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ARTICLES

Characteristics of DNA Replication in Isolated Nuclei Initiated by an Aprotinin-Binding Protein

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Abstract Isolated cell nuclei were used as the source of template DNA to investigate the role of a cytosolic aprotinin-binding protein (ADR) in the initiation of eukaryotic DNA replication. Computerized image cytometry demonstrated that the DNA content of individual nuclei increased significantly following incubation with ADR-containing preparations, and the extent of DNA synthesis is consistent with that allowed by the limiting concentration of dTTP. Thus, dTTP incorporation into isolated nuclei represents DNA synthesis and not parent strand repair. We found that dTTP incorporation into the isolated nuclei is dependent on DNA polymerase α (a principal polymerase in DNA replication) but that DNA polymerase β (a principal polymerase in DNA repair processes) does not play a significant role in this system. Finally, neither aprotinin nor a previously described cytosolic ADR inhibitor can block the replication of nuclease-treated calf thymus DNA, while both strongly inhibit replication of DNA in isolated nuclei. This result, coupled with the relative ineffectiveness of nuclease-treated DNA compared with nuclear DNA to serve as a replicative template in this assay, argues against a significant contribution from repair or synthesis which initiates at a site of DNA damage. These data indicate that ADR-mediated incorporation of ^{3}H-dTTP into isolated nuclei results from DNA replicative processes that are directly relevant to in vivo S phase events.

Key words: eukaryotic DNA replication, isolated nuclei

Much of the recent information about eukaryotic DNA replication proteins and their roles in the initiation and elongation mechanisms has been obtained using in vitro viral DNA replication systems [Challberg and Kelly, 1989; Stillman, 1989; Thommes and Hubscher, 1990]. In vivo, these mammalian DNA tumor viruses use cellular proteins for DNA replication under the control of a few viral regulatory proteins. Among the best-studied is the SV40 virus, which replicates its 5423-bp genome from a defined origin sequence (ori) and requires only one virus-encoded protein, the large T antigen (T Ag) for viral DNA replication. Several laboratories have replicated SV40 DNA in vitro using T Ag and seven to eight purified cellular proteins or protein complexes, including DNA polymerases α and δ, DNA primase, PCNA (proliferating cell nuclear antigen, a cofactor for DNA polymerase δ), two DNA binding protein complexes, and DNA topoisomerase I or II [Tsurimoto et al., 1990; Weinberg et al., 1990]. In these reconstituted systems, each of the proteins has at least one experimentally defined role in the replication of the SV40 DNA, and it is likely that these proteins play parallel roles in the replication of SV40 DNA in vivo and in cellular DNA replication during the normal cell cycle.

While this viral replication system permits detailed functional analysis of both the DNA sequences and protein components, there are several inherent drawbacks with this approach to understanding cellular DNA replication. First, two key elements of this system, the DNA replication origin sequence and the multifunctional T Ag, are viral products, and cellular homologs of these components are either not well defined or unknown. Second, the virus-encoded molecules (both the proteins and the DNA) can potentially alter the interactions between cellular components. Thus, how a cellular protein functions in the in vitro replication of viral DNA may not exactly duplicate how that protein functions during cellular DNA replication. Finally, the in vitro systems lack the peripheral and internal nuclear matrix structures which are the sites of DNA

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replication in intact cells [Dijkwel et al., 1986; Jackson and Cook 1986; Nakayasu and Berezney, 1989; Pardoll et al., 1980; Berezney, 1991; Cook, 1991]. Several of the proteins required for DNA replication are tightly bound to the nuclear matrix [Collins and Chu, 1987; Adachi et al., 1989]. Interphase DNA is bound to the nuclear matrix through sequences termed matrix attachment regions (MARs) [Smith et al., 1984]. These attachment regions appear to be the sites of initiation of cellular DNA replication [Dijkwel and Hamlin, 1988; Ito and Sakaki, 1987; Razin, 1987; Van der Velden et al., 1984; Van der Velden and Wanka 1987]. These results all demonstrate that interactions of DNA replication proteins and DNA with nuclear matrix are important for the normal progression of S phase events during the cell cycle.

