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Involvement of Proliferating Cell Nuclear Antigen (Cyclin) in DNA Replication in Living Cells†

MARGIT ZUBER,[1]‡ ENG M. TAN,[1] AND MASARU RYOJI[2]*

W. M. Keck Autoimmune Disease Center[1] and Division of Biochemistry,[2] Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037

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Proliferating cell nuclear antigen (PCNA) (also called cyclin) is known to stimulate the activity of DNA polymerase δ but not the other DNA polymerases in vitro. We injected a human autoimmune antibody against PCNA into unfertilized eggs of *Xenopus laevis* and examined the effects of this antibody on the replication of injected plasmid DNA as well as egg chromosomes. The anti-PCNA antibody inhibited plasmid replication by up to 67%, demonstrating that PCNA is involved in plasmid replication in living cells. This result further implies that DNA polymerase δ is necessary for plasmid replication in vivo. Anti-PCNA antibody alone did not block plasmid replication completely, but the residual replication was abolished by coinjection of a monoclonal antibody against DNA polymerase α. Anti-DNA polymerase α alone inhibited plasmid replication by 63%. Thus, DNA polymerase α is also required for plasmid replication in this system. In similar studies on the replication of egg chromosomes, the inhibition by anti-PCNA antibody was only 30%, while anti-DNA polymerase α antibody blocked 73% of replication. We concluded that the replication machineries of chromosomes and plasmid differ in their relative content of DNA polymerase δ. In addition, we obtained evidence through the use of phenylbutyl deoxyguanosine, an inhibitor of DNA polymerase α, that the structure of DNA polymerase α holoenzyme for chromosome replication is significantly different from that for plasmid replication.

Proliferating cell nuclear antigen (PCNA) was initially recognized as a nuclear autoantigen which reacts with autoimmune sera from a certain population of patients with systemic lupus erythematosus (47; for recent reviews, see references 60 and 61). This antigen is an acidic nuclear protein with a molecular weight of about 36,000 (13, 42, 51, 58). It is readily extractable from whole-cell or nuclear preparations and is expressed mainly in mitotically active cells of various tissues and cell lines (58). Independently, Celis and his colleagues identified, by two-dimensional gel electrophoresis, a protein called cyclin which is abundant in proliferating cells (for reviews, see references 13 and 16). It was later shown that cyclin and PCNA are identical (42). The complete amino acid sequence of this protein was determined by cDNA cloning in two independent laboratories (1, 43). Immunofluorescence studies with human autoantibodies against PCNA have demonstrated that PCNA expression increases during late G1 and early S phase, immediately preceding the onset of DNA synthesis (14, 36, 48, 55, 58). During S phase, the distribution of PCNA changes dramatically within the nucleus (5, 7, 14, 36, 41, 55, 58, 61), following closely the sites of chromosomes which are pulse-labeled with [^{3}H]thymidine or bromodeoxyuridine (8, 41). These observations strongly suggest an association of PCNA with the DNA replication apparatus. It has also been proposed that PCNA might be involved in DNA repair synthesis, because UV damage of DNA caused an increase in the nuclear PCNA staining of non-S-phase cells (15).

More-direct studies on the roles of PCNA have been reported recently in connection with a novel species of DNA polymerase, called DNA polymerase δ (12; for reviews of polymerase δ, see references 10, 22, and 35). Three groups of investigators have concluded that PCNA is identical in structure and function to the protein known as the auxiliary protein of DNA polymerase δ (6, 53, 59, 62). DNA synthesis in vitro, utilizing purified DNA polymerase δ together with poly(dA)-oligo(dT) as a template and primer, indicated that PCNA or the auxiliary protein is required for DNA synthesis when the primer/template ratio is low (59, 62). PCNA was not needed for the DNA polymerase α-driven synthesis of DNA in this system (59, 62). This protein has also been shown to stimulate, severalfold, the replication of plasmid DNA carrying the simian virus 40 (SV40) replication origin in a fractionated cell extract supplemented with SV40 large T antigen (52, 53). More recently, it has been reported that the induction of DNA synthesis in isolated nuclei, by some components present in an extract of proliferating cells, is also blocked by an anti-PCNA antibody (67). In regard to the function of PCNA, there is evidence that PCNA increases the processivity of DNA polymerase δ (53, 59). Despite these reports, equivalent studies in vivo are lacking at present.

DNA replication in activated eggs of *Xenopus laevis* provides a unique opportunity to investigate the role of PCNA in living cells. Upon activation by pricking, the eggs undergo several rounds of the cell cycle. In parallel, replication of egg chromosomes as well as injected foreign DNA takes place (24, 27). Due to the large size of the eggs, it is possible to introduce foreign proteins and other substances easily into the cells for examination of their effects on DNA replication. We took advantage of the availability of autoimmune and monoclonal antibodies which recognize different epitopes on PCNA (49–51). We injected these antibodies into eggs and asked whether they can inhibit DNA replication by their ability to bind to various domains in PCNA. In view of the recent findings that PCNA is specifically required for DNA polymerase δ activity in vitro (59, 62), we also inves-

* Corresponding author.
† Publication no. 5302 BCR.
‡ Present address: Department of Internal Medicine and Nephrology, Steglitz Medical Center, Free University of Berlin, 1000 Berlin 45, Federal Republic of Germany.

tigated the relative contributions of DNA polymerases α and δ to DNA replication in living cells.

