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A neutralizing antibody against human DNA polymerase ε inhibits cellular but not SV40 DNA replication

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ABSTRACT

The contribution of human DNA polymerase ε to nuclear DNA replication was studied. Antibody K18 that specifically inhibits DNA polymerase activity of human DNA polymerase ε *in vitro* significantly inhibits DNA synthesis both when microinjected into nuclei of exponentially growing human fibroblasts and in isolated HeLa cell nuclei. The capability of this neutralizing antibody to inhibit DNA synthesis in cells is comparable to that of monoclonal antibody SJK-132-20 against DNA polymerase α. Contrary to the antibody against DNA polymerase α, antibody K18 against DNA polymerase ε did not inhibit SV40 DNA replication *in vitro*. These results indicate that DNA polymerase ε plays a role in replicative DNA synthesis in proliferating human cells like DNA polymerase α, and that this role for DNA polymerase ε cannot be modeled by SV40 DNA replication.

INTRODUCTION

Yeast genetics can be readily applied to address the requirement of gene products in specific cellular processes. These studies have shown that three DNA polymerases (Pols) α, δ and ε are required for viability of *Saccharomyces cerevisiae* cells due to their essential role in DNA replication (reviewed in 1). Animal models and animal cells are less accessible to genetic analysis. Conceivably, our knowledge of animal cell DNA replication comes mainly from studies *in vitro*. The most useful model system has been SV40 DNA replication reconstituted *in vitro*. Pols α and δ are required for DNA synthesis in this system, whereas Pol ε does not seem to play any role (2). This view on SV40 DNA replication was supported by crosslinking Pols to nascent DNA in virus-infected cells *in vivo* (3). On the other hand, indirect evidence was presented in the same study that Pol ε, in addition to Pols α and δ, is involved in cellular DNA replication. In addition, Pol ε was found to copurify with a multiprotein complex that supports DNA replication *in vitro* (4). Nevertheless, the question about the involvement of Pol ε in replication of the mammalian genome has remained controversial. One approach to address the function of cognate animal cell proteins *in vivo* is the microinjection of neutralizing antibodies to specifically inhibit biological activities of target proteins. This method has been successfully applied especially for studying the influence of cell cycle regulators and checkpoint proteins such as cyclin A (5), cdk2 (6,7), p53 (8,9) and MCM2 (10) on cell cycle progression and replicative DNA synthesis in mammalian cells. We studied the contribution of Pol ε to DNA replication by microinjection of neutralizing antibodies into nuclei of proliferating human cells, and by monitoring the DNA synthesis in permeabilized HeLa cell nuclei in the presence of these antibodies. We present evidence that Pol ε synthesizes DNA during cellular but not SV40 DNA replication.

MATERIALS AND METHODS

Cell culture

IMR-90 human fetal lung fibroblasts (ATCC CCL 186) were from the American Type Culture Collection (Rockville, MD). Cells were grown at 37°C in 5% carbon dioxide atmosphere in Eagle's minimum essential medium supplemented with Earle's salts, 10% fetal bovine serum, non-essential amino acids, L-glutamine and antibiotics (Gibco BRL). HeLa S3 cells were cultivated in suspension as described (11).

DNA polymerases

Pols α and ε were purified from HeLa cells to step V (hydroxylapatite) as described (12). Pol δ was purified from calf thymus according to Weiser *et al*. (13). PCNA was purified from HeLa cells as described (14). Recombinant Pol β was a generous gift from Samuel H. Wilson. Pol δ was assayed with poly(dA)/oligo(dT) as primer–template in the presence of

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PCNA as described (13), with the exception that KCl was omitted from the reaction mixture. The Pol ε assays were performed as described by Syväoja and Linn (12). Pol α activity was measured with poly(dA)/oligo(dT) as primer–template in the presence of 1 mM $MgCl_2$ under conditions otherwise identical to the Pol ε assays. Pol β activity was measured with poly(dA)/oligo(dT) as primer–template as described (15). All polymerase assay reactions were performed in duplicate.

