Pol ϵ has been found to be the main leading strand replicase whereas Pol δ replicates the bulk of the lagging strand. Considering the central importance of DNA replication for cell function, one could expect a similar share of labor also at the human replication fork. On the other hand, studies of replication dynamics of human DNA polymerases have left the possibility that Pol ϵ may be more strongly involved in either early S phase DNA synthesis (20), or in replication of heterochromatin in late S phase (21). As an attempt to address this issue, we first studied enrichment of the DNA from two replication origins, the *LB2* gene origin (31) and the UPR origin at the *MCM4* gene (30) in nucleoprotein complexes derived by the ChIP method (see Experimental Procedures) with Orc2, Mcm3 and Pol ϵ antibodies. In HeLa cells arrested in early S phase by double thymidine block, *LB2* DNA was strongly associated with immunoprecipitates of Orc2, Mcm3 and Pol ϵ (Fig. 1A), the enrichment being 10-20 fold compared to proximal regions. At time points of 2 and 4 hours after the release from the thymidine arrest enrichment of the *LB2* DNA in Mcm3 immunoprecipitates disappeared whereas Orc2 immunoprecipitates still showed about 3-4 fold enrichment even at 4 hours after release. In contrast, enrichment of the *LB2* DNA in Pol ϵ immunoprecipitates was very low already at 0.5 hours and practically disappeared at 2 hours after release. Enrichment of the DNA of the UPR region of the *MCM4* gene in immunoprecipitates was

not as pronounced as the enrichment of the DNA of the *LB2* region. As seen for *LB2*, *MCM4* UPR enrichment was also highest in immunoprecipitates of Orc2 and Mcm3 from G1/S arrested cells at 0 hours showing about 20 fold *MCM4* UPR enrichment for Orc2 immunoprecipitations and 3-7 fold for Mcm3 immunoprecipitations (Fig. 1A). These results confirmed previous studies that found Orc2 (30) and Mcm3 (32) bind to the *MCM4* UPR origin already in G₁ phase and remain bound with the origin DNA in early S phase. In contrast, no enrichment of *MCM4* UPR was detectable at 0 hours in Pol ϵ immunoprecipitates. Instead, enrichment of *MCM4* UPR origin DNA in Pol ϵ immunoprecipitates peaked at 2 hours after the release from the arrest and its qPCR detection showed 4-6 fold higher level when compared to proximal regions of *MCM4* UPR origin, although its enrichment in Orc2 and Mcm3 immunoprecipitates had already decreased down to 4 fold and 2 fold, respectively. Therefore, loading of Pol ϵ to and subsequent release from this origin of replication takes place significantly later than at the *LB2* origin. These findings suggest that the *MCM4* UPR origin fires about 2 hours later than the *LB2* origin. The latter was already previously shown to be an early firing origin in HeLa cells (33). As enrichment of the *MCM4* UPR origin DNA in Orc2 and Pol ϵ immunoprecipitate persists up to 4 hours after the release from the thymidine arrest (Fig. 1A and 30), this origin fires in mid S phase, and probably with less efficiency than the *LB2* origin.

*DNA polymerases α , δ and ϵ are all loaded to and released from both *LB2* origin and the *MCM4* gene UPR origin.* We then analyzed the origin DNA of nucleoprotein complexes purified from precipitates of each replicative DNA polymerase. All three Pols α , δ and ϵ associated strongly with the *LB2* origin in G1/S arrested cells at 0 hours (Fig. 2). Enrichment of nascent DNA was about 20 fold for each enzyme complex. The replicative DNA polymerases were rapidly released from the *LB2* origin after cells proceeded in S phase since enrichment of *LB2*

origin DNA dropped rapidly to only 2-3 fold as early as 0.5 hours after release from the double thymidine block. Obviously the three DNA polymerases moved away from the origin along with proceeding replication forks. The firing of the origin and progression of the forks at G1/S arrested cells immediately after release of the block obviously takes place with a relatively high synchrony, as the flanking regions did not show any enrichment after 0.5 hours. It seems that all three DNA polymerases are involved in the early DNA synthesis from this early origin of replication. For the later firing origin in the UPR of the *MCM4* gene, the enrichment of the origin fragment reached a maximum at 2 hours with a 3-6 fold increase of that of the control DNA, and this applied equally to immunoprecipitates of all three DNA polymerases (Fig. 2). In contrast, no enrichment of the *MCM4* UPR origin DNA is found at 0 or 4 hour time points. Enrichment of the *MCM4* UPR origin DNA at 2 hours in precipitates of all three replicative DNA polymerases provides further evidence that this origin fires later than the *LB2* origin. These results suggest that the three replicative DNA polymerases are all involved in early synthesis of DNA from both the *LB2* origin and the *MCM4* UPR origin that fires about 2 hours later than the *LB2* origin. Altogether, loading and release of all three replicative DNA polymerases at these two origins of replication suggests that they are all involved in the initiation and progression of DNA replication from both origins. It is therefore not likely that the share of labor at the fork differs in the later firing *MCM4* UPR origin from the early firing *LB2* origin. Therefore, these results do not provide an explanation for the more active role of Pol δ in overall replicative DNA synthesis in late S phase when compared to Pol ϵ (20).