MATERIALS AND METHODS

Preparation of monospecific human anti-PCNA IgG. Serum from a patient with systemic lupus erythematosus was used to prepare monospecific anti-PCNA immunoglobulin G (IgG). The IgG fraction was isolated by DEAE-Sephacel column chromatography (Pharmacia, Piscataway, N.J.). To obtain monospecificity to PCNA, the IgG fraction was absorbed with rabbit kidney extract covalently coupled to CNBr-activated Sepharose 6B (Pharmacia). Since saline extract of rabbit kidney contains only a negligible amount of PCNA but large amounts of other autoantigens such as Sm (a group of autoantigens comprising the U1 through U6 small nuclear ribonucleoproteins), ribonucleoprotein (RNP), histone, and DNA (47), this absorption procedure can remove autoantibodies other than those against PCNA. The absorption was performed by the method described previously (51). Nonautoimmune human IgG (Miles Scientific, Naperville, Ill.) was used as a control. Preparations of the human autoantibody (AK IgG) and human control IgG were dialyzed against phosphate-buffered saline (PBS), pH 7.2, and stored at −70°C at a concentration of 20 mg/ml.

Monoclonal anti-PCNA antibodies. The supernatant of two hybridomas (19A2 and 19F4) producing monoclonal antibodies against PCNA was collected (49). Concentration and purification of the antibodies was performed by precipitation in 50% saturated ammonium sulfate and then by hydroxylapatite chromatography (57). Monoclonal antibody 19A2 (IgM) was further purified by gel filtration, while monoclonal antibody 19F4 (IgG) was subjected to protein A column chromatography (26). Both antibodies were made to 20 mg/ml in PBS and stored at −70°C. Nonimmune mouse IgG (Miles Scientific) and mouse myeloma IgG MOPC-21 (Pharmacia) served as controls. The relative anti-PCNA activities of the monoclonal and autoimmune anti-PCNA antibody preparations were determined by blotting the electrophoretically separated proteins of egg extract onto nitrocellulose membrane and then probing with serial dilutions of the anti-PCNA antibody preparations. Monoclonal antibody SJK132-20 against DNA polymerase α of human KB cells (63) was purchased from Pharmacia.

Preparation of purified PCNA. PCNA was purified from rabbit thymus as described previously (51). Final protein concentration was adjusted to 0.7 mg/ml in PBS.

Protein determination and immunoblotting. Protein concentration was determined by the Bradford method with human IgG or bovine serum albumin as a standard (4). Immunoblot analysis of antibody specificity was performed by a standard method (65) with ^{125}I-protein A as a detectant. Egg extract for immunoblotting was prepared as follows. Eggs of *Xenopus laevis* were dejellied (see below), washed 10 times in OR-2 medium (5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.6], 1 mM Na_2HPO_4, 82 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ [66]), homogenized in 4 μl of extraction buffer (50 mM Tris hydrochloride [pH 8], 85 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) per egg, and centrifuged three times at 10,000 × *g*. The final supernatant was used as the extract.

Chemicals, enzymes, plasmids, and frogs. N^2-(*p*-*n*-Butylphenyl)-2'-deoxyguanosine (BuPdGTP) was a gift from George E. Wright, University of Massachusetts Medical School (68). *Bam*HI and DNase I were purchased from Promega Biotec, Madison, Wis. Aphidicolin was purchased from Sigma Chemical Co., St. Louis, Mo. It was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Plasmid pUC9 DNA was obtained from Bethesda Research Laboratories, Bethesda, Md. [α-^{32}P]dCTP (10 mCi/ml, 3,000 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Adult females of *Xenopus laevis* were obtained from Nasco, Fort Atkinson, Wis.

Microinjection into unfertilized eggs. Unfertilized *X. laevis* eggs were obtained as described by Gurdon (23) with modifications. Human chorionic gonadotropin (1,000 IU; Sigma) was injected into the dorsal lymph sac of female frogs. Eggs were laid into OR-2 medium (66). Prior to injection, eggs were dejellied for 5 min in a solution containing 0.23 M NaOH, 0.1% papain, and 3% L-cysteine (54), washed five times with OR-2 medium, transferred to 0.5× OR-2 medium, and injected within 1 h. At least 10 eggs were injected per condition. After injection, eggs were incubated in 0.5× OR-2 medium for 1.5 to 4.5 h at 20°C. The injection volume was 10 to 80 nl. Incubated eggs were harvested one by one in microcentrifuge tubes and frozen immediately in dry ice-methanol. The radioactivity of each frozen egg was monitored by a Geiger counter. For reproducible and quantitative analysis, it is important to discard the eggs with radioactivities significantly lower or higher than the expected value.

Extraction of DNA. Injected eggs were combined and homogenized in 10 μl of lysis buffer (30 mM Tris hydrochloride [pH 7.4], 1% sodium dodecyl sulfate, 20 mM EDTA), containing 2 μg of proteinase K, per egg and incubated for 1 h at 37°C. Samples were extracted with phenol-chloroform, and the DNA was precipitated twice with ethanol. The precipitate was collected by centrifugation and dissolved in 5 μl of TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, pH 8) per egg.

TCA precipitation. A portion of DNA extracted from the injected eggs was placed on a Whatman GF/F filter and allowed to dry. Each filter was washed once for 15 min in ice-cold 10% trichloroacetic acid (TCA) and twice in 5% TCA, rinsed in ethanol, and allowed to dry. Radioactivities of the filters were determined by liquid scintillation counting in the presence of Econofluor (New England Nuclear). Background radioactivity without replication was determined from the TCA-precipitable radioactivity in the total nucleic acids of uninjected eggs which were homogenized with an equivalent amount of [α-^{32}P]dCTP.

Agarose gel electrophoresis. DNA was separated by 0.8% agarose gel electrophoresis in TPE buffer (50 mM Tris-phosphate [pH 7.2], 1 mM EDTA). In a typical experiment, the DNA from one egg was applied per lane. After electrophoresis, gels were dried and exposed to X-ray film.

