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Multistep pathway for replication-dependent nucleosome assembly

(DNA replication/chromatin)

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ABSTRACT **We have used cell-free DNA replication to study the relationship between DNA replication and chromatin assembly. As others have reported, we find that DNA replication facilitates nucleosome assembly. We show here that replication-dependent nucleosome assembly occurs in at least two steps. The first step requires replicating DNA; the second step occurs after replication has been completed and is promoted by a nuclear extract. Consistent with this multistep model, we observe that the replicated simian virus 40 minichromosome is organized into a repeating array of DNA–protein particles that are structurally distinct from mature nucleosomes. These particles may be precursors in a pathway of nucleosome assembly since in the second, replication-independent step the nuclear extract converts this nascent chromatin into nucleosomes.**

Nucleosome assembly is coupled tightly to DNA replication. Newly replicated DNA will, within 20 min, organize into a chromatin structure indistinguishable from total cellular chromatin (1). Two processes contribute to chromatin assembly during DNA synthesis—redistribution of preexisting histones and formation of new nucleosomes. Preexisting histone octamers (parental nucleosomes) are stable; they do not disassemble and mix with newly synthesized histones (ref. 2 but see ref. 3). In some cases it has been observed that parental nucleosomes segregate to both the daughter DNA molecules (4–8). On the other hand there is also evidence that parental nucleosomes may segregate in long patches to just one of the two daughter DNA molecules (9, 10). More specifically, these parental nucleosomes segregate with the DNA strand that is the template for leading-strand synthesis at the replication fork (11).

Nascent DNA is organized within 1–3 min into a chromatin structure resembling bulk nucleosomal DNA, as shown by electron microscopy (12). However, velocity sedimentation analyses suggest that for about 20 min the structure of nascent chromatin may differ significantly from bulk chromatin (13–15). In support of this, nascent chromatin is more susceptible to nuclease digestion than bulk chromatin (13–20). Also, the repeat length for nascent nucleosomal DNA is approximately 20 base pairs shorter than that of bulk chromatin (16, 19, 20). The biochemical relationship between nascent and mature chromatin is not well understood, but observations in a number of systems suggest that intermediates in assembly might result either from a stepwise association of histones to form the nucleosome core or result from an ordered series of post-translational histone modifications (refs. 21 and 22; see *Discussion*). In addition, some of the changes in chromatin organization immediately after DNA replication may reflect the assembly of mononucleosomes into higher order levels of chromatin packaging.

A cell-free replication-dependent chromatin assembly system has been described (23, 24). This system requires a closed circular plasmid DNA template containing the simian virus 40 (SV40) origin of replication, purified SV40 tumor (T) antigen (which functions as the replication initiator protein), and a cytosolic extract prepared from any human or monkey cell that supplies all the other factors necessary for DNA replication. Replication faithfully copies the DNA template producing closed-circular relaxed daughter molecules as products. If the reaction is supplemented with a nuclear extract, the replication products are highly supercoiled (24), reflecting assembly of the daughter molecules into nucleosomes (25). The organization of nucleosomes assembled on SV40 DNA *in vitro* is indistinguishable from SV40 DNA assembled into chromatin *in vivo* (25, 26). We have used the SV40 system as a model to study the mechanism coupling nucleosome formation to DNA replication by analyzing the chromatin structure of newly replicated DNA.

MATERIALS AND METHODS

***In Vitro* Replication.** The nuclear extract and S-100 from cytosol were prepared from suspension cultures of Manca cells, a human Burkitt lymphoma cell line as described (25, 27). Replication reactions were performed as described (27) and replication products were analyzed by electrophoresis through agarose gels (22, 24). In some cases, replication reactions were preceded by a 30-min preincubation containing all replication factors except deoxy- and ribonucleoside triphosphates. Nuclear extract was added to the replication reaction mixture at a concentration of 0.9 μg of nuclear extract protein per pmol of dCMP incorporated into replicated DNA.

