Characterization of early DNA synthesis in *Xenopus* eggs after injection of circular plasmid DNA

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ABSTRACT

Circular plasmid DNA is known to replicate when injected into unfertilized eggs of *Xenopus laevis*. We characterized early DNA synthesis which precedes the replication. Incorporation of a radioactive precursor into covalent closed circular DNA becomes detectable as early as 2 minutes after injection. Judging from the sensitivity to aphidicolin, replicative DNA polymerase α or δ is involved in this reaction. However, analysis of density-labeled product as well as detection of newly synthesized, unmethylated strands by restriction endonuclease DpnI digestion, both indicated that most if not all, of the synthesized DNA is present as short repair-like patches in the injected DNA molecules. They are present randomly in all the HaeIII fragments of injected DNA, and transcriptional activation of the 5S RNA gene inserted in the plasmid does not affect the distribution. Only a minor fraction of injected DNA is utilized for this reaction, and 12% of such DNA molecules are further chased into replicated progeny DNA. This efficiency of replication is nearly the same as that of bulk injected DNA which has not been subjected to the early DNA synthesis. We concluded that, despite the common use of replicative DNA polymerase, the early DNA synthesis and the subsequent DNA replication are mutually independent processes.

INTRODUCTION

Unfertilized eggs of *Xenopus laevis* are capable of synthesizing DNA directed by injected foreign plasmid DNA (1). When injected eggs are incubated in hypotonic medium, they become parthenogenically activated, and 10 to 15% of injected circular DNA molecules undergo multiple rounds of replication in the next several hours (2). Replication of injected DNA takes place synchronously with endogenous chromosome replication, indicating that it is under the control of cell cycle (3). Based on the appearance of replicative intermediate-like DNA which has lower electrophoretic mobility than the injected DNA, it was suggested that replication of injected DNA starts about 35 minutes after injection (3). In support of this notion, 4% of injected radioactive DNA were detected in the heavy-light fractions when newly synthesized DNA was density-labeled for the initial 1 hour (2). However, it was not clear whether they represent true heavy-light DNA or diffused material from the light-light DNA peak.

Although the major mode of DNA synthesis is due to replication, it is preceded by a low but significant level of DNA synthesis which is also directed by injected DNA (3). This minor DNA synthesis which occurs soon after DNA injection was assumed to be a repair-type reaction (3), and has not been characterized any further. In considering the fact that, once replication is initiated, the rate of replication fork progression in eukaryotes (about 2.6kb per minute, see ref. 4) is fast enough to complete replication of small plasmid

DNA within a minute, this early DNA synthesis may not necessarily represent repair synthesis. It was reported that DNA molecules, with an eye-like structure bearing some resemblance to the early theta structure of replication intermediate, are generated in a fraction of injected DNA within 15 minutes after injection (5). This observation also points to the possibility that at least initial steps of replication might be seen as early as 15 minutes after injection. In this paper, we characterized in detail the early DNA synthesis, and examined whether it has any connection to the subsequent DNA replication.

MATERIALS AND METHODS

Enzymes and Chemicals

Restriction endonucleases BamHI, HindIII, and HaeIII were purchased from Promega Biotech, Madison, WI. Restriction endonuclease DpnI was from New England Biolabs, Beverly, MA. Aphidicolin, Ca^{2+} -ionophore A23187 and BrdUTP (bromodeoxyuridine triphosphate) were obtained from Sigma, St. Louis, MO. (α - ^{32}P)dCTP (10 mCi/ml, 3,000 Ci/nmol) and (α - ^{32}P)GTP (10 mCi/ml, 800 Ci/nmol) were supplied by New England Nuclear, Boston, MA.

Plasmid DNA and Radiolabeling Procedures

Plasmid pXbsF201 is a recombinant construct of pUC9 (6) in which a 249bp HindIII-BamHI fragment is inserted (7). This fragment, which was obtained from plasmid pXbs201 (8), carries the *Xenopus borealis* somatic-type 5S RNA gene and part of the surrounding spacer sequences isolated and characterized by Bogenhagen *et al.* (8). Plasmid DNA was isolated from a culture of *E. coli* JM101/pXbsF201 or JM101/pUC9 by the alkaline lysis method as described (9). Supercoiled DNA was further purified twice by CsCl-ethidium bromide equilibrium density gradient centrifugation (9). RNase was not used throughout the procedure. ^{32}P -labeled circular pXbsF201 was prepared by growing *E. coli* JM101/pXbsF201 in $^{32}\text{PO}_4$ -containing medium.

