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Transfer of Nucleosomes from Parental to Replicated Chromatin

TORSTEN KRUDE AND ROLF KNIPPERS*

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Federal Republic of Germany

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Simian virus 40 (SV40) minichromosomes were used as the substrate for in vitro replication. Protein-free SV40 DNA or plasmids, carrying the SV40 origin of replication, served as controls. Replicated minichromosomal DNA possessed constrained negative superhelicity indicative of the presence of nucleosomes. The topological state of replicated minichromosomal DNA was precisely determined by two-dimensional gel electrophoresis. We show that most or all nucleosomes, present on the replicated minichromosomal DNA, were derived from the parental minichromosome substrate. The mode and the rate of nucleosome transfer from parental to minichromosomal daughter DNA were not influenced by high concentrations of competing replicating and nonreplicating protein-free DNA, indicating that nucleosomes remain associated with DNA during the replication process. The data also show that parental nucleosomes were segregated to the replicated daughter DNA strands in a dispersive manner.

The replication of the eukaryotic genome is accompanied by the assembly of chromatin. This process requires the formation of nucleosomes, the repeating units of chromatin, and includes the wrapping of consecutive 145-bp stretches of DNA around histone cores. The octameric nucleosomal core is composed of one histone H3-H4 tetramer and two histone H2A-H2B dimers (reviewed by Van Holde [29]).

The histone octamers on newly replicated DNA are from two sources. The "old" octamers are transferred from the unreplicated chromatin stem to the replicated DNA branches, whereas the "new" octamers are assembled from soluble histones, freshly synthesized during the DNA replication phase of somatic cells.

The assembly of new nucleosomes has been well studied in vivo (see reference 11 and references therein) and in in vitro systems (7, 25). In the in vitro systems, simian virus 40 (SV40) DNA or plasmids carrying the SV40 origin region are used as templates for replication requiring SV40 large T antigen as an initiator protein and cytosolic extracts as a source for all additional replication functions. The cytosol also contains soluble histones, which are used for the formation of nucleosomes on newly replicated DNA. This assembly reaction requires the function of a chromatin assembly factor, which is normally present in nuclear protein extracts (21). The nuclear assembly factor is necessary to deposit histone H3-H4 tetramers on DNA. In a second step, these tetramers are complemented by the addition of H2A-H2B dimers, giving complete histone octamers (22).

The transfer of old nucleosomes to replicated DNA has been less well studied in biochemical assay systems. Earlier in vivo data were interpreted to indicate that old histone octamers are transmitted as intact units from parental to progeny DNA. However, according to more recent data, it appears instead that H3-H4 tetramers are transmitted to the replicated branches and that these tetramers are then converted to an intact octamer by the addition of two H2A-H2B dimers (11).

Despite much experimental work, it has not been determined whether old nucleosomes appear only on the leading strand or on both strands of cellular replication forks (for a thoughtful presentation and discussion of the published data, see chapter 9 of van Holde [29]).

However, biochemical and electron-microscopic studies have provided compelling evidence for a random or distributive type of nucleosome segregation at the replication forks of SV40 minichromosomes (5, 23). Sogo et al. (23) were able to determine the distribution of nucleosomes around the replication forks of SV40 minichromosomes by using the psoralen cross-linking technique and examining the resulting structures in the electron microscope. It could be shown that replication forks move up to and even into nucleosome-associated DNA regions, excluding an early dissociation of prefork histone complexes. However, the first nucleosomes on the newly replicated DNA branches were visible at distances of 220 to 285 bp behind the fork. This was interpreted to indicate that prefork nucleosomes may dissociate at some time during the progression of the replication fork and reassociate later on the replicated double-stranded DNA.

However, this interpretation is problematic since the psoralen cross-linking method may not detect nucleosomes with an altered conformation. Changes in the nucleosome conformation could occur when nucleosomes and components of the replication apparatus bind to closely adjacent or overlapping sections of DNA. Furthermore, the psoralen cross-linking method is also inadequate to detect histone complexes on single-stranded DNA at replication forks. Thus, the possibility could not be rigorously excluded that nucleosomes remain bound to DNA during the passage of the replication fork. In fact, Bonne-Andrea et al. (2) have recently shown that, at least in principle, this could very well be the case. These workers used artificially assembled nucleosomes on a DNA substrate suitable for phage T4 replication enzymes and demonstrated that the assembled nucleosomes remained bound to DNA during the passage of the phage T4 replication apparatus. The nucleosomes remained bound to replicating DNA even in the presence of high concentrations of competing DNA to which histones should bind when they were transiently released at replication forks (2). However, these elegant experiments were performed with an entirely artificial system involving a DNA substrate with a few scattered nucleosomes that were assembled by an unphysiological method, and replication was carried out by bacteriophage replication factors which never

* Corresponding author.

encounter nucleosomes in their physiological environment. In addition, the T4 replication apparatus has evolved to replicate unusual DNA structures, namely glucosylated phage DNA. Therefore, it may be asked whether the conclusions drawn from these experiments are significant for considerations concerning the replication of eukaryotic chromatin. This can be decided only by using eukaryotic chromatin replicated by homologous eukaryotic replication enzymes.

Experiments of this kind became feasible after it had been shown that SV40 minichromosomes are efficient in vitro templates which replicate in the presence of the virus-encoded initiator protein T antigen and mammalian replication factors (6, 28).

In this communication we report experiments with in vitro-replicating SV40 minichromosomes and provide evidence showing that, under these more physiological replication conditions, nucleosomes do not appear to dissociate during the replication process.

MATERIALS AND METHODS

Preparation of SV40 minichromosomes. The SV40 chromosomes were prepared essentially as described by Su and DePamphilis (27) and Sugasawa et al. (28). Briefly, 10 subconfluent plates (145 mm in diameter) containing CV-1 cells were infected with wild-type SV40 at a multiplicity of infection of 10. For labeling of SV40 chromosomal DNA, the cells were incubated for 16 h in medium containing 10 μCi of [^{14}C]thymidine (Amersham) per plate. At 40 h after infection, the DME culture medium (Dulbecco modified Eagle medium without antibiotics) was removed. The cells were washed twice with ice-cold TSS buffer (20 mM Tris-Cl [pH 7.4], 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 250 mM sucrose) and three times with ice-cold LS buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, potassium salt [K-HEPES; pH 7.8]; 5 mM potassium acetate; 0.5 mM $MgCl_2$; 0.5 mM dithiothreitol). All subsequent steps were carried out at 4°C. The cells were scraped off the plates and disrupted with 10 to 15 strokes in a type S glass Dounce homogenizer (Braun). The integrity of the nuclei was checked by light microscopy. The nuclei were pelleted for 5 min at 1,000 × *g*, resuspended in 2 ml of LS buffer containing 500 mM potassium acetate, and incubated on ice for 2 to 3 h with occasional shaking. The nuclei were removed by centrifugation, and the eluted SV40 minichromosomes were recovered in the supernatant. The supernatant was cleared of debris by centrifugation at 20,000 × *g* for 10 min. The SV40 minichromosomes were then concentrated by pelleting at 300,000 × *g* for 1 h. The pellet was washed twice in LS buffer and resuspended in 0.4 ml of LS buffer. Insoluble material was removed by centrifugation at 20,000 × *g* for 5 min. The supernatant was frozen and stored in aliquots at −70°C. One microliter of this preparation usually contained 0.5 μg of protein (as determined by using the Bio-Rad system) and 0.3 μg of SV40 DNA (determined by measuring the A_{260}).

Preparation of DNA. SV40 DNA was prepared by the method of Hirt (9), and plasmid DNA was prepared by the alkaline lysis method, followed by CsCl isopycnic centrifugation (17). Plasmid pSVMO1 contains the SV40 minimal origin cloned into pUC18 (17). For some purposes, the plasmid DNA was relaxed in vitro by calf thymus topoisomerase I in 200 mM NaCl–10 mM Tris-Cl (pH 7.5) at 37°C.

