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DNA polymerase epsilon may be dispensable for SV40- but not cellular-DNA replication

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The contributions of DNA polymerases α, δ and ε to SV40 and nuclear DNA syntheses were evaluated. Proteins were UV-crosslinked to nascent DNA within replicating chromosomes and the photolabelled polymerases were immunopurified. Only DNA polymerases α and δ were detectably photolabelled by nascent SV40 DNA, whether synthesized in soluble viral chromatin or within nuclei isolated from SV40-infected cells. In contrast, all three enzymes were photolabelled by the nascent cellular DNA. Mitogenic stimulation enhanced the photolabelling of the polymerases in the α>δ>ε order of preference. The data agree with the notion that DNA polymerases α and δ catalyse the principal DNA polymerisation reactions at the replication fork of SV40 and, perhaps, also of nuclear chromosomes. DNA polymerase ε, implicated by others as a cell-cycle checkpoint regulator sensing DNA replication lesions, may be dispensable for replication of the small, fast propagating virus that subverts cell cycle controls.

Keywords: aphidicolin/butylphenyl-dGTP/cell cycle checkpoint/DNA polymerases α and δ/protein–DNA UV-crosslinking

Introduction

Three distinct DNA polymerases (pols), designated α, δ and ε, are essential for replication of nuclear chromosomes in *Saccharomyces cerevisiae* and this conclusion may apply to eukaryotic cells in general (Morrison *et al.*, 1990; Araki *et al.*, 1992; Budd and Campbell, 1993). Yet, the specific replicative reactions catalysed by each of the three pols are known only in part. Studies on the replication of SV40 DNA, a major eukaryotic paradigm, have implicated pol α and its associated primase with synthesis of short RNA–DNA precursor chains (DNA primers) reaching ~34 nucleotides (Nethanel *et al.*, 1988; Nethanel and Kaufmann, 1990; Bullock *et al.*, 1991; Nethanel *et al.*, 1992). DNA primers deposited at the replication origin (*ori*) prime the synthesis of the leading DNA strands. Those arising later, as the replication fork progresses, are converted into more advanced lagging strand intermediates, the Okazaki pieces (Murakami *et al.*, 1986; Prelich and Stillman, 1988; Bullock *et al.*, 1991; Nethanel *et al.*, 1992; Waga and Stillman, 1994). The switch from DNA primer synthesis to the subsequent reactions depends on the processivity factors RFC and PCNA that replace pol α with pol δ at the primer:template junction (Tsurimoto *et al.*, 1990). Complete enzymatic replication of DNA from SV40 *ori* has been achieved with highly purified proteins, including pol α, pol δ, RFC and PCNA (Weinberg and Kelly, 1989; Lee *et al.*, 1991b; Waga *et al.*, 1994). Therefore, pol δ may be responsible for continuous elongation of the leading strand and conversion of DNA primers into Okazaki pieces. However, because pol ε also interacts with RFC and PCNA (Burgers, 1991; Podust *et al.*, 1992) and partially substitutes pol δ in reconstituted SV40 DNA replication systems (Lee *et al.*, 1991b; Waga *et al.*, 1994), participation of pol ε in reactions attributed to pol δ has not been excluded.

In the present study we assessed the relative contributions of pols α, δ and ε to SV40 and cellular chromosome replication, using as a criterion the ability of the three enzymes to crosslink nascent DNA synthesized within the respective chromosomes. The data agree with the notion that pols α and δ catalyse the principal reactions of DNA synthesis at the SV40 replication fork. Pol ε, implicated in cell-cycle checkpoint regulation (Navas *et al.*, 1995), may be dispensable for a virus that subverts cell-cycle control.

Results

Detection of pols δ in replicating SV40 chromosomes by UV-crosslinking and immunopurification

The 'DNA polymerase trap' technique tags a pol with its DNA product. The DNA is pulse-labelled with radioactive and photoreactive dNTP precursors and then UV-crosslinked to the enzyme (Insdorf and Bogenhagen, 1989). This protocol was adapted to identify pols that partake in replication of SV40 and host cell chromosomes in a cellular systems, adding an immunopurification step. The specificity of the combined protocol was tested by studying the crosslinking of pol δ to nascent SV40 DNA pulse-labelled within isolated SV40 chromatin with BrdUTP and [α-^{32}P]dATP. The viral chromatin was then UV-irradiated and treated exhaustively with DNase I. Free DNA oligonucleotides were removed from the photolabelled proteins by phenol extraction. Subsequently, the catalytic subunit of pol δ was immunoprecipitated using the monoclonal antibody (MAb) 38B5 (Zeng *et al.*, 1994). Resolution of the immunoprecipitate by SDS–PAGE,