Micrococcal Nuclease Digestion. After DNA replication, the unincorporated nucleoside triphosphates were removed by chromatography through Sephadex G-50 containing 40 mM Hepes·KOH (pH 7.5), 8 mM $MgCl_2$, and 20 mM NaCl. Fractions containing the replicated DNA were adjusted to a final concentration of 40 mM Hepes·KOH (pH 7.5), 8 mM $MgCl_2$, 20 mM NaCl, 1 mM $CaCl_2$, 3 mM ATP, in a final volume of 100 μl. Products were digested at room temperature with either 1.6 units (replicated and unreplicated minichromosome) or 5.3 units (assembled minichromosome) of micrococcal nuclease (Worthington). Aliquots (20 μl) were removed at the indicated time points and reactions were terminated by adding EGTA to 10 mM. To analyze DNA–protein complexes, the reaction products were loaded on 4% polyacrylamide [acrylamide/*N*, *N'*-methylene bisacrylamide 40:2 (wt/vol)] and run in Tris acetate buffer (0.04 M Tris/0.02 M sodium acetate/2 mM EDTA, pH 8). To analyze DNA, the reaction mixtures were digested with proteinase K (0.2 mg/ml) for 30 min at 37°C in the presence of EDTA and SDS. The samples were extracted once with phenol/chloroform and analyzed on 6% polyacrylamide gels.

Isolation of Replicated Complexes. Duplicate 50-μl replication reaction mixtures were chromatographed at 4°C in 2-ml disposable pipettes (0.5 × 14 cm) on Sepharose CL-6B

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Abbreviations: SV40, simian virus 40; T, tumor.

(Sigma) equilibrated with 40 mM Hepes·KOH, pH 7.5/8 mM $MgCl_2$/5% (vol/vol) glycerol/1 mM ATP. Fractions of 100 μl were collected and fractions containing replicated minichromosomes were pooled. Twenty microliters of the pooled fractions was further incubated at 37°C in assembly reaction mixtures (50 μl) containing 40 mM Hepes·KOH (pH 7.5), 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 3 mM ATP, 40 mM creatine phosphate, creatine phosphokinase (1 μg/ml), nuclear extract and/or cytosolic S-100 for the indicated time.

RESULTS

Nucleosome Assembly *in Vitro* Is Facilitated by DNA Replication. In our initial experiments we confirmed that DNA replication is required for nucleosome assembly in this system. The products of a standard *in vitro* replication reaction were predominantly covalently closed circular DNA molecules (Fig. 1*A*). If an extract prepared from Manca cell nuclei was included in the reaction mixture, the replication products