TFIIIA Purification

Transcription factor TFIIIA was purified from immature ovaries of *Xenopus laevis* (Nasco, Fort Atkinson, Wisconsin) following the method of Smith *et al.* (10). After purification, the protein fractions were pooled and dialyzed against a buffer containing 50 mM Tris-HCl pH 7.5, 5 mM MgCl_2 , 0.5 mM dithiothreitol, 10 μM ZnCl_2 , 0.1 M KCl and 20% glycerol.

*Microinjection into Unfertilized Eggs of *Xenopus laevis**

Large adult females of *Xenopus laevis* were purchased from Nasco. Unfertilized eggs were obtained from the frogs by injection of human chorionic gonadotropin (1,000 IU, Sigma) into the dorsal lymph sac (11). Eggs were laid in OR-2 medium (12) and used within one hour. Prior to injection, a batch of freshly laid eggs were dejellied by the method as described (13), and stored in OR-2 medium until injection. A small number of eggs were transferred into (0.5 $\times$) OR-2 medium, and were then injected with 40 nl of solutions containing DNA, (α - ^{32}P)dCTP and transcription factor TFIIIA as indicated in the legends to the figures. Injection was performed into the animal half by using a calibrated capillary needle. At least 20 eggs were injected within 10 minutes for each experimental point. Injected eggs were individually incubated in (0.5 $\times$) OR-2 medium at 20°C. After a precise time of incubation, the eggs were harvested one by one in microcentrifuge tubes, frozen immediately in dry ice-methanol, and stored at -20°C until further analysis. The radioactivity of each frozen egg was monitored by a Geiger counter. Eggs with radioactivities significantly lower or higher than the mean value were discarded in order to get quantitative data.

Extraction of DNA and Electrophoresis

Injected eggs were combined and homogenized in 30 μ l per egg of a solution containing 30 mM Tris-HCl pH 7.4, 20 mM EDTA, 1% SDS (sodium dodecylsulfate) and 0.75 μ g/ml proteinase K, and incubated for 30 minutes at 37°C. Samples were extracted with phenol/chloroform, and DNA was precipitated with ethanol. The precipitate was collected by centrifugation and dissolved in 5 μ l per egg of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA). DNA was separated either by 1% agarose gel electrophoresis in (2/3 $\times$) TPE buffer (50 mM Tris phosphate pH 7.2, 1 mM EDTA), or by 5% acrylamide gel electrophoresis in (1/2 $\times$) TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). In most cases, DNA from two eggs was applied per lane. When Dpn I digests were analyzed, SDS was included in the loading buffer to the final concentration of 0.1 to 0.3% (see the text).

CsCl Equilibrium Density Gradient Centrifugation

The experiment was done as described by Harland and Laskey (2) with modification. A 40 nl mixture containing 10 mM BrdUTP, 7.5 ng DNA and 200 nCi (α -³²P)dCTP was injected into eggs. DNA was extracted from the eggs after various lengths of incubation time. Samples were made to 32 ml with TE buffer containing CsCl. The density of the solution was adjusted to 1.748. It was centrifuged in the Beckman VTi 50 rotor at 45,000 rpm for 21 hr. About 1 ml fractions of the gradient were collected and the densities of the fractions were determined by weight. To avoid precipitation of CsCl, each fraction was diluted with 1 ml TE buffer containing 10 μ g calf thymus DNA. Trichloroacetic acid was then added to a final concentration of 10%, and DNA was precipitated for 30 minutes on ice. The samples were filtered through nitrocellulose membrane (Gelman, GN-6) followed by extensive washing with ice-cold 10% trichloroacetic acid. The radioactivities of the filters were determined by a liquid scintillation counter.

Analysis of 5S RNA Transcription

A 40 nl mixture of 7.5 ng pXbsF201, 100 nCi (α -³²P)GTP and various doses of TFI_{IIA} was injected into eggs. RNA was extracted in the same manner as DNA. ³²P-labeled transcripts were separated in 8% polyacrylamide gels as described (14).