For these reasons, we have used isolated nuclei in experiments designed to characterize a cytosolic protein (ADR) that appears to play a key role in the initiation of eukaryotic DNA replication [Coffman et al., 1989]. ADR (activator of DNA replication) is a heat-labile protein of MW >90 kD that is tightly associated with a protease activity [Gutowski et al., 1984; Wong et al., 1987]. ADR-mediated nuclear dTTP incorporation can be blocked by several protease inhibitors, the most effective being aprotinin, a 6-kD polypeptide inhibitor with the physiological role of protecting alveoli from damage following the release of neutrophil proteases [Werle et al., 1968]. To define further the replicative events that occur in these isolated nuclei in response to ADR, and to allow comparison with in vitro viral DNA replication systems, we undertook a series of experiments to examine the various parameters of nuclear dTTP incorporation and how it is affected by various agents. To determine the relative contributions of daughter strand DNA formation and parent strand repair to dTTP incorporation, we measured the DNA content of nuclei before and after incubation by digital image analysis. Several inhibitors were utilized to assess the relative contributions of DNA polymerase α, a required component of the in vitro plasmid replication systems which likely plays a parallel role in vivo, and DNA polymerase β, a principal polymerase in DNA repair processes. Finally, parallel replication assays in which either isolated nuclei or nuclease-treated calf thymus DNA served as template DNA were used to assess the contribution of DNA damage to incorporation seen using isolated nuclei.

METHODS

Reagents

^{3}H-dTTP was obtained from ICN (Irvine, CA). Aphidicolin, aprotinin, and ddTTP were obtained from Sigma (St. Louis, MO). The hybridoma secreting the anti-DNA polymerase alpha monoclonal antibody SJK-287-38 [Shigeaki et al., 1982], and the human T-cell leukemia cell line MOLT-4 were obtained from ATCC. The antibody was isolated from SJK-287 culture supernatants by ammonium sulfate precipitation, followed by protein A–Sepharose chromatography.

Cell Culture

MOLT-4 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Gaithersburg, MD), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in 5% CO_2/95% humidified air.

ADR-Containing Preparations

Cytosolic extracts were prepared as described previously [Gutowski et al., 1984]. MOLT-4 cells were centrifuged and resuspended at a density of 5×10^7 cells/ml in ice-cold solution E (20 mM HEPES pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT). After an 8-min incubation on ice, the cells were homogenized by 20 strokes of a Dounce homogenizer (large pestle) and the homogenate centrifuged at 3000*g* for 10 min. The supernatant was adjusted to 0.1 M sucrose/10 mM KCl and centrifuged at 140,000*g* for 60 min. The supernatant (cytosolic extract) was transferred to a sterile 50-ml centrifuge tube on ice, along with sufficient solid ammonium sulfate (AS) to bring the solution to 30% AS saturation. The AS was dissolved by gentle rocking at 4°C. Following 1-h incubation at 4°C, the contents were transfered to autoclaved Corex tubes and centrifuged at 17,000*g* for 10 min. The supernatant was transferred to another 50-ml tube, brought to 50% AS saturation, and the above procedure repeated. The precipitate (30–50% AS ppt) was resuspended in solution E (one-half the original volume), dialyzed against solution E overnight at 4°C, and then assayed, aliquoted, and stored at −70°C. The 30–50% AS ppt contains 80–90% of the recoverable ADR activity and does not contain the cytosolic ADR inhibitor present in the 50–70% AS ppt [Coffman et al., 1991].

Preparation of Isolated Nuclei

Adult frogs (*Xenopus laevis*) were purchased from Nasco (Ft. Atkinson, WI). Isolated nuclei were prepared by detergent lysis of single spleen cell suspensions, as described previously [Gutowski et al., 1984]. DNA replication can be initiated in nuclei from human, murine, and *Xenopus* cells by ADR-containing preparations from human cells, indicating that ADR is not species-specific [Gutowski and Cohen, 1983; Gutowski et al., 1984].