Antibodies

Rabbits were immunized according to standard protocols with protein fragment representing different regions of the catalytic subunit of human Pol ε. The antigens were prepared as described by Uitto *et al*. (16). The antiserum of rabbit K18 that was immunized against a peptide representing amino acid residues 269–503 of human Pol ε (GenBank accession no. 3192938) showed specific immune response to Pol ε in western analysis of human HeLa cell extract (data not shown). This antiserum was subsequently found to neutralize the DNA polymerase activity of Pol ε and is further described below. K18 antibodies were purified by protein A–Sepharose affinity chromatography (Pharmacia, Sweden).

Microinjection

For microinjections, filtered antibodies were used at a concentration of 4–5 mg/ml in injection buffer (100 mM KCl, 5 mM HEPES–KOH, pH 7.25). IMR-90 cells were grown on Bellco photo-etched coverslips to ~50% confluency and all cells within a certain square of the grid were injected into the nucleus under an Axiovert 405M inverted phase contrast and fluorescence microscope (Zeiss, Oberkochen, Germany) using an Eppendorf Micromanipulator 5171 microinjector (Zeiss). Efficiency of injection was monitored by administration of fluorescent dye during the set-up of the method. All injected nuclei contained the dye indicating efficient microinjection (data not shown). During injections cells were kept warm in Medium 199 supplemented with Hank's salts (Gibco BRL). Cells were placed into fresh culture medium containing 100 μM bromodeoxyuridine (BrdU) after injection and grown for 24 h.

Immunocytochemistry

Cells on coverslips were fixed at room temperature for 20 min with 3% paraformaldehyde in phosphate-buffered saline (PBS). The cells were permeabilized by treatment with 0.1% Triton X-100 in PBS for 10 min, and DNA was denatured for 25 min in the presence of 4 M HCl. After blocking for 1 h in 0.2% gelatin in PBS, cells were incubated with FITC-conjugated mouse anti-BrdU monoclonal antibody (Boehringer Mannheim; dilution 1:60 in 0.2% gelatin in PBS) for 3 h. DNA was visualized by staining with Hoechst dye 33258 (Sigma). The coverslips were mounted with Immu-mount (Shandon).

Image capture and analysis

BrdU incorporation was measured by fluorescence microscopy of the stained cells using a low-light-level Extended Isis video camera (Photonic Science, Mountford, UK). Images were digitized using a DT5831 frame grabber from Data Translation (Marlboro, MA). The injected cells and non-injected control cells from another square on the same cover slip were photographed. The intensities of the stained nuclei and the surrounding cytoplasm were quantified using the NIH Image program (17). The intensity of the cell cytoplasm was subtracted from the nucleus intensity of the same cell, and average intensities and standard deviations for each series of measurements were calculated. Student's *t*-test was performed to assess the statistical confidence of results.

Preparation of permeabilized HeLa cell nuclei and cytoplasmic extracts and DNA replication assay in isolated nuclei

HeLa cell nuclei and cytoplasmic extract preparation and subsequent permeabilization with lysolecithin of the nuclei were performed as described (18). Nuclei were permeabilized immediately before use, washed, and suspended by 10 strokes with a loose-fitting pestle. Standard DNA replication reactions in isolated nuclei were performed in 50 μl mixtures containing 20 μl cytoplasmic extract (200–250 μg of protein), 100 μM dNTPs, 100 μM each of GTP, CTP and UTP, 4 mM ATP, an ATP-regenerating system, 2 μCi of [α-^{32}P]dCTP, 30 mM HEPES, pH 7.8, 7 mM $MgCl_2$ and 4–9 × 10^5 nuclei/reaction. Reaction mixtures were prepared on ice and started by transferring to 37°C. After 2 h of incubation, reactions were stopped by addition of 300 μl lysis buffer (100 mM NaOH, 10 mM EDTA, 0.5% SDS and 0.1 mg/ml of salmon sperm DNA). DNA was released and RNA degraded by incubation at 65°C for 30–60 min. DNA was precipitated by addition of 1 ml of ice-cold 10% trichloroacetic acid and incubated for at least 10 min on ice. The precipitate was collected and washed on GF/C glass fiber filters (Whatmann) as described (12). Incorporation of radioactivity was measured by liquid scintillation counting of the dried glass fiber filters. All reactions were performed in duplicate.