RESULTS

Time Course of Plasmid DNA-Dependent DNA Synthesis in Injected Eggs

The DNA we used is plasmid pXbsF201 which consists of vector plasmid pUC9 and a cloned *Xenopus* 5S RNA gene as illustrated in Fig. 1 (see Materials and Methods). To monitor the kinetics of DNA synthesis, we injected a mixture of pXbsF201 and (α -³²P)dCTP. As shown in Fig. 2 (A, lanes under 'undigested'), incorporation of (α -³²P)dCTP became detectable as early as 2 minutes after injection of plasmid pXbsF201. It is noted that a significant fraction of radioactive DNA was already supercoiled to intermediate levels, which gave rise to bands between forms I and II. By 15 minutes, this population became the major one, and highly supercoiled DNA was also generated. Analysis of the BamHI-linearized products by alkaline agarose gel electrophoresis revealed that at least 50% of the denatured radioactive DNA migrated as the intact plasmid size with the rest of the material running close to it as smears (data not shown). Therefore, if the newly synthesized products are short DNA fragments (see below), most of them must be covalently linked to the injected DNA template rather than simply associated with the injected DNA by hydrogen bonds. The intensities of DNA bands then increased gradually 2 to 3 fold for the subsequent 45 minutes (Fig. 2B). Although major bands represent topoisomers of monomeric circular DNA of plasmid pXbsF201, the bands corresponding to dimeric

Figure 1. Restriction map of pXbsF201. 5S RNA gene and part of its flanking sequences are inserted in the BamHI and HindIII sites of plasmid pUC9. The heavy arrow indicates the 5S RNA gene position and the direction of transcription. The bars projecting outward represent HaeIII sites. The numbers outside of the circle show the lengths of HaeIII fragments in bp.

circular DNA were also evident. They were synthesized from the dimer template originally present as a minor component in the injected DNA. At 60 and 120 minute time points, higher molecular weight bands and smears appeared further above the dimer bands (Fig. 2B, lanes under 'undigested'). They could be either higher multimers or replicative intermediates of plasmid DNA. Alternatively, they might represent replicating chromosomal DNA, although we did not characterize them any further. In order to quantitate the level of plasmid-dependent DNA synthesis, we linearized the product with BamHI which has a unique recognition site in pXbsF201 DNA (see Fig. 1). As expected, most of the radioactive bands were converted to 3kb monomeric linear DNA (Fig. 2, lanes under 'BamHI'). Incorporation into plasmid DNA gradually increased for the first 60 minutes, and then there seemed to be an abrupt increase of incorporation between 60 and 120 minutes. This jump of the incorporation rate was also observed by Mechali and Kearsey (3) who interpreted it as the start of the replication mode of DNA synthesis.

Aphidicolin Sensitivity of Early DNA Synthesis

There are at least four species of DNA polymerase present in eukaryotic cells. DNA polymerase α and δ have been shown to be the major species responsible for replication, while DNA polymerase β and γ function primarily for DNA repair and mitochondrial DNA replication, respectively (for a recent review, see ref. 15). Various inhibitors have been found to affect differentially the activity of each polymerase. Aphidicolin is a very frequently used inhibitor which preferentially blocks DNA polymerase α and δ activities, but not β activity under appropriate concentrations (16–18). We examined the sensitivity of the DNA synthesis to aphidicolin 10 minutes and 2 hours after injection of DNA and (α - 32 P)dCTP (Fig. 3). The latter time point serves as an internal control representing the replication mode because, according to our own studies (see below) and those by others

Figure 2. Time course of plasmid DNA-dependent DNA synthesis in *Xenopus* eggs. Eggs were injected with 7.5 ng pXbsF201 and 100 nCi (α - 32 P)dCTP, and incubated for various periods of time as indicated. DNA was extracted and subjected to agarose gel electrophoresis with or without BamHI digestion. These results were obtained from two independent experiments covering 2 to 15 minutes (in A) and 15 to 120 minutes (in B). I, Supercoiled monomer; III, linear monomer; Ir, relaxed, closed circle monomer; II, nicked circle monomer; I* supercoiled dimer.

(see above discussion), DNA synthesis by replication becomes dominant relative to the initial reaction at this time point. With 100 ng per egg of aphidicolin, incorporation was reduced to about 10% of control at both time points. Lower doses, e.g., 20 ng per egg, did not cause a significant level of inhibition (data not shown). In addition, the available *in vitro* data indicated that this concentration of aphidicolin did not inhibit DNA polymerase β (19). Thus the early synthesis of DNA seems to be carried out by DNA polymerase α or δ whose primary function is DNA replication. The reported dose response of these species of DNA polymerase to aphidicolin *in vitro* ranges from 2 μ g/ml to 50 μ g/ml for 90% inhibition (16, 20). The difficulty in solubilizing aphidicolin in aqueous solvents seems to contribute to such a wide variation (16). The inhibitory concentration of aphidicolin reported here (100 μ g/ml, assuming that the volume of an egg is 1 μ l) is rather high relative to those obtained from *in vitro* studies. However, direct comparison is difficult because of the wide variation of the data reported and because of the *in vivo* uncertainty of how injected aphidicolin is distributed within the egg.