RESULTS

Anti-PCNA antibodies. Autoimmune sera from patients with systemic lupus erythematosus very often contain a set of antibodies which react with various antigens present in the cell nucleus (60). Upon immunoblot analysis of a HeLa cell extract, the serum from a lupus patient (AK IgG) showed reactivity with 36-kilodalton (kDa) PCNA as the major antigen in addition to two or three minor polypeptides (data not shown). We absorbed out these other antibodies (see Materials and Methods) to obtain an IgG fraction operationally monospecific to PCNA. We used this preparation to examine whether PCNA is present in the frog egg extract. Although PCNA is widespread in a variety of mammals (14), the presence or absence of this protein in

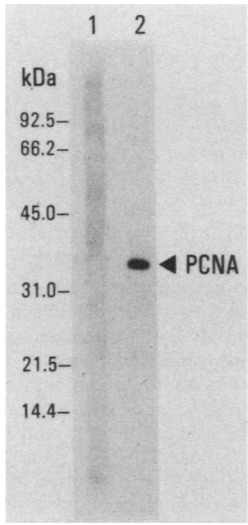

FIG. 1. Specificity of anti-PCNA IgG (AK IgG). Egg extract of *Xenopus laevis* was separated by electrophoresis, and proteins were transferred to nitrocellulose. They were then probed with human anti-PCNA antibody preparation (AK IgG) from human autoimmune serum (lane 2) and nonautoimmune human IgG (lane 1). Molecular mass markers are shown on the left.

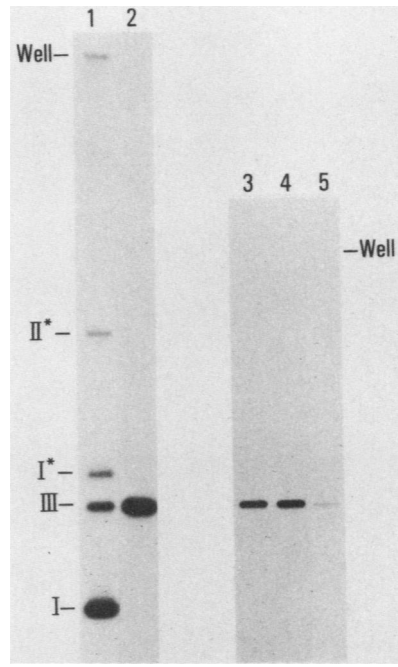

FIG. 2. Analysis of replicated plasmid pUC9 DNA. Eggs were injected with 10 ng of pUC9 and 100 nCi of [α-^{32}P]dCTP, and they were incubated for 3 h. DNA was extracted and subjected to agarose gel electrophoresis. Lane 1, unrestricted DNA; lane 2, DNA digested with *Bam*HI. In the experiment shown in lanes 3 to 5, double injection was performed as follows: 10 nl of H_2O (lane 3), 10 nl of DMSO (lane 4), or 10 nl of aphidicolin (10 mg/ml in DMSO, lane 5) was injected 1 h before injection of pUC9 and [α-^{32}P]dCTP. The injected eggs were then incubated for 2 h. DNA was extracted and subjected to agarose gel electrophoresis after *Bam*HI digestion. Relative densities of the bands were 85% (lane 3), 100% (lane 4), and 7.2% (lane 5). Relative TCA-insoluble radioactivity of the material applied in each lane was 87% (lane 3), 100% (lane 4), and 19% (lane 5), which was determined as described in Materials and Methods. I, Supercoiled monomer DNA; III, linear monomer DNA; I*, supercoiled dimer DNA; II*, relaxed dimer or supercoiled trimer DNA.

amphibia has not been described before. The immunoblot analysis of the *Xenopus* egg extract displayed a single band (Fig. 1) which comigrated with PCNA purified from rabbit thymus. We concluded that PCNA is present in *Xenopus* eggs and that the preabsorbed IgG from the lupus patient did not react with any other protein in the eggs at significant levels. Monoclonal antibodies 19A2 and 19F4 raised against rabbit thymus PCNA (49, 50) also detected the 36-kDa protein in the egg extract, indicating that there is substantial homology between PCNAs from mammals and amphibia (data not shown).

Replication of plasmid DNA. Frog eggs have the ability to replicate injected foreign plasmid DNA (24). Replication does not require specific DNA sequences, and virtually any circular DNA can be replicated to various extents (27, 28, 44). Incorporation of a radioactive precursor into DNA takes place linearly for the first several hours after DNA injection (44). Analysis by CsCl equilibrium centrifugation has established that most, if not all, of the incorporation is due to semiconservative replication and not repair synthesis (27, 44). As reported by others (27, 28, 44), replicated [^{32}P]DNA was composed of several bands separated by agarose gel electrophoresis, with the supercoiled monomer DNA as the most prominent (Fig. 2, lane 1). Multimeric forms of the DNA were also present at significant levels. Replicative-intermediate DNA was not evident in the gel, although the residual material in the well and faint smears in the high-molecular-weight region might contain such a structure.

To quantitate the total replication efficiency of such a mixture of multimeric forms, we linearized the replicated DNA with *Bam*HI, which has a single recognition site in pUC9 DNA. As expected, all the bands were converted to 2.7-kilobase (kb) monomer-size linear DNA (Fig. 2, lane 2). To ascertain that the incorporation of [α^{32}P]dCTP seen here was mediated by replicative DNA polymerase α or δ, we injected a well-known inhibitor, aphidicolin, which inhibits both DNA polymerases to the same extent but not DNA polymerases β and γ (30, 38). As shown in Fig. 2 (lanes 3 to 5), replication was reduced to less than 10% of the control (compare lanes 4 and 5). The estimated aphidicolin concentration within the injected egg was 100 μg/ml. Although this concentration is not yet inhibitory to DNA polymerases β and γ (69), it is much higher than the reported value for the inhibition of DNA polymerase α in vitro (2 μg/ml [30]). Lower concentrations of aphidicolin, for example 20 μg/ml, did not cause significant inhibition (data not shown). However, it is noted that in vitro doses for inhibition vary from report to report (for example, over 50 μg/ml for 90% inhibition in reference 25). This variation might have come from the fact that aphidicolin is not soluble in aqueous solvents (30). Andrews and Brown (2) also reported that *Xenopus* eggs require a higher concentration of aphidicolin for inhibition of replication. From these considerations, we concluded that the observed incorporation of [α-^{32}P]dCTP into pUC9 DNA is mostly carried out by DNA polymerase α or δ.