Assay for Initiation of DNA Synthesis in Isolated Nuclei (ADR Assay)

Assays were performed in triplicate in 96-well microtiter plates as previously described [Gutowski et al., 1984]. Each well receives 40 μl of solution 4 (25 mM HEPES pH 7.8, 2% (w/v) dextran, 0.25 M sucrose), 50 μl of the 30–50% AS ppt, and 10 μl of nuclei (2×10^7 nuclei/ml). The reaction is initiated by the addition of 50 μl of an incorporation mixture (38 mM HEPES pH 7.8, 12.5 mM phosphoenolpyruvate, 5 mM ATP, 10 U/ml pyruvate kinase, 100 mM KCl, 125 mM $MgCl_2$, 2 mM dithiothreitol, 125 mM sucrose, 0.5 mM dATP, 0.5 mM dGTP, and 0.5 mM dCTP) and 3 μl [methyl-^{3}H]dTTP (1.5–3 μCi/well), and the plates were incubated for 90 min at 37°C. Control triplicate wells measured the incorporation of DNA alone and 30–50% AS ppt alone. Duplicate time-zero plates measured nonspecific incorporation, and these values were subtracted from the postincubation counts. At the end of the incubation, the reaction was stopped with NaOH, and the DNA TCA-precipitated and harvested as described [Gutowski et al., 1984].

Assay for DNA Polymerase α Activity

Each well received 50 μl of solution 4, 25 μl of the 30–50% AS ppt and 25 μl of 0.83 mg/ml activated calf thymus DNA (Sigma) in solution 4. The reaction is initiated by the addition of 50 μl of incorporation mixture and 3 μl [methyl-^{3}H]dTTP (1.5 μCi/well). The plates were incubated for 120 min at 37°C and harvested as above.

FPLC Mono Q Chromatography

A HR 10/10 Mono Q column connected to an FPLC system (Pharmacia) in a refrigerated cabinet was sequentially washed with 30 ml of buffer E, 30 ml of buffer E + 0.5 M NaCl, and 60 ml buffer E; 9 ml of 30–50% AS ppt was loaded onto the column and the nonbinding proteins removed by washing with 20 ml buffer E. A 60-ml linear gradient from 0–0.5 M NaCl in buffer E was applied to the column and 1-ml fractions collected. Selected fractions were dialyzed against buffer E at 4°C and assayed as described above.

Image Analysis

Reactions were prepared as described above, except an equal concentration of unlabeled dTTP was used in place of ^{3}H-dTTP. Following incubation, the reactions were transferred to Eppendorf tubes and the nuclei isolated by centrifugation. Nuclei were fixed, stained with Feulgen reagent, and the fluorescence of DNA–Feulgen complexes recorded and analyzed by digital image analysis using a CAS 2000 system (Cell Analysis Systems, Elmhurst, IL) [Bacus et al., 1990; Taylor et al., 1989]. Nuclei that had been incubated in solution 4 alone were used as controls.

RESULTS

Nuclear Incorporation Parallels DNA Polymerase α Activity

DNA polymerase α is one of the principal DNA polymerases mediating eukaryotic DNA replication [So and Downey 1988], functioning as the initial polymerase in in vitro SV40 DNA replication [Collins and Kelly, 1991; So and Downey, 1988; Tsurimoto and Stillman, 1991]. We used several approaches to examine the role of this polymerase in the incorporation of ^{3}H-dTTP by nuclei in vitro. First, we examined the effects of a neutralizing anti-DNA polymerase alpha monoclonal antibody (SJK-287) [Shigeaki et al., 1982] on nuclear dTTP incorporation. Parallel reactions were performed using nuclease-treated calf thymus DNA (CT DNA), the usual in vitro template for DNA polymerase α [Kaguni and Lehman 1988]. As shown in Figure 1, the inhibition profiles in both nuclei and CT DNA assays are similar. Next, we examined how incorporation varies as a function of pH when nuclear DNA or CT DNA is the template. As shown in Figure 2, the loss of activity with increasing pH is nearly identical in both systems. Optimal activity for incorporation using either nuclei or CT DNA is maximal at pH 7 and falls progressively until at pH 8.2 only 10–20% of the initial activity is seen. Finally, we examined the sensitivity