DpnI Analysis of Early DNA Synthesis

Our observation that the initial incorporation of (α - 32 P)dCTP depends on the same DNA polymerase species as those required for DNA replication prompted us to examine whether it is in fact repair-type DNA synthesis as suggested by others (3). We analysed the 32 P-

Figure 3. Aphidicolin sensitivities of early and late DNA synthesis. Eggs were injected first with either 10 nl DMSO (dimethyl sulfoxide) or 10 nl aphidicolin (10 ng/nl in DMSO). Subsequently, a mixture of plasmid pUC9 DNA (10 ng per egg) and (α - ^{32}P)dCTP (100 nCi per egg) was injected into the same eggs, which were then incubated for 10 min or 2 hr as indicated. DNA was extracted from the injected eggs, as the intact form, and subjected to agarose gel electrophoresis as described in Materials and Methods. The autoradiographic exposures were adjusted so that DNA bands in the 10 min and 2 hr were of comparable intensities. II*, nicked circle dimer; I*r, relaxed, closed circle dimer.

labeled DNA using restriction endonuclease DpnI. This enzyme cuts the sequence GATC if the adenine residue is methylated by *E. coli* dam methylase (21). Under 0.15 to 0.2M NaCl, it can only cut this sequence if the adenine residues on both strands are methylated (22,23). Since dam methylase is not present in eukaryotes, the first cycle of plasmid replication in *Xenopus* eggs results in a loss of a methyl group from one of the two adenine residues in this sequence, which then generates DpnI-resistant DNA. Since there are over 10 DpnI sites distributed throughout the plasmid pXbsF201, synthesis of several repair-like patches at random sites would rarely convert the parental DNA into the DpnI-resistant form at all sites. Thus, preservation of the intact length of DNA after the DpnI reaction is taken as the evidence that at least one of the parental strands have been completely replaced by a replicated, unmethylated strand (23). ^{32}P -labeled DNA from the injected eggs, which were incubated for 15 to 60 minutes and for 2 hours, was first linearized with BamHI (Fig. 4, lanes 1, 3, 5, and 7), and then digested with DpnI under the conditions where hemimethylated DNA is insensitive to this enzyme (Fig. 4, lanes 2, 4, 6, and 8). We found that DNA isolated from the eggs, which had been incubated for up to 60 minutes, was still sensitive to DpnI, and was converted almost completely into short DNA fragments

Figure 4. Analysis of ^{32}P -labeled DNA by restriction endonuclease DpnI. Plasmid pXbsF201 (7.5 ng per egg) was injected along with $(\alpha\text{-}^{32}\text{P})\text{dCTP}$ (100 nCi per egg). Injected eggs were then incubated for various lengths of time as indicated. DNA was extracted and digested with BamHI/DpnI (lanes 2, 4, 6, 8, and 9) or BamHI alone (lanes 1, 3, 5, and 7). DpnI digestion was carried out in the presence of 0.2 M NaCl (lanes 2, 4, 6, and 8) or 50 mM NaCl (lane 9). Reaction mixtures were directly subjected to agarose gel electrophoresis. Note that the samples at 120 minutes were run in a separate gel, and the band positions were shifted upwards relative to the other time points.

(Fig. 4, lanes 2, 4, and 6). Hence, the incorporation in the initial 60 minutes is a result of the synthesis of short repair-like patches in DNA. On the other hand, DNA isolated after 2 hours of incubation generates only a small amount of shorter fragments, and the majority remained as the intact size DNA (Fig. 4, lane 8; note that DNA migration in this lane was slightly retarded presumably due to a tight association of Dpn I with DNA). Thus, DNA is replicated at least once within 2 hours after injection, which agrees with the data reported by others (2, 3). However, another round of replication was rare on the same DNA molecule, because DpnI digestion under 50 mM NaCl resulted in fragmentation of all the labeled DNA (Fig. 4, lane 9); if progeny DNA generated by multiple rounds of replication is present, it should be fully unmethylated and thereby becomes refractory to DpnI even at lower salt concentrations like 40–50 mM NaCl (21; Ryoji, unpublished observation). This data also showed that, at 50 mM NaCl, DpnI is able to cut hemimethylated sequences produced by the first round of replication. Such a change in the specificity of DpnI by salt concentrations was also noted by others (23).