Replication of chromosomal DNA. *Xenopus* egg microinjection has an advantage in that one can compare foreign and chromosomal DNA replication in the same environment. Incorporation of a radioactive precursor into replicated chromosomal DNA was about 10% of the amount observed in replicated plasmid DNA, although the quantity of chromosomal DNA (6 pg per egg [64]) was less than 0.1% of the

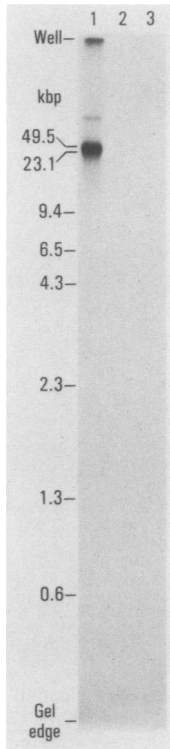

FIG. 3. Analysis of replicated chromosomal DNA. Eggs were injected with 100 nCi of [α-^{32}P]dCTP per egg and incubated for 4 h. DNA was extracted and treated with increasing amounts of DNase I for 2 h at 37°C. Digested and undigested materials were then analyzed by agarose gel electrophoresis and by TCA-insoluble radioactivity. DNase I was used at (lane 1) 0 ng (1,316 cpm per 7 eggs), (lane 2) 100 ng (927 cpm), and (lane 3) 300 ng (164 cpm).

injected plasmid DNA (10 ng per egg). This implies that only about 1% of injected plasmid DNA molecules are utilized as replication templates, if the speed of the replication fork movement is the same in both cases. The size of chromosomal DNA, though it must be much smaller than intact size after many steps of isolation, is still beyond the resolving capacity of agarose gel electrophoresis, and radioactive DNA migrated as a relatively broad band to a position near the 23.1- and 49.5-kb markers (Fig. 3, lane 1). Other investigators have reported a similar migration pattern of chromosomal DNA (44). rRNA genes are amplified in early stages of oogenesis and are present as multicopy extrachromosomal DNA whose size ranges from 13 to 234 kb (29). The amount of this DNA (25 pg per egg) is much more than the chromosomal DNA. However, it no longer replicates after oocyte maturation (3, 9). Mitochondrial DNA (15.8 kb) is also present in large quantity (3.8 ng per egg), but replication of this DNA does not occur until hatching (17, 18). Thus, the radioactive band seen in Fig. 3 does not correspond to either of the extrachromosomal DNAs. We noticed an additional band above the major one, but the radioactivity of this band was negligibly small. These radioactive bands were further confirmed to be DNA because they disappeared upon digestion with RNase-free DNase I and were converted mostly to TCA-soluble material (Fig. 3, lanes 2 and 3). Based on these considerations, we concluded that the broad radioactive band migrating between 23.1 and 49.5 kb represents replicated chromosomal DNA.

Effects of anti-PCNA antibodies on plasmid DNA replication. With the above analytical methods, we examined

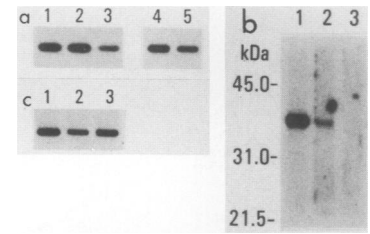

FIG. 4. (a) Effects of human anti-PCNA IgG on replication of plasmid pUC9 DNA. Eggs were injected with 10 ng of pUC9, 100 nCi of [α-^{32}P]dCTP, and two different doses of antibodies. They were incubated for 3 h. DNA was extracted, digested with *Bam*HI, and subjected to agarose gel electrophoresis. Lane 1, 30 nl of PBS buffer; lane 2, 600 ng of nonautoimmune human IgG; lane 3, 600 ng of human anti-PCNA IgG; lane 4, 1,200 ng of nonautoimmune human IgG; lane 5, 1,200 ng of human anti-PCNA IgG. Relative densities of bands: 85% (lane 1), 100% (lane 2), and 33% (lane 3); 100% (lane 4) and 57% (lane 5). (b) Titration of anti-PCNA IgG with purified PNA. Protein blot on nitrocellulose described in the legend to Fig. 1 was probed with 6.0 μg of AK IgG alone (lane 1), a mixture of 6.0 μg of AK IgG and 70 ng of PCNA (lane 2), or a mixture of 6.0 μg of AK IgG and 140 ng of PCNA (lane 5). Relative densities of the PCNA bands: 100% (lane 1), 8% (lane 2), and 0% (lane 3). (c) Neutralization of the inhibitory effect of anti-PCNA IgG by purified PCNA. Eggs were injected with 10 ng of pUC9 and 100 nCi of [α-^{32}P]dCTP along with either 600 ng of nonautoimmune human IgG (lane 1), 600 ng of human anti-PCNA IgG (lane 2), or a mixture of 600 ng of human anti-PCNA and 7 ng of purified PCNA (lane 3). Injected eggs were incubated for 2 h. Extracted DNA was subjected to agarose gel electrophoresis after *Bam*HI digestion. Relative densities of bands: 100% (lane 1), 48% (lane 2), and 88% (lane 3).