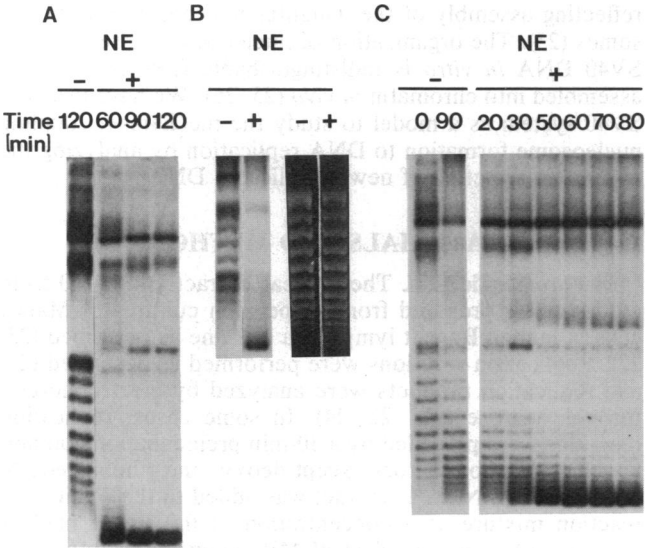

FIG. 1. (*A*) Addition of nuclear extract (NE) during DNA replication produces highly supercoiled replication products. DNA purified from a standard replication reaction mixture was analyzed by agarose gel electrophoresis and the replication products were visualized by autoradiography. The replication reactions were carried out in the absence (−) or presence (+) of 1.8 μg of nuclear extract protein. Covalently closed circular products (form I) from reaction mixtures lacking nuclear extract have low superhelical densities and migrate as a ladder of topoisomers. Replication products from reaction mixtures containing nuclear extract are highly supercoiled. Incubation times are indicated. (*B*) Replication reactions were carried out in the absence (−) or presence (+) of 1.8 μg of nuclear extract protein (NE). DNA was purified from the reations and fractionated by agarose gel electrophoresis. Total DNA was visualized by ethidium bromide staining (*Left*) and the replication products were visualized by autoradiography (*Right*). Note that in this experiment sufficient T antigen was added to replicate just 2–5% of the total DNA to demonstrate more dramatically the strong preference of assembly for replicated DNA. (*C*) Nuclear extract will induce supercoiling of DNA after replication has been completed. DNA was preincubated with cytosolic S-100 and T antigen for 30 min and then replication was initiated by addition of ribo- and deoxynucleoside triphosphates. After 30 min of replication, the reaction was chased with a 100-fold excess of unlabeled dCTP for the indicated times. Alternatively, 1.8 μg of nuclear extract protein (NE) was added during the chase and aliquots were removed at the indicated times (+). Reaction products were fractionated on agarose gels and visualized by autoradiography. Replication products formed prior to the dCTP chase (0 min) and after a 90-min chase without nuclear extract (−) are shown.

were highly supercoiled (Fig. 1*A*), reflecting assembly of the daughter molecules into nucleosomes (see below and ref. 25).

Under standard replication conditions only 10% or less of the input plasmid template replicated, due to limitations in the abundance of essential replication factors. It was possible to discriminate between replicated and unreplicated DNA by including [α-^{32}P]dCTP in the replication reaction. During replication the bulk of the input unreplicated DNA, which was visualized by ethidium bromide staining, remained relaxed even in the presence of Manca nuclear extract (Fig. 1*B*). However, the subpopulation of molecules that had replicated (i.e., those that had incorporated [^{32}P]dCMP) became highly supercoiled (Fig. 1*B*). This directly demonstrated that the replicated DNA was preferentially supercoiled in the presence of nuclear extract. Control experiments proved that all labeled DNA was the product of semi-conservative DNA replication. (*i*) The reaction products were resistant to digestion with *Dpn* I, which assays the methylated state of the DNA. (*ii*) All radiolabeled molecules are of either heavy or hybrid density in CsCl equilibrium density gradients after replication in the presence of BrdUTP, indicative of molecules having replicated twice or once, respectively.

Replicated Minichromosomes Were Marked. One interpretation of the above results was that some feature of actively replicating DNA, such as the unique structure of the replication fork, facilitated nucleosome assembly. Alternatively, it was possible that DNA replication stably altered some property of the minichromosome and that this alteration could be recognized, after replication had been completed, by the nucleosome assembling machinery. To distinguish between these models, we asked whether fully replicated daughter molecules remained preferred targets for nucleosome assembly.

To follow the assembly of nucleosomes on fully replicated daughter minichromosomes a replication reaction mixture was pulse-labeled with [α-^{32}P]dCTP for 30 min. Then, at the time of addition of Manca nuclear extract, the reaction mixture was chased with a 100-fold excess of unlabeled dCTP. This prevented any molecules that initiated DNA replication after the addition of the nuclear extract from becoming labeled. Fig. 1*C* shows that most of the labeled DNA molecules rapidly supercoiled upon addition of the nuclear extract; as shown below, supercoiling reflected the assembly of these molecules into nucleosomes. Since all labeled molecules became supercoiled, nucleosome assembly was not restricted to templates that were at intermediate stages of replication at the time of addition of nuclear extract but must have occurred on fully replicated molecules as well. As a further control, we added aphidicolin (an inhibitor of DNA polymerases α and δ) at the time of addition of nuclear extract. Again, the nuclear extract rapidly induced supercoiling of the replicated DNA (data not shown). In the absence of added nuclear extract the replicated minichromosome underwent a small increase in superhelical density. This most likely reflected slight contamination of the cytosolic S-100 with nuclear components.