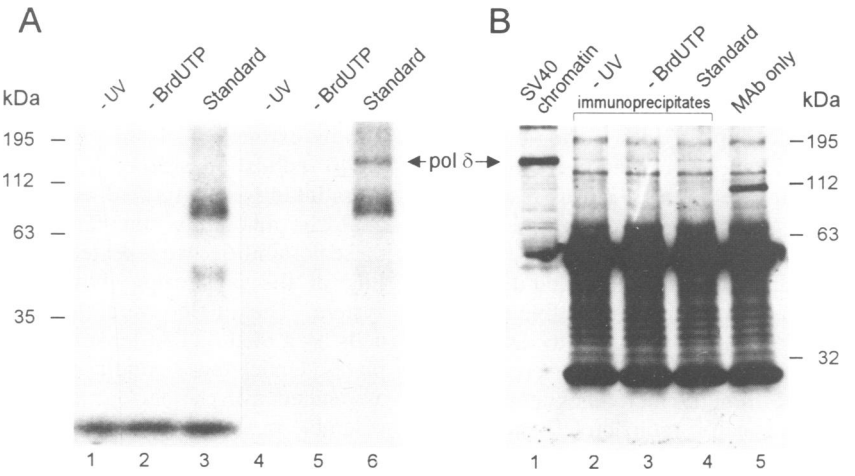

Fig. 1. Photolabelling of pol δ in replicating SV40 chromatin by nascent DNA. SV40 chromatin was pulse-labelled with radioactive and photoreactive precursors and subjected to UV-crosslinking. Following digestion with DNase I, phenol extraction of the proteins and pre-clearing immunoprecipitation, pol δ was immunoprecipitated with anti-pol δ MAb 38B5 and separated by electrophoresis on two 6% SDS–polyacrylamide gels, as detailed in Materials and methods. (**A**) Autoradiography of photolabelled pol δ: preclearing immunoprecipitates (lanes 1–3), MAb 38B5 immunoprecipitates (lanes 4–6). Non-irradiated control (lanes 1 and 4), control with dTTP instead of BrdUTP (lanes 2 and 5), standard mixture (lanes 3 and 6). (**B**) Western analysis of immunoprecipitated pol δ: SV40 chromatin sample (lane 1), immunoprecipitates of lanes 4–6 of panel A (lanes 2–4 respectively), control of anti-pol δ MAb 38B5 released from protein A–Sepharose beads (lane 5).

followed by parallel autoradiography and immunoblotting, revealed a specific photolabelled band (Figure 1A, lane 6) not seen with the non-specific pre-clearing Ab (lane 3). This band coincided with the ECL signal of pol δ (Figure 1B). This radioactive band was seen neither without UV-irradiation (Figure 1A, lane 4) nor when BrdUTP was replaced by dTTP (lane 5). The efficiency of immunoprecipitation was similar, regardless of treatment (Figure 1B, lanes 2–4).

Next, we examined the responses of the crosslinking signal of pol δ to two specific inhibitors of DNA synthesis. One, butylphenyl-dGTP (BuPdGTP), is a specific inhibitor of pol α (Syväoja *et al.*, 1990; Wright *et al.*, 1994) and DNA primer synthesis (Nethanel and Kaufmann, 1990). The other, aphidicolin, inhibits all three pols (Syväoja *et al.*, 1990; Wright *et al.*, 1994). However, in replicating SV40 chromosomes, aphidicolin selectively inhibits the syntheses of Okazaki pieces and leading strand DNA but allows accumulation of DNA primers (Nethanel *et al.*, 1988). Addition of BuPdGTP to the pulse-labelling mixture did not change the subsequent photolabelling of pol δ (Figure 2, compare lanes 2 and 3). In contrast, aphidicolin reduced this signal by 60% (compare lanes 2 and 4). The resistance of the photolabelling signal to BuPdGTP and its sensitivity to aphidicolin are consistent with the participation of pol δ in the synthesis of nascent DNA species other than DNA primers.

The crosslinking signal of pol δ was also monitored in pulse–chase kinetics. Chasing the pulse-labelled SV40 chromatin with non-radioactive dATP for 30 s sufficed to abolish the signal (Figure 3, lanes 3 and 4) but 75% of it persisted when further replication was prevented by including BuPdGTP and aphidicolin in the chase mixture (lane 5).

The specificity of the photolabelling method was also evidenced by failure to tag the pre-mRNA-splicing factor U1A with nascent DNA. This protein, which is unrelated to DNA replication, abounded in the SV40 chromatin

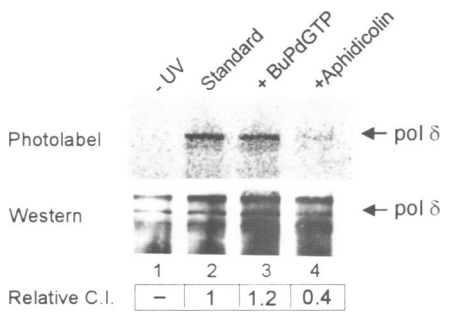

Fig. 2. Effect of inhibitors of DNA synthesis on crosslinking pol δ to nascent SV40 DNA. SV40 chromatin was pulse-labelled in the presence of the indicated inhibitors. Pol δ was monitored as in Figure 1 except that the sample was separated on one gel only. The immunoblot was developed first by ECL and was then autoradiographed, after the ECL signal had decayed. Non-irradiated control (lane 1), standard mixture (lane 2), standard mixture + 50 μM BuPdGTP (lane 3), standard mixture + 10 μg/ml aphidicolin (lane 4). The crosslinking intensity (CI) of the samples was determined by phosphorimaging and densitometry as described in Materials and methods. The value, 1.0, given to the standard situation is arbitrary.

preparation and was efficiently immunoprecipitated from it (not shown).

Nascent SV40 DNA preferentially photolabels pols α and δ

A similar protocol was used to compare the interactions of pols α, δ and ε with nascent SV40 DNA. Antibodies specific to pol α (1Ct 102 and 2Ct 25, Dornreiter, 1991), pol δ (38B5, Zeng *et al.*, 1994) and pol ε (93G1A, Uitto *et al.*, 1995) were used to sequentially immunoprecipitate the catalytic subunits of the respective pols from the UV-irradiated, DNase I-digested SV40 chromatin. The same antibodies were used in Western blotting, to assess the efficiency of the immunoprecipitation (see Materials and methods). As shown in Figure 4A, all three pols were present in the isolated SV40 chromatin. The catalytic

subunit of pol α appeared on the immunoblots in two bands migrating below the 195 kDa marker (Dornreiter *et al.*, 1990). The pol δ subunit appeared as a single band slightly above the 112 kDa marker. The pol ε subunit of the viral chromatin sample appeared as a single band above the 195 kDa marker. However, at the lower amounts of the protein in the immunoprecipitates, it was resolved into doublet bands (Uitto *et al.*, 1995). Although all three pols were immunopurified in similar yields (Figure 4A), only pols α and δ yielded detectable photolabelled derivatives. The corresponding bands were of comparable intensities (Figure 4B, lanes 2 and 4). The failure to detect a radioactive band corresponding to pol ε is underscored by the fact that each of the three immunoprecipitates contained a similar amount of a non-specific radioactive band that migrated below the 63 kDa marker.