In G1/S arrested cells all three replicative DNA polymerases are present in the same nucleoprotein complexes, but in late S phase Pol ϵ behaves distinct from Pol α/δ . If Pol δ and ϵ act at all replication forks as proposed by studies on yeast cells (13-14) and confirmed here for selected origins in human

cells, it could be expected that the two replicases act as a complex that is responsible for simultaneous synthesis of both strands like Pol III in *E. coli* cells (for review see 22). We therefore studied the presence of all three replicative DNA polymerases, their ORC subunit Orc2, and the Mcm helicase component Mcm3 in highly purified nucleoprotein complexes isolated by ChIP at different stages of S phase. We included also Cdc45, since this protein forms on one hand an integral part of the Cdc45-MCM-GINS (CMG) complex considered the active form of the replicative DNA helicase (10,34-35). On the other hand, Cdc45 also appears to mediate the contact between the MCM proteins and the replicative DNA polymerases (27, reviewed in 36). Orc2, Mcm3 and Cdc45 were present in nucleoprotein complexes in cells arrested in G1/S and separately precipitated individually with antibodies against the three replicative DNA polymerases (Fig. 3A), although the Orc2 signal was very weak in Pol δ precipitates. ChIP performed late in S phase at 6 hours, showed that Mcm3 and Cdc45 were still present in all three DNA polymerase precipitates, whereas Orc2 was absent. These results are consistent with earlier studies revealing that origin recognition complex proteins are not present at progressing replication forks (37,38) while Mcm3 and Cdc45 travel along with the forks (37). At 6 hours, firing of origins is rare compared to progressive forks and therefore, the contacts between ORC complex proteins and elongation proteins have obviously been lost. In contrast, Mcm3 and Cdc45 are expected to be present in crosslinked nucleoprotein complexes purified by immunoprecipitation with antibodies against replicative DNA polymerases if these DNA polymerases were present at progressing forks. As this is the case (Fig. 3A), the results suggest that all three replicative DNA polymerases act at forks, or close to forks still at 6 h time point. It should be noted though that the length of the DNA at crosslinked nucleoprotein complexes is 200-1000 bp, and therefore the presence of the three replicative DNA

polymerases in these complexes may not indicate a direct interaction between them.

If all three DNA polymerases are associated with stable replication complexes comparable to the Pol III holoenzyme in *E. coli*, nucleoprotein complexes should have a comparable protein composition, including replicative DNA polymerases and other replication factors, independent of the polymerase antibody that was used for ChIP. To address this we studied the presence of Pol δ and ϵ in nucleoprotein complexes precipitated with Pol α antibodies. Pol δ and Pol ϵ were both present in these immunoprecipitates from G1/S arrested cells at 0 hours (Fig. 3B) as can be expected on their obvious presence at early firing replication origins at this time (see above). Pol δ was present at all later time points studied 2, 4 and 6 hours in Pol α immunoprecipitates, but Pol ϵ was no longer detectable at 4 and 6 hours, indicating, that in late S phase Pol α and Pol δ were still present in the same nucleoprotein complexes, suggesting that they still acted at the same forks, but Pol ϵ was essentially no longer present in these complexes. Consistently, when nucleoprotein fragments were immunoprecipitated with Pol ϵ antibodies, Pol δ was present in seemingly large quantities at 0 hours and in smaller quantities still at 2 hours, but despite the very high sensitivity of the antibodies used here for detection of Pol δ , only a weak band is detectable at 4 and 6 hours (Fig. 3C). The extensive purification procedure for the isolation of the nucleoprotein includes a CsCl density gradient centrifugation that efficiently separates the nucleoproteins from free protein complexes and free nucleic acids. Therefore, the ChIPs analysed here represent the chromatin-associated fractions of the DNA polymerases, and not soluble pools of non-productive enzymes. Taken together these results suggest that the three replicative DNA polymerases are all loaded to the origin of replication and co-exist in nucleoprotein complexes purified with DNA polymerase antibodies, but already at 4 hours in S phase, Pol α and Pol δ are processing DNA in

complexes that are essentially free of Pol ϵ . Altogether, these results suggest that in late S phase Pol ϵ and Pol α/δ act essentially independently, although the three DNA polymerases are all still in nucleoprotein complexes containing Mcm3 and Cdc45 proteins.

DNA polymerase ϵ , but not DNA polymerase δ is associated with nuclear matrix throughout the cell cycle. We studied nuclear association of Pol δ and ϵ by fractionation of human T98G glioblastoma cells to further analyze the distinct contexts in which Pol δ and ϵ may act. T98G cells respond to serum deprivation and can therefore be synchronised without interference with the checkpoint response. Hypotonic extraction in the presence of Nonidet NP-40 was performed to release soluble proteins including the nucleoplasmic fraction. Nuclear remnants were then treated with DNase I to release chromatin-bound proteins and the remaining insoluble material was classified as “matrix” fraction representing largely the nuclear matrix. Total cell extract was prepared from parallel plates and all extracts were analysed by Western blotting using marker antibodies (Table 1, Fig. 4A). β -tubulin was completely found in the soluble fraction indicating that bound and matrix fractions were largely free of soluble proteins. Mcm2 appeared both in soluble and DNA bound fractions as expected (39). Lamins A/C could be detected almost exclusively in the matrix fraction, whereas the soluble and chromatin-bound fractions were largely free of lamins A/C, demonstrating that these fractions were substantially free of contaminating nuclear matrix. In asynchronous cells PCNA, Cdc45, and Pol δ were predominantly in the soluble fraction, a minor part of each being bound to chromatin, and none to the matrix (Fig. 4A). Pol ϵ behaved in a different manner: The majority of the enzyme was in the matrix fraction, a small amount was released by DNase I and only very little was found to be soluble.