T antigen and cytosolic HeLa cell extracts. T antigen was prepared by immunoaffinity chromatography as described by Simanis and Lane (20), with the modification described by Klausing et al. (13).

Cytosolic cell extracts were prepared essentially by the method of Li and Kelly (16), with the following modifications. HeLa-S3 cells were grown to 90% confluency as monolayer cultures on 145-mm plates in DME medium without antibiotics. The cells were washed twice with ice-cold TSS buffer and twice with ice-cold LS buffer. All subsequent steps were performed at 4°C. The cells were allowed to swell for 10 min in 10 ml of LS buffer per plate, and the excess buffer was removed by aspiration. The cells were scraped and immediately homogenized as described above. The cell lysate was centrifuged at 20,000 × *g* for 15 min. The supernatant was flash frozen in liquid nitrogen and stored at −70°C. One microliter contained 12 μg of protein.

SV40 chromatin replication reactions. The standard SV40 chromatin in vitro replication reaction was adapted from the literature (6, 28) and slightly modified. The reaction mixture contained the following components, added to the the 50-μl reaction volume in the order specified below. Distilled H_2O to adjust the final volume was added first, followed by 12 μl of cytosolic extract (ca. 150 μg of protein), 1 μg of SV40 large T antigen, a mix of nucleotides and salts (yielding final concentrations of 2 μCi of [α-^{32}P]dATP [Amersham], 40 mM K-HEPES [pH 7.8; Fluka], 4 mM $MgCl_2$, 3 mM ATP, 0.1 mM each GTP, CTP, and UTP; 25 μM dATP, 0.1 mM each dGTP, dCTP, and dTTP, 0.5 mM dithiothreitol, 20 mM creatine phosphate, and 5 μg of phosphocreatine kinase [all from Boehringer Mannheim]). The reaction was mixed on ice and started with the addition of 2 μl of the chromatin preparation at 37°C. DNA was replicated under the same reaction conditions.

For density labeling, dTTP was substituted by 0.1 mM bromo-dUTP. The labeled DNA was extracted and investigated by $CsSO_4$ equilibrium centrifugation.

In some experiments, we interrupted the T-antigen-dependent replication by using the monoclonal antibody Pab 204 (31; kindly provided by H. Stahl).

Processing of the replication products. (i) Determination of the incorporation of α-^{32}P-deoxynucleoside triphosphates into replicated DNA. The in vitro replication reactions were stopped by the addition of 50 μl of 2× stop mix (2% sarcosyl, 0.2% sodium dodecyl sulfate, 20 mM EDTA), and the solution was mixed thoroughly and transferred to ice. An aliquot was added to 1 ml of 10% trichloroacetic acid on ice and incubated for 10 min to precipitate the incorporated radioactivity, which was determined by scintillation counting.

(ii) Processing for agarose gel electrophoresis. The reaction mixtures from the stopped reactions were phenol-chloroform extracted, ethanol precipitated, dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 7.5]), and loaded onto agarose gels.

(iii) Micrococcal nuclease digestion. The in vitro replication reaction mixture (0.05 ml) was transferred to an ice bath, and 0.450 ml of ice-cold 3 mM $CaCl_2$ and 4.25 A_{260} U of micrococcal nuclease (Boehringer Mannheim) were added. The reaction mixture was transferred to 37°C and aliquots were withdrawn at the times indicated below. The resistant DNA was analyzed by both trichloroacetic acid precipitation and agarose gel electrophoresis.

Electrophoresis. Agarose gel electrophoresis was performed at room temperature in a low-salt TBE buffer (45 mM Tris-borate, 0.5 mM EDTA [pH 8.4]) at 3.5 V/cm.

Two-dimensional electrophoresis was carried out by the method of Peck and Wang (19), with adjustments. The DNA

was applied to a round slot 1 mm in diameter, and the gel (0.75% agarose) was run for the first dimension as described above. For the second dimension, the lane of the gel was cut out as a 5-mm thick slice and turned by 90°C. It was placed at the top of the apparatus, and a new gel was then poured around it. Chloroquine at 0.35 μM was added to the agarose at 50°C before pouring. The second-dimension electrophoresis was carried out in the same way as the first one, except that 0.35 μM chloroquine was present in the running buffer. The DNA was visualized by autoradiography of the dried gel or by staining with ethidium bromide.

The topological markers used for two-dimensional gel electrophoresis were prepared by relaxing negatively supercoiled SV40 DNA with topoisomerase I in the presence of ethidium bromide as described by Keller (12).

RESULTS

Minichromosome replication. We used isolated SV40 minichromosomes as substrates for in vitro replication and cytosolic proteins, supplemented with isolated T antigen, as the source of enzymes. Minichromosomes are usually prepared by incubating nuclei from SV40-infected cells in a buffer of low osmolarity. Minichromosomes leak from the nuclei and can conveniently be recovered in the supernatant after centrifugation. The eluted structures sediment through sucrose gradients as dense nucleoprotein particles at about 75S (14, 18, 27).

In our experiments, these 75S particles were poor substrates for in vitro replication since they contained an associated nucleolytic activity degrading some of the in vitro-synthesized polynucleotide strands (results not shown). However, we obtained suitable chromatin substrates without this endonuclease when the minichromosomes were eluted from the nuclei in buffers containing 0.5 M potassium acetate (28). It is known from earlier studies that treatment with 0.5 M NaCl removes histone H1 and some nonhistone proteins and converts the dense nucleoprotein particle into more extended structures carrying the full complement of 25 to 28 nucleosomes per SV40 DNA (18).

In pilot control experiments we confirmed the nature of the template to be chromatin by sucrose gradient fractionation and subsequent testing of each fraction for its ability to support DNA replication in vitro. We found that the template activity cosedimented with SV40 chromatin (and not with protein-free SV40 DNA [data not shown, but see reference 28]). Furthermore, in micrococcal nuclease digestions of SV40 chromatin used for replication, we observed up to three or four multimers of nucleosomes, as previously shown (14).

The replication of protein-free SV40 DNA has been extensively studied in vitro (reviewed in references 4 and 26). We therefore used protein-free SV40 DNA in our experiments as a control to assess the efficiency at which SV40 minichromosomes are replicated in vitro. For this purpose, SV40 DNA and an equivalent amount of SV40 chromatin were incubated in parallel under the conditions known to be optimal for SV40 DNA replication (see Materials and Methods). As shown in Fig. 1, the replication of SV40 minichromosomes started after a lag period of 15 to 30 min and then continued at a linear rate for at least 120 min. The amount of replicated minichromosomal DNA, determined as incorporated nucleotides, was one-third to one-half of the amount of replicated protein-free DNA (Fig. 1B and C).

The time course of in vitro replication was monitored by agarose gel electrophoresis and autoradiography. With both

