Minichromosome assembly accompanying repair-type DNA synthesis in *Xenopus* oocytes

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ABSTRACT

Minichromosomes were assembled by injection of circular DNA into the nucleus of *Xenopus* oocytes. We observed that, in the course of DNA supercoiling and chromatin assembly, a small percentage of the injected DNA molecules incorporated a radioactive precursor. This DNA synthesis was carried out by aphidicolin-sensitive DNA polymerase, and generated short repair-like patches covalently linked to the injected DNA. We found that the DNA thus repaired was rapidly supercoiled almost to completion within 15 to 30 min after injection, whereas 60 to 120 min were required to supercoil the intact, bulk DNA molecules. Such differential supercoiling kinetics was also observed when UV-damaged DNA was injected. Chromatin assembly, which was characterized by DNA fragment sizes protected from micrococcal nuclease digestion, was consistent with the rapid DNA supercoiling and proceeded more efficiently on the repaired DNA. These results indicate that there are at least two kinetically distinct ways of assembling minichromosomes in the oocyte nucleus, and that the repaired DNA molecules preferentially follow the faster pathway.

INTRODUCTION

The assembly of chromatin-like structures on small circular DNA is a useful model to understand how histones are placed on DNA in an orderly fashion. A number of studies have been carried out both *in vivo* and *in vitro*. Some of the findings relevant to our present work can be summarized as follows: (a) Topoisomerase activities are required to wrap the circular DNA around the nucleosome core thereby generating negative supercoils in the DNA (1). (b) While purified histones can directly associate with DNA, and are reconstituted into a nucleosomal structure *in vitro* under appropriate conditions, the nucleosomes thus assembled are not regularly spaced (1–5). On the other hand, the assembly of minichromosomes in some cell-free extracts gives rise to a regular array of nucleosomes resembling the structure of bulk chromatin observed in the cell (6–10). (c) In such extracts, histones may be deposited on DNA through a transient association with acidic carrier proteins, or they may be subjected to temporary modifications until maturation of the nucleosomes (9,11,12). (d) ATP appears to be involved in the DNA supercoiling reaction and the formation of a regular array of nucleosomes (6,7). (e) It is noteworthy that, in the cell-free extracts or in living cells, linear or nicked circular DNA does not seem to serve as a good substrate for minichromosome assembly, because the nucleosome-like structure thus formed is neither regularly spaced nor stably associated with DNA (6,13).

The actual chromatin assembly process in the cell primarily occurs during chromosome replication. The parental DNA is unwound to form a single-stranded region as the replication fork proceeds. Core histones and then histone H1 are sequentially placed on DNA (14) soon after a short stretch of the complementary strand is synthesized (15). Since a newly

synthesized strand has at least one free end at the replication fork, the DNA in this region is not under strict topological constraint. Such a relatively flexible structure of DNA, together with the presence of the large 'replisome' complex in the same area (16), is likely to affect the process of chromatin assembly. Recent studies on the replication of circular DNA *in vitro* have in fact shown that the minichromosome assembly is tightly coupled with the simultaneous occurrence of DNA replication (17–19). Similar interactions of chromatin assembly and DNA synthesis may be seen when DNA is damaged by various agents. Nucleosomes appear to be either released from DNA or at least disrupted during the formation of nicks and gaps in the DNA (20–23). Then, they are reassembled as the gap is filled by repair DNA synthesis. In this communication, we describe repair-type DNA synthesis which occurs when circular DNA is injected into the nucleus (germinal vesicle) of the *Xenopus laevis* oocyte. We then examine how such a DNA template is assembled into the minichromosome structure.

MATERIALS AND METHODS

Chemicals, enzymes and frogs

($\alpha^{32}\text{P}$)dCTP was purchased from New England Nuclear. Dideoxythymidine triphosphate (ddTTP) and aphidicolin were obtained from Sigma. Restriction enzymes and micrococcal nuclease were supplied by Promega and Calbiochem, respectively. A nick translation kit (Bethesda Research Laboratories) was used to prepare hybridization probes following the manufacturer's instructions. Nylon membrane (Zeta probe) was obtained from Bio-Rad. Large females of *Xenopus laevis* were purchased from Nasco.

Plasmid and marker DNA

Plasmid pUC9 has been described by Vieira and Messing (24). Plasmid pXbsF201 is a derivative of pUC9 with an insert of a *Xenopus borealis* somatic-type 5S RNA gene (25) between the Hind III and BamHI sites (26). Supercoiled DNA was prepared by alkaline lysis of host *E. coli* cells, and then purified through two cycles of CsCl-ethidium bromide equilibrium density gradient centrifugation. DNA was finally dissolved to a concentration of 0.75 $\mu\text{g}/\mu\text{l}$ in a buffer containing 5 mM Hepes (pH 7.6), and 80 mM NaCl. The 123 bp ladder used for molecular size markers was purchased from Bethesda Research Laboratories.