A fully replicated minichromosome but not the replicated DNA itself is the preferred target for nucleosome assembly. ^{32}P-labeled DNA was purified after replication by digestion with proteinase K in the presence of 0.1% SDS, extraction with phenol/chloroform, and precipitation with ethanol. This DNA was not supercoiled upon incubation with the Manca nuclear extract or upon addition of Manca S-100 plus nuclear extract (Fig. 2*A*). Control experiments showed that if the replicated DNA was allowed to replicate again (i.e., by including both T antigen and nucleoside triphosphates during the incubation with cytosolic extract) then it could be supercoiled upon addition of the nuclear extract.

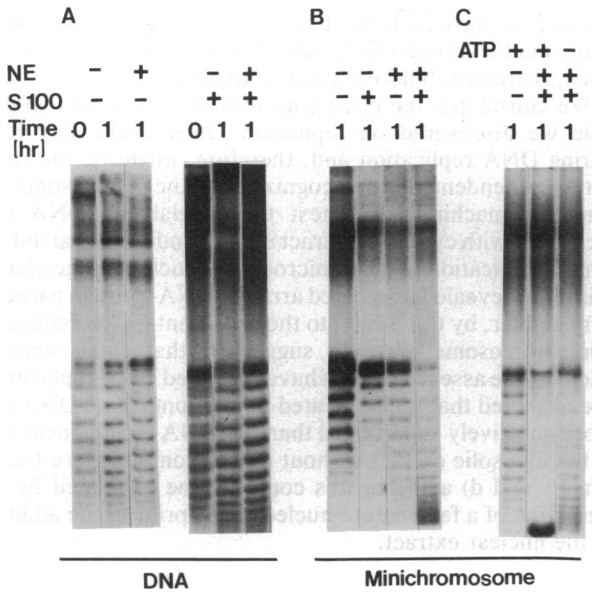

FIG. 2. (*A*) Deproteinized replicated DNA is not a substrate for nuclear-extract-induced supercoiling. DNA purified from a standard replication reaction mixture was incubated in a reaction mixture without T antigen and cytosolic S-100 for times indicated at the top of the figure either without or with 1.8 μg of nuclear extract protein (NE). Alternatively, DNA purified from a replication reaction mixture was preincubated with cytosolic S-100 for 1 hr prior to incubation for the indicated time with nuclear extract. (*B*) Supercoiling of isolated minichromosomes. Replicated minichromosomes were isolated by chromatography on Sepharose 6B-CL and further incubated in 125 μg of cytosolic S-100, 1.8 μg of nuclear extract protein, or both for 1 hr as indicated. Controls without nuclear extract or cytosolic S-100 at 1 hr are shown. (*C*) Reactions were carried out as in *B* and the isolated replicated minichromosome was further incubated with both cytosolic S-100 and nuclear extract, with or without ATP as indicated.

In contrast to purified replicated DNA, the isolated replicated minichromosome is an efficient substrate for nucleosome assembly. After purification on Sepharose 6B or Bio-Gel A-5, supercoiling of the replicated minichromosome (hence assembly of the replicated minichromosome into nucleosomes) required the addition of both the nuclear and cytosolic extracts (Fig. 2*B*) and was ATP-dependent (Fig. 2*C*). No replication occurred during the assembly reaction. The contributions of cytosolic and nuclear components to the assembly reaction have not been identified at present; however, the cytosolic extract contained a significantly greater histone pool than the nuclear extract (data not shown). The above results argue that replication-dependent nucleosome assembly recognized some feature of the replicated minichromosome that was not retained on purified replicated DNA. Furthermore, this distinguishing feature must form during DNA replication since it was not reestablished upon incubation of deproteinized replicated DNA with the cytosolic extract in the absence of replication.

Nucleosome Assembly on the Replicated Minichromosome. Incubation of the replicated minichromosome with a nuclear extract caused the minichromosome DNA to become highly supercoiled, which reflected assembly of the minichromosome into a regularly repeating nucleosome array. This was shown as follows. The assembled minichromosome was partially digested with micrococcal nuclease and the reaction products were either separated as DNA–protein complexes by electrophoresis through low percent polyacrylamide gels (Fig. 3*A*) or deproteinized and displayed as DNA on higher percent polyacrylamide gels (Fig. 3*B*). When the digestion products were analyzed as DNA–protein complexes, a repeated array was observed that was highly suggestive of particle ladder consisting of monomer, dimer, trimer, and higher order nucleosome oligomers. Indeed, after deproteinization the digestion products formed a DNA ladder with 160- to 180-base-pair periodicity, demonstrating assembly of the DNA into regularly spaced nucleosomes. When the assembled minichromosome was incubated with topoisomerase I

