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Human DNA polymerase ε is expressed during cell proliferation in a manner characteristic of replicative DNA polymerases

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ABSTRACT

In order to shed light on the role of mammalian DNA polymerase ε we studied the expression of mRNA for the human enzyme during cell proliferation and during the cell cycle. Steady-state levels of mRNA encoding DNA polymerase ε were elevated dramatically when quiescent (G_0) cells were stimulated to proliferate (G_1/S) in a similar manner to those of DNA polymerase α. Message levels of DNA polymerase β were unchanged in similar experiments. The concentration of immunoreactive DNA polymerase ε was also much higher in extracts from proliferating tissues than in those from non-proliferating or slowly proliferating tissues. The level of DNA polymerase ε mRNA in actively cycling cells synchronized with nocodazole and in cells fractionated by counterflow centrifugal elutriation showed weaker variation, being at its highest at the G_1/S stage boundary. The results presented strongly suggest that mammalian DNA polymerase ε is involved in the replication of chromosomal DNA and/or in a repair process that may be substantially activated during the replication of chromosomal DNA. A hypothetical role for DNA polymerase ε in a repair process coupled to replication is discussed.

INTRODUCTION

There is genetic evidence that DNA polymerases α, δ and ε of the yeast *Saccharomyces cerevisiae* (formerly DNA polymerases I, III and II, respectively) are all required for the replicative synthesis of chromosomal DNA (1–3). Both human and yeast DNA polymerase ε are also involved in the repair of UV damaged DNA (4–6). Calf thymus DNA polymerase ε has also been implicated in recombinative repair of deletions and double-strand breaks (7). As demonstrated by the best available *in vitro* replication system for modeling mammalian replication, SV40 DNA replication *in vitro* (8), α and δ are the only polymerases required for complete replication of this DNA (9). In this model system DNA polymerase α/primase synthesizes RNA–DNA primers for initiation at the origin and for priming Okazaki fragments. Both the leading and lagging strands are subsequently synthesized by DNA polymerase δ. DNA polymerase ε can substitute for δ under some conditions (10), but is not really required in this system. Two possible explanations for these seemingly contradictory results between yeast *in vivo* experiments and *in vitro* work with purified mammalian proteins are: (i) *in vitro* replication of SV40 DNA does not represent replication of the host cell chromosomal DNA in a manner that is able to reveal the cellular role of DNA polymerase ε in it; (ii) unlike yeast DNA polymerase ε, its mammalian counterpart is not involved in the replication of chromosomal DNA. The latter explanation, assuming that DNA polymerase ε has different roles in yeast and mammals, is suggested by the different subunit structures of the two enzymes (11,12).

Since growth factors or serum regulate the growth of fibroblasts and related cells in culture, the stimulation of serum-deprived quiescent cells to proliferate by adding serum results in the appearance of numerous mRNAs or increases in their steady-state levels. The genes activated to produce mRNAs by such growth factors can be divided into two classes, early growth-regulated genes and late growth-regulated genes. Examples of the former genes are c-*fos* and c-*myc* (13,14) and of the latter the genes encoding proteins involved either in nucleotide metabolism, e.g. thymidine kinase (15), or in DNA replication, e.g. proliferating cell nuclear antigen (PCNA) (16). In continuously dividing cells the mRNA levels of the late growth-regulated genes, e.g. PCNA, vary little throughout the cell cycle (17). As is typical of replication proteins, the steady-state levels of mRNAs for the mammalian replicative DNA polymerases α and δ show a dramatic increase when quiescent cells are stimulated to proliferate with serum, but little or no variation in continuously dividing cells (18–20). DNA polymerase β, which has been implicated in DNA repair (21), is expressed constitutively in quiescent cells stimulated to proliferate and throughout the cell cycle (22), but its message levels are elevated by several DNA damaging agents (23).