In vitro SV40 DNA replication assay

The DNA synthesome was isolated from human leukemia cells (HL-60) according to published procedures (19). The DNA synthesome is a protein complex containing several replication proteins including Pols α, δ and ε (4,20). SV40 DNA replication *in vitro* was measured as described (20), except that 30 mM HEPES (pH 7.5) was used as reaction buffer. Replication reactions including 2.5–3 μg SV40 large T-antigen (21), 20 μg synthesome protein fraction, 50 ng plasmid pSVO containing the SV40 replication origin (22) were incubated at 37°C for 4 h, stopped by adding 100 μg yeast RNA in 1% SDS, followed by proteinase K digestion. After phenol–chloroform extraction, DNA replication products were separated by electrophoresis in 1% agarose gels in TBE buffer and analyzed by autoradiography of the dried gels.

RESULTS

In order to assess the role of Pol ε in DNA synthesis *in vivo*, we prepared antibodies by immunizing rabbits with fragments of Pol ε catalytic subunit and screened their antisera for the ability to recognize Pol ε polypeptide in western analysis and to specifically neutralize Pol ε activity. One of these antisera, K18, was neutralizing. The purified IgG fraction from this serum inhibited >90% of the activity of purified human Pol ε *in vitro* at a concentration of 16 μg/ml (Fig. 1). In contrast, no inhibition of purified human Pol α, recombinant human Pol β nor calf thymus Pol δ was observed at antibody concentrations up

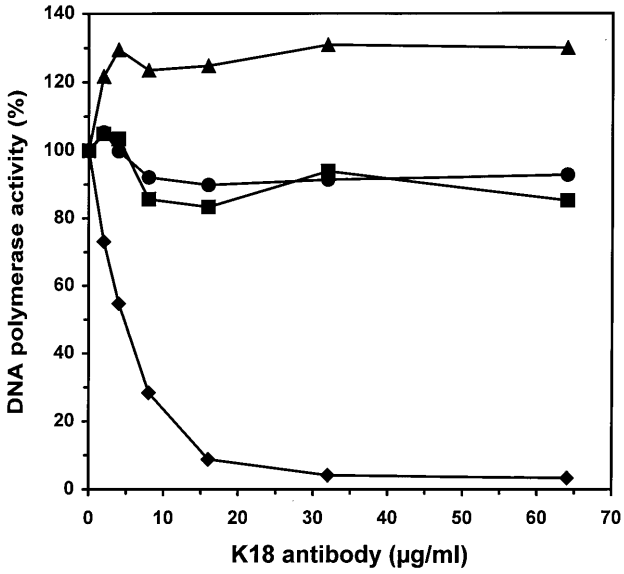

Figure 1. Specific inhibition of DNA polymerase ε by the polyclonal antibody K18. IgG fraction from the serum of an immunized rabbit was purified as described in Materials and Methods. Aliquots of 0.15, 1.5, 0.1 and 0.25 U of DNA polymerases α (circles), β (triangles), δ (squares) and ε (diamonds) respectively, were incubated with antibody on ice for 2–3 h and then assayed for DNA polymerase activity. Results represent the averages of two independent experiments.