Upon close inspection of an overexposed film of the 15–60 minute samples, we noticed that there was Dpn I-resistant DNA which migrated as a faint smear in the higher molecular weight region of the gel. As will also be described in a following section, this smear became a single band corresponding to the BamHI-linearized DNA when SDS concentration in the sample loading buffer for electrophoresis was raised to 0.3% in order to release DpnI possibly bound to DNA (see Fig. 10, lane 1+). This DpnI-resistant band (about 5% of the total) is not due to incomplete digestion, since ^{32}P -pXbsF201 labeled in *E. coli* did not give rise to the residual band when it was digested with DpnI under identical conditions (see Fig. 10, lane 4+). We concluded that there are DNA molecules of which at least one strand is displaced with newly synthesized DNA at all DpnI sites. However, we do not know whether the DpnI-resistant DNA is caused by extensive synthesis of repair-like

Figure 5. Analysis of ^{32}P -labeled DNA by CsCl equilibrium density gradient centrifugation. Eggs were injected with 7.5 ng pXbsF201, 400 pmole BrdUTP and 200 nCi ($\alpha\text{-}^{32}\text{P}$)dCTP. Incubation was carried out for 15 minutes (filled circles), 60 minutes (open circles) or 5 hours (filled squares). DNA was extracted and subjected to CsCl isopycnic centrifugation as described in Materials and Methods. ^{32}P -labeled circular pXbsF201 was used as a standard for unreplicated 'light-light' DNA (LL; open squares). HL, position of 'heavy-light' DNA; HH, position of 'heavy-heavy' DNA.

patches or it actually represents replication (see below).

Density Substitution Analysis of Early DNA Synthesis

To ascertain the above conclusions drawn from the DpnI analysis, we carried out CsCl equilibrium density gradient centrifugation of DNA which was density-labeled with BrdUTP in the newly synthesized sequences (Fig. 5). The peak radioactivity of the 15 minute sample was located in the fractions of almost the same density as that of uninjected LL DNA ($\rho = 1.678$). The density distribution became broadened, and shifted toward the HL position after 60 minutes. However, the mean density ($\rho = 1.688$) was still lower than that of the HL standard ($\rho = 1.707$). Taken together, the data demonstrated that the DNA synthesis for the first 60 minutes is due mostly to the formation of repair-like patches in the injected DNA. It should be pointed out that, in the 15 minute sample, a small but significant amount of radioactive DNA is present in the tail fractions near the HL position (Fig. 5, filled circles). Although the relatively high background radioactivities distributed throughout the gradient preclude one from assessing these fractions quantitatively, they might account

Figure 6. Distribution of repair-like patches in pXbsF201 DNA. Eggs were injected with 7.5 ng pXbsF201, and 100 nCi (α - 32 P)dCTP. They were incubated for 15 minutes or 3 hours. Extracted DNA was digested with HaeIII, and subjected to acrylamide gel electrophoresis as described in Materials and Methods. The autoradiographic exposures were adjusted so that the bands in both samples were of comparable intensities. They were exposed for 60 hours (left) and 6 hours (right). The sizes of HaeIII restriction fragments are indicated in bp on the left. The 3' half of the 5S RNA gene is located in the 147 bp fragment (see Figure 1).

for the weak DpnI-resistant band described above. It is also noted in this figure that only a minor HH peak was present in the 5 hr product (lower panel, filled square). This agrees with a conclusion from the above DpnI analysis that multiple rounds of replication is rare under our experimental conditions.