To analyse the nuclear dynamic of replication proteins during S phase, T98G cells were starved in G₀ phase by serum

deprivation and induced to grow by re-addition of serum. Samples from indicated time points were analysed by flow cytometry to verify synchronous re-entry into the cycle (Fig. 4B). PCNA and Pol δ became bound to chromatin after cells had entered S phase at 16 h. Cdc45 remained bound to chromatin throughout the cell cycle, but there was increase in binding towards late S phase. It appears that chromatin association of Cdc45 preceded that of PCNA and Pol δ , consistent with the role of the former in initiation of DNA replication (40). Pol ϵ behaved completely different from the other proteins analysed. In contrast to these replication proteins that were bound to chromatin or were in the soluble fraction (Fig. 4B), Pol ϵ was mainly in the insoluble matrix fraction resistant to detergent and DNase extraction, and its abundance in the bound fraction peaked in mid S phase (Fig. 4B, 22 h). Only a small portion of Pol ϵ was soluble or remained chromatin-bound throughout the cell cycle. Notably, the amount of a form of Pol ϵ with reduced electrophoretic mobility increased in the chromatin fraction whereas the form with normal electrophoretic mobility decreased at the same time (Fig. 4A). The observed difference in the association with nuclear fractions and its time course is consistent with our previous study on replicative Pols during S phase (20) and with the results presented above. In particular, it is noteworthy that Pol ϵ , unlike Pol δ remains essentially bound to nuclear matrix throughout the cell cycle (Fig. 4C).

The three replicative DNA polymerases associate with lamins A/C at G1/S arrest, but only DNA polymerase ϵ remains bound to lamins in late S phase. Association of Pol ϵ with nuclear matrix prompted us for studying whether lamins would be components of replicative nucleoprotein complexes purified by ChIP. We found that in nucleoprotein complexes purified by immunoprecipitation with DNA polymerase antibodies from G1/S arrested cells, lamins A/C were abundant in precipitates of all three replicative Pols (Fig. 5). This is not surprising taking into account that Pol ϵ is continuously

bound to nuclear matrix, and all three replicative Pols seem to be bound to replication initiation sites in these conditions (above). When nucleoprotein complexes were immunoprecipitated with antibodies against lamin A/C that are structural proteins and hence very much more abundant than replicative Pols, signals of Pol ϵ and δ , and Mcm3, but not of Pol α , were still obtained.

At 6 hours a strong signal from lamins A/C was obtained in the Pol ϵ ChIP, but essentially no signal in Pol α and Pol δ immunoprecipitates. Consistently, reciprocal ChIPs with lamin A/C antibodies resulted in signals for Pol ϵ and Mcm3, but not for Pol α and Pol δ . These results indicate that binding of Pol ϵ to nuclear matrix (Fig. 4) is most likely mediated by lamins. Control precipitates utilising a nonspecific IgG fraction analysed on the same membrane did not yield a discernible signal for any of the proteins analysed, thereby confirming the specificity of the ChIPs.

Analysis of the DNA of the nucleoprotein complexes isolated from G1/S arrested cells by lamin A/C antibodies revealed about 20 fold enrichment of *LB2* origin DNA when compared to proximal regions, but at 6 hours this enrichment had essentially disappeared (Fig. 5). Obviously, since associated with DNA elongating replication complexes, also lamins A/C were released from the origin of replication after firing, and, as suggested above, retained its association with Pol ϵ but not with Pol α/δ .

Thus, whereas in early S phase, nucleoprotein complexes containing all three replicative Pols, Mcm3, Cdc45, Orc2 and lamin A/C were detectedable, in late S phase, Pol α/δ and Pol ϵ represented distinguishable complexes, the latter marked by the presence of lamin A/C (Fig. 3 and 5).

DISCUSSION

Applying the ChIP technique, we found that in HeLa cells all three replicative DNA polymerases, Pol α , δ and ϵ are bound to and released from both early firing *LB2* origin and *MCM4* UPR origin that was found to fire 2

hours later in mid S phase (Fig. 1 and 2). Nucleoprotein complexes purified by immunoprecipitation with Pol α , δ or ϵ antibodies from S phase cells were highly enriched with DNA fragments representing the two origins at the time preceding the firing, followed by rapid decrease after firing. Therefore, both replicases are loaded to the two origins and disappear together with Mcm3, when replication forks have moved away from the origin. This is consistent with studies in yeast suggesting that Pol ϵ acts mainly at leading strand and Pol δ mainly at lagging strand synthesis (for review see 3).

All three DNA polymerases were associated with the same nucleoprotein complexes in G1/S arrested cells, as can be expected if they all bind to origins of replication and are associated with each other or are even physically coupled at the replication fork at this time. In late S phase the three polymerases are all still likely to be at replication forks, as shown by the presence of Mcm3 and Cdc45 proteins in nucleoprotein complexes purified by immunoprecipitation with cognate Pol antibodies (Fig. 3A). However, in late S phase Pol δ and α seem to be essentially devoid of Pol ϵ , and *vice versa*, suggesting that Pol α/δ and Pol ϵ are now mainly associated with distinct complexes. This suggests that Pol δ and ϵ are physically uncoupled in late S phase. This is consistent with the fact that Pol ϵ alone remains associated with nuclear matrix, obviously through lamin A/C, while Pol α and δ are only associated with lamins in early S phase, most likely as components of replication complexes.