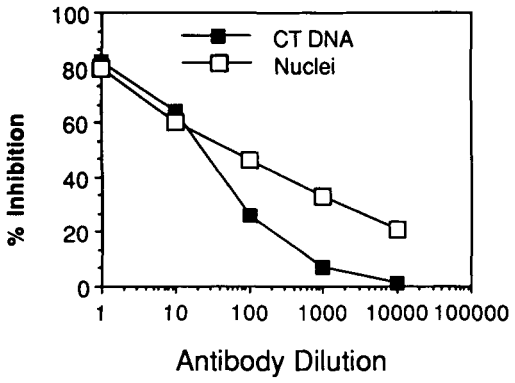

Fig. 1. Inhibition of ADR and DNA polymerase α activity by an anti-DNA polymerase α monoclonal antibody. ^{3}H-dTTP incorporation was measured in assays using either isolated nuclei (ADR activity) or nuclease-treated calf thymus DNA (CT DNA, a substrate for DNA polymerase α) as DNA templates. Dilutions of purified IgG fractions from SJK-287 hybridoma supernatants were added to MOLT-4 30–50% AS ppt fractions and assayed as described.

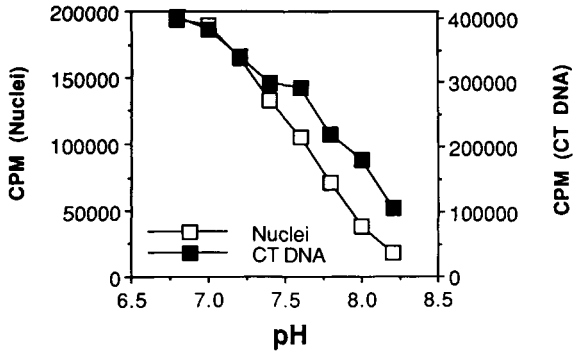

Fig. 2. ADR and DNA polymerase α activities as a function of pH. Reactions were performed using assay solutions prepared at the indicated pH values, and 30–50% AS ppt preparations were dialyzed overnight against solution E adjusted to the appropriate pH.

of nuclear incorporation to aphidicolin, a potent inhibitor of mammalian DNA polymerases α, δ, and ε but a relatively ineffective inhibitor of DNA polymerase β [Syvaoja et al., 1990]. As shown in Figure 3, aphidicolin effectively inhibits incorporation of ^{3}H-dTTP into isolated nuclei.

The Inability of ddTTP to Inhibit ^{3}H-dTTP Incorporation by Nuclei Indicates That DNA Polymerase β Is Not Involved

DNA polymerase β appears to function as a principal polymerase enzyme in DNA strand repair processes [Randahl et al., 1988]. This polymerase differs from polymerases α and δ in several ways, including sensitivity to a number

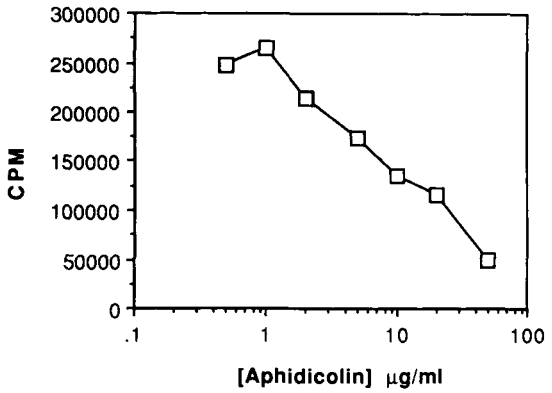

Fig. 3. Inhibition of ADR activity by aphidicolin. Reactions were performed as described but with each well containing 45 μl 30–50% AS ppt and 5 μl of concentrated aphicolin stock solution, which gave the indicated final aphidicolin concentrations. Control reactions in the absence of aphidicolin incorporated 266,609 ± 5,852 cpm.

of inhibitors, such as aphidicolin [Syvaoja et al., 1990]. Another such inhibitor is ddTTP, which completely inhibits DNA polymerase β at 10 μM concentrations but has no effect on DNA polymerases α, δ, and ε at that concentration [Syvaoja et al., 1990]. As shown in Figure 4, ddTTP at concentrations up to 10 μM had essentially no effect on ^{3}H-dTTP incorporation directed by either nuclei or activated calf thymus DNA.