whether anti-PCNA antibodies can block plasmid pUC9 replication. In a preliminary experiment, injection of 60 ng of AK IgG did not affect pUC9 replication (data not shown). At a dose of 600 ng of AK IgG, we were able to detect the inhibitory effect of this antibody (Fig. 4a, lane 3). Nonimmune human IgG, on the other hand, was slightly stimulatory at this dose compared with the control, into which the same volume of PBS buffer was injected (compare lanes 1 and 2). The inhibitory effect of AK IgG did not become pronounced by increasing the dose (compare lanes 3 and 5). We also attempted to inject the antibody 1 h before plasmid DNA injection. This double-injection protocol, in which antibodies might have a better chance of capturing PCNA in the egg before replication starts, did not increase the inhibitory effect (data not shown). The inhibitory effect varied from experiment to experiment despite the technical precautions taken to get reproducible results (see Materials and Methods), which ranged from 35 to 67% inhibition, with an average of 56%, according to 13 similar experiments we performed. We think that this variation came from various sources. Most notable was the tendency of antibodies to be inactivated gradually after repeated freezing and thawing. It is also likely that each batch of eggs contains a different quantity of endogenous PCNA to be neutralized.

We were concerned about the problem that the products which were incompletely replicated and migrated as smears in the gel were not included in this analysis. To account for all of the replication products, we measured total TCA-precipitable radioactivity in the homogenate of injected eggs (see Materials and Methods). However, the inhibitory effects determined by this analysis were almost the same as those obtained by densitometer tracing of the *Bam*HI band (see the legend to Fig. 7). It appears that accumulation, if any, of replicative intermediate possibly caused by AK IgG

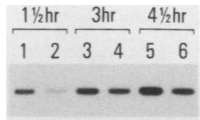

FIG. 5. Time course of the inhibitory effect of human anti-PCNA IgG on plasmid pUC9 DNA replication. Eggs were injected with 10 ng of pUC9, 100 nCi of [α-^{32}P]dCTP, and either 600 ng of nonautoimmune human IgG as a control (lanes 1, 3, and 5) or 600 ng of human anti-PCNA IgG (lanes 2, 4, and 6). They were incubated for 1.5 h (lanes 1 and 2), 3 h (lanes 3 and 4), or 4.5 h (lanes 5 and 6). DNA was extracted, digested with *Bam*HI, and subjected to agarose gel electrophoresis. Relative densities of bands: 100% (lane 1) vs. 25% (lane 2); 100% (lane 3) vs. 68% (lane 4); 100% (lane 5) vs. 54% (lane 6).

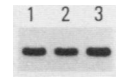

FIG. 6. Effects of monoclonal antibodies against PCNA on replication of plasmid pUC9 DNA. Eggs were injected with 10 ng of pUC9, 100 nCi of [α-^{32}P]dCTP, and 150 ng of antibodies. They were incubated for 3 h. DNA was extracted, digested, and subjected to agarose gel electrophoresis. Lane 1, Control mouse IgG; lane 2, monoclonal 19A2; lane 3, monoclonal 19F4. Relative densities of bands: 100% (lane 1), 95% (lane 2), and 110% (lane 3).

is negligible. This is in contrast to the inhibitory effect of aphidicolin, which gave a significantly different result in the two methods (see the legend to Fig. 2). From these data, we concluded that this anti-PCNA antibody can inhibit up to 67% of pUC9 replication and that a significant level of replication is insensitive to anti-PCNA antibody (at least 33%).

Although our preparation of AK IgG seems to have strong specificity for PCNA (see Fig. 1), the inhibitory effect on replication could be due to contaminating antibodies which react weakly with other antigens. Alternatively, the inflammatory activity possibly associated with autoimmune sera might produce general toxic effects on many biological reactions. We have some evidence to rule out the latter possibility; (i) the same dose of AK IgG did not interfere at all with 5S RNA gene transcription in *Xenopus* oocytes (Ryoji, unpublished observation), and (ii) another preparation of autoimmune IgG which reacts with different autoantigens did not inhibit plasmid replication in this system (Ryoji, unpublished observation). To assess the former possibility, i.e., the specificity of AK IgG, we tested whether the inhibitory activity can be neutralized by coinjection of purified PCNA. First, we determined the amount of PCNA necessary to block anti-PCNA antibody in the AK IgG preparation (Fig. 4b). We mixed AK IgG with two different doses of PCNA and used them for immunoblot analysis. When PCNA was mixed at the ratio of 7 ng of PCNA per 600 ng of AK IgG, the intensity of the PCNA band was reduced to 8% of the control (Fig. 4b, lane 2). The band was eliminated completely at the ratio of 14 ng of PCNA per 600 ng of AK IgG (Fig. 4b, lane 3). Therefore, 600 ng of AK IgG contains anti-PCNA antibody which can bind to a little more than 7 ng of PCNA. We then injected 7 ng of PCNA premixed with 600 ng of AK IgG per egg and examined how plasmid replication was affected. Figure 4c demonstrates that 7 ng of PCNA almost restored the replication activity of the injected egg to the control level. We concluded that binding of AK IgG to PCNA in the egg is the cause of inhibition of plasmid replication.

Replication of plasmid DNA takes place synchronously with chromosomal replication (44). The first round of replication is completed 90 min after pricking, and subsequent rounds occur every 30 min (27, 44). We determined the time point in this replication cycle which was optimum for observing the effect of AK IgG. We isolated DNA after various periods of incubation time and compared the inhibitory effect of AK IgG (Fig. 5). The amount of ^{32}P-labeled plasmid DNA in control eggs (lanes 1, 3, and 5) increased as the eggs were incubated longer. The inhibitory effect seemed to be somewhat weaker at later time points (compare lane 2 with lanes 4 and 6). Although we do not know at present why the inhibitory effect decreases at later time points, we would like to offer two possible explanations. The simplest explanation would be that antibodies might gradually degrade in the injected eggs and become less effective at later times. Alternatively, we have evidence that one egg contains about 70 ng of PCNA (Zuber et al., submitted for publication), whereas antibodies effective to neutralize only 7 ng of PCNA were needed to observe maximum inhibition (Fig. 4b). It is conceivable that the majority of PCNA molecules in eggs might be stored in an inactive as well as nonantigenic form, perhaps as a complex with other components, and then they might be gradually activated in the course of egg cleavage, thereby overcoming the inhibitory effect of AK IgG at later time points.