How can the lack of association between Pol α/δ and Pol ϵ in late S phase be explained? First, the share of labor could be different in late compared to early S phase, such that late S phase forks have a different DNA polymerase composition. The colocalization of Pol ϵ with sites of DNA synthesis in late, but not early S phase (21), would be consistent with an augmented role of Pol ϵ in late S phase replication. Nevertheless, profiling of replication errors

generated by an asymmetric mutator variant of Pol δ in the budding yeast indicate that Pol δ synthesizes the lagging-strand throughout the genome (13), and argue strongly against such a model. We found in a previous study that in late S phase, Pol δ is associated more strongly with newly synthesized DNA and chromatin than Pol ϵ , and that replication in isolated nuclei is more strongly inhibited in late S than in early S phase by a monoclonal antibody inhibiting Pol δ (20). Therefore, alternatively, this can be explained in a manner proposed by Pavlov and Shcherbakova (14). In their model Pol ϵ in principle replicates the leading strand, but is switched to Pol δ at pause sites or sites of translesion synthesis (TLS). In this way, Pol ϵ would synthesize most of the leading strand, but Pol δ would synthesize the parts of the leading strand after replication fork arrest and the restart of DNA synthesis. The fact that the Pol domain of Pol ϵ is not essential for viability of yeast (15) and that Pol ϵ is not needed for SV40 DNA replication (6,8,9) are two examples of the ability of Pol δ to substitute for Pol ϵ at the leading strand. Alternatively, it is possible that replicative Pols α , δ and ϵ in late S phase are increasingly involved in tasks that are no longer closely associated with the replication fork. It has been reported that a considerable amount of DNA translesion synthesis may be performed after and independent of DNA replication (41). There may also be a differential requirement of DNA polymerases e.g. for the termination of DNA replication, or for the rescue of stalled and reversed forks by recombinational processes.

Finally, Pols δ and ϵ may be physically separated in late S phase but are still synthesizing DNA at the lagging and leading strand of the same forks. We consider the possibility that synthesis of the lagging strand is distributive, i.e. a new molecule of Pol α and Pol δ is recruited from a nucleoplasmic pool, and that the lagging strand replication machinery may be released from the fork during initiation of the following lagging strand. In this way, maturation of several

successive Okazaki fragments may be ongoing simultaneously, and Pol δ molecules may remain associated with chromatin after completion and joining of Okazaki fragments. Such a model would explain both the increasing association of Pol δ with chromatin (Fig. 4B) as well as the increased involvement in DNA synthesis as S phase progresses (20). In contrast, the leading strand would be synthesized largely progressively by the same Pol ϵ molecule attached to the nuclear scaffold. There is no evidence on heterodimer formation by Pol δ and ϵ (see e.g. 42) that would represent a human counterpart of the *E. coli* Pol III holoenzyme dimer or trimer that is stably associated with the core replication proteins (43,44) and carries out simultaneous synthesis of both strands. Pols α , δ and ϵ are all accumulated at replication roadblocks (45), but they did not form a stable complex with a replication progression complex (46). And although coupling of Pol α and ϵ with

the Cdc45-Mcm2-7-GINS (CMG) helicase complex has been described (47,48), replicases are easily uncoupled from DNA unwinding after inhibition of DNA synthesis (49) or after DNA damage (50). Therefore, transient association and dissociation of the replicative DNA polymerases with the CMG complex is likely to allow their flexible utilization for the duplication of complex human genome.

It is possible that a combination of all three, different involvement of the Pols δ and ϵ in DNA replication-associated processes such as DNA translesion synthesis, increasing involvement of the replicative DNA polymerases in late S phase in tasks that are no longer closely associated with replication forks, and repeated recruitment of new Pol δ molecules from a nucleoplasmic pool for the lagging strand synthesis, account for the dynamics of Pols α , δ and ϵ in their mutual association described here.

Table 1. Antibodies used in this study

Target	Clone/Name	Species/type	Source/purification	Application^a	Reference
Pol α catalytic subunit	p140	Rabbit polyclonal	Protein A	ChIP/WB	(24)
Pol δ catalytic subunit	K30	Rabbit polyclonal	Protein A	ChIP/WB	(20)
	K31, K32, K33	Rabbit polyclonal	Protein A	ChIP/WB	this study
	7B4	Rat Monoclonal	Hybridoma supernatant	WB	(20)
Pol ϵ catalytic subunit	G1A, H3B, E24C	Mouse monoclonal	Protein G	WB	(25)
	K19	Rabbit polyclonal	Protein A	ChIP	(20)
	K27	Rabbit polyclonal	Protein A	ChIP	(26)
PCNA	PC10	Mouse monoclonal	Sigma/Zymed	WB	
Mcm2	N19	Rabbit polyclonal	Santa Cruz Biotechnologies	WB	
Mcm3	N19	Rabbit polyclonal	Santa Cruz Biotechnologies	WB	
Lamin A/C	N18	Goat polyclonal	Santa Cruz Biotechnologies	ChIP/WB	
Cdc45	3G10	Rat monoclonal	Hybridoma supernatant	WB	(27)
Orc2	3B7	Mouse monoclonal	Stressgen	WB	
β -tubulin	KMX-1	Mouse monoclonal	Chemicon	WB	
Cyclin A	H-432	Rabbit polyclonal	Santa Cruz Biotechnologies	WB	

^aAbbreviations: ChIP - chromatin immunoprecipitation, WB - Western blot

Table 2. Primers used in this study

Primer	Sequence (5' to 3')	Amplicon length (bp)	Annealing temp (°C)	Reference
LB2C4F LB2C4R	ACACCGTGAGACGCGTTTGACC CAACAACCCATGAGCACCCCTGG	138	63	This study
LB2C3F LB2C3R	CATTTCTTGGGCAAATGCCTAGG AGATGGGGTTTCTCCATGTTGG	160	63	This study
LB2C1F LB2C1R	GTTAACAGTCAGGCGCATGGGCC CCATCAGGGTCACCTCTGGTTCC	240	64	(30)
LB2F LB2R	GGCTGGCATGGACTTTCATTTTCAG GTGGAGGGATCTTTCTTAGACATC	232	66	(30)
LB2C2F LB2C2R	TTCTGACCTCCAGCCCTGCAG ACCCAGTAGAAAGCTGCCCTC	107	63	This study
Ex9F Ex9R	TCTCAGAGGGTACCTGGTTTGG GGAATGTAGGGAGCTGCGGTG	90	60	This study
UPRF UPRR	GGTCAAGAGTTCCAAGTTTGTTCCT TGCAGGCGGGCATCAC	72	60	This study
In6F In6R	GACATTCTGCTTCCATAGATGTGG GTTGGGAAAGATGTCATCATCAGG	346	55	(30)
In7F In7R	GAGGAATGCCAGAATTTCCAGAGG TTCCATCTGGAATGAGATCCCAGC	327	58	(30)