Distribution of Repair-Like Patches in the Template

We asked which region of the injected DNA preferentially incorporates a radioactive precursor in the early DNA synthesis. In particular, the 5S RNA gene sequence present in the plasmid DNA might behave differently from the rest of the vector sequences, because the gene is transcribed in injected eggs. Plasmid pXbsF201 was labeled with (α - 32 P)dCTP in the injected eggs for 15 minutes, and then digested with HaeIII. This restriction endonuclease generates 14 DNA fragments of plasmid pXbsF201 (Fig. 1). We compared the radioactivity distribution in these restriction fragments with those of DNA 32 P-labeled in injected eggs for 3 hours (Fig. 6). The latter DNA which has gone through one to two cycles of replication presumably contains 32 P at all cytosine residues equally throughout the plasmid. It is seen that the distributions of radioactivity from these two DNAs are indistinguishable, demonstrating that the repair-like patches are present randomly throughout

Figure 7. Effect of TFIIIA prebinding on 5S RNA transcription. Eggs were injected with 7.5 ng pXbsF201, 100 nCi (α - 32 P)GTP, and the indicated molar ratios of TFIIIA. They were then incubated for 15 minutes. RNA was extracted and subjected to polyacrylamide gel electrophoresis as described in Materials and Methods.

the plasmid. The presence of a 5S RNA gene did not cause any uneven distribution of repair-like patches. Furthermore, we tested whether the injection of plasmid DNA along with purified transcription factor TFIIIA results in any changes in the distribution of the repair-like patches. TFIIIA is one of the transcription factors required for 5S RNA gene transcription (24). Coinjection of TFIIIA at various doses increased the transcription of the 5S RNA gene by at least 10-fold (Fig. 7). However, the distribution of repair-like patches was not affected; the relative intensity of the DNA fragment carrying the 5S RNA gene (Fig. 8; see the 249bp fragment in lanes 1 and 3, and 147 bp fragment in lanes 2 and 4) remained the same in the presence or absence of TFIIIA. It is noted that the total level of repair-type DNA synthesis increased in the presence of TFIIIA (Fig. 8, compare lanes 1 and 3). Our extensive studies revealed that the stimulation of DNA synthesis by TFIIIA was reproducible only when we used a certain population (about 30%) of frogs (Yasui and Ryoji, unpublished observation). Thus we do not know at present the biological significance of this phenomenon. Nevertheless, activation of 5S RNA transcription (Fig. 7) and the random distribution of the repair-like patches were always seen regardless of whether TFIIIA stimulated DNA synthesis or not (data not shown).

Effects of Egg Activation on the Early DNA Synthesis

DNA replication in eggs requires activation of the injected eggs (2), which can be achieved by a variety of methods (25). We examined whether the activation also triggers the repair-type DNA synthesis. Although it is not possible to block activation completely once eggs are pricked for injection, the timing of activation can be altered by salt concentrations of the medium or by treatment of eggs with Ca^{2+} -ionophore A23187. Hypertonic medium such as (1.5 $\times$)OR-2 seemed to delay the activation reaction, because the cortical furrow formation for egg cleavage did not become apparent even after incubation for 2 hours in this medium (data not shown). On the other hand, Ca^{2+} -ionophore is known to

Figure 8. Effects of TFIIIA binding on the distribution of repair-like patches. Eggs were injected with 7.5 ng pXbsF201, 100 nCi (α - 32 P)dCTP, and TFIIIA (the molar protein:DNA ratio = 1), and incubated for 15 minutes. DNA was extracted, digested with BamHI/HindIII (lanes 1 and 3) or HaeIII (lanes 2 and 4), and subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. The sizes of HaeIII restriction fragments are indicated in bp on the right.

accelerate the contraction of the pigmented cortical layer in the animal half, which is characteristic of activated eggs (26). We found that neither condition significantly affects the level of DNA synthesis observed 15 minutes after DNA injection (Fig. 9, A and B). It appears that the initial repair-type DNA synthesis does not depend on egg activation. This conclusion is consistent with the observation that a very similar type of DNA synthesis takes place in oocytes which are not activatable by pricking (Ryoji *et al.*, manuscript in preparation).

Entry of Repaired DNA into Replication

We have shown that the early DNA synthesis which generates repair-like patches is carried out by replicative DNA polymerase α or δ (Fig. 3). It is possible that those polymerases which have participated in the early DNA synthesis might stay on DNA, and carry out the subsequent replication steps. Consequently, the template for the early DNA synthesis might be utilized for the subsequent replication more efficiently than the non-template DNA. We analysed what fraction of DNA molecules carrying the repair-like patches can replicate in the subsequent replication reaction. Injected DNA was pulse-labeled for 15 minutes through the initial repair-type DNA synthesis, and then further incorporation of the radioactive precursor was blocked by subsequent injection of excess cold dCTP. After