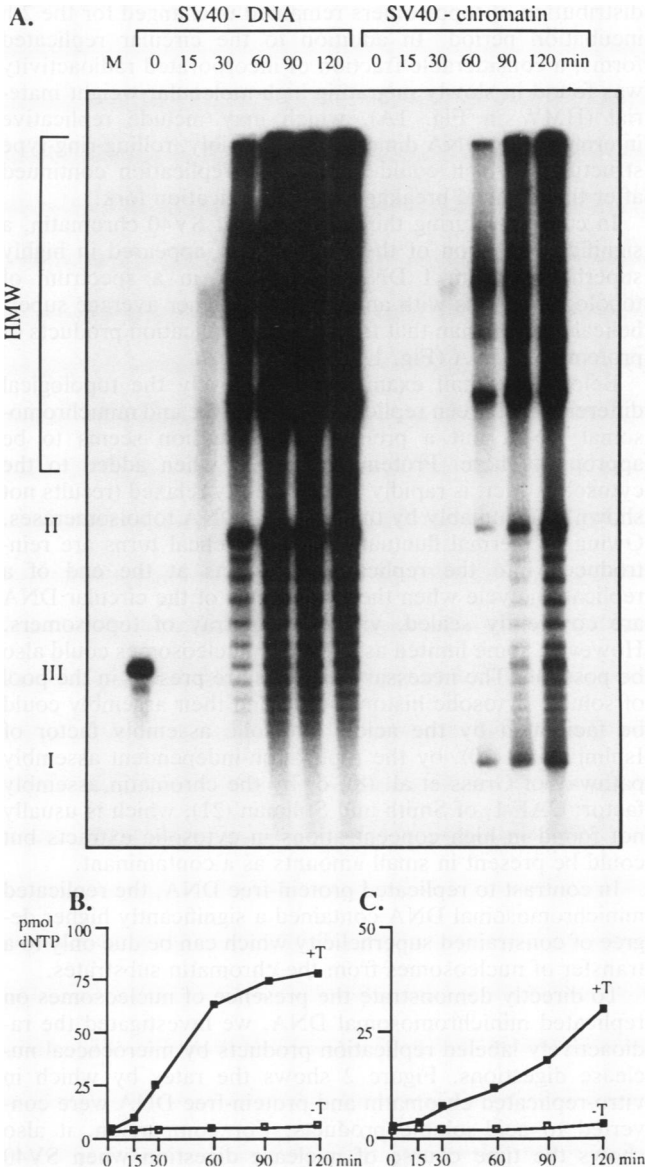

FIG. 1. Replication of SV40 DNA and SV40 chromatin. (A) A 100-ng amount of SV40 DNA (left) and 2 μl of the SV40 minichromosome preparation [ca. 600 ng of DNA] (right) were replicated in vitro for the indicated times. The products were processed for agarose gel analysis and autoradiography. In lane M, linearized and ^{32}P-end-labeled SV40 DNA was run as a size marker. HMW denotes high-molecular-weight DNA. DNA forms I (supercoiled), II (relaxed and open circular), and III (linear unit length) are indicated. (B and C) Incorporation of [^{32}P]dATP into replicated DNA. The incorporation was determined by precipitation with trichloroacetic acid. The reactions were performed with either SV40 DNA (B) or SV40 chromatin (C) as a template in the presence (+T) or absence (−T) of SV40 large T antigen. dNTP, deoxynucleoside triphosphate.

templates, we detected the first radioactively labeled material in the part of the gel where replicative intermediates were expected to appear (Fig. 1A; 15 and 30 min for SV40 DNA and chromatin, respectively).

With continued replication of SV40 DNA, more and more radioactivity appeared in circular, relaxed form II DNA as well as in DNA with up to about 10 superhelical turns. This

distribution of topoisomers remained unchanged for the 2-h incubation period. In addition to the circular replicated forms, a considerable fraction of incorporated radioactivity was found in slowly migrating high-molecular-weight material (HMW in Fig. 1A), which may include replicative intermediates, DNA dimers, and, possibly, rolling-ring-type structures, which could arise when replication continued after the artificial breakage of one replication fork.

In contrast, during the replication of SV40 chromatin, a significant fraction of the radioactivity appeared in highly superhelical form I DNA and, later, in a spectrum of topological forms with an apparently higher average superhelical density than that found in the replication products of protein-free DNA (Fig. 1A).

Below, we shall examine more closely the topological differences between replicated protein-free and minichromosomal DNA, but a preliminary discussion seems to be appropriate here. Protein-free DNA, when added to the cytosol extract, is rapidly and efficiently relaxed (results not shown), presumably by the action of DNA topoisomerases. Owing to thermal fluctuations, superhelical turns are reintroduced into the replication products at the end of a replication cycle when the two strands of the circular DNA are covalently sealed, yielding an array of topoisomers. However, some limited assembly of nucleosomes could also be possible. The necessary histones are present in the pool of soluble cytosolic histones (21), and their assembly could be facilitated by the acidic cytosolic assembly factor of Ishimi et al. (10), by the replication-independent assembly pathway of Gruss et al. (8), or by the chromatin assembly factor, CAF-1, of Smith and Stillman (21), which is usually not found in high concentrations in cytosolic extracts but could be present in small amounts as a contaminant.

In contrast to replicated protein-free DNA, the replicated minichromosomal DNA contained a significantly higher degree of constrained superhelicity which can be due only to a transfer of nucleosomes from the chromatin substrates.

To directly demonstrate the presence of nucleosomes on replicated minichromosomal DNA, we investigated the radioactively labeled replication products by micrococcal nuclease digestions. Figure 2 shows the rates by which in vitro-replicated chromatin and protein-free DNA were converted to acid-soluble products. For comparison, it also shows the time course of nuclease digestion when SV40 minichromosomes were used before replication. We found that unreplicated minichromosomes were most resistant against nuclease attack and that replicated minichromosomes were more resistant than replicated protein-free DNA (Fig. 2). This result was expected since the unreplicated minichromosomes carried more nucleosomes and should therefore be more efficiently protected against the nuclease than minichromosomes which had been replicated.

Replication products treated for various lengths of time with micrococcal nuclease were deproteinized and investigated by gel electrophoresis. The autoradiograms (Fig. 3) show that most of the replicated protein-free DNA was already degraded after a 2-min incubation to products smaller than the 123-bp marker. In contrast, a significant fraction of replicated chromatin yielded DNA fragments which corresponded in size to DNA stretches associated with one or with two consecutive nucleosomes. After a 5-min treatment with micrococcal nuclease, replicated protein-free DNA was largely degraded, whereas a significant fraction of replicated minichromosomal DNA survived as fragments of mononucleosomal lengths.

However, a closer inspection of Fig. 3 shows that a small

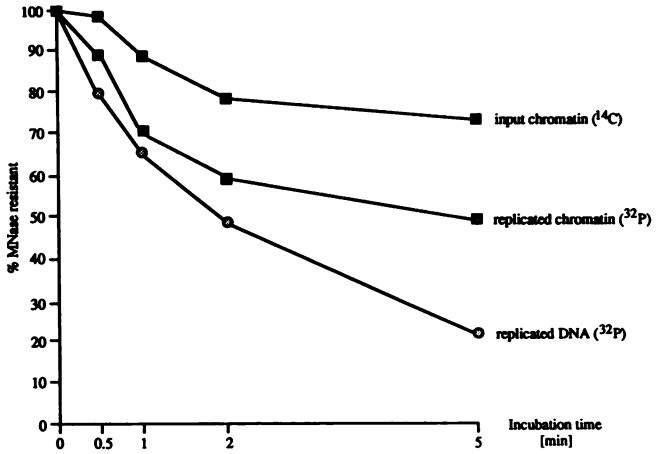

FIG. 2. Degradation by micrococcal nuclease (MNase). The figure compares the degradation kinetics of template SV40 chromatin, replicated SV40 chromatin, and replicated SV40 DNA. Nuclease-treated DNA was precipitated with trichloroacetic acid. The resistant radiolabeled DNA was determined by scintillation counting. The results are expressed as a percentage of the resistant DNA, compared with the untreated DNA (100%). The template SV40 chromatin was labeled with [^{14}C]thymidine in vivo, and 2 μl of this preparation was digested under the same conditions as the ^{32}P-labeled products of the in vitro replication reaction (as described in Materials and Methods). The mean values of three independent experiments are shown.

percentage of replicated protein-free SV40 DNA appeared to be resistant against a 2-min attack by micrococcal nuclease and migrated within the bracket of the mononucleosomal DNA fragments. This could be the result of a limited nucleosome assembly, as discussed above. We shall consider this possibility below.