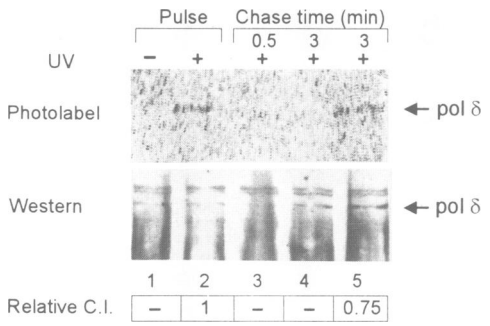

Fig. 3. Behaviour of pol δ's photolabel in pulse–chase kinetics. SV40 chromatin was pulse-labelled and UV-irradiated as described in Figure 1 and was chased with non-radioactive dATP, ± the indicated inhibitors. Photolabelled pol δ was monitored as in Figure 2. Reaction mixtures: non-irradiated (lane 1), pulse-labelled (lane 2), pulse-labelled and chased for 30 s (lane 3) or 3 min (lane 4), pulse-labelled and chased for 3 min in the presence of 50 μM BuPdGTP and 10 μg/ml aphidicolin (lane 5). CI was determined as in Figure 2.

The preferential photolabelling of pols α and δ could be taken to indicate that pol ε plays little or no role in SV40 replication. However, other possibilities were also considered. Thus, inadvertent loss of nuclear factor(s) during extraction of the viral chromatin could render SV40 replication independent of pol ε. Alternatively, the crosslinking reactivity of pol ε towards nascent DNA could be inherently constrained. To distinguish between these possibilities we repeated the comparative photolabelling of the pols using, however, a nuclear replication system. The nuclei were prepared from SV40-infected cells by a method that leaves them attached to the culture plates. Under these conditions, SV40 chromatin remains associated with the nuclear matrix and its replication may resemble more closely the *in vivo* process, compared with that of isolated SV40 chromatin (Schirmbeck and Deppert, 1991). Moreover, the crosslinking signals obtained with nuclear monolayers were stronger than those seen with nuclei in suspension (not shown), probably due to the lower self-absorption of UV-light by the monolayer. After UV-crosslinking, the nuclear monolayer was treated with DNase I and lysed. The proteins were extracted from the lysate and the pols were immunoprecipitated and identified by immunoblotting and autoradiography as above. As shown in Figure 5, each one of the three pols was photolabelled. This result refuted the possibility that the crosslinking of pol ε to nascent DNA is inherently constrained. However, because the source of the crosslinked DNA, viral or cellular, was uncertain, it was necessary to determine which pols crosslinked specifically SV40 DNA.

To this end, the UV-irradiated nuclei were lysed and low molecular weight viral DNA, including any covalently-linked proteins, was isolated and digested with DNase I. The three pols were then sequentially immunoprecipitated from this fraction. However, photolabelling signals were detected only for pols α and δ (Figure 6). Thus, pol ε must have been labelled largely or only by

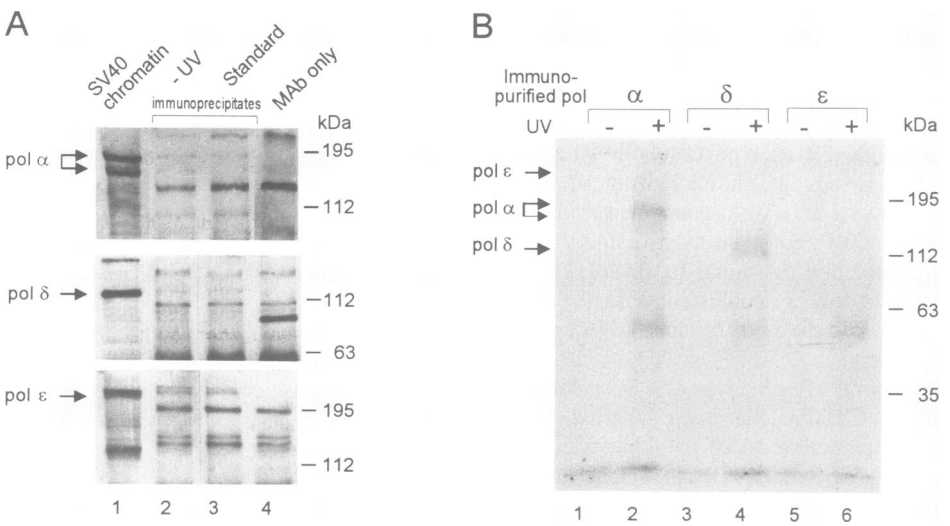

Fig. 4. Preferential photolabelling of pols α and δ by nascent DNA in isolated SV40 chromatin. Proteins were UV-crosslinked to nascent DNA within isolated SV40 chromatin and extracted. Pols α, δ and ε were sequentially immunoprecipitated and then monitored essentially as described in Materials and methods and Figure 1. (**A**) Western analysis of the indicated pols: isolated SV40 chromatin (lanes 1), immunoprecipitate of non-irradiated (lanes 2) and irradiated samples (lanes 3), controls of the respective MAbs released from protein A–Sepharose beads (lanes 4). (**B**) Autoradiography of the separated immunoprecipitates of pol α (lanes 1 and 2), pol δ (lanes 3 and 4) and pol ε (lanes 5 and 6), from non-irradiated (lanes 1, 3 and 5) or UV-irradiated samples (lanes 2, 4 and 6).