Aprotinin and the MOLT-4 Inhibitor Activity Inhibits Nuclear Incorporation But Not Incorporation Into Activated CT DNA

It has been previously shown that the initiation of nuclear DNA synthesis in vitro can be inhibited by aprotinin and by a factor found in the 50–70% AS ppt of MOLT-4 cytosol [Coffman et al., 1989; Wong et al., 1987]. One potential mechanism for this occurrence is that aprotinin and the MOLT-4 inhibitor could simply act as polymerase inhibitors. We then tested the ability of these effectors to inhibit DNA polymerase α activity using activated calf thymus DNA as a substrate, in parallel with their effect on isolated nuclei. As shown in Figure 5, neither aprotinin nor the MOLT-4 inhibitor was capable of inhibiting DNA polymerase α activity, while both inhibit 90% of the incorporation into isolated nuclei.

Image Analysis Shows Increased Nuclear DNA Content Following Incubation

A definitive indication of replication is the formation of daughter DNA. Image analysis al-

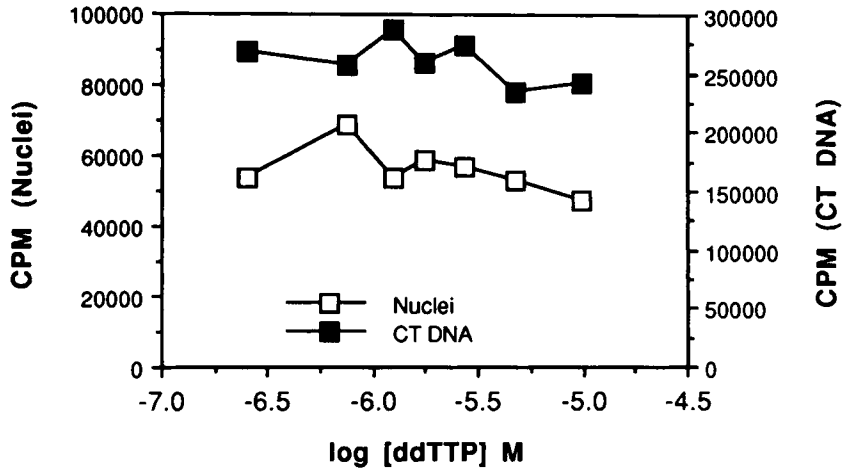

Fig. 4. The effect of ddTTP on incorporation, using either isolated nuclei or nuclease-treated calf thymus DNA (CT DNA) as templates. Reactions were performed as described but with increasing amounts of ddTTP added as part of the ^{3}H-dTTP solution. Control reactions in the absence of ddTTP incorporated 69,466 ± 13,037 cpm using nuclei and 270,029 ± 3,342 cpm using CT DNA.

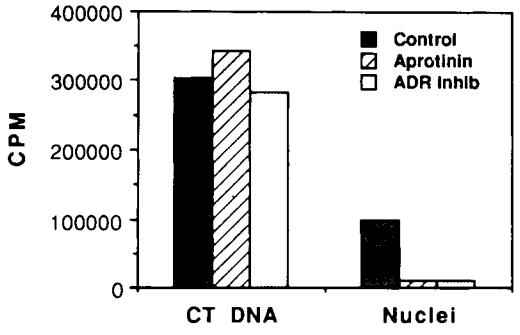

Fig. 5. The effect of aprotinin and the MOLT-4 ADR inhibitor on ADR and DNA polymerase α activities; 25 μl of an aprotinin stock solution (100 μM final concentration) or a concentrated 50–70% MOLT-4 AS ppt, which contains the ADR inhibitor activity [Coffman et al., 1991] was added to 25 μl of 30–50% AS ppt and the mixture assayed for each activity as described.

lows the direct visualization and quantitation of DNA in groups of individual nuclei based on intercalation of the Feulgen reagent [Bacus et al., 1990; Taylor et al., 1989]. Thus, both the distribution and extent of replication can be observed. The results are shown in Figure 6. The modal peak DNA content of isolated nuclei before the assay is 6.3 pg, with a mean DNA content of 6.41 ± 0.06 pg (Fig. 6A). Following a 90-min incubation with ADR preparations, there was an increase in the modal peak value to 7.4 pg, with a corresponding increase in the mean DNA per cell to 7.21 ± 0.08 picograms (Fig. 6B). This represents a net increase of 12% in mean DNA content per nucleus.