Nucleosome transfer. The results in Fig. 1 suggested a transfer of parental nucleosomes to replicated progeny minichromosomes. However, the one-dimensional gel electrophoresis system was inadequate to investigate this process in quantitative detail since SV40 DNA molecules containing more than 15 superhelical turns could not be resolved and since completely relaxed monomeric molecules comigrate with open-circular molecules that can arise artifactually from every topoisomeric species by nicking one DNA stand. We therefore tried to obtain more quantitative results by using a two-dimensional (2D) gel electrophoresis system. Such a system has not been used before to study DNA replication in vitro. We first compared the products of a 90-min replication process with either protein-free SV40 DNA or SV40 minichromosomes as the template. As shown in Fig. 4, high-molecular-weight material (HMW) remained close to the start point of the 2D gel, whereas replicative intermediate DNA, relaxed open-circular form II DNA, and linear DNA (including unit-length form III DNA) were arranged along a diagonal. These DNA forms are not topologically fixed. They can rotate freely, and they possess similar electrophoretic mobilities in the absence and presence of intercalating chloroquine.

In contrast, chloroquine changes the topology of closed-circular DNA molecules by introducing positive superhelical turns. These topologically fixed DNA forms change their electrophoretic mobility in the presence of chloroquine. After several initial trials, we determined a chloroquine concentration allowing all topoisomers to migrate on a single arc.

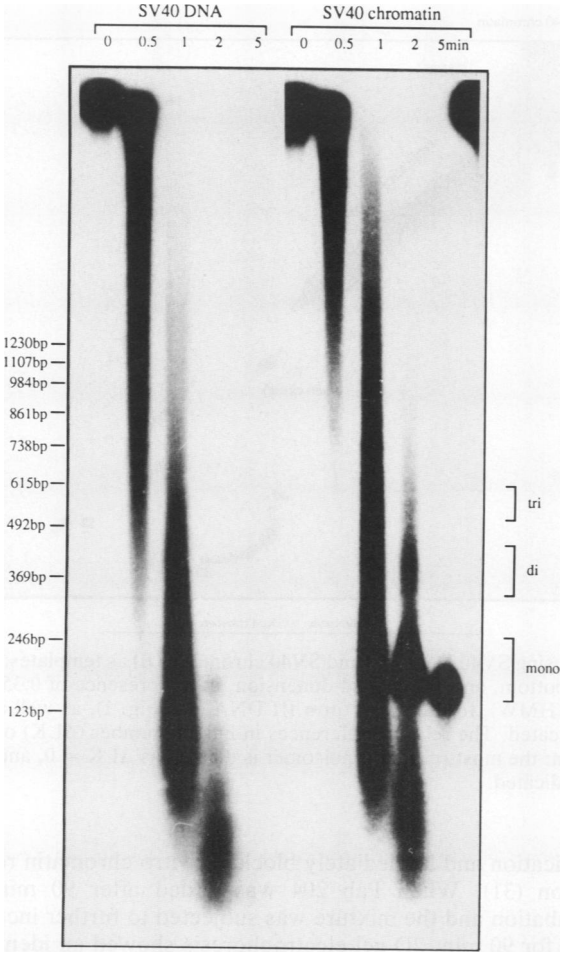

FIG. 3. Micrococcal nuclease digestion products of in vitro-replicated SV40 DNA and SV40 chromatin. The conditions for nuclease treatment were identical to those described in Fig. 2 (digestion times are indicated at the top of the figure). The autoradiogram of a 2% agarose gel is shown. A marker of multimeric 123-bp fragments (obtained from BRL) was run on the same gel. The positions of these fragments are indicated on the left. The brackets on the right denote the positions of DNA fragments of mono-, di-, and trinucleosomal lengths.

For an orientation, we have marked the position of fully relaxed closed-circular DNA (marked $\Delta LK = 0$) as a reference point (Fig. 4). Molecules traveling faster in the second dimension had originally one or more positive superhelical turns ($\Delta LK > 0$), whereas those with lower electrophoretic mobility had one or more negative superhelical turns ($\Delta LK < 0$).

As shown in Fig. 4A, replicated SV40 DNA possessed up to eight positive superhelical turns, which, as explained above, were most probably introduced by thermal fluctuations at the time of ring closure at the end of a replication cycle. Since this is a statistical process, we would expect the same number and the same distribution of negative superhelical turns. In fact, the number of negative supercoils was larger, about 12 superhelical turns. A simple explanation is that a few nucleosomes were assembled on replicating DNA (see above), inducing a net excess of four negative supercoils in the replication products of protein-free DNA. This could indicate a limited assembly of four nucleosomes on the replicated DNA. Our replication system therefore has a limited background capacity to assemble a few nucleosomes on protein free DNA.

Another possible explanation of this shift in mobility of the topoisomers in the gel is the presence of different ionic strengths and pH values in the reaction mixture compared with the gel buffer. We consider this latter possibility to be less relevant since the data in Fig. 3 also suggest a limited nucleosome assembly reaction (see above).

However, the topology of DNA in replicated SV40 chromatin was significantly different. No relaxed-circular or positively superhelical DNA could be detected (see the area marked ΔLK in Fig. 4B). Instead, most DNA in replicated minichromosomes possessed between 10 and 20 negative supercoils. Since replicating DNA incubated under identical conditions aquired only up to four nucleosomes, we therefore conclude that the nucleosomes present on replicated chromatin were efficiently transferred from the parental chromatin.

Using the 2D electrophoresis system, we were able to monitor the distribution of nucleosomes quantitatively during the course of minichromosome replication. As an important control and reference, we first determined the topology of SV40 minichromosomes used as the template for replication by 2D electrophoresis. For this purpose, unreplicated minichromosomal DNA was compared with an overlapping set of SV40 DNA topology markers. We determined an average of 24 to 26 negative superhelical turns per minichromosomal DNA (Fig. 5), in good agreement with the number of nucleosomes identified by electron microscopy (23).

Next, we used 2D gel electrophoresis to determine the superhelicity of minichromosomal DNA at different times of in vitro replication. The results of this experiment are shown in Fig. 6, which presents only the part of the autoradiographed gel containing the various topological forms of completely replicated DNA.

After 30 min of incubation, the replication products carried an average of 20 to 25 negative supercoils (Fig. 6). With continued incubation, the majority of replication products accumulated carried a smaller number of negative supercoils (Fig. 6). After 150 min of replication, we found a wide distribution of supercoils ranging from more than -25 to positive values up to 7. This distribution was significantly broader than the distribution of nucleosomes on the parental template chromatin. It was centered at an average linking number of -10 to -15, indicating that a replicated daughter molecule statistically received half the complement of the parental nucleosomes.

Why is the picture different at very early replication times? We propose that, owing to the presence of small amounts of a contaminating nuclear assembly factor, we obtained a limited assembly of few nucleosomes in addition to the nucleosome transfer. This occurred only at the beginning of the incubation period and corresponded to the limited assembly of nucleosomes on bare DNA, as discussed above (Fig. 4).

Furthermore, we also considered the possibility that some nucleosomes were continuously assembled from soluble histones, present in the cytosol (8, 21), or transferred from the excess of nonreplicating chromatin. It could be argued that this process remained undetectable at later replication times because in vitro-assembled nucleosomes could be unstably bound and lost again during prolonged incubation. We have excluded this possibility by inhibiting chromatin replication with the antibody Pab 204, which only and specifically inhibits the T-antigen-dependent steps of DNA