These earlier reports and other related experiments demonstrate that the influence of cell proliferation on steady-state levels of a particular mRNA provides valuable information on the role of a protein involved in DNA metabolism. We therefore studied the expression of DNA polymerase ε in mammalian cells. This study was rendered possible by our previous cloning of the cDNA that encodes the 261 kDa catalytic subunit of human DNA polymerase ε (24,25; GenBank/EMBL accession no. L09561).

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The results strongly favor the idea that this enzyme is either directly involved in the replication of chromosomal DNA in mammalian cells and/or in a repair process closely associated with replication.

MATERIALS AND METHODS

Bacterial strains, cell lines and cell cultivation

Escherichia coli strain XL1-Blue was used for plasmid propagation. The human cell lines used were HeLa S3 (ATCC CCL 2.2) and IMR-90 (ATCC CCL 186). The HeLa S3 cells were grown in suspension in Joklik's modification of minimal essential medium (Flow Laboratories, USA) supplemented with L-glutamine, penicillin, streptomycin and 5% fetal calf serum. The IMR-90 fibroblast cells were cultivated as monolayers in a 5% carbon dioxide atmosphere in minimal essential medium supplemented with Earle's salts, L-glutamine, non-essential amino acids, penicillin, streptomycin and 10% fetal calf serum. The mouse strain used was C57BL.

Preparation of RNA probes for protection assays

The template used for *in vitro* transcription when preparing an antisense RNA probe for human DNA polymerase ε was a 355 bp fragment, representing nucleotides 1309–1663 of the respective cDNA (24), subcloned into the Bluescript KS plasmid vector. The RNA probe template for human DNA polymerase α was made by RT–PCR (Dynazyme PCR polymerase; Finnzymes, Finland) from HeLa poly(A)$^+$ RNA and represented nucleotides 3238–3614 of the published sequence (26). The 382 bp product was subcloned into Bluescript KS. Similarly, the 201 bp RT–PCR product of DNA polymerase β (nucleotides 31–231) (27), the 310 bp RT–PCR product of human cytoplasmic β-actin (nucleotides 35–344) and the 210 bp product of human histone H3 (nucleotides 628–838), all three derived from HeLa poly(A$^+$) RNA, were subcloned into the same plasmid. Antisense RNA probes were then prepared by incubating 1 µg linearized template DNA in 40 mM Tris–HCl, pH 7.5, containing 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 500 µM ATP, GTP and UTP, 2.5 µCi/µl [α-^{32}P]CTP (800 Ci/mmol) and ribonuclease inhibitor with 1 U T3 RNA polymerase (Promega, USA) for 30 min at 25°C. The DNA template was then digested with 3 U RNase-free DNase (Promega, USA) at 37°C for 15 min. The full-length RNA probe was purified by 4% PAGE in the presence of 8 M urea.

RNase protection assays

For RNA analysis, total RNA was isolated as described (28). The ribonuclease protection assay was performed with a RPA II™ kit (Ambion, USA). Briefly, 5–10 µg target RNA was hybridized with a ^{32}P-labeled RNA probe at 42°C for 15 h and the remaining single-stranded RNA was digested with a RNase A/RNase T_1 mixture. The protected fragments were separated by SDS–PAGE and visualized by autoradiography.

Western analysis

The monoclonal antibody 93G1A against human DNA polymerase ε has been described elsewhere (29). The monoclonal antibody against human PCNA (clone PC10) and the rabbit antiserum against tubulin were from Boehringer (Mannheim, Germany) and Clontech (Palo Alto, USA) respectively. The extracts for Western blots were prepared as described (30), except that the buffers were supplemented with the protease inhibitors phenylmethylsulfonyl fluoride and pepstatin A at concentrations of 1 mM and 1 µg/ml, respectively. The protein samples were separated by SDS–PAGE and blotted onto nitrocellulose filters (Sleicher & Schüll, Germany). After transfer, the filters were washed with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) and blocked with 5% non-fat milk in TBS for 1 h. The samples were incubated overnight with monoclonal antibodies at 1.5 µg/ml or antiserum at a dilution of 1:1000 in TBS supplemented with 0.05% Tween-20. The blots were first incubated for 2 h with goat anti-mouse IgG (BioRad, USA) or goat anti-rabbit IgG (Sigma, USA) conjugated to alkaline phosphatase (1:3000 dilution in TBS/Tween solution) and then with the colour developing reagents 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 100 mM Tris–HCl, pH 9.5.