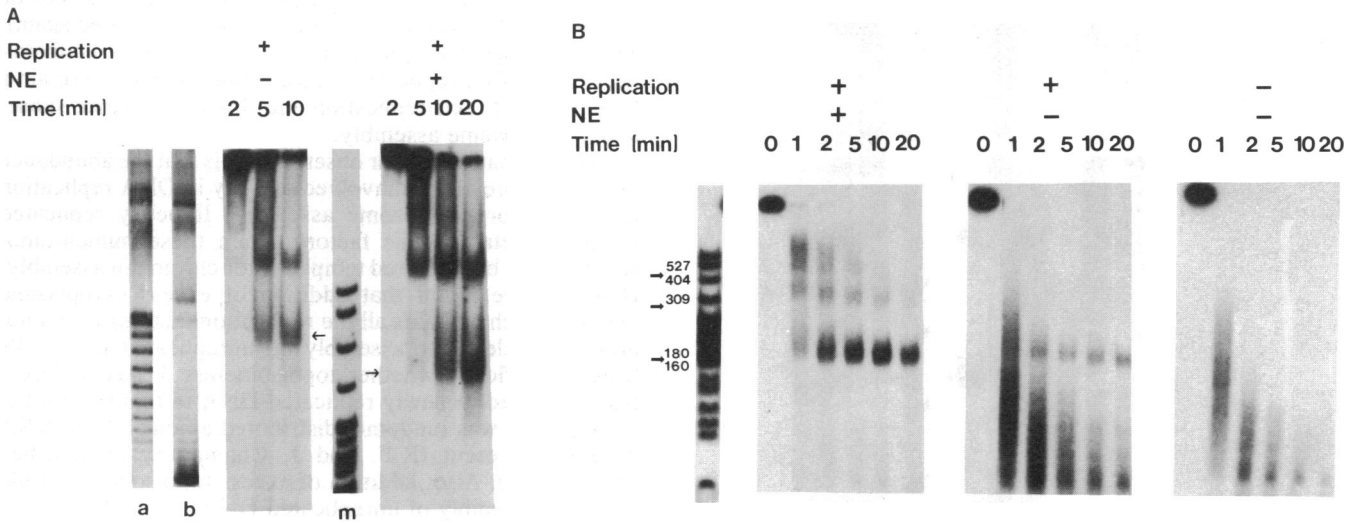

FIG. 3. Analysis of chromatin structure with micrococcal nuclease. (*A*) After 60 min of replication, reaction products were digested with micrococcal nuclease for 2, 5, 10, or 20 min and fractionated without deproteinization on 4% polyacrylamide gels. The digestion products from reactions carried out without (−) or with (+) 1.8 μg of nuclear extract protein (NE) were fractionated as native DNA–protein complexes on 4% acrylamide gels and visualized by autoradiography. Limit digestion products (monomer particles) are indicated by arrows. DNA size markers (*Msp* I-digested pBR322) are shown (lane m). In addition, prior to micrococcal nuclease digestion, an aliquot of each reaction was removed and DNA was purified. The replication products from the reaction without (lane a) or with (lane b) nuclear extract were analyzed on agarose gels and visualized by autoradiography. Note that the DNA–protein particles formed with or without nuclear extract were analyzed on the same gel and have been separated for display in this figure. Typically, the mature mononucleosome migrates between DNA markers of 404 and 309 base pairs and the monomer particle formed without nuclear extract between the DNA markers of 527 and 404 base pairs. (*B*) Reaction products were digested with micrococcal nuclease for various times, the DNA was purified, fractionated on a 6% polyacrylamide gel, and visualized by autoradiography. Digests were performed on DNA replicated with or without nuclear extract and on prelabeled DNA incubated in reaction mixture B (minus T antigen) and cytosolic S-100. The positions of the monomer, dimer, and trimer are indicated by arrows.

for 30 min prior to deproteinization, the purified DNA remained supercoiled, demonstrating that the supercoils were constrained in the minichromosome (presumably by the DNA–nucleosome interaction, data not shown). In contrast, naked supercoiled DNA was fully relaxed under identical reaction conditions.