UV-irradiation of DNA

Supercoiled DNA was irradiated with UV light (254 nm) under the Spectroline high-intensity lamp (Spectronics Corp., Westbury, New York). The UV-dosage was determined using the Spectroline digital radiometer (*idid.*).

Microinjection into Xenopus oocytes

Supercoiled DNA (7.5 ng per oocyte) was injected into the nucleus of stage VI oocytes together with 100 nCi per oocyte of ($\alpha^{32}\text{P}$)dCTP by the method previously described (27,28). Injected oocytes were incubated at 20°C for various periods of time as indicated, harvested one by one in microcentrifuge tubes, and frozen immediately in dry ice-methanol. Radioactivity of each oocyte was monitored by a Geiger counter, and those containing far above or below the mean radioactivity were discarded. DNA was extracted as described previously (29).

Micrococcal nuclease digestion

A group of ten oocytes, each of which had been incubated for 30 ± 2 min or for 120 ± 4 min after injection, were gently homogenized in 100 μl of ice-cold chromatin digestion buffer (28). Then CaCl_2 was added to a concentration of 1 mM. Digestion was started

by adding 13 units per oocyte of micrococcal nuclease. The mixture was incubated for either 2 or 6 min at 24°C, and then 100 μ l of a solution containing 30 mM Tris-HCl (pH 7.4), 20 mM EDTA (pH 8.0), 1% sodium dodecylsulfate and 200 μ g proteinase K was added to stop the reaction. DNA was phenol-extracted, ethanol-precipitated, and subjected to electrophoresis.

Agarose gel electrophoresis

Separation of circular or linearized DNA was carried out in 1% agarose gels with TPE buffer (33 mM Tris-phosphate [pH 7.2] and 0.7 mM EDTA). Separation of denatured DNA was performed in 1% agarose gel using the alkaline electrophoresis buffer (30 mM NaOH and 1 mM EDTA) described by McDonnell *et al.* (30). Micrococcal nuclease digests were separated in 1.5% agarose gels with Tris-glycine buffer (50 mM Tris base, 384 mM glycine, and 1 mM EDTA). For direct autoradiography, gels were dried and exposed to X-ray films. In the case of the micrococcal nuclease digests, DNA was transferred first to a nylon membrane as described below, which was then exposed to x-ray film.

Southern blot analysis

After electrophoresis, DNA was blotted by capillary action onto a nylon membrane using 0.5 M NaOH, and then the blot was probed with 100 ng of nick-translated 32 P-pXbsF201 or 32 P-pUC9 (about 10^7 cpm) in 10 ml of the hybridization buffer according to the method described (31).

RESULTS

Time course of DNA synthesis in oocytes

The *Xenopus* oocyte nucleus can support a low level of DNA synthesis upon injection of foreign double-stranded DNA, despite the fact that the replication of its own chromosomes is blocked (32,33). As of yet, the nature of this DNA synthesis has not been well documented. We separated the products from background material by gel electrophoresis and examined how the reaction proceeds after injection of plasmid pXbsF201 DNA. The DNA for injection was prepared through two cycles of CsCl-ethidium bromide equilibrium centrifugation, thus consisting mostly of the supercoiled form. A time course of the rate of DNA synthesis is depicted in Fig. 1. (α^{32} P)dCTP was incorporated into various topoisomers of the injected circular DNA, and the incorporation was most pronounced during the initial 30 min. At later time points, the rate of DNA synthesis measured by a 30 min pulse of (α^{32} P)dCTP declined to very low levels. This reduction is due either to the completion of DNA synthesis or to the formation of a histone-DNA complex causing steric hindrance to the association of DNA polymerase with DNA. It is likely that histones are already associated with DNA 30 min after injection, although maturation of the minichromosomes requires a much longer time (9,28).

We previously reported that a similar type of DNA synthesis occurs in *Xenopus* eggs prior to the onset of DNA replication (34). We showed that it is a repair-type DNA synthesis carried out by DNA polymerase α or δ . However, oocytes are very different from eggs in several respects. When oocytes mature into eggs, the nuclear envelope is dissolved, and the content of the germinal vesicle (GV) is diffused into the cytoplasm (for a review, see ref. 35). In parallel, protein synthesis is stimulated causing a two-fold increase of DNA polymerase activities (36). A series of events leading to DNA replication and mitosis are triggered by activation of the egg, which further makes the eggs distinct from the oocytes. Therefore DNA synthesis in oocytes may not necessarily be identical in nature to the repair-type reaction previously observed in eggs, so we decided to further characterize this reaction.