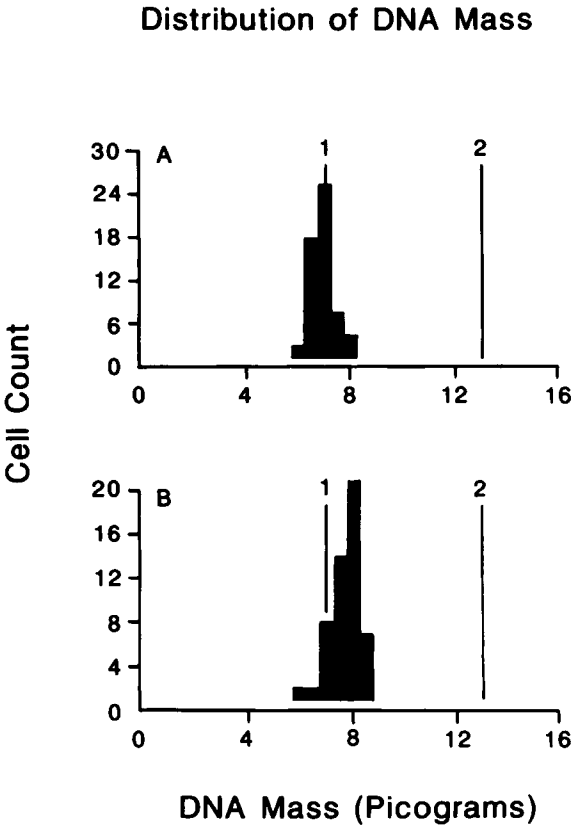

Fig. 6. Changes in nuclear DNA mass during the ADR assay, as measured by image analysis. Nuclei before and after the ADR assay were fixed and stained and the fluorescence from Feulgen-stained DNA measured and digitized. Nuclei prior to incubation have a mean DNA content of 6.41 ± 0.06 pg DNA (A), while after incubation the mean DNA content shifts to 7.21 ± 0.08 pg DNA (B).

DISCUSSION

To establish that in vitro incorporation of ^{3}H-dTTP directed by the DNA in isolated nuclei represents physiologically relevant DNA replication, several criteria must be met. First, the template DNA must be replicated to produce daughter DNA strands. Second, this process must be mediated by the same proteins that mediate DNA replication in vivo. Finally, the replication events must initiate at physiologically relevant DNA origin regions, and not at DNA strand breaks. Our results have shown that DNA replication does occur in this system, and that this replication is mediated by DNA polymerase enzymes, which have been implicated in S phase events. In addition, the patterns of inhibition suggest that DNA strand breaks do not play a significant role in this process.

The 12% increase in mean DNA content per nucleus following incubation with ADR represents a highly significant increase, given the limiting concentrations of dTTP in the assay. Since it is unlikely that all replication origins in all nuclei would initiate simultaneously, the resulting distribution would include some nuclei which have replicated a significant portion of their DNA, and others with little evidence of increased DNA content, which is precisely the observed result as seen in the histograms.

The inhibition of nuclear incorporation by the anti-DNA polymerase α monoclonal antibody and aphidicolin demonstrate that DNA polymerase α is required for in vitro nuclear DNA replication. DNA polymerase α is the best-studied of the mammalian DNA polymerase enzymes [Lehman and Kaguni 1989], and current evidence indicates that this polymerase mediates the replication of lagging DNA template strands in vivo and that it is not involved in DNA repair processes [Dresler and Frattini, 1986]. DNA polymerase α also initiates in vitro SV40 DNA replication at the replication origin [Tsurimoto and Stillman, 1991; Weinberg et al., 1990]. In the in vitro SV40 system, DNA polymerase δ displaces DNA polymerase α on the leading template strand [Tsurimoto et al., 1990].