Analysis of DNA synthesis *in vivo*

DNA synthesis *in vivo* was monitored as described (31), with minor modifications. Briefly, cells cultured on 24-well microtiter plates were pulse-labeled with [methyl-^{3}H]thymidine (48 Ci/mmol) at 25 µCi/ml for 1 h before harvesting. They were then washed with PBS containing 0.4 mg/ml thymidine, lysed with 0.1 M NaOH, 10 mM EDTA, 0.5 % SDS and 100 µg/ml DNA as a carrier at 68°C for 30 min and the DNA was then precipitated with 3 vol ice-cold 5% trichloroacetic acid. The precipitate was collected on GF/C paper (Whatmann, USA) and the radioactivity measured using a liquid scintillation counter. Three independent samples were measured for each time point.

Serum deprivation experiments

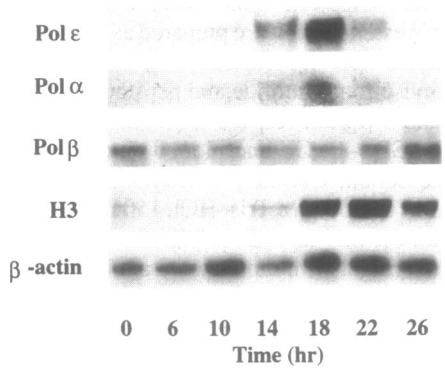
IMR-90 cells at 21 doubling passages were cultivated either in 150 cm^2 tissue culture flasks or on 75 cm^2 plates to 40% confluency in the presence of 10% fetal calf serum. They were then incubated for 96 h in medium containing only 0.25% fetal calf serum. The quiescent cells were subsequently re-stimulated to proliferate by elevating the serum concentration to 10%. RNA samples were isolated at given intervals.

Cell synchronization by double thymidine block and nocodazole

Logarithmically growing IMR-90 cells were synchronized by double thymidine block (32,33). The cells were first arrested by incubating with 2 mM thymidine for 18 h and the block was then released by incubating the cultures with fresh medium not containing thymidine for 10 h. A second 18 h thymidine block was then applied, after which the cells were released and RNA samples isolated at given intervals. The HeLa S3 cells were synchronized by adding nocodazole to a concentration of 0.1 µg/ml and incubating for 15 h, after which the block was released by changing the medium (34).

Counterflow centrifugal elutriation

Log phase HeLa S3 cells were fractionated into semisynchronized populations by counterflow centrifugal elutriation in a Beckman JE-6 elutriator rotor (35). Cells (2×10^8) were loaded into a separation chamber with a loading speed of 2800 r.p.m. Eight 100 ml fractions were collected by increasing the flow rate