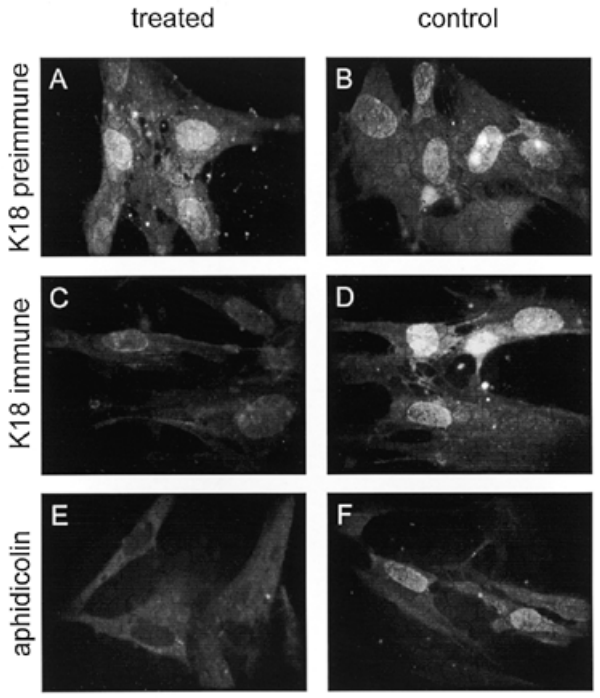

Figure 2. Microphotographs of exponentially growing IMR-90 cells microinjected with K18 antibody. The images represent cells microinjected with K18 pre-immune (**A**) or immune (**C**) antibodies, and neighboring, non-injected control cells (**B** and **D**, respectively), and cells cultivated in the presence (**E**) or absence (**F**) of aphidicolin. All nuclei in images (A) and (C) were injected with the respective antibody. The microphotographs present incorporation of BrdU detected by immunocytochemistry as described in Materials and Methods.

to 64 µg/ml. Purified IgG fraction from pre-immune serum of the same rabbit did not inhibit the activity of any of the Pols (data not shown).

We next addressed the question of whether we could reliably monitor the inhibition of DNA synthesis in human cells. Exponentially growing IMR-90 cells were cultured in the presence of increasing concentrations of aphidicolin, a well-characterized inhibitor of the polymerase activity of the replicative Pols α, δ and ε (reviewed in 23) and the BrdU incorporation into DNA was measured (Fig. 2). DNA synthesis decreased in a dose-dependent manner and only ~15% of BrdU incorporation was detected at an aphidicolin concentration of 2 µg/ml (data not shown), demonstrating that DNA synthesis can be reliably measured by this method.

Table 1. Effect of microinjected antibodies on DNA synthesis in exponentially growing IMR-90 fibroblasts

Antibody	Inhibition (%)	*n*	Significance
K18 [anti-Pol ε]	27	65/47	$P \leq 0.05$
	50	84/28	$P \leq 0.001$
	36	66/90	$P \leq 0.001$
K18 preimmune	8	101/41	–
	4	105/107	–
SJK-132–20 [anti-Pol α]	33	140/107	$P \leq 0.001$
	28	197/38	$P \leq 0.05$
	36	19/22	$P \leq 0.05$
93G1A [anti-Pol ε]	3	97/81	–
	–37	76/87	–
93H3B [anti-Pol ε]	7	130/74	–
	8	81/64	–
93E24C [anti-Pol ε]	3	78/91	–
	–5	75/57	–
Mouse IgG[a]	–9	25/21	–
	–12	7/12	–
Injection buffer	1	73/68	–
	2	78/91	–

The nuclei of exponentially growing IMR-90 human fetal lung fibroblasts were microinjected with antibodies and subsequent DNA synthesis was measured as described in Materials and Methods. Inhibition of DNA synthesis in injected cells was measured relative to neighboring non-injected control cells. *n* indicates the number of cells analyzed (injected/control). The statistical significance was calculated by Student's *t*-test. 93G1A, 93H3B and 93E24C are non-neutralizing monoclonal antibodies against human DNA polymerase ε catalytic subunit (16).

[a]Purchased from Sigma.