The effectiveness of aphidicolin and the inability of ddTTP to inhibit ^{3}H-dTTP incorporation into isolated nuclei demonstrates that DNA polymerase β is not involved in this process. Since the assay measures ^{3}H-dTTP incorporation and DNA polymerase β is a principal polymerase involved in the excision repair of thymine dimers [Randahl et al., 1988], it was important to determine whether DNA polymerase β participated in this system. Our data suggest that DNA polymerase β does not play a significant role in dTTP incorporation into isolated nuclei under assay conditions.

Because of its ability to replicate long DNA sequences without dissociating, DNA polymerase δ is believed to be a leading strand polymerase in vivo [Crute et al., 1986; Focher et al., 1988] and is a required component of the in vitro SV40 DNA replication systems [Tsurimoto et al., 1990; Weinberg et al., 1990]. We have previously shown that a monoclonal antibody against PCNA (proliferating cell nuclear antigen) blocks ADR activity [Wong et al., 1987], and PCNA is a cofactor for DNA polymerase δ [Celis et al., 1987; Tan et al., 1986]. Thus, DNA polymerase δ is an integral component of the in vitro nuclear replication system, in addition to DNA polymerase α.

The monoclonal antibody inhibition data suggest that DNA polymerase α and δ do not function as independent entities in the nuclear replication assay. The anti-DNA polymerase α antibody inhibited 80% of the total incorporation into isolated nuclei, while the anti-PCNA antibody inhibited 70% of the total incorporation. These results suggest that replication in isolated nuclei is mediated by a complex containing both polymerases and that antibodies that bound to either component could interfere with replication mediated by that complex. This appears to be the case in intact cells, as active replication complexes containing both enzymes have been isolated from such systems [Focher et al., 1988; Sabatino et al., 1988]. In addition, DMSO progressively inhibits DNA polymerase α while concentrations of DMSO from 2–10% significantly stimulate DNA polymerase δ [Lee and Toomey 1986]. At low DMSO concentrations (2–10%), ^{3}H-dTTP incorporation into isolated nuclei is significantly stimulated, while DMSO levels $>15\%$ inhibits nuclear incorporation [Coffman, unpublished data]. Thus, the response of nuclear incorporation to DMSO resembles that of DNA polymerase δ at low DMSO concentrations and resembles that of DNA polymerase α at higher DMSO concentrations.

Several lines of evidence argue that damaged DNA is not a significant initiation point for the observed replication. First, both aprotinin and the cytosolic ADR inhibitor block nuclear incor-

poration but have no effect on incorporation directed by nuclease-treated calf thymus DNA. The nuclease-treated DNA is a much poorer substrate for the ADR-containing preparations, as the amount of calf thymus DNA required for the DNA polymerase assay (20 μg) is more than 30 times the amount of DNA in the assay using isolated nuclei (0.6 μg). In addition, the polymerase requirements strongly favor replication over repair. The principle repair enzyme (DNA polymerase β) is not a significant participant in this system, while the two polymerase enzymes which are required for nuclear dTTP incorporation also mediate leading and lagging DNA strand replication in other in vitro systems and are believed to have similar roles in vivo. While no roles in repair processes have been found for DNA polymerase α, DNA polymerase δ was recently implicated in excision repair [Shivji et al., 1992]. However, this activity was unaffected by antibodies that neutralize DNA polymerase α, in direct contrast to the incorporation seen in this system. Taken together, these data strongly suggest that damaged DNA does not play a significant role in this in vitro replication system.

In summary, we have shown that the ^{3}H-dTTP incorporation mediated by ADR-containing preparations in isolated nuclei is consistent with the synthesis of daughter strand DNA. Incorporation can be blocked by monoclonal antibodies and inhibitors that block the activities of DNA polymerases α and δ, which are believed to replicate the lagging and leading template DNA strands in vivo. DNA polymerase β, a principal DNA repair enzyme, does not appear to function in this system. Finally, DNA replication does not appear to depend on the presence of broken DNA strands. Thus, ADR-mediated ^{3}H-dTTP incorporation in the isolated nuclei system appears to represent physiologically relevant DNA replicative processes.

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