Figure 1. Time course of the rate of DNA synthesis. Supercoiled pXbsF201 was injected into groups of twelve oocytes. After indicated times of incubation, each oocyte received ($\alpha^{32}\text{P}$)dCTP, and was then incubated for additional 28 to 30 min. A portion of DNA equivalent to one oocyte was separated by electrophoresis and detected by autoradiography (lanes under -). Another portion of DNA was linearized with BamHI, and analyzed in parallel with the undigested DNA (lanes under +). I, supercoils; Ir, covalently closed, but relaxed circles; II, nicked circles; III, linear; I*, dimer supercoils.

DpnI digestion of the product

To examine whether the DNA synthesis as described above represents DNA repair or replication, we digested the radioactive products with the restriction endonuclease DpnI. This enzyme is known to cleave GACT sequences only when the adenine is methylated in both strands (37). DNA isolated from wild-type *E. coli* strains is fully methylated due to the *E. coli* dam methylase (37). If one of the DNA strands in this region is displaced with newly synthesized DNA lacking methylated adenines, DpnI can no longer cleave this hemimethylated sequence under 0.2M NaCl (34,38). Since more than eleven DpnI sites are distributed throughout the plasmid pUC9 which was used in this analysis, repair synthesis even at several of these sites will not convert the plasmid DNA to the DpnI-resistant form. On the other hand, progeny DNA produced by replication should be completely refractory

Figure 2. DpnI digestion of ^{32}P -labeled DNA. A mixture of pUC9 DNA and $(\alpha^{32}\text{P})\text{dCTP}$ was injected into two groups of seven oocytes. Each group was incubated for either 10 or 30 min. A portion of extracted DNA equivalent to one oocyte was digested either by BamHI alone or by BamHI and DpnI. The digests were electrophoresed and detected by autoradiography. The 30 min sample in the right panel represents a shorter exposure of the one shown in the left panel.

to this enzyme. We first linearized the product at the unique BamHI site, and then digested it further with DpnI in the presence of 0.2M NaCl (Fig. 2). The product synthesized during the initial 10 min was converted completely to short fragments (see the lanes under '10 min' in the left panel), indicating that the synthesis was of a repair type similar to the one we observed in the egg (34). It is likely that a minor fraction of the injected DNA molecules, which already carried nicks and gaps even before injection, were subjected to the repair reaction. It is also possible that such damages in DNA might have been generated in oocytes after injection. A further incubation gave rise to a small amount of DpnI-resistant DNA (see lanes under '30 min' in the left panel). A shorter autoradiographic exposure to the level of the '10 min' lane still gave a visible DpnI-resistant band (Fig. 2, right panel), indicating that the emergence of the DpnI-resistant DNA after 30 min was not simply due to an increase in the total level of DNA synthesis, but to a qualitative change in the mode of DNA synthesis. Although we do not have any further evidence, an infrequent, but processive reaction like the nick translation might have completely displaced one of the parental strands with a newly synthesized one. A rare replication event is also a possibility. A prolonged incubation of up to 2 hr did not increase the DpnI-resistant DNA to a significant extent (data not shown). Thus, both modes of DNA synthesis, i.e., DpnI-sensitive and -resistant ones, occur mostly in the initial 30 min after injection of DNA.

Alkaline gel electrophoresis of the product

The result of the DpnI digestion does not rule out an abortive replication in which labeled DNA fragments are hydrogen-bonded to the unwound parental strands. Such fragments, if any, can be released from the parental DNA by alkaline denaturation. On the other hand, the repair-like patches covalently linked to the parental DNA cannot be freed by

Figure 3. Alkaline gel analysis of the ^{32}P -labeled DNA. Oocytes were injected with pUC9 DNA and $(\alpha\text{-}^{32}\text{P})\text{dCTP}$, and were incubated for 30 min. Extracted DNA was electrophoresed using the regular TPE buffer (lane 2) or the alkaline buffer (lane 4) as described in Materials and Methods. As a control, pUC9 DNA which was ^{32}P -labeled by nick-translation was also applied in the same gels (lanes 1 and 3). Nonlabeled linear and supercoiled pUC9 DNAs were used as position markers, which were detected by ethidium bromide staining as indicated on the side of each autoradiograph. I*, I_r*, II*, and III*, the dimer forms of DNA corresponding to those without asterisk seen in Fig. 1; BPB and BCB, the migration positions of bromophenol blue and bromocresol blue, respectively.