We also tested monoclonal antibodies raised against PCNA (49, 50). Monoclonal antibodies 19F4 and 19A2 have been shown to react with a domain located in the central region of the PCNA molecule, whereas epitopes near the N- and C-termini are specific to the AK antibody (50). Thus, it was of interest to determine whether monoclonals can also block the function of PCNA. In the experiment shown in Fig. 6, we injected 150 ng of purified monoclonals. It is clear that neither monoclonal inhibited replication of pUC9 to a significant level. Lack of the inhibitory effect was not because we injected a smaller amount of monoclonals than of AK IgG, since the concentration of antibodies specific to PCNA was 16-fold greater in the monoclonal preparations than in AK IgG (see Materials and Methods). From these data, we concluded that the epitopes for the monoclonals either do not represent the functional domains of PCNA or are not accessible to antibodies when PCNA is in the native form.

Inhibition of plasmid replication by an anti-DNA polymerase α antibody. It has recently been demonstrated in vitro that PCNA is an auxiliary protein of DNA polymerase δ and is required for DNA polymerase δ-mediated, but not DNA polymerase α-mediated, DNA synthesis (59, 62). This specificity of PCNA for DNA polymerase δ and the observed inhibition of replication by the anti-PCNA antibody prompted us to investigate whether DNA polymerase α is also working in the present system. Monoclonal antibody SJK132-20 is one of the clones which were raised against human KB cell DNA polymerase α (63). This clone has potent activity in neutralizing the DNA polymerase α activity in vitro and in vivo (32, 46) but does not cross-react with DNA polymerase δ (1, 39). We injected this monoclonal antibody into the eggs to study its effects on plasmid DNA replication. As shown in Fig. 7A, this antibody reduced plasmid replication to 40% of the control. Like the effect of anti-PCNA antibody, the blockage by the anti-DNA polymerase α antibody was not complete, and a significant level of plasmid DNA replication remained after inhibition by anti-DNA polymerase α antibody. We simultaneously injected both of the antibodies to test whether a mixture of the

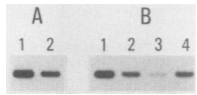

FIG. 7. Effect of anti-DNA polymerase α antibody on plasmid replication. (A) Injection of anti-DNA polymerase α antibody alone. Eggs were injected with 10 ng of pUC9 and 100 nCi of [α-^{32}P]dCTP together with either 200 ng of control mouse myeloma IgG (lane 1) or 200 ng of monoclonal antibody SJK 132-20 against DNA polymerase α (lane 2). Injected eggs were incubated for 3 h. DNA was extracted, digested with *Bam*HI, and separated by agarose gel electrophoresis. A portion of the same material was also subjected to TCA precipitation as described in Materials and Methods. Relative densities of the bands: 100% (lane 1) vs. 42% (lane 2). Relative radioactivity of TCA-insoluble material: 100% (lane 1) vs. 37% (lane 2). (B) Coinjection of anti-PCNA and anti-DNA polymerase α antibodies. Eggs were injected with pUC9 and [α-^{32}P]dCTP as in A. In addition, the following combinations of antibodies were injected simultaneously: lane 1, 200 ng of control mouse myeloma IgG and 600 ng of control human IgG; lane 2, 200 ng of control mouse myeloma IgG and 600 ng of human anti-PCNA IgG; lane 3, 200 ng of mouse monoclonal IgG against DNA polymerase α and 600 ng of human anti-PCNA IgG; lane 4, 200 ng of mouse monoclonal IgG against DNA polymerase α and 600 ng of control human IgG. After 3 h of incubation, DNA was processed as in A. Relative densities of the bands were 100% (lane 1), 32% (lane 2), 7% (lane 3), and 35% (lane 4). Relative radioactivities of TCA-insoluble material were 100% (lane 1), 38% (lane 2), 5% (lane 3), and 38% (lane 4).

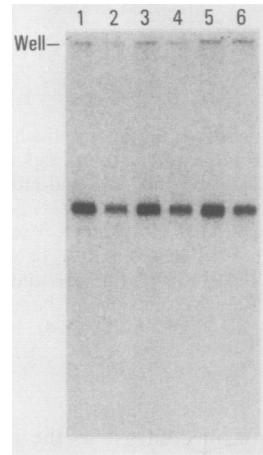

FIG. 8. Anti-PCNA antibody dose response of chromosomal replication. Eggs were injected with 400 ng (lane 1), 800 ng (lane 3), or 1,400 ng (lane 5) of nonautoimmune human IgG or 400 ng (lane 2), 800 ng (lane 4), or 1,400 ng (lane 6) of human anti-PCNA IgG 1 h prior to injection of 100 nCi of [α-^{32}P]dCTP. They were incubated for 2 h, and DNA was extracted and subjected to agarose gel electrophoresis. Relative densities of bands: 100% (lane 1) vs. 45% (lane 2); 100% (lane 3) vs. 57% (lane 4); 100% (lane 5) vs. 64% (lane 6).

two different antibodies would completely shut off replication. We observed that plasmid DNA replication was further reduced to about 7% of the control (Fig. 7B, compare lanes 1 and 3), while either antibody alone was able to reduce replication to about 35% of the control (lanes 2 and 4). These results indicate that there are at least two independent pathways for plasmid replication, one mediated by a PCNA-dependent enzyme, presumably DNA polymerase δ, and the other carried out by DNA polymerase α. It is also important to note that, although each antibody alone did not abolish replication (68% inhibition by anti-PCNA antibody and 65% inhibition by anti-DNA polymerase α antibody [Fig. 7B]), these two effects added up to more than 100%. Therefore, the inhibition of the activity of DNA polymerase α appears to cause partial blockage of DNA polymerase δ-mediated replication and vice versa. In other words, there seems to be a third mode of replication in which both DNA polymerases function cooperatively. We will consider a possible mechanism for such cooperativity in the Discussion.