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Time course of association of replication proteins with two human origins of DNA replication. **A.** Association with replication origins at the *LB2* gene (left panels) and at the UPR of the *MCM4* gene (right panels) with nucleoprotein complexes isolated by immunoprecipitation of S phase Orc2, Mcm3 and Pol ϵ . HeLa cells were synchronized by double thymidine block to early S phase (0 h) and released to proceed in S phase (0.5h, 2h and 4h). The regions analyzed by quantitative PCR (qPCR) are indicated in the panels and their locations are shown below the panels, for *LB2* origin and UPR origin see the left and right panel, respectively. The *LB2* gene is on left site of the replication origin as indicated by an arrow. The *MCM4* gene is on left and *PRKDC* gene on right site of the replication origin as presented by arrows. The *MCM4* gene encodes the minichromosome maintenance protein 4 and the *PRKDC* gene the catalytic subunit of the DNA-dependent protein kinase. The origin regions are shown as gray boxes below the axis. Black bars above the axis represent regions that were amplified by qPCR. **B.** Cell cycle progression of G1/S arrested cells released to progress into S phase verified by FACS of propidium iodide-treated cells.

FIGURE 2. Time course of the association of origin of replication DNA within the *LB2* gene (left panels) and the UPR of *MCM4* gene (right panels) with nucleoprotein complexes isolated by immunoprecipitation of S phase Pol α , δ and ϵ . The regions analyzed by qPCR are indicated in the panels and their locations are shown below the panels, *LB2* origin in the left and UPR origin in the right panel. The origins are shown as gray boxes. Black bars represent regions that were amplified by quantitative PCR. The genomic organization and the location of the PCR products are identical to Fig.1. The cells were synchronized by double thymidine block to early S phase (0 h) and released to proceed in S phase (0.5h, 2h and 4h).

FIGURE 3. A. Association of Orc2, Mcm3 and Cdc45 with S phase nucleoprotein complexes isolated by precipitation with antibodies against replicative DNA polymerase. **B.** Association of Pol δ and Pol ϵ with early S phase nucleoprotein complexes immunoprecipitated with Pol α antibodies. **C.** Association of Pol δ with S phase nucleoprotein complexes immunoprecipitated with Pol ϵ antibodies. The cells were synchronized by double thymidine block to G1/S (0 hours) and released to proceed in S phase. The antibody used for immunoprecipitation is indicated above each panel and the antibody used for Western blotting on left site of the panels. Nucleoproteins were isolated and treated as described under "Experimental Procedures" for chromatin immunoprecipitation. In,

nucleoprotein sample taken for immunoprecipitation; S, supernatant of the immunoprecipitation; P, immunoprecipitate.

FIGURE 4. Chromatin and matrix association of replicative DNA polymerases during S phase. T98G cells synchronised by serum stimulation were subjected to subnuclear fractionation and relevant proteins were analysed by Western blotting. **A.** Analysis of marker proteins and selected replication factors in fractionated, asynchronous T98G cells. T, total cell extract; S, soluble fraction; B, chromatin bound fraction and M, matrix fraction. **B.** Cell cycle progression of the cell synchronisation of T98G cells employed for fractionation. Propidium iodide stained cells at indicated times after serum stimulation were analysed by flow cytometry. **C.** Time course for the occurrence of Pol δ , Pol ϵ and Cdc45 in fractions from serum-stimulated synchronous T98G cells. Times after the addition of serum are presented on the top of the panel.

FIGURE 5. Association of DNA polymerases with lamins A/C in S phase. The cells were synchronized by double thymidine block to early S phase (0 hours) and released to proceed in S phase (6 hours). The antibody used for immunoprecipitation is indicated on top and the antibody used for Western blotting on left site of the panels. Nucleoproteins for chromatin immunoprecipitation were isolated and treated as described under “Experimental Procedures”. In, nucleoprotein sample taken for immunoprecipitation; S, supernatant of the immunoprecipitation; P, immunoprecipitate. Bottom left: Association of *LB2* origin DNA with lamins A/C at 0 and 6 hours after release from double thymidine arrest in nucleoprotein complexes of human cells.