denaturation. Thus, the DNA which had been labeled with ^{32}P for 30 min in oocytes was separated in alkaline and neutral gels side by side (Fig. 3). As expected, the majority of the labeled DNA migrated to the position of supercoiled DNA in the neutral gel (lane 2). In the alkaline gel, the major radioactive band was also seen at the position of the supercoiled DNA marker detected by ethidium bromide staining (lane 4). The material migrating as a smear behind the supercoiled DNA in this lane is presumably a mixture of single-stranded linear and circular DNA both generated by denaturation of forms II and III DNA. Nick-translated DNA applied in these gels as a control displayed forms II and III in the neutral gel (lane 1), whereas in the alkaline gel, there was no discrete band except for a faint smear of short single-stranded DNA fragments (lane 3). Most of such fragments were lost upon drying the gel. From these results, we concluded that the newly synthesized DNA is present in the form of repair-like patches covalently linked to the template. We noted that the intensity of the supercoiled DNA in the alkaline gel (lane 4) was somewhat stronger than that in the neutral gel (lane 2), although we applied the same amount of material in both cases. The intermediately supercoiled DNA molecules, which were resolved between the positions of forms I and II in the neutral gel (lane 2), seemed to comigrate with the highly supercoiled ones in the alkaline gel thereby contributing to the increase of the intensity (lane 4).

Figure 4. Aphidicolin sensitivity of DNA synthesis. A 40 nl mixture of pUC9 DNA, ($\alpha^{32}\text{P}$)dCTP, and aphidicolin (two different doses as indicated) was injected into groups of ten oocytes. Since aphidicolin was dissolved in 10 nl of DMSO, the control injection (0 ng aphidicolin) also contained the same volume of DMSO. They were incubated for 30 min. A portion of DNA equivalent to one oocyte was electrophoresed with (+) or without (-) BamHI digestion, and detected by autoradiography.

Effect of aphidicolin

There are at least three species of DNA polymerase, α , β , and δ , which are involved in DNA repair synthesis in eukaryotes (39–41; for a review, see ref. 42). DNA polymerases α and δ are also responsible for chromosome replication (for reviews, see ref. 16 and 43). While it is difficult at present to distinguish α from δ , various inhibitors are available to differentiate α and δ activities from β . For example, aphidicolin is known to inhibit α and δ , but not β activities (44,45). Fig. 4 shows a dose response of the DNA synthesis to aphidicolin. Since dimethyl sulfoxide (DMSO) was necessary to solubilize aphidicolin (44), the injection shown in the control lane also contained the same volume of DMSO without aphidicolin. We found that DMSO itself was inhibitory, which resulted in an increase of the relative amount of form II DNA (compare Fig. 1, the lane under 0 min and Fig. 4, the lane under 0 ng), yet the experiment should reveal the effect of aphidicolin. It is seen in this figure that 2 ng of aphidicolin per oocyte reduced the DNA synthesis occurring in the initial 30 min to about a 25% level. If this amount of aphidicolin is dispersed homogeneously within a 50 nl GV, the concentration of aphidicolin will be about 40 $\mu\text{g}/\text{ml}$, which is close to the highest among the reported doses for the inhibition of DNA polymerase α and δ *in vitro* (2 to 50 $\mu\text{g}/\text{ml}$, see ref. 44 and 45). Yet this concentration is not inhibitory to DNA polymerase β *in vitro* (42). In addition, we think that the actual concentration within the GV was somewhat lower than the estimated value, because aphidicolin appeared to diffuse gradually into the cytoplasm as evidenced by partial recovery of DNA synthesis

Figure 5. Time course of DNA supercoiling. A mixture of pXbsF201 DNA and ($\alpha^{32}\text{P}$)dCTP was injected into groups of ten oocytes. They were incubated for 2 to 120 min as indicated. A portion of extracted DNA equivalent to two oocytes was separated by electrophoresis, and the dried gel was subjected to autoradiography for 48 hrs (lower panel). Another portion of the extracted DNA equivalent to one oocyte was separated in the same gel as above. This part of the gel, however, was processed for Southern blot analysis as described in Materials and Methods. The autoradiographic exposure was 1 hr (upper panel). At this level of exposure, the radioactive, repaired DNA bands, though present without hybridization, were not detectable. M, plasmid pXbsF201 DNA before injection.

1 hr after preinjection of aphidicolin (data not shown). From these considerations, we suggest that either polymerase α or δ , but not β is the one which carries out the plasmid-dependent DNA synthesis in oocytes.