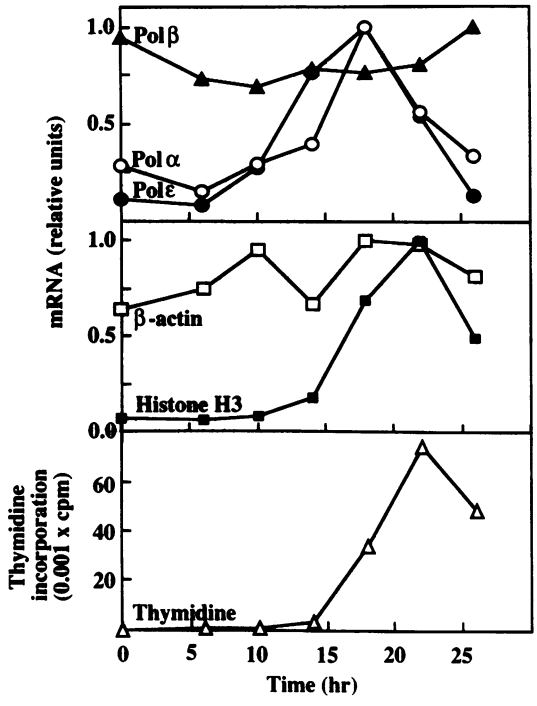


Figure 1. Steady-state mRNA levels of DNA polymerases α, β and ε, histone H3 and β-actin in serum-deprived IMR-90 cells stimulated to proliferate. Serum was added to quiescent cells and RNA samples were isolated for RNase protection assays at the times indicated. The intensities of the autoradiograms from the protection assays were measured by densitometric scanning and are presented as relative intensities by taking the maximum intensity of each message as 1. The level of *in vivo* DNA synthesis was determined in parallel cultures for each time point.

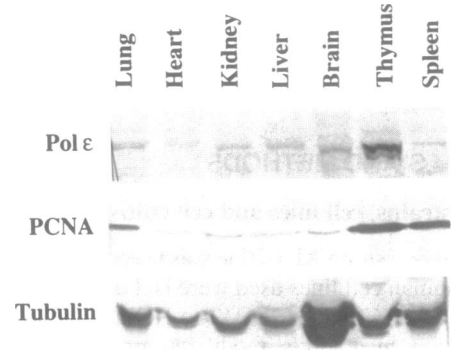


Figure 2. Western analysis of DNA polymerase ε, PCNA and tubulin in extracts from various mouse tissues. Samples of tissue extracts containing 50 μg protein were separated on a 5% SDS–polyacrylamide gel, transferred onto nitrocellulose filters and stained with a monoclonal antibody (93G1A) against human DNA polymerase ε. Tubulin was analyzed as a control. It is known to be equally abundant in all tissues analyzed, with the exception of brain, in which it is more abundant.

stepwise from 24 to 74 ml/h. The first fraction and the last two fractions were discarded. Aliquots of each fraction were fixed and stained with DAPI (36) and their cell cycle distribution was analyzed by flow cytometry on a PAS II flow cytometer (Partec, Münster, Germany).

RESULTS

Expression of DNA polymerases α, β and ε and proliferative state of the cells

When the cultivated human fibroblast cell line IMR-90 was brought to a G_0-like state by serum deprivation and then re-stimulated to proliferate by adding serum, DNA synthesis increased 18 h after stimulation and reached its maximum at 22 h (Fig. 1). The DNA polymerase ε message level started to increase at 10 h and rose to its maximum at 18 h, the maximum level being 5- to 8-fold relative to that in non-stimulated cells. A very similar stimulation profile was obtained for the steady-state message levels of DNA polymerase α, while the level of DNA polymerase β message remained practically constant upon serum stimulation of quiescent cells. Earlier experiments with DNA polymerase α (18) and DNA polymerase β (22) have led to similar results. The message levels of β-actin and histone H3 were measured as controls, as the former is known to be expressed constitutively and the latter almost synchronously with DNA synthesis. To confirm the finding that the message levels of DNA polymerases α and ε increase synchronously upon serum stimulation of cultivated cells, we repeated the experiment for the mRNAs of these two DNA polymerases with the cell line WI-38 and obtained almost identical results (data not shown).