When cells were microinjected with neutralizing polyclonal antibody K18 against human Pol ε, inhibition of DNA synthesis was apparent (Fig. 2). On average, DNA synthesis was reduced by 38% (Table 1), whereas no inhibition was detected after microinjection of K18 preimmune antibodies or injection buffer alone (Table 1; Fig. 2). The reduction in BrdU incorporation by K18 antibodies was statistically significant in several independent experiments (Table 1) although we generally

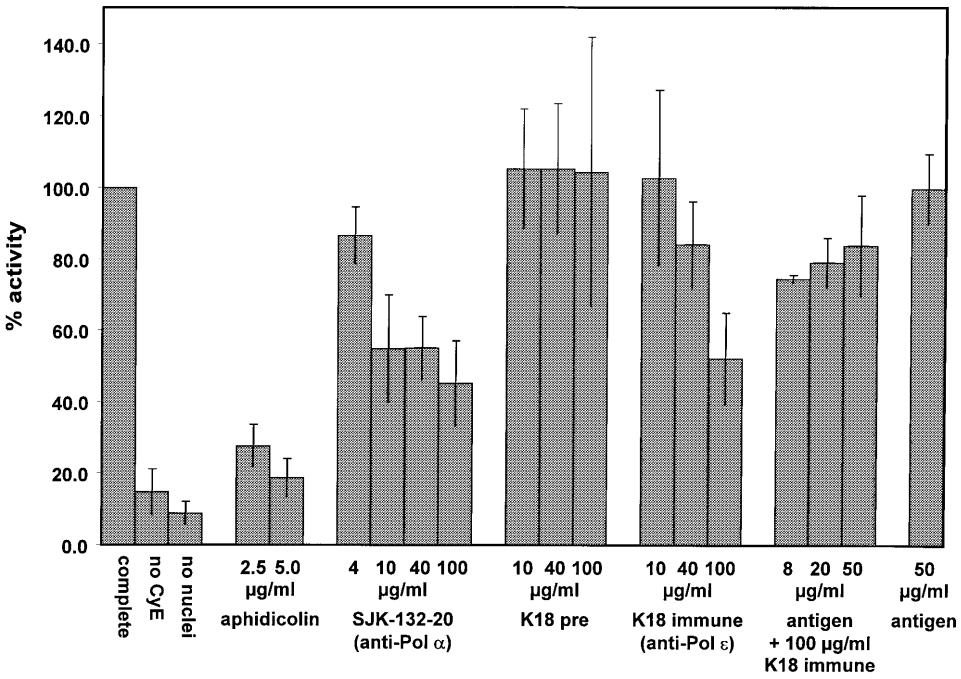

Figure 3. Replicative DNA synthesis in isolated, permeabilized HeLa cell nuclei is inhibited by neutralizing antibodies against DNA polymerases α and ε. Replicative DNA synthesis in isolated nuclei was measured by incorporation of radioactive dCMP into newly synthesized DNA as outlined in Materials and Methods. Nuclei and cytoplasmic extract were preincubated for 2 h on ice in the presence of the indicated concentrations of aphidicolin, antibody and/or antigen prior to the replication reactions. Antigen designates the Pol ε fragment that was used to raise the antibody K18. Activities were calculated relative to the complete reactions and the data shown represent the averages of at least two independent experiments with standard deviations indicated by error bars.

observed some variation in the incorporation of BrdU in different nuclei (Fig. 2) since we utilized asynchronous cultures. We also studied the influence of the non-neutralizing monoclonal antibodies 93G1A, 93H3B and 93E24C against the catalytic subunit of Pol ε (16). These antibodies had no significant effect on DNA synthesis when microinjected into growing fibroblasts (Table 1). These results indicate that the inhibitory effect on DNA synthesis is specific for antibody K18 and can be best explained by its neutralizing activity.

In an earlier study it has been shown that replicative DNA synthesis could be inhibited by microinjection of neutralizing antibodies against human Pol α (24). These results are expected, as Pol α is known to play a major role in replication of DNA in eukaryotic cells. We therefore studied, for comparison, the ability of neutralizing monoclonal antibody SJK-132-20 against human Pol α (25) to inhibit DNA synthesis. Microinjection of this antibody into nuclei of growing IMR-90 cells caused, on average, 33% inhibition of DNA synthesis (Table 1), which is comparable with the level of inhibition by K18 antibodies.