Supercoiling of repaired DNA

The assembly of minichromosomes is accompanied by DNA supercoiling (1). We noticed that most of the repaired DNA was already supercoiled in the initial 30 min (Fig. 1). We followed this reaction more closely, and compared the supercoiling kinetics with that of unrepaired DNA. Our previous studies on a similar type of DNA synthesis in *Xenopus* eggs indicated that the repaired DNA is only a minor fraction which was indirectly estimated to be somewhere between 5 and 30% of injected DNA (34). If the repaired DNA is indeed a minor population of injected DNA in oocytes as well, then the behavior of the total DNA would essentially represent that of the unrepaired DNA. The supercoiling kinetics of the repaired DNA was analyzed by direct autoradiography of a dried gel, while total DNA from the same group of injected oocytes was monitored by Southern blot analysis performed simultaneously (Fig. 5). Although both analyses employed a ^{32}P -label, we found that the band intensity detected by Southern analysis was at least 90-fold higher than that produced by the repair synthesis in this experiment (see the figure legend). Therefore, the signal

from the repair synthesis did not interfere with the Southern analysis. Consistent with the result in Fig. 1, the repair reaction of plasmid pXbsF201 almost reached a plateau after 30 min (Fig. 5, lower panel). At this time point, the supercoiling of DNA was also near completion. By contrast, the total DNA, probed with nick-translated ^{32}P -pXbsF201, exhibited much slower supercoiling kinetics subsequent to the rapid relaxation in the first 2 min (Fig. 5, upper panel). It took almost 2 hr for the bulk DNA to reach the supercoil level obtained by the repaired DNA within 30 min. Note that the bands representing lower supercoils were still evident in the 1 hr sample of the total DNA. In this figure, the autoradiographic exposure was adjusted so that the DNA migrating in the region of form I DNA gave comparable intensities in total and repaired DNA. To examine the highly supercoiled topoisomers accumulating at the position of form I DNA in this gel, we employed a chloroquine-containing gel (28). A concentration of 15 $\mu\text{g}/\text{ml}$ of chloroquine phosphate in the gel brings about two major changes in the migration pattern of DNA. While nicked circular DNA (form II) remains to be the slowest species, fully supercoiled DNA (form I) such as those isolated from *E. coli* migrates only a little ahead of form II (Fig. 6). Instead, form Ir (covalently closed, but relaxed circles) becomes the fastest species. As the supercoiling reaction proceeds, the migration position shifts upward to the site indicated by I in this figure. It is evident that the supercoil level of repaired DNA almost reached a plateau after a 30 min incubation, and only a few more turns of supercoils were introduced upon 60 min incubation (Fig. 6, lower panel). The faint Ir band present in the 120 min sample appears to be generated by an artifactual relaxation of DNA during the oocyte homogenization as reported previously (46). On the other hand, the bulk DNA became relaxed to forms II and Ir within 2 min, and then slowly resupercoiled in the next 2 hr (Fig. 6, upper panel). After the 2 hr incubation, there was no clear difference in the mean superhelicity between the repaired and bulk DNA. The distinct difference detected in the early supercoiling kinetics between the repaired and the bulk DNA confirmed the notion that the repaired DNA represents only a minor fraction, perhaps less than 10% of the injected DNA molecules. It is important to point out that the slower supercoiling kinetics of the bulk DNA is due to the supercoiling reaction itself, but not to the slow ligation of nicked form II DNA. As seen in this figure, form II DNA constituted only a minor portion of the bulk DNA after 5 min incubation (upper panel), and the majority of the DNA molecules were present as form Ir or as intermediately supercoiled ones. Thus most of the initially supercoiled DNA molecules become relaxed as intact, covalently closed circles upon injection, and then proceed slowly to the chromatin assembly reaction in this system. It should be mentioned that the results described here do not depend on the 5S RNA gene insert present in the plasmid pXbsF201. We observed that the parental plasmid pUC9 was also supercoiled rapidly within 30 min when it was repaired (see Fig. 3, lane 2 and Fig. 7A, lane 3). Note that the result in Fig. 4 (lane under 0 ng) is not appropriate to verify this point, because DMSO present in the injection mixture appeared to increase the form II DNA as discussed in the preceding section.