Figure 1A

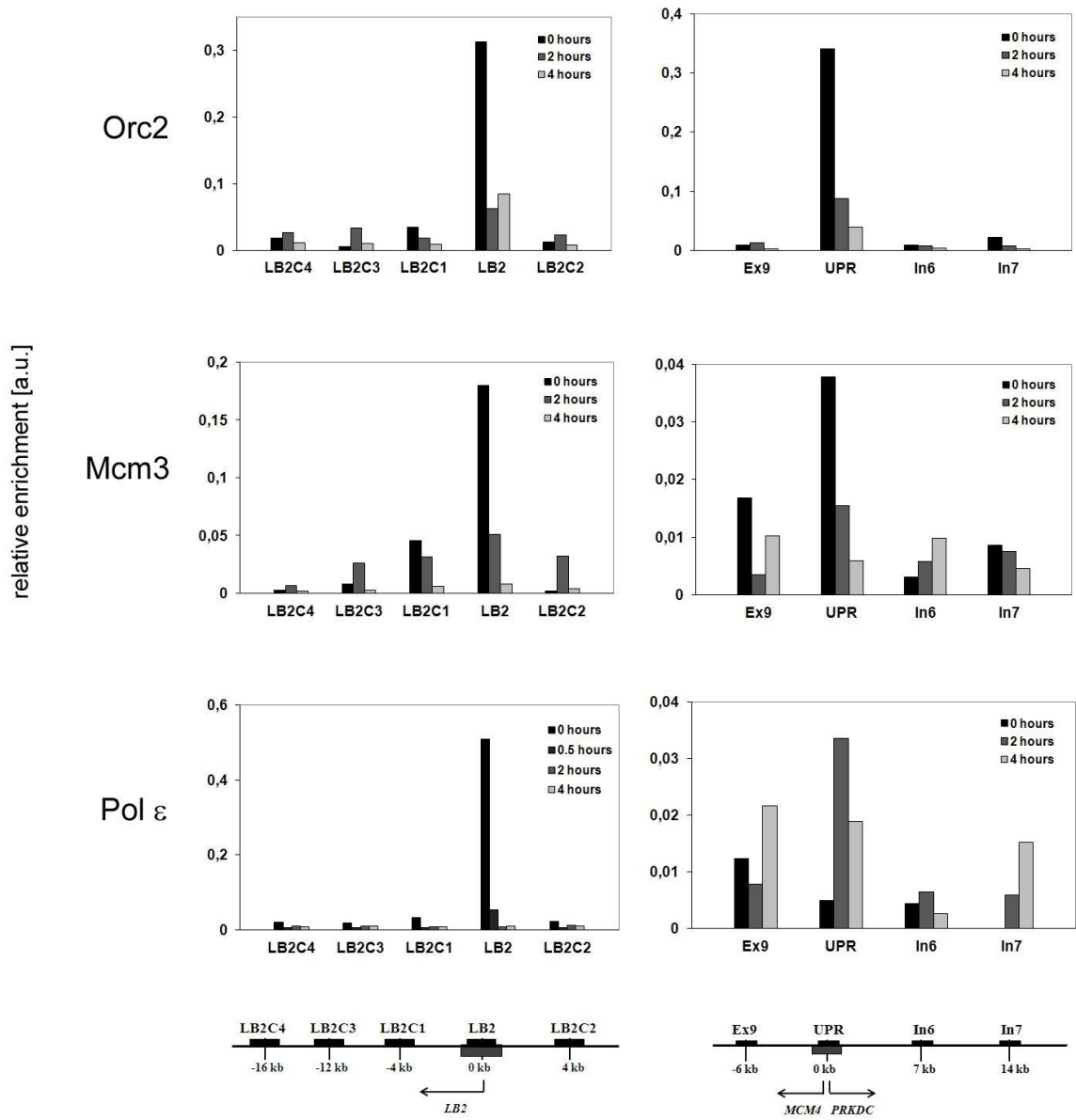


Figure1B

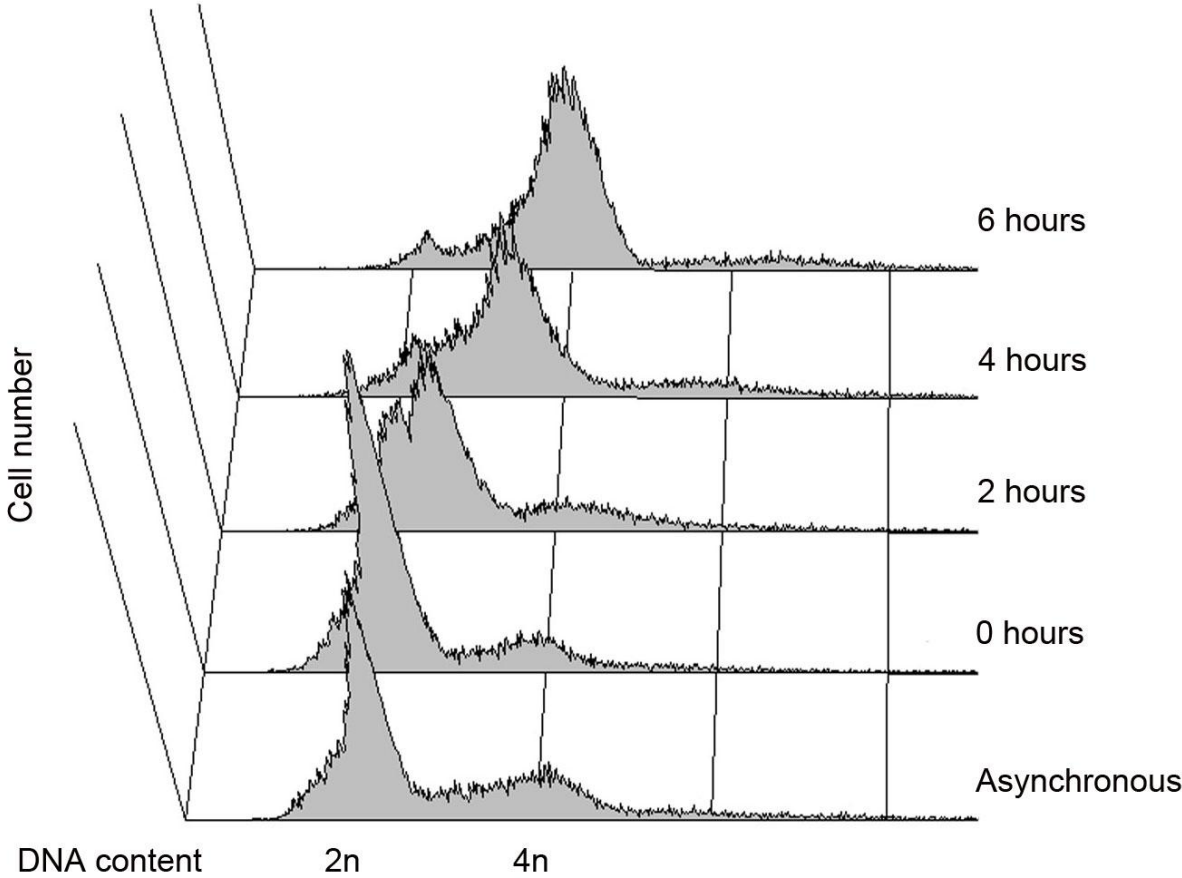


Figure 2

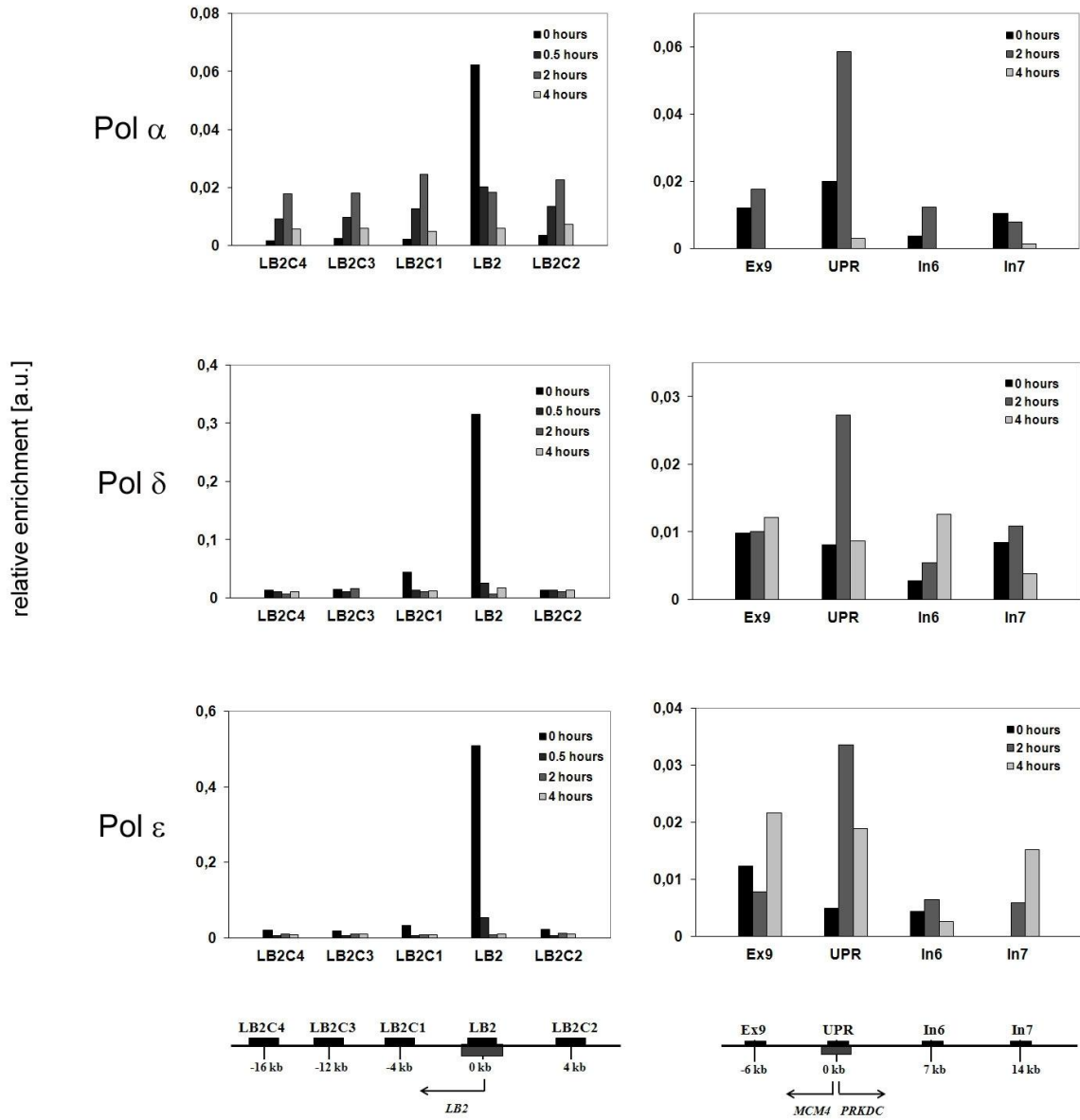