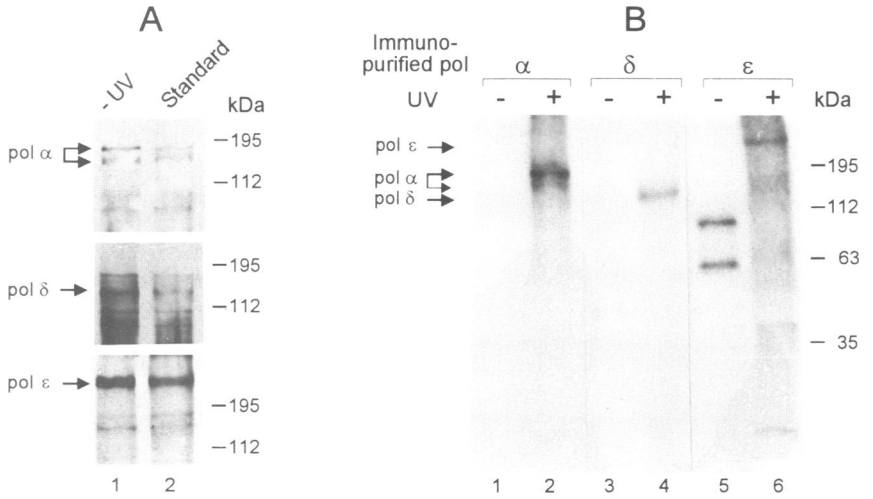

Fig. 5. Photolabelling of pols α, δ and ε in nuclei isolated from SV40-infected cells. Proteins were crosslinked to nascent DNA within nuclei prepared from SV40-infected cells. The nuclei were treated with DNase I, lysed, and the proteins were extracted. Pols α, δ, ε and their photolabelled derivatives were monitored as described in Materials and methods and Figure 4. (**A**) Western analysis of the indicated immunoprecipitates of samples derived from non-irradiated (lanes 1) and UV-irradiated (lanes 2) reaction mixtures. (**B**) Autoradiogram of immunoprecipitated pol α (lanes 1 and 2), pol δ (lanes 3 and 4) and pol ε (lanes 5 and 6), from non-irradiated (lanes 1, 3 and 5) or UV-irradiated samples (lanes 2, 4 and 6). The two radioactive bands of lane 5 were not reproduced in other experiments.

cellular DNA. This experiment included a control of nuclei that had not been incubated at 30°C before the UV-irradiation at 4°C. This control revealed that SV40 DNA synthesized during the UV-crosslinking period, part of which could be the product of repair DNA synthesis, contributed no more than 15% or 6% to the overall signals of pol α or pol δ respectively.

Mitogenic stimulation enhances the photolabelling of the pols in the α>δ>ε order of preference

Next we evaluated the contributions of the three pols to replicative nuclear DNA synthesis. To this end, we compared the crosslinking intensities (CI) of each pol in nuclei from cultures differing in the proportions of cells that replicate DNA. A high level of replication was achieved by serum deprivation/replenishment (see Materials and methods). A near-confluent culture of CV-1 cells was used as a reference with a low level of replication (nuclei from serum-deprived cells did not yield detectable crosslinking signals for any of the pols). Nuclei from SV40-infected cells were also examined in this experiment. As shown in Figure 7, nuclei of mitogenically stimulated cells yielded for each pol CI values that were invariably higher than the corresponding values obtained with the non-stimulated cycling cells. However, the increases were not equal, being 9.6 for pol α, 5.8 for pol δ and 2.6 for pol ε (compare lanes 1 with 2, 4 with 5 and 7 with 8). SV40 infection elicited a similar order of preference. In this case the respective ratios were 8.7, 4.9 and 1.9 (compare lanes 1 with 3, 4 with 6 and 7 with 9).

Discussion

Pols α and δ may suffice for SV40 DNA replication

UV-crosslinking of DNA bases to amino acid residues in proteins occurs at 'zero distance' between the interacting macromolecules and, therefore, is highly specific (Hockensmith *et al.*, 1993). This property has been exploited to identify the catalytic subunit of a pol by photolabelling it with its DNA product (Insdorf and Bogenhagen, 1989). We have adapted this method to evaluate the contributions of pols α, δ and ε to the replication of SV40 and cellular chromosomes. The pols were photolabelled within the respective replicating chromosomes and isolated by immunoprecipitation. The crosslinking intensity (CI), i.e. the photolabelling signal normalized to the amount of pol protein, is considered a reflection of the synthetic activity of a given pol. The specificity of this procedure and its relevance to the problem at hand were demonstrated by the pharmacological and kinetic attributes of the photolabelling signal of pol δ within soluble, replicating SV40 chromatin (Figures 1–3).

In the subsequent comparative studies, only pols α and δ were detectably photolabelled by nascent SV40 DNA, whether synthesized within the soluble viral chromatin (Figure 4) or in nuclei isolated from infected cells (Figure 6). Yet all three pols could be immunoprecipitated from the viral chromatin in comparable yields. On the other hand, all three pols, including ε, were photolabelled by newly-made cellular DNA (Figures 5 and 7). We infer from these results that pol ε contacts the replicating viral chromosome poorly, if at all. Consequently, pols α and δ may be the principal pols that operate in SV40 DNA replication. Nonetheless, further studies will be needed to rule out the possibility that pol ε partakes, after all, in SV40 DNA replication without being catalytically active. The assignment of pols α and δ as the only pols needed for SV40 replication has been proposed before, based on complete enzymatic replication of DNA from the SV40 *ori* in systems reconstituted from defined components, including pols α and δ but devoid of pol ε (Weinberg and Kelly, 1989; Lee *et al.*, 1991b; Waga *et al.*, 1994). The present data, obtained with authentic replicating chromosomes, add physiological significance to this proposal.

