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Role of Amino-Terminal Histone Domains in Chromatin Replication

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Simian virus 40 minichromosomes were treated with trypsin to specifically remove the amino-terminal histone domains (tails). Trypsin treatment does not affect the spacing and the number of nucleosomes on minichromosomes but induces a more extended conformation, as shown by the reduced sedimentation coefficient of trypsinized minichromosomes compared with the untreated controls. Trypsinized minichromosomes replicate more efficiently than control minichromosomes in in vitro replication assays. The increased template efficiency appears to be due to higher rates of replicative fork movement. In vitro replication in the presence of protein-free competitor DNA shows that replicating trypsinized minichromosomes do not lose nucleosomes and replicating competitor DNA does not gain nucleosomes. This finding suggests that tailless nucleosomes are transferred from the unreplicated prefork stem to replicated DNA branches and excludes a participation of the basic histone domains in nucleosome transfer.

The replication of eukaryotic genomes is accompanied by an assembly of chromatin. This reaction consists of two fundamental processes, the transfer of parental nucleosomes from the unreplicated DNA stem to replicated DNA branches and the assembly of new nucleosomes from newly synthesized histones. Numerous studies in vivo and in vitro indicate that both processes proceed in two consecutive steps: an early deposition of histone H3-H4 tetramers followed by the association of two histone H2A-H2B dimers (reviewed in references 15, 16, 25, and 48).

Several basic facts concerning the fate of parental nucleosomes during replication have been discovered through in vitro experiments. Using phage DNA templates with in vitro-assembled nucleosomes and phage T4 replication enzymes, Bonne-Andrea et al. (7) first showed that histone octamers remain on DNA when a replication fork passes and do not dissociate in solution even in the presence of competing DNA. These results were confirmed with simian virus 40 (SV40) minichromosomes as natural templates, using extracts from proliferating human HeLa cells as a source for replication functions (27, 46). Similarly, replication of chromatin, assembled in vitro on SV40 DNA, was shown to be accompanied by a direct transfer of parental nucleosomes from prefork to replicated DNA in a distributive manner (37).

Electron microscopic examination of replicative intermediate SV40 minichromosomes revealed that replication forks move up to the next prefork nucleosome and that new nucleosomes appear on the replicated DNA branches at average distances of about 250 nucleotides behind the fork (43). Randall and Kelly (37) combined the results of the chromatin replication experiments in vitro and the electron microscopic observations and proposed an interesting model to explain how a replication machine can move along the template DNA without displacing the nucleosomes lying in its path. They suggested that advancing replication forks release positively charged amino acid side chains in histones of the prefork nucleosome. Released positively charged amino acids then immediately gain contact with newly synthesized DNA in daughter strands. Thus, a transient intermediate structure may exist in which parental DNA and newly synthesized DNA are bound to the same nucleosome. The model requires a position side by side of prefork and mature replicated DNA which could be achieved by looping out the intervening stretch of 250 nucleotides.

Positively charged amino acids are concentrated in the flexible amino-terminal histone domains which contain roughly 20% of all residues in core histones and include all sites for posttranslational modifications such as acetylations and phosphorylations. All four core histones have random coiled unstructured amino-terminal domains, and histone H2A has an additional unstructured carboxy-terminal arm (reviewed in reference 35). A prediction, based on the transfer model, is that histone octamers lacking the flexible domains should not be transferred from parental to progeny DNA, and it may be even possible that chromatin with truncated histones does not function at all as a replication template. We have tested the model by using SV40 minichromosomal templates that were treated with trypsin under conditions known to remove the charged flexible tails without attacking the central globular histone domain (6).

Many previous experiments had shown that trypsinized nucleosomes remain remarkably stable under a variety of in vitro conditions (see reference 3 and references therein). Trypsinized and untreated control minichromosomes can therefore be directly compared as templates under in vitro replication conditions. We found that trypsinized minichromosomes serve well as templates and that the amino-terminal histone domains are not required for the transfer of parental nucleosomes to replicated DNA strands. Interestingly, trypsinized minichromosomes replicate more efficiently than untreated control minichromosomes.

MATERIALS AND METHODS

Templates. Plasmid pSVori (2,643 bp) (53) and SV40 DNA (20) were purified according to published procedures.