Supercoiling of UV-damaged DNA

So far we have shown that DNA which has been subjected to repair-type DNA synthesis is supercoiled more rapidly than the intact DNA. We do not know the nature of the damages which were repaired in this reaction, although they were likely to be nicks or gaps presumably present in a small portion of the injected DNA molecules even after two cycles of CsCl-ethidium bromide equilibrium centrifugation (see Materials and Methods). We wanted to know whether the repair of other, better defined damages would also be accompanied by accelerated supercoiling. Recently, Legerski *et al.* (47) reported that UV-

Figure 6. Analysis of DNA supercoiling kinetics by chloroquine-containing gel. The same materials as described in Fig. 5 were separated by a chloroquine-containing gel (see Materials and Methods). Lower panel, a portion of DNA equivalent to three oocytes was applied in the gel, and bands were detected in the dried gel by direct autoradiography for 39 hr; Upper panel, a portion of DNA equivalent to one oocyte was applied in the gel, and bands were detected by Southern blotting. The exposure time for autoradiography was 1 hr; M, plasmid pXbsF201 DNA before injection; I, position of form I DNA before injection.

induced lesions in circular DNA were efficiently repaired in the oocyte nucleus. Moreover, we noticed in their data that the repaired DNA seemed to be supercoiled rapidly within 30 min, although the above authors were not interested in such details of the supercoiling kinetics (see Fig. 4b of ref. 47). The low sensitivity method they adopted to detect bulk DNA also precluded one to closely compare the supercoiling kinetics between the repaired and bulk DNA (see Fig. 4a, *ibid.*). We repeated their experiment but replaced the ethidium bromide staining with the more sensitive Southern analysis. We irradiated plasmid pUC9 DNA with UV light (254 nm) at a fluence of 200 J/m². Further damages caused incomplete repair resulting in an increase of form II DNA after a 30 min incubation in oocytes (data not shown). At this dose of irradiation, the repair synthesis was stimulated about 4-fold relative to the control (compare lanes 3 and 6 in Fig. 7A). This is not due to variations in the DNA recovery from the injected oocytes, because comparable quantities of total DNA were present in all the samples (Fig. 7B). Despite the additional lesions in the the DNA caused by UV irradiation, supercoiling of the repaired DNA proceeded

Figure 7. Supercoiling kinetics of UV-damaged DNA. (A) Supercoiling of repaired DNA. Plasmid pUC9 DNA was irradiated with UV light at a fluence of 200 J/m². DNA (7.5 ng per oocyte) was then injected into oocytes along with ($\alpha^{32}\text{P}$)dCTP. The injected oocytes were harvested after the indicated periods of time. A portion of the extracted DNA equivalent to 2 oocytes was separated by agarose gel electrophoresis and detected by autoradiography. The exposure time was 35 hr. Lanes 1 to 3, unirradiated DNA control; lanes 4 to 6, irradiated DNA. (B) Comparison of DNA recovery. A portion of the extracted DNA equivalent to 0.2 oocytes was linearized by BamHI, and then subjected to Southern blot analysis using ^{32}P -labeled pUC9 DNA as a probe. The autoradiographic exposure time was 25 min. The material in each lane corresponds to that in (A). (C) Supercoiling of bulk DNA. A portion of the extracted DNA equivalent to 0.4 oocytes was subjected to Southern blot analysis without BamHI digestion. Only the material from the 30 min-incubated oocytes was analyzed. The autoradiographic exposure was 25 min at room temperature. Lane 1, unirradiated DNA control; lane 2, irradiated DNA.

as rapid as the unirradiated control (compare lanes 3 and 6 in Fig. 7A), except that slightly more form II DNA was accumulated at the beginning of the repair of the UV-damaged DNA (compare lanes 2 and 5 in Fig. 7A). We concluded that the UV-induced repair also accompanies accelerated DNA supercoiling kinetics. The supercoil level of the bulk, UV-damaged DNA at 30 min postinjection was indistinguishable from that of the unirradiated control (Fig. 7C), suggesting that the bulk of the UV-damaged DNA molecules were supercoiled without removal of the lesions. It is noted that the formation of thymine dimers partially unwinds DNA and changes the electrophoretic mobility of each topoisomer (47–49). One thymine dimer untwists DNA by about 12° (48,49). We did not clearly detect such a band shift of either uninjected (data not shown) or injected DNA (Fig. 7C). Thus, the DNA we used did not appear to be damaged severely.

Nucleosome assembly on the repaired DNA

We then asked whether the accelerated DNA supercoiling of repaired DNA (unirradiated) was also reflected in the kinetics of nucleosome assembly. Minichromosomes labeled with ^{32}P through the DNA repair synthesis in the initial 30 min were digested with micrococcal

nuclease to two different levels. The digests were subjected to electrophoresis, and the gel blot was analyzed by direct autoradiography without hybridization (Fig. 8A, left panel). This analysis showed that there were protected DNA fragments of about 200, 360 and