If pol ε has no principal role in SV40 DNA replication,

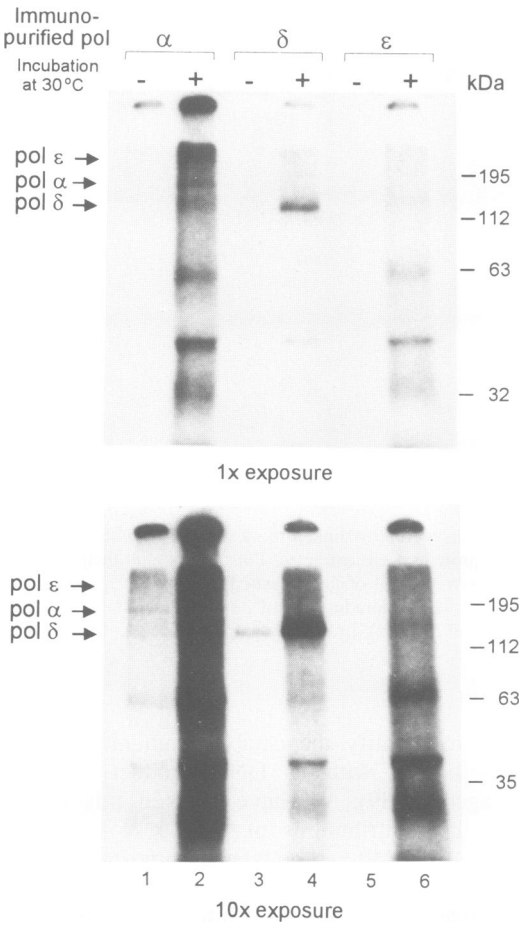

Fig. 6. Preferential photolabelling of pols α and δ by nascent SV40 DNA synthesized in isolated nuclei. Proteins were UV-crosslinked to nascent DNA within nuclei prepared from SV40-infected cells as in Figure 5. Photolabelled pols co-purifying with the low molecular weight viral DNA fraction were monitored as described in Figure 5. Immunoprecipitates of pol α (lanes 1 and 2), pol δ (lanes 3 and 4) and pol ε (lanes 5 and 6), without (lanes 1, 3 and 5) or with incubation at 30°C (lanes 2, 4 and 6). The lower autoradiogram was exposed 10 times longer than the upper. The upper part of the gel includes the stacking portion.

elongation of the leading DNA strand and completion of Okazaki pieces must be catalysed by pol δ alone. This conclusion is supported by reduction of the photolabelling signal of pol δ by aphidicolin (Figure 2), a selective inhibitor of the two PCNA-dependent reactions during replication of SV40 DNA in isolated nuclei (Nethanel *et al.*, 1988, 1992; Nethanel and Kaufmann, 1990) or isolated viral chromatin (Zlotkin, 1994). In contrast, the photolabelling of pol δ was refractory to BuPdGTP (Figure 2), a specific inhibitor of pol α (Syväoja *et al.*, 1990; Wright *et al.*, 1994) and of DNA primer synthesis (Nethanel and Kaufmann, 1990; Nethanel *et al.*, 1992). It is noteworthy in this regard that BuPdGTP abolishes the photolabelling of pol α (unpublished data). Acting on both arms of the replication fork, pol δ may be considered a functional counterpart of dimeric prokaryotic replicases (Stillman, 1994). Accordingly, pol α-primase and associated viral T-antigen helicase (or putative cellular counterpart) constitute an analogue of the prokaryotic primosome, albeit one bolstered with limited capacity to synthesize DNA.

Pols α and δ may perform parallel tasks at SV40 and nuclear replication forks

SV40 and cellular DNA replication are characterized by similar chromatin templates and reaction intermediates (DePamphilis and Bradley, 1986 and unpublished data). Therefore, pols α and δ may perform parallel tasks in the two processes, whereas pol ε may exercize function(s) specific to cellular DNA replication. Alternatively, in nuclear DNA replication, pol ε catalyses a principal PCNA-dependent reaction, elongation of leading strands and/or completion of Okazaki pieces, but this role is taken over by pol δ in SV40 DNA replication. Arguing in favour of the first possibility are the unequal CI enhancements, in the α>δ>ε order of preference, induced by mitogenic stimulation (Figure 7). The relevant effect of the mitogenic stimulation could be to increase the proportion of S-phase cells and, hence, of replicative versus repair DNA synthesis. This effect would yield the observed pattern of CI enhancements if pol α catalyses mainly replicative DNA synthesis, pol ε mainly repair DNA synthesis and pol δ has an intermediate status (reviewed in Friedberg *et al.*, 1995). Thus, DNA excision repair in permeabilized cells is refractory to BuPdGTP (Dresler and Frattini, 1988) and purified pol α does not support this reaction in an *in vitro* system reconstituted from purified components (Aboussekhra *et al.*, 1995). On the other hand, PCNA could be isolated as a DNA excision repair factor (Nichols and Sancar, 1992) and pairwise combinations of pol mutations in yeast indicate that both pols δ and ε are involved in DNA repair synthesis (Budd and Campbell, 1995). Moreover, pol ε complements DNA excision repair DNA synthesis both in acellular systems (Nishida *et al.*, 1988; Wang *et al.*, 1993) and in a system reconstituted from defined components (Aboussekhra *et al.*, 1995).