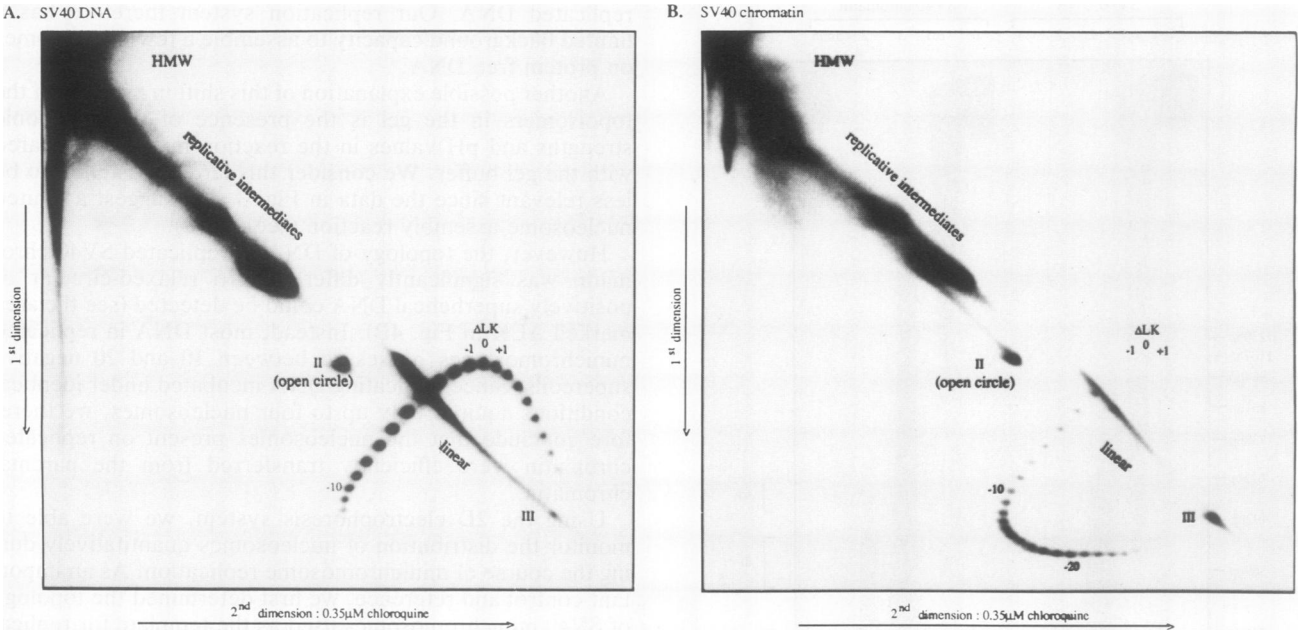

FIG. 4. 2D agarose gel electrophoresis of the in vitro replication products using SV40 DNA (A) and SV40 chromatin (B) as templates. The autoradiograms of the gels are shown. The first dimension was from top to bottom, and the second dimension, in the presence of 0.35 μM chloroquine, was from left to right. The positions of high-molecular-weight (HMW), form II, and form III DNA (see Fig. 1), as well as of replicative intermediates and non-unit-length linear DNA fragments, are indicated. The relative differences in linking number (ΔLK) of the topoisomers of monomeric circular DNA molecules are shown for orientation: the most relaxed topoisomer is defined as ΔLK = 0, and the positions of positive (ΔLK > 0) and negative (ΔLK < 0) topoisomers are indicated.

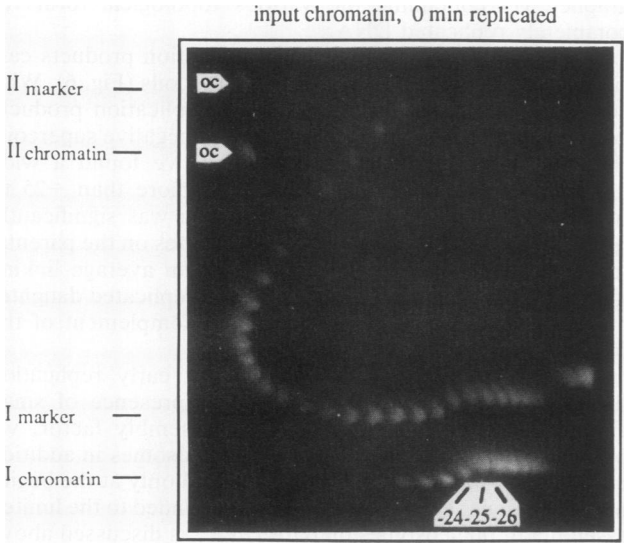

FIG. 5. Linking-number determination of the template SV40 minichromosomes. We have used a single 2D gel with two slots, 2 cm apart, in the same lane for the first dimension. The upper slot (marker) was loaded with a mixture of SV40 DNA topoisomers prepared in vitro (12), differing in linking number from +5 to approximately −40. The lower slot (chromatin) was loaded with SV40 DNA, obtained from the minichromosome preparation after deproteinization. An alignment in the first dimension of these topoisomers with the markers allows a direct determination of its average linking number as labeled in the figure. The positions, in the first dimension, of the form I, form II, and open-circular (oc) DNA are indicated.

replication and immediately blocks in vitro chromatin replication (31). When Pab 204 was added after 90 min of incubation and the mixture was subjected to further incubation for 90 min, 2D gel electrophoresis showed an identical distribution of topoisomers before and at 90 min after replication inhibition by the antibody (data not shown, but see Fig. 6). This experiment demonstrates the stability of nucleosomes on in vitro-replicated chromatin.

Second rounds of replication which could further dilute the number of nucleosomes per replicated minichromosome do not occur in vitro (28; our unpublished results). Therefore, the data in Fig. 5 and 6 indicate a dispersive distribution of nucleosomes with a more or less random transfer of parental nucleosomes to either one of the two emerging daughter DNA strands. The results of this linking-number analysis are not compatible with an exclusive transfer of nucleosomes to only one strand at the replication fork (conservative segregation). In this case, we would expect a narrow distribution of topoisomers of the replicated daughter molecules, carrying about half the complement of nucleosomes found on template chromatin. This was clearly not the case.

The important question remains of whether nucleosomes remain bound when replication forks move through nucleosome-associated DNA. In the experiments reported so far, nucleosomes could dissociate at the fork and reassociate again at some distance behind the fork at the only free DNA sequences available, namely those produced during the replication process. If this were the case, histones released at the advancing replication forks should also bind to additional free DNA sequences added as a competitor to the minichromosome replication assay.

Competition with protein-free DNA. The rationale of the experiments reported in this section, is that a loss of nucle-

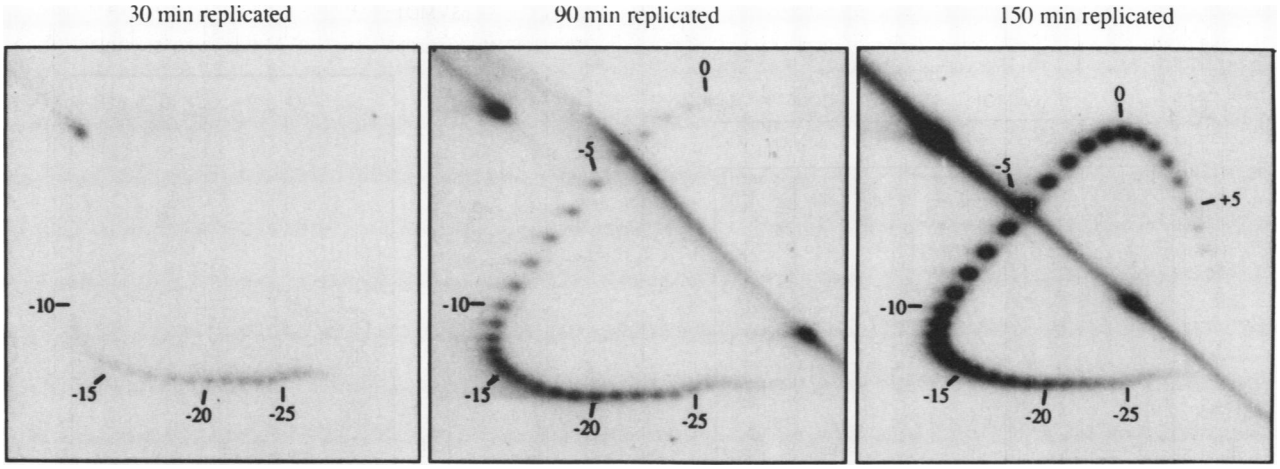

FIG. 6. Time course experiment of SV40 chromatin replication in vitro examined by 2D gel analysis. The procedure was described in the legend to Fig. 4. We show the part of the gel with monomeric topoisomers. The linking-number positions along the arc of topoisomers are indicated in the figure.

osomes from replicating minichromosomes should be detectable by a reduction in the number of superhelical turns in the replicated minichromosomal DNA and, conversely, that competing circular DNA molecules should acquire an increased number of negative superhelical turns if they received nucleosomes from minichromosomes.