The dramatic increase in the mRNA level of DNA polymerase ε ~10 h after serum stimulation and 5 h prior to DNA synthesis *in vivo* is typical of late growth-regulated genes. The synchronous increase in the steady-state mRNA level of DNA polymerase ε with that of replicative DNA polymerase α strongly suggests that the former is also one of the prerequisites for replication of chromosomal DNA in mammalian cells. If it were needed for the maintenance repair of DNA only, it would be expected to be expressed constitutively in a manner that is independent of the proliferative state of the cells, like DNA polymerase β (22). To further study the possibility that DNA polymerase ε may be needed for cell growth, we analyzed its presence in extracts from various tissues of 3-month-old (adult) mice with the monoclonal antibody 93G1A against the human enzyme (Fig. 2). This antibody recognizes the enzyme widely in samples from numerous eukaryotic species (29). Immunoreactive DNA polymerase ε with a molecular weight of >200 kDa was present in extracts from all the mouse tissues analyzed, but by far the highest concentration was found in thymus extracts. Its existence in non-proliferating tissues suggests that it has a housekeeping role, probably in the maintenance repair of DNA. This is consistent with a report that it is involved in the repair of UV damage in fibroblast cells

from density-inhibited cultures (4). Thymus and spleen extracts can be considered to represent proliferating lymphocytes, rather than non-proliferating organs. A high concentration of DNA polymerase ε was detected in thymus. For comparison, PCNA and tubulin were also analyzed. PCNA is a replication and repair factor (37,38). As expected, it is abundant in extracts from both thymus and spleen (Fig. 2), the two tissues containing proliferating cells. The intensity of the tubulin band is known to be approximately the same in each tissue, with the exception of brain, in which the band is more intense due to the prevalence of microtubules (37).

Steady-state levels of DNA polymerases β and ε mRNA through the cell cycle

Earlier experiments have indicated that the steady-state mRNA levels of replicative DNA polymerases α and δ show little or no variation at different stages of the cell cycle (see Introduction), with only slight elevation prior to S phase and a decline in the G_2 phase. To study whether the steady-state level of DNA polymerase ε mRNA was dependent on the cell cycle stage, we employed two different synchronization methods, a double thymidine block, which arrests the cells at S phase, and nocodazole, which arrests them at M phase. The steady-state level of the mRNA was also studied in log phase cells fractionated by counterflow centrifugal elutriation. To confirm the expected arrest of the cells with thymidine and nocodazole, DNA synthesis *in vivo* was measured by analyzing the incorporation of labelled thymidine after the block had been released. The cell cycle distribution of the fractions from centrifugal elutriation were analyzed with flow cytometry. When IMR-90 fibroblast cells synchronized by double thymidine block were released to proliferate by removing the thymidine, replication was resumed, as indicated by its incorporation (Fig. 3). At 15 h the cells started to enter S phase again. Both DNA polymerase ε and β message levels remained high through G_1 phase. No significant decline occurred after the first S phase nor any increase prior to the second S phase. When the experiment was repeated with HeLa S3 cells grown in suspension, the results were almost identical (data not shown), but when the HeLa S3 cells were synchronized with nocodazole a 2-fold increase in the DNA polymerase ε message level occurred prior to S phase (Fig. 4). Roughly 2-fold mRNA levels were also measured in G_1/S cells (fractions 2 and 3) from centrifugal elutriation when compared with those from G_1 phase- (fraction 1) and S phase-enriched cells (fraction 4) (Fig. 5). According to these results, steady-state mRNA levels of DNA polymerase ε probably show a slight variation through the cell cycle, being highest slightly before S phase. This variation is weak, however, compared with the dramatic increase in message levels that occurs upon stimulation of serum-deprived cells with serum. The apparent independence of the steady-state level of DNA polymerase ε mRNA with respect to the stage in the cell cycle in cells synchronized with a double thymidine block may be due to an unknown mechanism by which a high concentration of thymidine could influence the regulation of replication.