Figure 9. Effects of egg activation on the early DNA synthesis. (A) Effect of Ca^{2+} -ionophore A23187 treatment. Eggs were injected with 7.5 ng pXbsF201 and 100 nCi (α - ^{32}P)dCTP. They were treated with or without Ca^{2+} -ionophore A23187 (0.2 $\mu\text{g}/\text{ml}$) in ($\times 1/2$) OR-2 medium for 5 minutes and then incubated for another 10 minutes without Ca^{2+} -ionophore. DNA was extracted, digested with BamHI and subjected to agarose gel electrophoresis. (B) Effect of hypertonic medium. Eggs were injected as described above. They were incubated in ($\times 1/2$) or ($\times 3/2$) OR-2 medium for 15 minutes. DNA was processed as above.

incubation of the injected eggs for 5 hours, replicated DNA was quantified as the amount of DpnI-resistant DNA. Densitometer tracing of Fig. 10 (lane 2) indicated that 12% of labeled DNA molecules became DpnI-resistant above the background generated even in the early DNA synthesis (Fig. 10, lane 1). Here we assume that all of the labeled DNA have equal chances for replication regardless of whether they carry extensive repair-like patches or only a few. The radioactivity in the DpnI-resistant band is not from the residual incorporation of radioisotope even in the presence of excess cold dCTP during the replication mode of DNA synthesis, because at this dose of cold dCTP, incorporation of the radioisotope was diluted out to less than 3% of the DpnI-resistant band (data not shown). We then determined the replication efficiency of the total injected DNA. The plasmid DNA which had been prelabeled with ^{32}P in *E. coli* was injected into eggs, and incubated for 5 hours without the radioactive precursor. DpnI analysis of the isolated DNA indicated that about

Figure 10. Replication efficiency of DNA carrying repair-like patches. Lanes 1+ and 1-, eggs were injected with 7.5 ng pXbsF201 and 100 nCi (α - ^{32}P)dCTP, and incubated for 15 minutes; Lanes 2+ and 2-, eggs were injected as lanes 1+ and 1-. After 15 minutes, they received an additional injection of 700-fold excess cold dCTP (2 nmoles per egg), and incubated for 5 hours in total; Lanes 3+ and 3-, eggs were injected with 7.5 ng pXbsF201 already labeled with ^{32}P (see text), and incubated for 5 hours in the absence of (α - ^{32}P)dCTP; Lanes 4+ and 4-, the same ^{32}P -labeled DNA was digested without injection as a control. In all cases, DNA was digested with either BamHI alone (in lanes with a minus) or BamHI/DpnI (in lanes with a plus). DpnI digestion was carried out in a buffer containing 0.2 M NaCl.

15% of the injected DNA was utilized for replication (Fig. 10, lane 3), which is very similar to the efficiency of DNA carrying the repair-like patches. Since the majority of injected DNA molecules do not experience the initial repair-type DNA synthesis (see Discussion), the data implies that the replication machinery selects the template randomly regardless of whether the template has gone through the early repair-type DNA synthesis or not. It should be mentioned that, in Fig. 4, a much larger percentage of DNA is DpnI-resistant (compare lanes 7 and 8). However, it does not contradict the data described here, because the radioactive band in lane 7 (Fig. 4) represents newly synthesized DNA in injected oocytes, but not total injected DNA which is not radioactive.