Studies on chromosomal replication. Early embryos of *Xenopus laevis* are known for their exceptional rate of chromosomal replication (37). We studied how anti-PCNA antibody affects this process. Response of chromosome replication to different doses of AK IgG is shown in Fig. 8. Although the major chromosomal bands in the gel did not correspond to the actual levels of inhibition determined by TCA-insoluble radioactivities, these two measurements correlated well with each other (see below). We therefore used the major chromosomal band as a convenient marker to estimate roughly the relative replication levels. In the range of doses we tested, we did not see significant differences in the inhibitory effect. Rather, there was a tendency for higher doses of AK IgG to reduce the inhibitory effect for unknown reasons. A similar tendency was also seen in plasmid replication (Fig. 4a, compare lanes 3 and 5). We then measured the actual level of replication inhibition by comparing TCA-precipitable radioactivities (Table 1). In contrast to plasmid replication, the inhibitory effect calculated from the density of the major band was always 10 to 20% larger than the value from the TCA analysis. It appears that replication products running as smears contribute significantly to the total replication level. The inhibition by 800 ng of AK IgG was found to be only 11% (Table 1, experiment 1). Injection of AK IgG 1 hour prior to the introduction of [α-^{32}P]dCTP enhanced the inhibitory effect to 30% (Table 1, experiment 2). Thus, anti-PCNA antibody is inhibitory to chromosomal replication, but the extent of inhibition is less than that for plasmid replication. Anti-DNA polymerase α antibody, on the other hand, inhibited chromosome replication by more than 70% (Table 1, experiment 3). This inhibition level is close to the value previously observed in permeabilized human fibroblasts (46). A similar result was also reported after microinjection studies of mammalian cell lines (32). It is clear from the present results that frog embryos also utilize DNA polymerase α for the replication of chromosomes.

Sensitivity of chromosomal and plasmid replication to BuPdGTP. As described above, plasmid and chromosomal replications are both inhibited to about 30% of the control level by anti-DNA polymerase α antibody. We also tested another inhibitor, BuPdGTP, which preferentially blocks DNA polymerase α but not δ at appropriate doses. BuPdGTP is a GTP analog synthesized by Wright and Dudycz (68). Subsequent studies by them and others demonstrated that this chemical can inhibit the activity of purified DNA polymerase α, including the one from *Xenopus laevis*, at concentrations much lower than that needed to inhibit purified DNA polymerase δ (11, 33, 40, 68). It is difficult and may even be erroneous to apply data obtained in vitro with purified enzymes to the in vivo situation and determine which species of DNA polymerase operates in vivo. However, comparison of the dose responses of chromosomal and plasmid replication in the same in vivo environment would produce meaningful data about the structural differences or similarities in the replication apparatus of the two cases. The results of anti-DNA polymerase α antibody injection predict that BuPdGTP would also inhibit both of the replications in similar fashions. We injected increasing doses of BuPdGTP along with [α-^{32}P]dCTP and examined

TABLE 1. Effects of anti-PCNA and anti-DNA polymerase α antibodies on chromosomal replication

Expt no.	Antibody injection[a]	Antibody injected (ng/egg)	Labeling time in h (nCi/egg)	TCA-insoluble radioactivity[b] (cpm/10 eggs)	Relative replication (% of control)
1	Simultaneous	Control[c] (800)	3.5 (200)	1,898	100
		Anti-PCNA[d] (800)		1,680	89
2	1 h before	Control[c] (800)	2.0 (200)	1,786	100
		Anti-PCNA[d] (800)		1,249	70
3	1 h before	Control[e] (200)	2.0 (70)	311	100
		Anti-pol α[f] (200)		85	27

[a] Time of injection in relation to labeling with [α-^{32}P]dCTP.
[b] Radioactivities per 10 eggs. Background radioactivities (409, 388, and 187 cpm for experiments 1, 2, and 3, respectively) were determined and subtracted from the total TCA-insoluble radioactivities as described in Materials and Methods.
[c] Nonautoimmune human IgG.
[d] Human anti-PCNA IgG (AK IgG).
[e] Mouse myeloma IgG.
[f] Mouse monoclonal anti-DNA polymerase α IgG.

how much of this inhibitor was necessary to block chromosomal or plasmid DNA replication. Against the above prediction, we found that chromosomal and plasmid replications responded quite differently to BuPdGTP (Fig. 9). The half-inhibition dose for chromosomal replication was about 5 μM, whereas that for plasmid replication was found to be more than 20 μM in the same environment. We will discuss the possible reasons for this discrepancy in the Discussion.