Figure 8. Micrococcal nuclease digestion of the minichromosomes. (A) 30 min minichromosomes. A mixture of unirradiated pXbsF201 DNA and ($\alpha^{32}\text{P}$)dCTP was injected into two groups of ten oocytes within 4–5 min. Each group was incubated for 30 min, and subjected to micrococcal nuclease digestion for either 2 or 6 min as indicated (see Materials and Methods). The digests were separated by electrophoresis and analyzed by Southern blotting using nick-translated ^{32}P -pXbsF201 as a hybridization probe (right panel). Autoradiography was carried out for 2 hr. At this level of exposure, the band intensities without hybridization (see below) were negligible. Duplicate samples were also blotted after electrophoresis, but autoradiography was performed for 40 hr without hybridization (left panel). Note that, in the lane of the 2 min digest (left panel), we accidentally applied a smaller amount of DNA. The numbers in bp on the right indicate positions of the size markers (123 bp ladder) detected by ethidium bromide staining. The numerals 1 to 3 between the two panels represent nucleosome monomers, dimers and trimers. I, position of supercoiled pXbsF201. (B) 2 hr minichromosomes. Injection and analyses were carried out as described in (A) except that the injected oocytes were incubated for 2 hr. Note that there is a small area between the mono- and dimer-size DNA (left panel) where the blotting was less efficient than in the other parts of the membrane.

530 bp over the background smear. They seemed to correspond to the monomer-, dimer- and trimer-sizes of nucleosomal DNA since a nucleosome monomer contains a 180–200 bp DNA fragment. It is also evident that the monomer-size DNA accumulated as digestion proceeded (see the 6 min lane). Thus the repair patches in the injected DNA molecules were mostly associated with nucleosomes or nucleosome-like structures 30 min after injection. Part of the repair patches were not fully protected, and gave rise to subnucleosome-size fragments, indicating that premature nucleosomes were also present on the repaired DNA. Such heterogeneous nucleosomal structures as well as somewhat irregular placement of nucleosomes contributed to the high level of background smears seen in both of the lanes. It was also reported that an assembly of regularly spaced nucleosomes depends upon the amount of DNA injected, and that the micrococcal nuclease ladder of minichromosomes assembled at a dose of 7.5 ng DNA per oocyte, as we used here, is not as clear as those observed when higher doses of DNA are injected (27,28). Note that the analysis described here is primarily limited to the localized regions of DNA molecules which carry the ^{32}P -labeled repair patches. The properties of the remaining portion of the same DNA molecules can potentially be assessed by the pattern of the longer DNA fragments produced during the 2 min digestion. Because of the high background smears discussed above, it did not show whether the nucleosomal ladder was present in the unlabeled regions as well. On the other hand, the nuclease sensitivity seen in this lane was very similar to the typical minichromosomes assembled on the bulk DNA after a 2 hr incubation as described below (see Fig. 8B, right panel), suggesting that the unlabeled region of the repaired DNA molecule did not possess any peculiar structures such as protein-free supercoils.

The bulk DNA detected by hybridization of the gel blot (Fig. 8A, right panel) displayed a similar pattern of digestion like that of the repaired DNA, except for much more monomer-size fragments produced upon 2 min digestion. This indicates that the bulk DNA carried a lower number of nucleosomes and therefore is more sensitive to micrococcal nuclease which attacks internucleosomal linker DNA. On the other hand, the monomer-size fragments were still minor species at this level of digestion of the repaired DNA (compare the lanes of 2 min digestion). It should be emphasized again that the DNA fragments labeled by the repair reaction were negligibly weak at this level of exposure necessary for detecting the fragments by hybridization, and did not interfere with the analysis (see the figure legend). These results demonstrated that the reaction of assembling nucleosomes was also faster on the repaired DNA than on the bulk DNA.

Further incubation of the injected oocytes appeared to produce more matured forms of minichromosomes. As seen in Fig. 8B, the repaired and the bulk minichromosomes after a 2 hr incubation both displayed a more distinct ladder of the micrococcal nuclease digest with reduced background smears. It was possible to detect up to tetramer-size DNA in both cases (see the lanes of 6 min digestion). In addition, the difference in the micrococcal nuclease sensitivity of repaired and total DNA became less evident in the 2 hr minichromosomes (compare the lanes of 2 min digestion). We concluded that the minichromosome assembly on the repaired and the bulk DNA only differs in the kinetics, but the final structure is indistinguishable at the level of micrococcal nuclease digestion analysis. This conclusion is consistent with the data of DNA supercoiling kinetics discussed above (Fig. 5 and 6).