DISCUSSION

We have described the nature of DNA synthesis observed immediately after injection of DNA in *Xenopus* eggs. Most of the products are short repair-like patches distributed randomly in the injected DNA. Although the plasmid DNA we used for injection was prepared as the supercoiled form through CsCl equilibrium density gradient centrifugation, it might have still contained open circular DNA carrying single-stranded gaps. Such a structure would be a good target for this type of DNA synthesis. However, as discussed below, nicked DNA or even intact ones might also be utilized for the repair-type DNA synthesis perhaps depending upon a mechanism similar to the nick translation (27). Whatever the structure of the template is, the repair-type DNA synthesis appears to occur to only a fraction of injected DNA. We can roughly estimate it to be 5 to 30% of the injected DNA molecules from the following reasons. Firstly, we calculated the total length of repair patches based on the level of (α - 32 P)dCTP incorporation, the size of the endogenous dCTP pool (28), and the GC content of the plasmid DNA. In order to assess the specific activity of the isotope after injection, we assumed that injected (α - 32 P)dCTP and endogenous dCTP are distributed similarly within the injected egg. The average size of repair was thus estimated to be equivalent to 3% of either strand (3 kb) of the template if the repair-like patches are evenly distributed in all the DNA molecules injected. Since we observed that the density shift of the BrdU-substituted DNA in the major peak is negligible in the initial 15 minute reaction (Fig. 5, filled circles), the synthesis cannot be attributed to a very small fraction, e.g., less than 5% of the injected DNA molecules; otherwise, more than 60% ($3\% \times 100/5$) of either strand would be displaced by the newly synthesized heavy strand, which should in turn have caused a detectable level of density shift. It should be mentioned that this estimated value (5%) has to be lowered considerably if the specific activity of the isotope in the injected egg is much higher than expected. For instance, such a discrepancy occurs when the endogenous dCTP is highly localized in a compartment not involved in DNA synthesis. Secondly, we noted that the density of the initially repaired DNA gradually shifts toward the HL position in the subsequent DNA synthesis occurring from 15 to 60 minutes after DNA injection: (Fig. 5, open circles). If a large portion, e.g., 30% of the injected DNA molecules are subjected to the repair-type DNA synthesis, one would expect a clear peak of DNA to be still present in the intermediate density fractions between the HL and LL positions of the gradient even after 5 hour incubation; this is because only 12% of the DNA carrying the repair-like patches are presumed to replicate and shift to the HL and HH positions during this period of time (Fig. 10, lane 2+). Although we do not have our own data, such intermediate density DNA was not seen in the similar experiment by others who injected 3 H-DNA and examined its density change after 5 hours (see Fig. 6A of ref. 2). Therefore, the template for the repair-like DNA synthesis does not comprise a large fraction like more than 30% of the injected DNA molecules.

As discussed above, the density of the DNA containing repair-like patches shifts from the LL position to the intermediate fractions between HL and LL during the period of 15 to 60 minutes after injection (Fig. 5, upper panel). This density shift is indicative of multiple repair patch formation in the same DNA molecule. Additional repair-type reactions during this period are mostly directed to those already carrying repair-like patches rather than to the majority of DNA which have not yet experienced the reaction. Otherwise, the density of the labeled material would remain close to the LL position just as the 15 minute product. It is possible that DNA polymerase remains on the DNA upon completion of the first repair-like patch, and then participate in subsequent rounds of DNA synthesis. Despite the formation of multiple repair-like patches in the same DNA molecules, the structure of DNA in the majority of the repaired DNA was already covalently closed circles after 15 minutes (Fig. 2A), and nicked or relaxed circular DNA did not accumulate in the subsequent reaction from 15 to 60 minutes after injection (Fig. 2B). Thus, the DNA must be religated and resupercoiled quickly upon completion of each repair-like patch. This observation further indicates that even intact DNA becomes a template for the repair-type DNA synthesis described here.

We observed that the early DNA synthesis is carried out by replicative DNA polymerase α or δ . Despite the use of common DNA polymerases in the early repair-type DNA synthesis described here and in the subsequent DNA replication, we found that these two reactions are mutually independent: The repair-type DNA synthesis is not affected by egg activation (Fig. 9), whereas the activation is required for DNA replication. Further, the initial association of DNA with DNA polymerase, which carries out the repair-type DNA synthesis, does not seem to facilitate the entry of the same DNA molecule into the subsequent replication mode (Fig. 10). The DNA molecules which are used for the initial DNA synthesis, and the ones which are not both replicate with similar efficiencies. However, it should be mentioned that even those DNA molecules which are not utilized for the initial repair-type DNA synthesis are mostly intact circles, since we purified DNA through CsCl density gradient. Thus, our data does not imply that damaged but unrepaired DNA is as active a template for replication as intact ones. In the case of yeast, there is a mechanism to arrest cells before S phase until chromosome damage is repaired (29).

We speculate that DNA synthesis which appears to insert short repair-like patches even in intact DNA as described in this paper may have some roles other than just repairing accidentally damaged DNA. For example, DNA repair synthesis following the formation of gaps and nicks in DNA would facilitate chromatin assembly and disassembly processes which require topological changes as well as flexibility of DNA (Ryoji, *et al.*, manuscript in preparation). Such chromatin rearrangement as might occur in the absence of chromosome replication could be important when cells switch the pattern of gene expression without going through S phase. The emergence of nuclease hypersensitive sites in the flanking sequences of activated genes (for a review, see ref. 30), and an increase of single-stranded nicks in differentiating cell chromosomes (for example, see ref. 31) could be relevant to this speculation.

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