In a first experiment, we added increasing amounts of plasmid pUC18 or of pSVMO1 to standard chromatin replication mixtures. The latter plasmid contained the SV40 origin of replication and was actively replicated under the assay conditions. Both plasmids are about 2.9 kbp, allowing the analysis of replicating minichromosomal SV40 DNA (5.2 kbp) and plasmid DNA on the same gel.

We found that the addition of plasmid DNA caused an inhibition of minichromosome replication (Fig. 7). As a possible explanation, we suggest that replication factors could be present in limiting amounts in the replication mixture and be trapped by an excess of competing plasmid DNA. The limiting factor was not T antigen, since the origin-minus plasmid (lacking a specific T-antigen-binding site) was as effective as pSVMO1 in inhibiting minichromosomal replication (Fig. 7) and an addition of excess T antigen did not release the block imposed by free DNA (results not shown).

Whatever the cause for replication inhibition may be, it can be seen from Fig. 7 that the topological state of the replicated pSVMO1 DNA was identical at different plasmid/minichromosome ratios, and, conversely, the number of superhelical turns in replicated minichromosomal DNA did not detectably decrease, at least at relatively low plasmid/minichromosome ratios when the inhibiting effect was less severe and the topology of replicated minichromosomal DNA could be assessed.

A comparable result was obtained with plasmid pUC18 as the competing DNA. This DNA did not replicate, and its topological state was determined by ethidium bromide staining of the gel (Fig. 7B, lanes 7 to 11). Plasmid pUC18 DNA is still relaxed after 120 min in the presence of replicating minichromosomes. Interestingly, the average linking number of pUC18 is more positive than that of pSVMO1, as judged by the presence of positive supercoils (minor bands in lanes 10 and 11). The average higher negative superhelicity of pSVMO1 may indicate a limited replication-dependent nucleosome assembly as discussed above. In summary, the data in Fig. 7. provide no evidence for a transfer of nucleosomes from replicating minichromosomes to free DNA.

However, the results from this experiment require further support evidence because (i) the amount of chromatin present in the experiment in Fig. 7 may have been insufficient for an efficient nucleosome transfer, (ii) it could be that nucleosomes are more readily transferred when competing DNA is added at a later stage of the minichromosomal replication process when many replication forks have arrived at nucleosome-associated DNA, and (iii), the one-dimensional gel system, used in the experiment in Fig. 7, may simply not be sensitive enough to detect nucleosome transfer to competing DNA. We have therefore performed experiments to address these points.

First, a constant amount of pSVMO1 was added to replication mixtures containing increasing concentrations of SV40 chromatin. The number of superhelical turns on replicating pSVMO1 DNA did not change during simultaneous replication of minichromosomes and plasmid DNA (Fig. 8). Similar data were obtained when nonreplicating pUC18 DNA was used as the competitor (results not shown). Thus, even a concentration of SV40 chromatin which was severalfold higher than that used in the previous experiment (Fig. 7) did not result in nucleosome transfer to protein-free DNA.

Second, minichromosomes were allowed to replicate for 60 min under standard conditions before increasing amounts of pSVMO1 were added. Replication products were then analyzed after an additional 45-min incubation under competing conditions. We again observed an inhibition of replication at high concentrations of competing DNA. However, significant changes in the number of supercoils on either the minichromosomal or the replicated protein-free DNA could be not be detected by standard gel electrophoresis (Fig. 9A) or by electrophoresis in the presence of chloroquine (Fig. 9B). We conclude that a transfer of nucleosomes to competing DNA does not seem to be facilitated by ongoing minichromosome replication.

Third, a more detailed analysis of the in vitro replication products was performed by using the 2D gel electrophoresis system described above. We compared two experimental conditions. In one experiment, minichromosomes and plasmid pSVMO1 were replicated in separate vials. The replica-

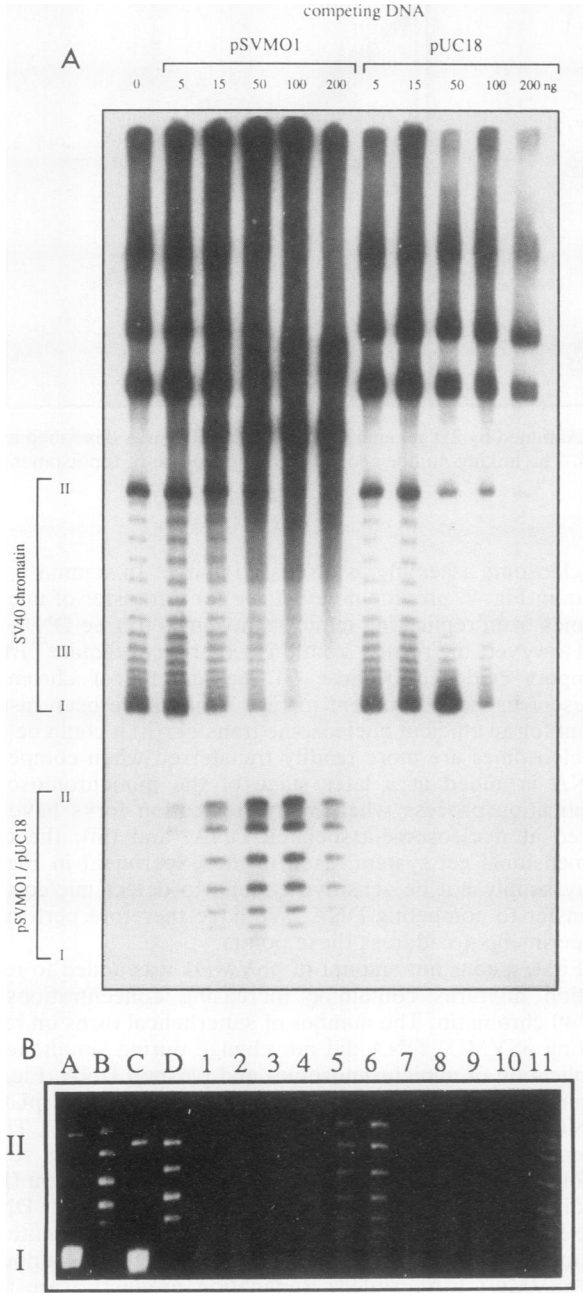

FIG. 7. In vitro replication of SV40 chromatin and competing plasmid DNA. In each reaction, 2 μl of the SV40 chromatin preparation was replicated in the presence of increasing amounts of competing plasmid DNA (the amount and type of the competitor are indicated at the top of the figure). The replication time was 90 min. (A) Autoradiogram of the products after gel analysis. The DNA forms I, II, and III of both competing plasmid DNA and SV40 chromatin DNA after deproteinization are indicated on the left. Note that the high-molecular-weight DNA of the competing replicating plasmid partially overlaps the position of replicated minichromosomal DNA. (B) Ethidium bromide stain of the same gel. Only the competing plasmids are shown (lanes 1 to 11). The markers are form I DNA (pSVMO1 [lane A] and pUC18 [lane C]) and relaxed plasmids prepared in vitro (pSVMO1 [lane B] and pUC18 [lane D]).