DISCUSSION

Our studies have demonstrated that PCNA (cyclin) is involved in DNA replication in living cells, which agrees with the data on DNA synthesis in various cell-free systems and in isolated nuclei (52, 53, 59, 62, 67). Given the known function of PCNA as the auxiliary protein of DNA polymerase δ (6, 53, 59, 62), our results further imply that DNA polymerase δ plays a role in DNA replication in vivo. However, DNA polymerase δ does not seem to be solely responsible for replication of all of the replicons, because the inhibition by anti-PCNA antibody did not exceed 70%. In particular, its effect on chromosomal replication was significantly smaller. It is unlikely that the injected antibodies might not have reached the sites in the egg where replication takes place, because anti-DNA polymerase α antibody exhibited a much stronger inhibitory effect on chromosomal replication (Table 1), and coinjection of anti-DNA polymerase α and anti-PCNA antibodies almost completely abolished pUC9 replication (Fig. 7B). One could alternatively argue that binding of anti-PCNA antibody might not necessarily lead to complete inhibition of PCNA function. However, we rule out this possibility based on the observation that this autoimmune antibody was able to shut off completely the DNA synthesis driven by DNA polymerase δ in vitro (62). Thus, it appears that replication which does not require PCNA, such as the one carried out by DNA polymerase α, occurs in many of the replicons or plasmids. The contribution of the replication which is insensitive to anti-PCNA antibody is estimated to be about 30% in the case of plasmid replication and about 70% in the case of chromosomal replication. The importance of DNA polymerase α for chromosomal replication in the cell has been firmly established from the studies introducing anti-DNA polymerase α antibodies into cell nuclei, as well as from analyses on DNA polymerase α mutants (21, 31, 32, 45, 46; this study). However, it does not necessarily mean that DNA polymerase α is the only polymerase needed for replication. The data presented here rather indicate that both of the DNA polymerases are involved in DNA replication in living cells. Dresler and Frattini (20) and Hammond et al. (25) also reached a similar conclusion from their studies on the sensitivity of chromosomal replication to BuPdGTP. More-direct evidence on the involvement of DNA polymerase δ must await the isolation of DNA polymerase δ mutants or the introduction of anti-DNA polymerase δ antibodies into living cells.

A novel feature of DNA replication which has emerged from the present study is the apparent cooperativity of DNA polymerases α and δ. It is evident from Fig. 7B that both DNA polymerases are not necessarily working as independent enzymes, because the inhibitory effects of anti-PCNA and anti-DNA polymerase α antibodies are not additive. Inhibition by either one of the antibodies was nearly 70% in the experiment shown in Fig. 7B. Although we obtained

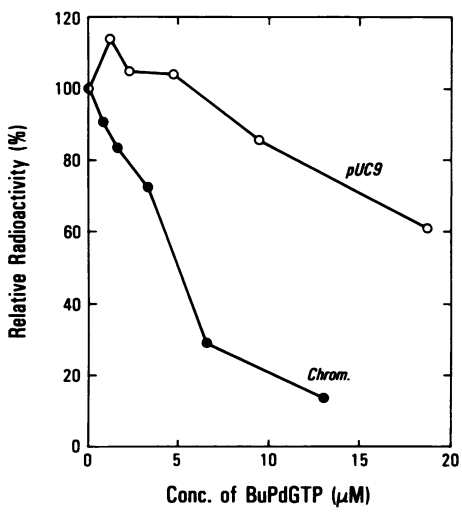

FIG. 9. Sensitivity of replication to BuPdGTP. Eggs were injected with 10 ng of pUC9, 100 nCi of [α-^{32}P]dCTP, and various doses of BuPdGTP. In the case of chromosomal (chrom.) DNA replication, pUC9 was omitted. Injected eggs were incubated for 4 h, DNA was extracted from 16 eggs (chromosome replication) or 20 eggs (plasmid replication), and TCA-insoluble radioactivity was determined as described in Materials and Methods. The concentrations of BuPdGTP in the injected egg (abscissa) were estimated on the assumptions that the diameter of an egg is 1.2 mm and that the injected BuPdGTP is homogeneously distributed within an egg. TCA-insoluble radioactivities (ordinate) were plotted after normalization against that with no BuPdGTP.

variable levels of inhibition by anti-PCNA antibody from experiment to experiment, the average of 13 experiments was 56%. On the other hand, anti-DNA polymerase α reproducibly exhibited over 62% inhibition (Fig. 7A and B). In considering the report that the same monoclonal antibody inhibited DNA polymerase α purified from *Xenopus* ovaries by only 60% at saturating doses (34), it is conceivable that injection of a more-specific antibody against *Xenopus* DNA polymerase α, if available, would result in 100% inhibition of replication. We must therefore assume that inhibition of one species of DNA polymerase causes at least partial blockage of replication carried out by the other species. This cooperativity is understandable if one considers the dimeric structure of the "replisome" which has been proposed by Sinha et al. (56) and by Kornberg (35). In this hypothetical model of the replisome, the two DNA polymerase holoenzymes for leading- and lagging-strand synthesis are spatially coupled rather than working separately. If this dimeric replisome is composed of DNA polymerase α and δ holoenzymes, one can imagine how the movement of DNA polymerase δ on the template is impeded when DNA polymerase α is blocked and vice versa. On the other hand, the cooperative mode appears to have a relatively small role in chromosomal replication, because the contributions by DNA polymerases α (73%) and δ (30%) add up close to 100%.

Another interesting finding in our present studies is that there are subtle differences in the structure of the replication apparatus for chromosomes and plasmid. Analysis by anti-PCNA antibody indicated that PCNA is involved more in plasmid replication than in chromosomal replication. Furthermore, replication of plasmids is more resistant than that of chromosomes to BuPdGTP, a preferential inhibitor of DNA polymerase α. It is important that the latter observation seems to contradict the data that anti-DNA polymerase α antibody inhibited both types of replication to similar extents (62 versus 73%). To reconcile this discrepancy, we suggest that the DNA polymerase α holoenzyme involved in plasmid replication might have a slightly different structure from the chromosomal counterpart. For example, each holoenzyme might contain a distinct accessory protein(s) which in turn affects sensitivity to BuPdGTP, as originally proposed by Decker et al. (19). It is also possible that there is a subspecies of DNA polymerase α or δ which is antigenically similar to α but is not as sensitive to BuPdGTP. These differences would be an important aspect to be considered in constructing structural models of chromosome replication based on studies mostly carried out with small circular DNA.

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