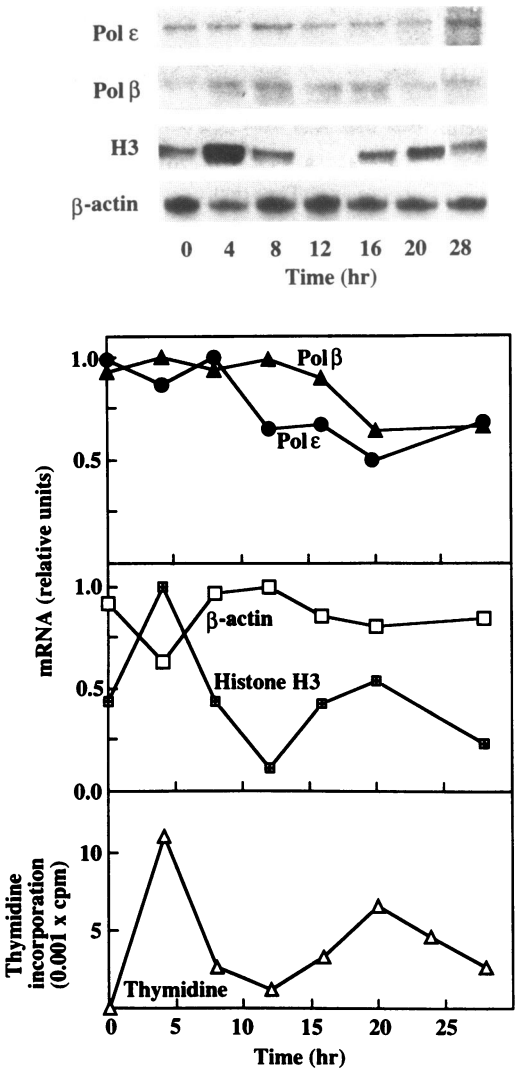


Figure 3. Steady-state mRNA levels of DNA polymerases β and ε, histone H3 and β-actin from IMR-90 cells synchronized by the double thymidine block method. Cells arrested by double thymidine block were released by removing the thymidine and RNA samples were taken at the time points shown. The intensities of the autoradiograms from the RNase protection assays were measured by densitometry and are presented as relative intensities by taking the maximum intensity of each mRNA as 1. DNA synthesis activity *in vivo* was determined in parallel cultures for each time point.

DISCUSSION

The results indicate that the expression of human DNA polymerase ε is dependent on cell proliferation, essentially in a similar manner to that of human DNA polymerase α (18) and δ (19), namely there is a dramatic increase in the steady-state mRNA level prior to the peak in DNA synthesis when serum-deprived cells are stimulated to proliferate by serum addition. The steady-state message level of DNA polymerase ε in cycling cells showed slight variation, being at its highest at the G_1/S stage boundary, in a similar manner to DNA polymerase α and δ (18,20). Furthermore, a significantly higher concentration of DNA polymerase ε protein was present in extracts from mouse thymus than in those from non-proliferating or slowly proliferating mouse organs.

The results indicate that mammalian DNA polymerase ε plays a role in the proliferation of cells, probably acting either at a replication fork itself or in a repair process that is substantially activated during the replication of chromosomal DNA. This