Figure 3A

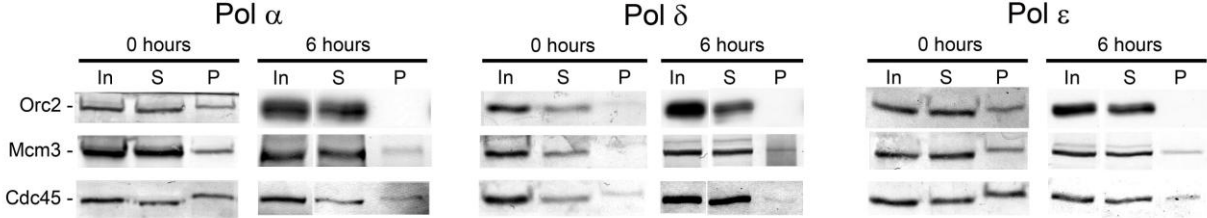


Figure 3B

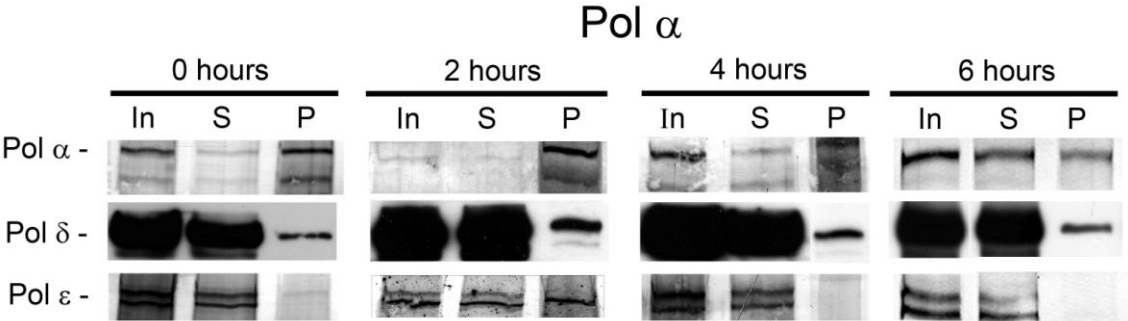


Figure 3C

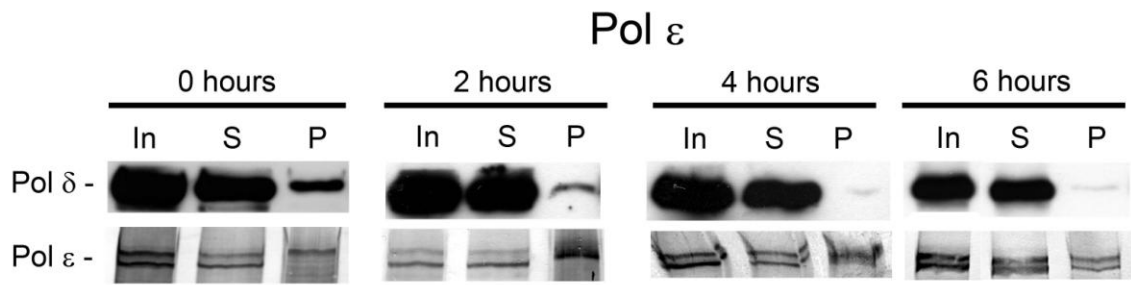


Figure 4A