We also consider the possibility that the pattern of CI enhancements was influenced by the specific features of newly initiated cellular replicons, reported to contain clusters of unidirectional 'microbubbles' likely to be enriched for pol α molecules (Micheli *et al.*, 1982; Burhans *et al.*, 1990; Linskens and Huberman, 1990; Vaughn *et al.*, 1990). If a considerable fraction of the replicons pause at this stage, pols δ and ε may be under-represented. In SV40 replicons the relative occupancy of pol α may be lower because they are primed at a unique site and are distributed largely at late stages of replication (Tapper and DePamphilis, 1978). In agreement, nascent SV40 DNA photolabelled pols α and δ similarly (Figure 6), whereas total nascent DNA of the SV40-infected cells labelled pol α preferentially (Figure 5).

Replication strategies of SV40 and cellular genomes

The yeast S-phase checkpoint gene *DUN2* coincides with the C-proximal domain of *POL2* encoding pol ε (Navas *et al.*, 1995). *dun2* mutants fail to activate DNA damage-inducible genes in response to inhibition of DNA synthesis and do not prevent entry into mitosis under these circumstances. The C-proximal domain of pol ε, dispensable for the polymerase function (Morrison *et al.*, 1990; Kesti *et al.*, 1993) may exert the checkpoint activity by sensing DNA replication defects, either directly or by cooperating with the N-proximal polymerase domain. Because other studies cited above have implicated pol ε in DNA repair

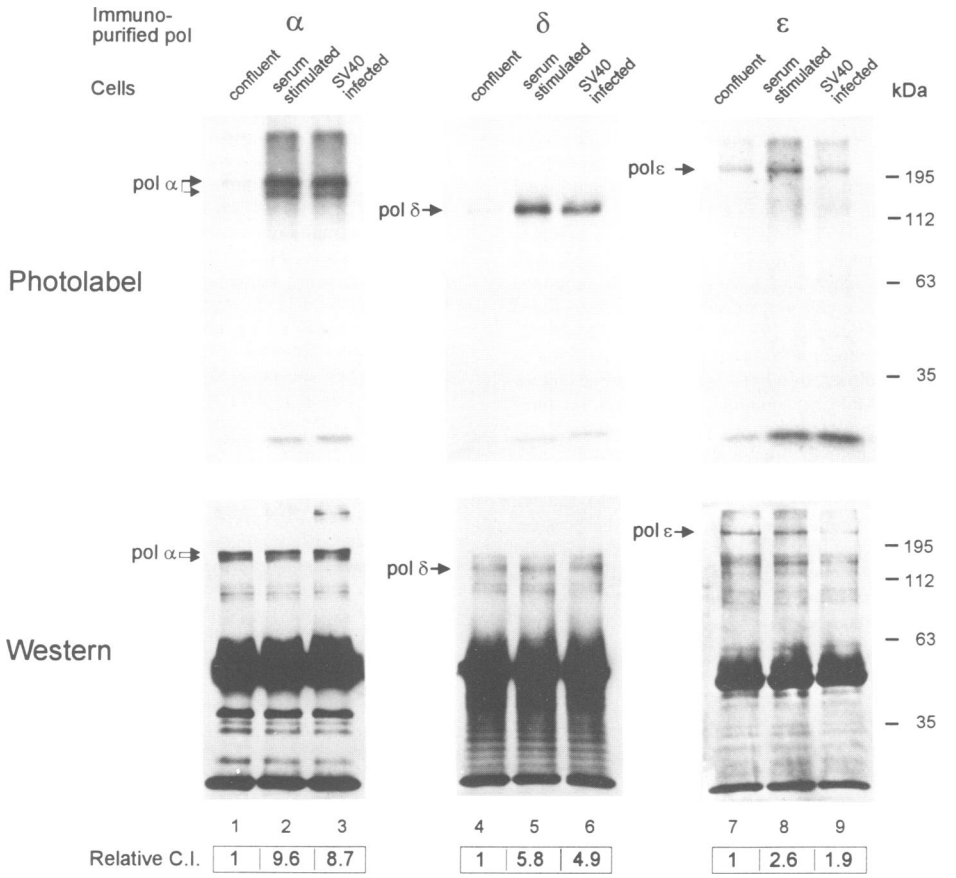

Fig. 7. Mitogenic stimulation or SV40 infection enhance photolabelling by nascent DNA in a pol α>δ>ε order of preference. Proteins were crosslinked to nascent DNA within nuclei prepared from the indicated cell cultures. Pols α (lanes 1–3), δ (lanes 4–6), ε (lanes 7–9) were monitored as described in Figure 5. The crosslinking intensity (CI) of the samples was determined by phosphorimaging and densitometry, as described in Materials and methods. The values, 1.0, given to confluent cells for each pol are arbitrary.

synthesis, the N-domain may also partake in replacement of aberrant DNA synthesized by the other two replicases. A relatively low contribution of pol ε to replicative DNA synthesis, deduced from the unequal CI enhancements (Figure 7), is consistent with participation of this enzyme mainly in post-replication repair and checkpoint activities. This conclusion may apply to both the yeast and mammalian enzymes, based on their sequence similarities (Morrison *et al.*, 1990; Kesti *et al.*, 1993).