In order to assess the effect of K18 antibodies on DNA replication in a different system, we also studied DNA replication in isolated permeabilized nuclei. It has been shown earlier that chromosomal DNA replication in isolated nuclei is stimulated by addition of excess cytoplasmic extract (26,27). We could confirm that both nuclei and cytoplasmic extract are required for efficient incorporation of nucleotides into precipitable DNA (Fig. 3). The fact that low concentrations of aphidicolin inhibited the DNA synthesis indicated that we were indeed measuring replicative DNA synthesis in nuclei and not mitochondrial replication or DNA repair synthesis by Pol β. The neutralizing antibody SJK-132-20 against Pol α inhibited DNA synthesis in this system, confirming earlier results by Heintz and Stillmann (26). As in microinjection experiments, antibody K18 inhibited replicative DNA synthesis almost as efficiently as SJK-132-20 (48 and 55% inhibition by K18 and SJK-132-20 at an antibody concentration of 100 µg/ml, respectively). Inhibition at lower antibody concentrations was less pronounced for K18 than for SJK-132-20. This is not surprising since SJK-132-20 appears to have a higher capability to inhibit the activity of purified polymerase compared to K18 antibodies (25). K18 preimmune antibodies had no effect in this assay. Furthermore, addition of the purified Pol ε fragment that was used to raise the K18 antibody restored the level of the DNA synthesis from 52% in the presence of 100 µg/ml antibody up to 84%. We take these data as a strong indication that the inhibition of DNA synthesis by antibody K18 is due to its specific binding to Pol ε rather than non-specific interaction with some other replication factor.

We next addressed whether K18 anti-Pol ε antibody would effect SV40 DNA replication. Waga and Stillmann (2) found that Pol ε was not required for this process in a system reconstituted from highly purified replication factors. We performed specific SV40 DNA replication *in vitro* with isolated DNA synthesome fractions (28). The formation of full-length nicked and supercoiled DNA daughter molecules (Fig. 4, lanes 1 and 2) indicates that DNA synthesome fraction supports origin-specific T-antigen-dependent viral DNA