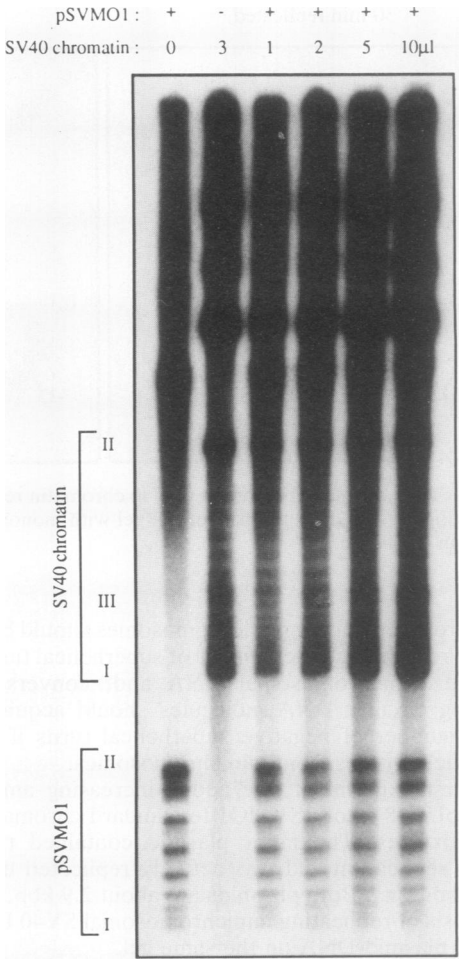

FIG. 8. Competition by pSVMO1 DNA at increasing amounts of SV40 chromatin. In each reaction, 10 ng of pSVMO1 DNA (marked + at the top of the figure) was replicated with the amounts of the SV40 chromatin preparations indicated. As a control, 3 μl of SV40 chromatin was replicated without plasmid DNA (marked − at the top of the figure).

tion products of the two assays were then mixed and investigated on one gel (Fig. 10A; SV40 chromatin and pSVMO1 replicated independently). In a second parallel experiment, both SV40 minichromosomes and pSVMO1 were replicated in one assay mixture and processed for gel electrophoresis (Fig. 10B; SV40 chromatin and pSVMO1 replicated simultaneously).

As is clearly evident in Fig. 10, the number of superhelical turns in minichromosomal DNA remained the same regardless of whether the DNA had been replicated in the presence or absence of pSVMO1 DNA. Conversely, no gain of negative supercoils could be detected in pSVMO1 DNA replicated in the presence of minichromosomes when compared with pSVMO1 DNA replicated in the absence of chromatin (Fig. 10).

To confirm this, micrococcal nuclease digestion experiments were conducted with the products of DNA and chromatin replication reactions, performed in the absence of a radioactive tracer. The micrococcal nuclease digestion products were analyzed on agarose gels and blotted onto nylon membrane (17). Hybridization with an SV40 DNA

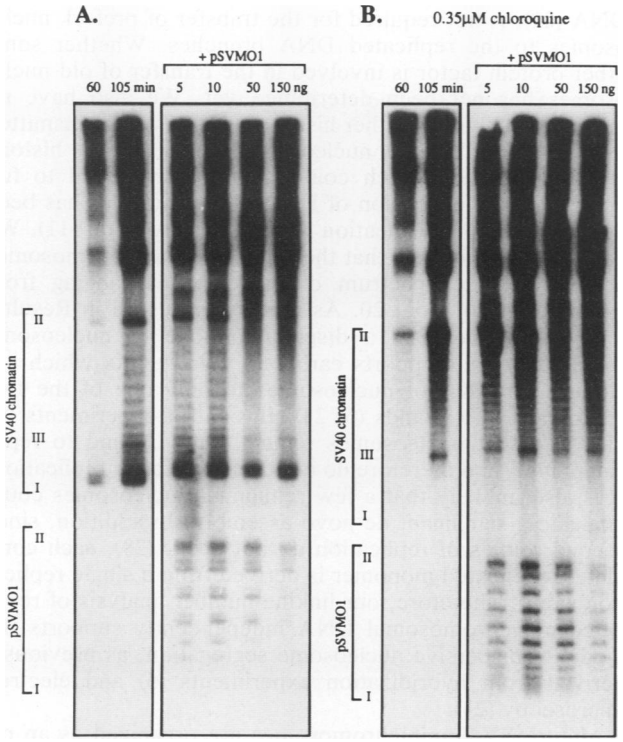

FIG. 9. Competition by pSVMO1 DNA added after the initiation of minichromosome replication. The figure shows autoradiograms of the products after gel analysis in the absence (A) or presence (B) of 0.35 μM chloroquine in the running buffer. In each reaction, 2 μl of SV40 chromatin was replicated for 60 min. The indicated amounts of competing pSVMO1 were then added to the reaction mixture, and the incubation was continued for a further 45 min for a total reaction time of 105 min. On the left of both panels A and B, the reaction products of SV40 chromatin in vitro replications without competitor are shown for comparison. On the right the reaction products with competing pSVMO1 are shown.

probe (17) always yielded nucleosomal DNA fragments like those shown in Fig. 3. Hybridization with a plasmid probe always revealed an essentially complete degradation of the replicated protein-free DNA (data not shown; see Fig. 3).

In conclusion, nucleosomes were efficiently transferred from parental to replicated minichromosomal DNA but not to competing replicating pSVMO1 DNA. It is therefore unlikely that nucleosomes dissociate from replicating chomatin during the replication process.

DISCUSSION

SV40 minichromosomes are well replicated in vitro when incubated under DNA replication conditions with T antigen as the initiator protein and a cytosolic extract from HeLa cells as a source of additional replication factors. Minichromosomal DNA is semiconservatively, bidirectionally, and discontinuously replicated beginning at the natural replication start point (6, 28) (confirmed by us [data not shown]) exactly like the replication of protein-free SV40 DNA or plasmid DNA carrying the SV40 origin (reviewed in references 4 and 26). Since only very limited assembly of new nucleosomes occurs in the absence of nuclear extracts (8, 21, 25), the in vitro replication of SV40 minichromosomes offers the unique possibility of investigating the fate of parental nucleosomes during the replication process.

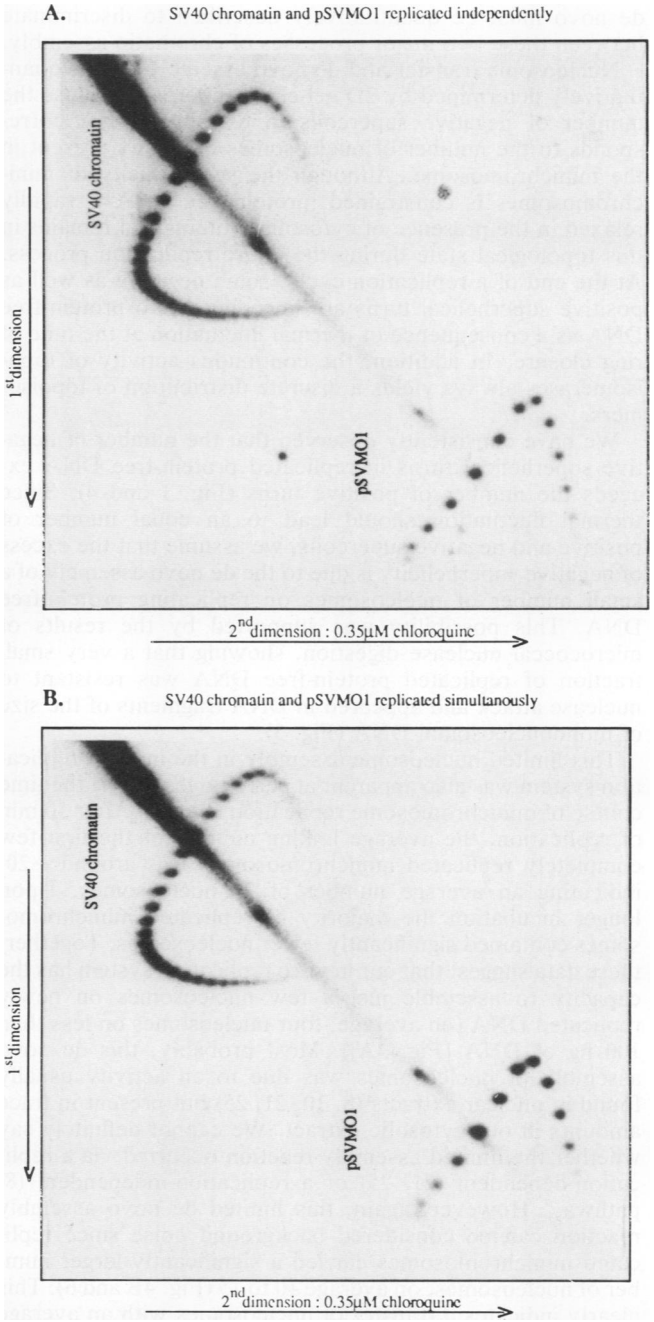

FIG. 10. 2D gel analysis of SV40 chromatin replication in the presence of competing pSVMO1 DNA. (A) Control experiment. Two reactions were performed in separate vials: in the first, 3 μl of chromatin was replicated in vitro, and in the second, 30 ng of pSVMO1 DNA was replicated in vitro. The products of the two reactions were mixed after deproteinization and applied to one 2D gel. (B) Competition experiment. Replication of SV40 chromatin and pSVMO1 took place in the same vial under competing conditions; 2.5 μl of SV40 chromatin and 10 ng of plasmid DNA were used as templates. The amounts of each template were chosen to yield identical incorporation rates. We show the autoradiograms of the parts of the gels where the monomeric replication products of the two templates were located.