Why may pol ε be dispensable for SV40- but essential for nuclear-DNA replication? A possible reason could be the different survival strategies exercised by the cellular and SV40 genomes. Faithful replication of the former depends on elaborate mechanisms that prevent individual DNA segments from replicating more than once during a cell division cycle, condition initiation of particular cell cycle events on successful completion of others and, under adverse circumstances, commit the cell to programmed death (Sancar, 1995). However, SV40 subverts cell cycle controls and offsets apoptosis (McCarthy *et al.*, 1994; Yanai and Obinata, 1994). Moreover, a miniscule genome and vast progeny may render SV40 more tolerant to DNA damage. Greater genetic instability can even be considered beneficial to the virus, due to a greater abundance of variants that may cope better with host countermeasures. In sum, proteins that guarantee highly accurate, cell cycle-regulated replication of nuclear DNA may be superfluous for SV40 replication. Pol ε may be a case in point.

Materials and methods

Materials

The preparation of anti-pol α MAb 1Ct 102 and 2Ct 25 was described (Dornreiter, 1991). The anti-pol δ MAb 38B5 used in this study is specific to the C-terminal peptide of the catalytic, 125 kDa subunit of the enzyme (Zeng *et al.*, 1994). Anti-pol ε MAb 93G1A (Uitto *et al.*, 1995) is specific to amino acids 242–474 of the published sequence of pol ε (Kesti *et al.*, 1993). An antibody directed against the mRNA splicing factor U1A was obtained from J.Sperling, the Weizmann Institute of Science. Anti-mouse myeloma IgG protein was from P-L Biochemicals Inc. Rabbit anti-mouse IgG (H+L) and horse radish peroxidase anti-mouse antibody conjugate were purchased from Jackson Immuno-Research. Enhanced chemilluminescence (ECL) reagents were purchased from Amersham. Protease inhibitors, BrdUTP, protein A–Sepharose and protein molecular size markers SDS-7B were purchased from Sigma. DNase I was purchased from Worthington. The sources of other materials were described (Nethanel *et al.*, 1988, 1992; Nethanel and Kaufmann, 1990).

UV-crosslinking of proteins to nascent DNA in isolated SV40 chromatin

SV40 chromosomes were isolated from nuclei of SV40 infected CV-1 monkey kidney cells 36 h post infection by a method adapted from that of Su and DePamphilis (1978). Four culture plates (240×240 mm) were washed twice with ice-cold isotonic buffer containing 20 mM Tris–HCl, pH 7.5; 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 250 mM sucrose and 1 mM phenylmethyl sulfonyl fluoride (PMSF); and once with ice-cold low salt buffer [20 mM HEPES–Na, pH 7.8; 5 mM K-acetate, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM PMSF]. The cells were scraped off the plates with a rubber policeman. Their lysis was completed in a Dounce homogenizer by four strokes of the tight-fitting pestle B. The lysate was centrifuged for 5 min at 1200 *g*. The nuclear pellet was suspended in 1.6 ml of low salt buffer and

incubated on ice for 2 h with occasional shaking. Nuclei and cellular debris were removed by centrifugation at 8000 *g* for 10 min and SV40 chromatin was concentrated by centrifugation of the nuclear extract at 220 000 *g* for 90 min. The pellet of crude SV40 chromatin was suspended in 320 µl of low salt buffer and stored in aliquots at –70°C. The replication reaction mixture of 20 µl contained 6 µl crude SV40 chromatin, 40 mM HEPES–Na, pH 7.8; 60 mM sucrose, 5 mM $MgCl_2$, 30 mM potassium acetate, 1 mM Na–EGTA, 250 mM ethylene glycol and 0.25 mM DTT. Non-labelled dGTP and dCTP were used at 2 µM, the photoreactive analogue of dTTP, BrdUTP, at 20 µM, to compete with any endogenous dTTP and [α-^{32}P]dATP was used at 1 µM. ATP was used at 2 mM and rNTPs other than ATP at 20 µM. To minimize degradation of pols α, δ and ε, eight protease inhibitors were included (Lee *et al.*, 1991a; Podust and Hübscher, 1993) as follows: 1 mM PMSF, 1.5 µg/ml aprotinin, 1 µg/ml leupeptin, 5 µg/ml chemostatine, 1 µg/ml pepstatine, 10 mM sodium bisulfite, 50 µg/ml *N'*-p-tosyl-l-lysine chloromethyl ketone (TLCK) and 10 mM benzamidine. The reaction mixtures were incubated at 30°C for 3 min, chilled on ice and irradiated for 5 min at 4°C on a TM-36 transilluminator (Ultra Violet Products) at 302 nM. Subsequently, 35 units of DNase I were added in two volumes of DNase buffer (40 mM Tris–HCl, pH 8.0; 6 mM $MgCl_2$, 2.25 mM $CaCl_2$), containing 0.4 mg/ml bovine serum albumin (BSA) and the protease inhibitors listed above. DNA digestion was performed at 37°C for 30 min and was terminated by adding one volume of 100 mM Tris–HCl, pH 7.5; 1% SDS, 140 mM β-mercaptoethanol. Further processing of the photolabelled proteins is described below.