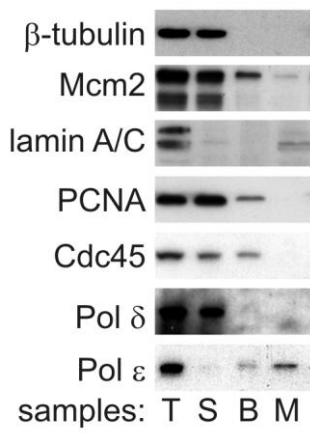


Figure 4B

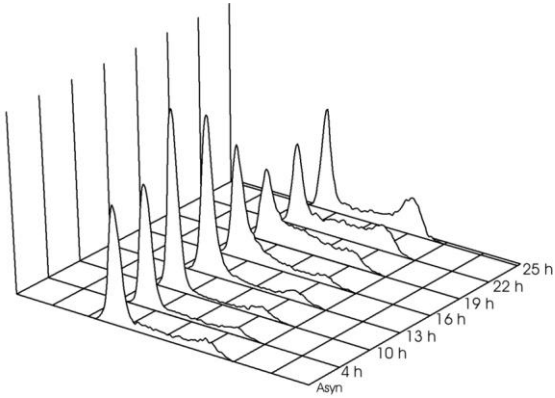


Figure 4C

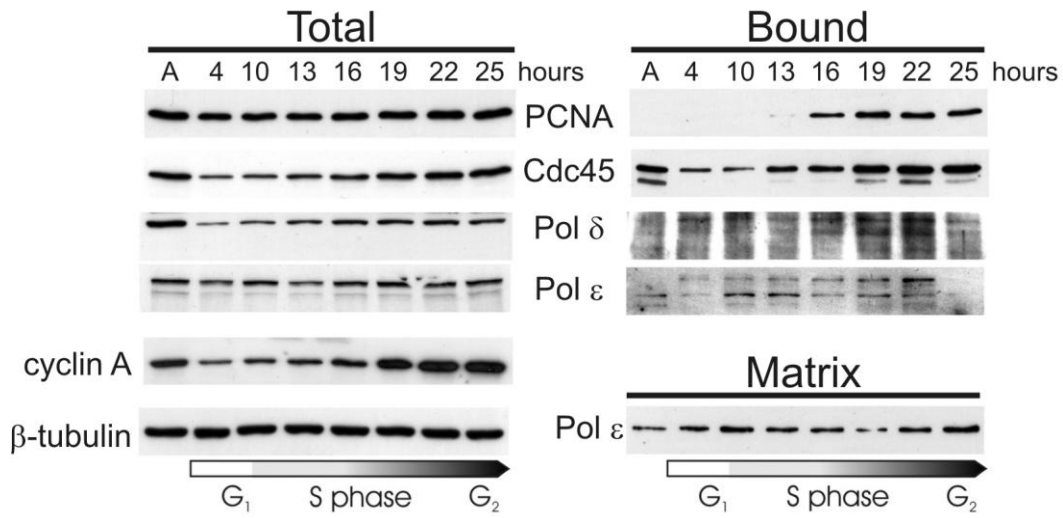


Figure 5