Chromatin Structure of Newly Replicated DNA. Since the replicated minichromosome is a precursor to the nucleosome-assembled minichromosome described above, we asked whether this precursor was organized into a structure that might help us understand its role as an assembly intermediate. Replicated minichromosomes before and after addition of nuclear extract were partially digested with micrococcal nuclease and the products were analyzed either as DNA–protein complexes or as DNA, as described above. Analysis of the DNA–protein complexes (Fig. 3*A*) revealed that the replicated minichromosome was organized into a repeating array of DNA–protein particles. However, these particles differed from mature nucleosomes in at least three ways. (*i*) The monomer particle (i.e., the limit digestion product) moved more slowly than the mature mononucleosome during electrophoresis through low percentage polyacrylamide gels, suggesting alterations in size, shape, or net charge. (*ii*) The DNA isolated from the replicated minichromosome is not extensively supercoiled, suggesting that the DNA does not follow a tight superhelical path about this particle. (*iii*) Newly replicated chromatin is much more sensitive to nuclease digestion than DNA assembled into nucleosomes. Analysis of deproteinized DNA obtained after partial digestion of the replicated minichromosome with micrococcal nuclease demonstrated rapid cleavage beyond the monomer size DNA fragment to subnucleosomal DNA pieces (Fig. 3*B*). Only small amounts of monomer-size DNA accumulated as a digestion intermediate. A major point is that micrococcal nuclease treatment of replicated minichromosomes produced monomer DNA–protein particles containing many double-strand DNA cuts. Protein–protein interactions must, therefore, hold the particle together.

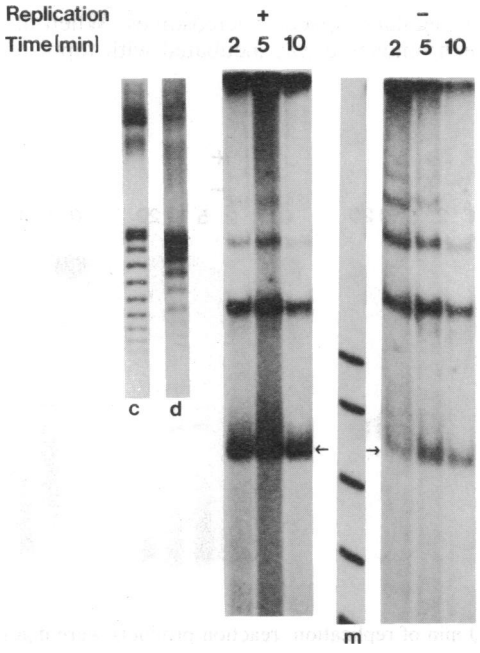

FIG. 4. Reactions were performed and analyzed as in Fig. 3*A*. Micrococcal nuclease digestion products of DNA replicated without nuclear extract and digestion products of purified replicated DNA that had been incubated in reaction mixture without T antigen but with cytosolic S-100 for 60 min without replication are shown. In addition, prior to nuclease digestion, an aliquot of each reaction mixture was removed, and the DNA was purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography. Lane c contains purified replicated DNA and lane d contains purified replicated DNA after incubation in cytosolic S-100.

We considered the possibility that the DNA–protein particle we discovered on replicated DNA could only form during DNA replication and, therefore, might be the replication-dependent mark recognized by the nucleosome assembling machinery. To test this, prelabeled DNA was incubated with cytosolic extract under conditions that did not permit replication. Partial micrococcal nuclease digestion of this DNA revealed a repeated array of DNA–protein particles very similar, by this assay, to those present on the replicated minichromosome (Fig. 4), suggesting that some steps in nucleosome assembly might have preceded DNA replication. We observed that the replicated minichromosome DNA was more negatively supercoiled than the DNA simply incubated in the cytosolic extract without replication (compare Fig. 4, lanes c and d) and that this could not be explained by the formation of a few mature nucleosomes prior to the addition of the nuclear extract.