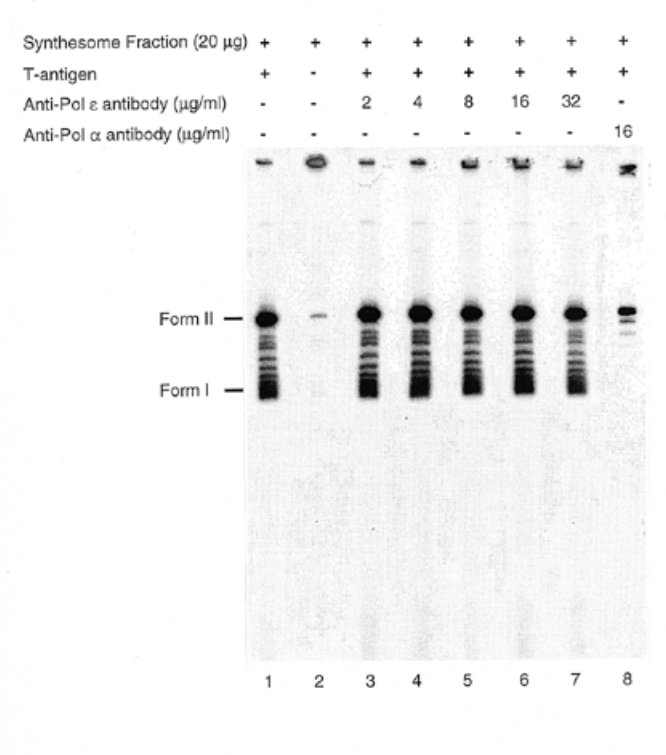

Figure 4. Neutral agarose gel analysis of the synthesome-mediated *in vitro* SV40 DNA replication products. SV40 DNA replication products formed in the absence or presence of increasing concentrations of K18 anti-DNA polymerase ε antibody. DNA replication products were isolated and analyzed as outlined in Materials and Methods. DNA synthesome fraction and antibodies were preincubated for 2 h at 4°C before the SV40 DNA replication reaction was started. Lane 1, DNA products isolated from a reaction mixture in the presence of T-antigen and absence of antibody; lane 2, DNA products isolated from a reaction performed in the absence of T-antigen and antibody; lanes 3–7, DNA products formed in the presence of T-antigen and 2, 4, 8, 16 and 32 µg/ml K18 anti-DNA polymerase ε antibody; lane 8, pruducts formed in the presence of T-antigen and 16 µg/ml of SJK-132-20 anti-DNA polymerase α antibody.

replication. Although Pol ε is present in the DNA synthesome fraction (4), antibody K18 against Pol ε, in concentrations ranging from 2 to 32 µg/ml, had no effect on the *in vitro* replication (Fig. 4, lanes 3–7). However, as shown previously by Malkas *et al.* (28), SJK-132-20 anti-Pol α antibody significantly inhibited SV40 DNA replication at a concentration of 16 µg/ml (Fig. 1, lane 8). Thus, our results indicate that Pol ε does not play an important role in SV40 DNA replication *in vitro*.

DISCUSSION

Our results provide evidence that Pol ε synthesizes a significant fraction of new DNA in proliferating human cells, thereby suggesting a central role for it in replication of chromosomal DNA. This is consistent with results from *S.cerevisiae* and *Schizosaccharomyces pombe*. In both yeasts, Pol ε is required for replication of chromosomal DNA (29–31). The decrease in DNA synthesis after microinjection of K18 antibodies is most likely attributed to the specific neutralization of Pol ε activity by these antibodies, since several other Pol ε-specific antibodies did not influence replicative DNA synthesis.

Our results are not in contradiction to the recent observation that the N-terminal portion of Pol ε carrying the polymerase and exonuclease domains is dispensable for viability of *S.cerevisiae* cells (32). The biochemical approach used in this study does not address whether the Pol ε polymerase activity is essential for viability of mammalian cells since inhibition of Pol α or ε by the addition of neutralizing antibodies is probably not complete. Therefore, some DNA synthesis by cognate Pols may still take place although at a reduced rate. Interestingly, the pol2-18 mutant in yeast (29) carries a point mutation in the N-terminal region and is replication deficient at restrictive temperature. This raises the possibility that the N-terminal part of Pol ε blocks or disrupts the replication fork when misfolded in the temperature-sensitive yeast mutant or when being bound by the neutralizing antibody in human, but may be substituted for when it is completely absent. This could explain the elongated S phase in yeast carrying the N-terminal Pol ε deletion (32). Finally, it cannot be ruled out that the requirements for replication of the large mammalian genome are more stringent than those of the relatively small yeast genome, making Pol ε activity dispensable for yeast but not for mammalian DNA replication. This view is supported by the fact that Pol ε activity is not required for replication of the small SV40 genome (3; this study).

It is not clear though whether the inhibition of DNA synthesis is due to the specific loss of DNA synthesis by Pol ε or is rather explained by impaired replication fork function due to the inhibition of Pol ε. The fact that K18 antibodies inhibited replicative DNA synthesis as effectively as neutralizing antibodies against Pol α favors the latter alternative, but does not rule out the former. Zlotkin *et al.* (3) evaluated the contributions of Pols α, δ and ε to nuclear DNA synthesis by cross-linking the enzymes to nascent DNA within replicating chromosomes. Their data indicated that although all three Pols catalyze DNA polymerization during replication, the contribution of Pol ε was less than those of Pols α and δ.

The results presented here demonstrate for the first time that a major fraction of DNA synthesis in growing mammalian cells is dependent on DNA polymerase activity of Pol ε.

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