UV-crosslinking of proteins to nascent DNA in monolayers of isolated nuclei

The conditions of cell growth and SV40 infection were described previously (Nethanel *et al.*, 1988). Nuclei were isolated by the *in situ* fractionation method of Schirmbeck and Deppert (1991) from SV40 infected CV-1 cells, near-confluent monolayers of uninfected CV-1 cells or CV-1 cells stimulated to proliferate by deprivation and replenishment of serum. In the latter case, the cells were grown in medium supplemented with 5% calf serum to 70% confluency and brought to quiescence by incubation for 72 h in a medium containing 0.25% calf serum. They were then stimulated to proliferate by elevating the serum concentration to 10%. Cellular DNA synthesis peaked 12–14 h later, evidenced by the rate of [methyl-^{3}H]thymidine incorporation (Tuusa *et al.*, 1995). The various cell monolayers were washed with ice-cold KM buffer [10 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 100 µg of leupeptin per ml, 10 mM Na-morpholinepropane-sulfonic acid (MOPS) buffer, pH 7.0]. The cells were lysed by incubation for 30 min at 2°C with KM buffer containing 0.5% Nonidet P-40, leaving a monolayer of isolated nuclei. The nuclear monolayers were then washed with 5 mM K-acetate, 0.5 mM $MgCl_2$, 2 mM DTT and 30 mM K–HEPES buffer, pH 7.4. They were gently agitated at 30°C for 3 min with 600 µl of a replication reaction mixture containing 50 mM K-acetate, 5 mM $MgCl_2$, 2 mM DTT, 100 µg/ml leupeptin, 0.05% Nonidet P-40, 2 µM each of dGTP and dCTP, 20 µM BrdUTP, 1 µM [α-^{32}P]dATP (3000 Ci/mmol), 2 mM ATP and other rNTPs at 20 µM. The reaction mixture was removed and the plates were chilled on ice. They were then placed open and facing down on the transilluminator and UV-irradiated. The irradiated nuclei were processed either for isolation of the bulk crosslinked proteins or those co-purifying with the low molecular weight viral DNA fraction. In the first instance the irradiated nuclei were treated for 30 min at 37°C with 100 units of DNase I in 2 ml of DNase buffer containing 1.5 mM $CaCl_2$, 0.4 mg/ml BSA and the protease inhibitors listed above. Following digestion of the DNA, the supernatant was discarded and the residual nuclear structures were lysed in 1 ml TES buffer (1% SDS, 2 mM EDTA, 20 mM Tris–HCl, pH 7.4). The lysate was extracted with aqueous phenol to separate proteins crosslinked to the short DNA adducts from free DNA oligonucleotides. The phenol phase and interphase were washed twice with TES buffer and twice with water and then mixed with six volumes of acetone pre-cooled at –20°C. After incubation at –20°C overnight, the proteins were sedimented at 20 000 *g* at 4°C for 15 min. The pellet was washed twice with 80% aqueous acetone and once with 100% acetone, both pre-cooled at –20°C. Residual acetone was evaporated under vacuum. In the second instance, low molecular weight viral DNA and any covalently linked proteins were isolated from the irradiated nuclei by extraction in SDS–salt essentially as described (Nethanel *et al.*, 1988). However, the DNA and associated proteins were precipitated and washed with acetone. The photolabelled proteins were released by DNase I treatment and precipitated again with acetone.

Immunochemical detection of individual crosslinked proteins

To prepare the proteins that were photolabelled in the SV40 chromatin or nuclear monolayers for immunoprecipitation, they were boiled for 10 min in 50 mM Tris–HCl, pH 7.5; 0.5% SDS, 70 mM β-mercaptoethanol and 1 mM of carrier dATP (to minimize non-specific binding of radioactive dATP). The proteins were partially renatured by diluting the samples with nine volumes of ice-cold washing buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl) containing 0.5% Nonidet P-40 as well as the protease inhibitors mentioned above but at 10-fold lower concentrations. At this level the inhibitors did not interfere with the immunoprecipitation. Preclearing immunoprecipitation was performed by incubating the samples with 10 µg myeloma IgG protein adsorbed to 4 mg protein A–Sepharose via 20 µg rabbit anti-mouse IgG for 3 h at 4°C on a rotator. Rabbit anti-mouse IgG was also used to enhance the binding of anti-pol α and anti-pol ε MAbs to protein A. Anti-pol δ antibodies that efficiently bound protein A were adsorbed without the IgG mediator. Sequential immunoprecipitation of pols α, δ and ε with the cognate antibodies were performed for 6–16 h each, using, in respective order, 1 ml each of anti-pol α 1Ct 102 and 2Ct 25 MAb hybridoma supernatants, 10 µg of anti-pol δ and 10 µg of anti-pol ε antibodies. The anti pol δ and anti pol ε MAbs were adsorbed to protein A–Sepharose in 300 µl of phosphate buffered saline (10 mM potassium phosphate buffer, pH 7.4; 0.15 M NaCl) containing 1% BSA. The precipitates were washed four times with washing buffer containing 0.2% Nonidet P-40. The proteins were released from the beads by heating at 95°C for 10 min in 40 µl of 2× sample buffer (Laemmli, 1974). They were divided into two aliquots separated in parallel on two 6% SDS–polyacrylamide gels. One gel was dried and the radioactive signals of the photolabelled proteins were monitored by autoradiography or phosphorimaging (see below). The second was blotted on a nitrocellulose membrane to monitor the efficiency of immunoprecipitation by ECL, using the cognate MAb and peroxidase-conjugated anti-mouse IgG. The blot was subsequently autoradiographed to determine whether the positions of the ECL and radioactive signals of the pols matched.

Evaluation of the crosslinking intensity

The radioactive signals of the crosslinked derivatives of the pols were detected and quantified by phosphorimaging (Fujix Bas 1000, Fuji) or autoradiography and densitometry (Hewlett Packard ScanJet 3p). TINA software (Raytest Isotopenmessgeräte GmbH), compatible with the TINA-PCBAS and TIFF files of the phosphorimager and the scanner respectively, was applied in both cases. The same tools were used for the densitometry of the ECL autoradiograms. The crosslinking signal was normalized toward the total amount of the respective immunoprecipitated protein (ECL-signal). The normalized value is referred to as the crosslinking intensity (CI). The value 1.0 given to the standard situation in the various experiments was arbitrary and served for comparing the behaviour of the same pol in the different situations

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