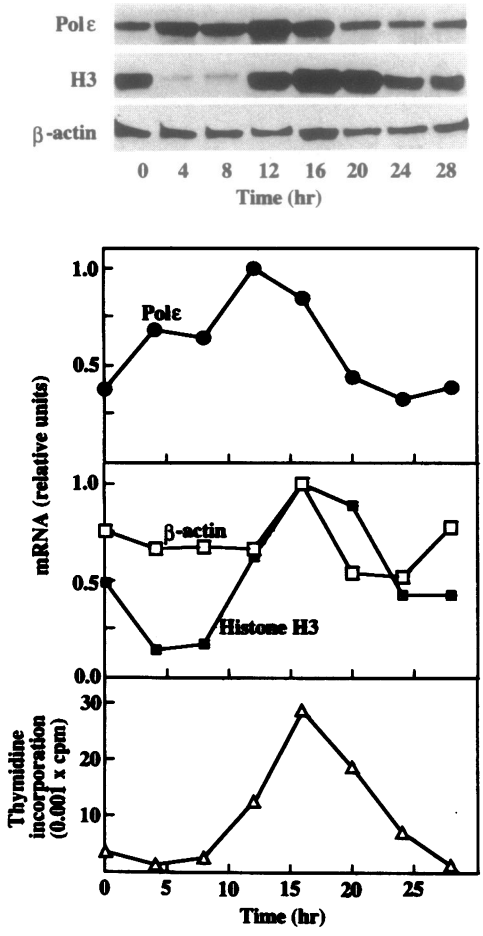


Figure 4. Steady-state mRNA levels of DNA polymerases ε, histone H3 and β-actin from HeLa S3 cells synchronized with nocodazole. Cells grown in suspension were arrested with nocodazole and released by removing the nocodazole. RNA samples were taken at the time points shown. The intensities of the autoradiograms from the RNase protection assays were measured by densitometry and are presented as relative intensities by taking the maximum intensity of each mRNA as 1. DNA synthesis activity *in vivo* was determined in parallel cultures for each time point.

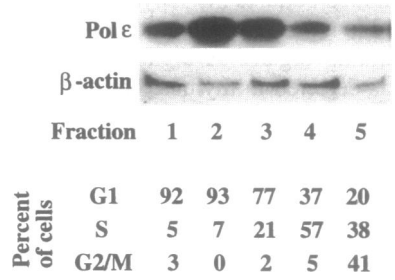


Figure 5. Steady-state mRNA levels of DNA polymerase ε and β-actin from HeLa S3 cells fractionated by counterflow centrifugal elutriation. Cell cycle distribution of the fractions were analyzed by flow cytometry. RNA samples from each fraction were analyzed by RNase protection assays.

function is essential, at least in yeast, since deletion of the gene encoding DNA polymerase ε is lethal for the cells (1). According to the results of *in vitro* replication of SV40 DNA, mammalian DNA polymerase ε does not act at replication forks (see Introduction). How can one explain the apparent contradiction between the present results and the *in vitro* work with SV40 DNA replication? One explanation could be that DNA polymerase ε plays a role that is absolutely essential for the advancement of replication forks, but that the enzyme does not act at the forks themselves. Such a function could be an essential repair function coupled to replication, so that if it cannot be completed advancement of the fork stalls. Indeed, it was recently reported that in *S.cerevisiae* DNA polymerase ε links the replication machinery to the S phase checkpoint by acting as a sensor that coordinates the transcriptional responses to DNA damage and preventing mitotic entry (40). Such a control mechanism is obviously important for eukaryotes, especially mammals, in order to prevent highly mutated cells from appearing. Repair of a form of damage occurring frequently during replication would necessitate an elevation in the expression of the participating repair proteins, including DNA polymerase ε. An important repair mechanism that must be associated with replication is mismatch repair, which is known to exist in both prokaryotic and eukaryotic organisms (41–43). Following recognition of the mismatched base pair, the new strand containing the mismatch is distinguished from the old one by a specific mechanism, removed through the mismatch and resynthesized by a DNA polymerase. For a eukaryotic organism to survive, error frequencies must be as low as 10^{-9}–10^{-10} per base replicated. The contribution of the mismatch repair system to this high fidelity is ~100-fold (44,45), indicating its importance for the maintenance of DNA integrity. DNA polymerase ε could be essential for the replication of chromosomal DNA due to its central role in the DNA synthesis step in the repair process required for fork advancement. A common intermediate in the repair of mismatches resulting from replication errors, and also from many other damage types, is a single-stranded gap and this may be a target of DNA polymerase ε. SV40 DNA replication *in vitro* in a reconstituted system, and possibly also *in vivo*, may not be dependent on DNA polymerase ε, e.g. if it is devoid of this kind of repair machinery or the factors coupling it to replication.

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