DISCUSSION

We have shown that the DNA synthesis, observed when undamaged, double-stranded DNA is injected in *Xenopus* oocytes, is primarily of a repair type. Although we do not have

any evidence, this reaction may be directed to the nicks and gaps presumably present in a very small portion of injected DNA molecules. Alternatively, the target of the repair reaction such as apurinic/apyrimidinic sites may have been artifactually produced in the course of plasmid isolation. We also detected a low level of DNA synthesis which appeared to extend over the entire strand of the template. At present it is not technically possible to determine whether such a replication-like synthesis represents true replication or just an extensive repair-type synthesis. Studies employing aphidicolin suggested that the DNA synthesis we observed was carried out by DNA polymerase α or δ . In light of the report by others that only α -type activities were detected in the oocyte extract (50), DNA polymerase α may be the major species carrying out the observed DNA synthesis. This tentative conclusion is also consistent with the observation that the repair reaction is weakly retarded by DMSO (Fig. 4), which is known to inhibit DNA polymerase α , but not δ *in vitro* (51).

An intriguing observation in the present study is that there are two kinetically distinct pathways for minichromosome assembly in oocytes. DNA molecules which are subjected to the repair reaction preferentially take the faster pathway. This does not necessarily infer that the accelerated assembly of chromatin is driven by the repair reaction, because the rapidly assembling chromatin may have some unknown peculiarity in the structure which allows more efficient repair synthesis. If the repair reaction is in fact the cause of the faster assembly, one may ask whether a blockage of the repair reaction slows down the chromatin assembly process. We were not able to address this question by injecting an appropriate repair inhibitor. This is due to the loss of the radioactive tag from the repair template, which makes it infeasible to trace the chromatin assembly process occurring on only a small fraction of the injected DNA molecules. Such an experiment would require a cell-free system which is enriched with the repair enzymes thereby allowing a large portion of the damaged DNA molecules to get repaired simultaneously during the period of chromatin assembly. Nevertheless, we favor the possibility that the repair reaction facilitates chromatin assembly, since it has been demonstrated clearly that replication significantly affects or even is required for chromatin assembly in certain cell-free extracts (17,18).

There are two conceivable mechanisms for the facilitated chromatin assembly coupled with DNA synthesis: Firstly, the free 3' end of DNA present at the site of DNA synthesis may increase the topological flexibility of DNA for wrapping itself around the nucleosome core. However, it should be pointed out that this type of flexibility does not necessarily provide a favorable condition for the nucleosome assembly. For instance, the histone association with linear DNA injected in *Xenopus* oocytes did not stably protect DNA from micrococcal nuclease (13). Minichromosome assembly with linear DNA in a cell-free extract also supported this conclusion (6). Formation of unstable nucleosomes on nicked circular DNA, observed in an ATP-depleted cell extract where ligation of the nick was inhibited (6), may also be interpreted as an indication that a free end(s) of DNA does not necessarily lead to accelerated minichromosome assembly. In addition, we recently found that, unlike the observation in the oocyte, the supercoiling kinetics of repaired DNA in the injected egg is not as fast as that seen in the oocyte (see Fig. 2B in ref. 34), and is very close to that of the bulk DNA (Ryoji, unpublished observation). This observation cannot be easily explained if the presence of free DNA ends is important. The second possible mechanism is that DNA synthesis itself may facilitate minichromosome assembly as previously proposed by others (17,18). It is conceivable that the DNA polymerase may be associated with some factors necessary for assembling nucleosomes. In support of this notion, Smith and Stillman

(19) have recently described a novel protein factor, which is required for the replication-driven chromatin assembly *in vitro*. On the other hand, DNA synthesis in our system takes place only in localized regions of circular DNA, and we must in addition invoke a *cis*-acting mechanism which gives rise to global effects on the entire DNA molecule. For instance, topoisomerase activities which are likely to be present in the DNA polymerase complex might swivel DNA more efficiently than without, thereby facilitating the association of nucleosomes throughout the plasmid. A direct action of free topoisomerases on DNA could be kinetically less favorable particularly when histones are already bound to DNA in various forms of premature nucleosomes. An association of the DNA polymerase complex with DNA may provide a better entry site for topoisomerases. According to the analysis involving topoisomerase inhibitors, neither topoisomerase I nor II seemed to be required for DNA repair synthesis (47). However, this does not imply that the assembly of minichromosomes on the repaired DNA does not utilize topoisomerases. In fact, the same inhibitor caused an increase of the forms II and III of the repaired DNA suggesting that topoisomerases were interacting with the repaired DNA molecules (see Fig. 8 and 9 in ref. 47). This second mechanism is compatible to the aforementioned slower supercoiling reaction in the early stage of the activated eggs, if one assumes that a different species of DNA repair polymerase, which is not associated with the assembling factor(s), operates in these cells. It is possible that DNA polymerases α and δ may be differentially expressed in oocytes and in the early stage of activated eggs. Further studies are necessary to determine which of the above mechanisms operates in this system, and to understand how the actual chromatin is assembled in the cell.

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