DISCUSSION

It has been suggested that unique structural features of actively replicating DNA facilitate the assembly of new nucleosomes (for review, see refs. 28 and 29). For example, the replication fork might serve as a swivel to remove the torsional stress caused by wrapping DNA about the nucleosome core. In addition, lagging-strand Okazaki fragments might each position one new nucleosome, with parental nucleosomes segregating to the leading strand of DNA synthesis. However, in studying the connection between DNA replication and nucleosome assembly, we found that, while replication stimulated nucleosome formation, the replicated minichromosome remained a preferred template for nucleosome assembly after replication had been completed. This was not a property of replicated DNA itself, since DNA purified from the replicated minichromosome could not be assembled into nucleosomes (unless it was replicated again). Thus, although nucleosome assembly can occur concomitantly with DNA replication, chromosomes can also retain a "memory" of their replication and this can enhance subsequent nucleosome assembly.

One explanation for our observations is that the abundance of one or more factors involved directly in DNA replication is limiting for nucleosome assembly. If newly replicated molecules retained this factor(s) then these minichromosomes would be preferred templates for chromatin assembly. However, we found that addition of excess cytoplasmic extract (which contains all the replication factors) would not promote nucleosome assembly on unreplicated DNA. We tested specifically whether topoisomerase I was preferentially localized to newly replicated DNA in this system but found that it was randomly distributed among all the DNA molecules present (R.F. and J. Champoux, unpublished observations). Also, addition of excess topoisomerase I did not drive assembly of unreplicated DNA.

Another explanation for our observations postulates a multistep pathway of nucleosome assembly in which formation of an essential intermediate can only occur during DNA replication. Consistent with this explanation, we find that replicated DNA is organized into a repeated array of nucleoprotein particles that differ from mature nucleosomes in at least three ways. (*i*) They had a different size, shape, and/or charge as assayed by their migration through low percentage polyacrylamide gels. (*ii*) They maintained a less intimate contact with the associated DNA, as demonstrated by the increased rate of nucleolytic degradation of the intraparticle DNA. (*iii*) The DNA was not coiled about the particle as it is

in a mature nucleosome since the deproteinized replicated DNA is not extensively supercoiled.

There are similarities between the chromatin structure we observe on DNA replicated *in vitro* and the chromatin structure observed *in vivo* on newly replicated DNA. One example is the accelerated nuclease digestion of nascent chromatin assembled *in vivo* or *in vitro*. In neither case does monomer-size DNA accumulate as a digestion intermediate; the DNA is rapidly digested beyond the monomer to subnucleosomal fragments. Also, the altered velocity sedimentation profile of nuclease-treated nascent chromatin might be consistent with the more extended particle structure we observe on replicated DNA. A discrete nucleosome assembly intermediate has also been observed on DNA assembled in *Xenopus* oocyte extracts (30). Interestingly, in that system, as in ours, nucleosome assembly is ATP-dependent.

In vivo, histones H3 and H4 are deposited rapidly on newly replicated DNA and histones H2A and H2B are deposited 2–10 min later (21). Perhaps related are the observations that H3 and H4 can be transferred to the DNA by carrier protein N1 and that H2A and H2B are transferred by a different carrier protein, nucleoplasmin (31). These data raise the interesting possibility that the chromatin present on newly replicated DNA, both *in vivo* and *in vitro*, may consist primarily of particles containing only H3 and H4. $(H3/H4)_2$ tetramers have been reconstituted onto DNA *in vitro* to form a particle with properties similar to those we report for the particle present on the *in vitro*-replicated minichromosome (32–36). The $(H3/H4)_2$ tetramer and 140 base pairs of DNA (32–34) form a highly elongated structure (32–34) with altered nuclease sensitivity (36), as compared to the mature nucleosome, in which the DNA traces a shallow coil (32) such that the template does not become extensively supercoiled (36). These particles can be converted to mature nucleosomes upon the addition of H2A and H2B (33–36). It is possible that the prenucleosome particle present on our *in vitro* replicated DNA may consist of H3 and H4.

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