To demonstrate the transfer of parental nucleosomes, the ability of the in vitro system to assemble new nucleosomes de novo must be quantitatively described to discriminate between these two major processes of chromatin assembly.

Nucleosome transfer and de novo assembly can be quantitatively determined by 2D gel electrophoresis because the number of negative supercoils in extracted DNA corresponds to the number of nucleosomes originally present in the minichromosome. Although the superhelicity of minichromosomes is constrained, protein-free DNA is rapidly relaxed in the presence of cytosolic proteins and remains in this topological state during the entire replication process. At the end of a replication cycle, some negative as well as positive superhelical turns are introduced into protein-free DNA as a consequence of thermal fluctuation at the time of ring closure. In addition, the continuous activity of topoisomerases always yields a discrete distribution of topoisomers.

We have consistently observed that the number of negative superhelical turns in replicated protein-free DNA exceeds the number of positive turns (Fig. 1 and 4). Since thermal fluctuation should lead to an equal number of positive and negative supercoils, we assume that the excess of negative superhelicity is due to the de novo assembly of a small number of nucleosomes on replicating protein-free DNA. This possibility was supported by the results of micrococcal nuclease digestion, showing that a very small fraction of replicated protein-free DNA was resistant to nuclease attack and appeared in DNA fragments of the size of mononucleosomal DNA (Fig. 3).

This limited nucleosome assembly in the in vitro replication system was also apparent at early stages during the time course of minichromosome replication (Fig. 6). After 30 min of replication, the average linking number of the first few completely replicated minichromosomes was around −20, indicating an average number of 20 nucleosomes. Upon longer incubation the majority of replicated minichromosomes contained significantly fewer nucleosomes. Together, these data suggest that our in vitro replication system has the capacity to assemble just a few nucleosomes on newly replicated DNA (on average, four nucleosomes on less than 100 ng of DNA [Fig. 4A]). Most probably, this de novo assembly of nucleosomes was due to an activity usually found in nuclear extracts (8, 10, 21, 25) but present in trace amounts in our cytosolic extract. We cannot definitely say whether the limited assembly reaction occurred via a replication-dependent (21, 25) or a replication-independent (8) pathway. However, again, this limited de novo assembly reaction can be considered background noise since replicated minichromosomes carried a significantly larger number of nucleosomes, on average 10 to 15 (Fig. 4B and 6). This clearly indicates a transfer of nucleosomes with an average delivery of half the complement of parental nucleosomes to the replicated daughter molecules. It is therefore possible to distinguish this process from the residual de novo assembly. In fact, de novo assembly becomes insignificant at replication times longer than 90 min, when the majority of chromatin participates in replication (Fig. 1A and C; also compare 30 and 150 min in Fig. 6).

In summary, previous experiments by Sugasawa et al. (28), as well as the experiments reported above, clearly show that a transfer of nucleosomes from the minichromosomal substrate to the replicated products occurs in the in vitro replication system.

Our data imply that the chromatin assembly factor CAF-1, needed for the assembly of new nucleosomes on replicated DNA (21), is not required for the transfer of prefork nucleosomes to the replicated DNA branches. Whether some other protein factor is involved in the transfer of old nucleosomes has not been determined yet. We also have no information about whether histone octamers are transmitted as intact units, as split nucleosomes (15, 30), or as histone H3-H4 tetramers which could later be converted to full octamers by the addition of H2A-H2B dimers, as has been discussed for the replication of cellular chromatin (11). We have noted, however, that the replicated minichromosomes possess a wide spectrum of nucleosomes ranging from essentially 0 to about 20. As already discussed in Results, this finding indicates a dispersive mode of nucleosome segregation and supports earlier in vivo results which excluded a transfer of nucleosomes to only one of the two emerging DNA strands (5, 23). In control experiments we observed that nucleosomes remain stably bound to replicated DNA and therefore do not dissociate after replication. It is also unlikely that a few remaining nucleosomes could nucleate a significant de novo assembly. In addition, since second rounds of replication do not occur (28), each completely replicated monomer is derived from a single replication event. Therefore, our linking-number analysis of replicated minichromosomal DNA independently supports the model of dispersive nucleosome segregation, as previously derived from hybridization experiments (5) and electron microscopy (23).

Mature SV40 minichromosomes are organized as an ordered array of spaced nucleosomes on the DNA (23). By using the technique of limited micrococcal nuclease digestion, the regular spacing of nucleosomes can be demonstrated as a ladder of multimeric nucleosome-size DNA fragments (reviewed in reference 28). Using replicated SV40 minichromosomes as the substrate, we were able to detect only monomeric and dimeric nucleosomal DNA fragments (Fig. 3). This probably indicates that the in vitro-transferred nucleosomes are probably not regularly spaced, but may rather appear as scattered mononucleosomes with a tendency to cluster locally, yielding dimers or possibly trimers (Fig. 3), as has previously been shown for SV40 chromatin replicated in vivo in cycloheximide-treated cells (5, 23).

An important question, addressed in the present work, is whether parental nucleosomes remain associated with DNA during minichromosome replication. This problem can adequately be investigated only in in vitro replication systems. In fact, Bonne-Andrea et al. (2) have previously presented a biochemical system to investigate this point and have presented evidence that nucleosomes could remain on replicating DNA. However, as mentioned in the Introduction, their experimental system is entirely artificial and their findings required an independent investigation in a more homologous system. We believe that this has been accomplished by the experiments described in this communication (Fig. 7 to 10), since we were able to demonstrate that an excess of protein-free DNA, added to the minichromosome replication mixture, did not change the mode of nucleosome segregation. This was found for competing plasmid DNA, which cannot replicate under the conditions of our experiments, as well as for origin-containing DNA, which replicated simultaneously with SV40 minichromosomes. Replicating DNA should be a highly specific and efficient competitor as it contains the structural elements at and behind the replication forks to which displaced nucleosomes (or their subunits) might bind. However, replicating minichromosomes did not lose constrained negative superhelicity in the presence of competing replicating DNA, and this is clear evidence that, at least in

replicating SV40 minichromosomes, histone complexes remain bound when the nucleosomal DNA is replicated. We do not know whether these histone complexes are intact octamers or histone H3-H4 tetramers (which can also induce constrained negative superhelicity in topologically fixed DNA [1, 3]).

This conclusion raises a number of further questions. We may ask, for instance, how histones are bound to the unwound DNA regions at replication forks. In fact, the precise conformation of histone complexes on single-stranded DNA remains to be studied. Furthermore, if only H3-H4 tetramers remain associated with replication forks, what is the mechanism for releasing H2A-H2B dimers at or ahead of the fork? Another interesting question concerns the simultaneous and overlapping binding of histones and elements of the replication machinery to nucleosomal DNA. More specifically, it would be interesting to know how histone-bound DNA could be unwound by helicases and function as a template for DNA polymerases. An investigation of these and related points will certainly be rewarding.

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