Salt-treated SV40 minichromosomes were extracted from SV40-infected cells as described previously (14, 27) and purified by centrifugation through a 5 to 30% sucrose gradient, containing 500 mM NaCl, for 3 h at 39,000 rpm in the Beckman SW40 rotor. The minichromosomes were concentrated by centrifugation through a 30% sucrose cushion (with 500 mM NaCl) and resuspended in LS buffer (5 mM potassium acetate, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8], 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol). Minichromosome preparations were stored in aliquots at −70°C. The concentrations of DNA in these preparations were determined after deproteinization by agarose gel electrophoresis in comparison with known amounts of SV40 DNA marker.

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Trypsinization. Salt-treated minichromosomes (1 μg of DNA) were incubated with 100 ng of freshly dissolved trypsin (tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK] treated; Sigma) in 10 μl of LS buffer for 10 min at 25°C. Proteins were analyzed on 15% denaturing polyacrylamide gels (29) and visualized by silver staining (52). In our initial experiments, we added hen egg white trypsin inhibitor (Boehringer) to block trypsinization of chromatin (8). However, the trypsin inhibitor negatively affected the replication efficiency of HeLa cell extracts. We therefore purified minichromosomes by sucrose gradient centrifugation to remove trypsin (see below). We noted, however, that trypsinized minichromosomes, added directly to the reaction mixture, served well as a replication template, and control experiments showed that the addition of equivalent amounts of trypsin to the replication reaction of control minichromosomes had no effect on replication (data not shown). For this reason, all replication experiments reported below were performed under these conditions.

Electron microscopy. Purified control and trypsinized minichromosomes were diluted into triethanolamine buffer (10 mM, pH 7.5) and fixed with glutaraldehyde (final concentration, 0.1%). The samples were processed by using the BAC (benzyldimethylalkylammonium chloride) spreading technique of Vollenweider et al. (49) as described in detail before (51).

Chromatin replication. T antigen was prepared from baculovirus-infected insect cells (30) and purified by immunoaffinity column chromatography (39). Cytosolic extracts were prepared from proliferating HeLa cells (44). In standard experiments, 500 ng of chromatin was incubated with 1 μg of T antigen and 240 μg of cytosolic extract in a 50-μl incubation mixture for 2 h at 37°C exactly as described previously (17). In competition experiments, competitor DNA was added 10 min after incubation. Reactions were stopped by adding 0.7% sodium dodecyl sulfate–20 mM EDTA. DNA was extracted after treatment with 40 μg of proteinase K per ml by phenol extraction and ethanol precipitation. DNA was analyzed by agarose gel electrophoresis and autoradiography as described previously (27). In some experiments, extracted replicated DNA was digested by incubation with restriction nucleases before agarose gel electrophoresis. The autoradiograms were evaluated by laser densitometry, using the Scanpack program of the manufacturer (Biometra).

Micrococcal nuclease digestion. DNA or minichromosomes were treated with micrococcal nuclease (Boehringer Mannheim) at room temperature for the times indicated. The amounts of micrococcal nuclease used are given in the relevant figure legends. DNA fragments were purified from the incubation mixture and investigated by agarose gel electrophoresis as described previously (17).

RESULTS

Properties of trypsinized minichromosomes. Viral minichromosomes were prepared from African green monkey kidney CV-1 cells infected with SV40. When extracted from infected cells under low-salt conditions, SV40 minichromosomes are densely packed nucleoprotein particles containing stoichiometric amounts of the four core histones H2A, H2B, H3, and H4 as well as the outer histone H1 and a variety of nonhistone chromatin proteins (14). Among the nonhistone proteins is the chromatin assembly factor, CAF1 (26), which promotes the replication-dependent assembly of soluble histones (40, 41). Since the purpose of the present study was an investigation of the transfer of parental nucleosomes to progeny DNA and not the assembly of new nucleosomes, we treated native minichromosomes with 0.5 M potassium acetate to remove most nonhistone proteins, including CAF1 (26). Most salt-treated minichromosomes carry between 22 and 26 nucleosomes, but 20 to 30% of all minichromosomes have 2 nucleosomes less and contain a nucleosome-free gap of about 400 bp which includes the viral genomic control region and the origin of replication (24, 38, 43).

Salt-treated minichromosome preparations were split into two parts; one served as the control, while the other was treated with trypsin under experimental conditions known to specifically cleave the basic histone tails without attacking the central histone domains (6). We obtained an end point of enzyme titration at 80 to 120 ng of trypsin per μg of minichromosomal DNA, as determined by denaturing polyacrylamide gel electrophoresis (29) (Fig. 1). A comparison with published proteolytic cleavage products of chromatin (1a, 3) reveals that the prominent polypeptides obtained after trypsin treatment of minichromosomes correspond to the trypsin-resistant globular histone domains. The minor polypeptides on the gel (Fig. 1)

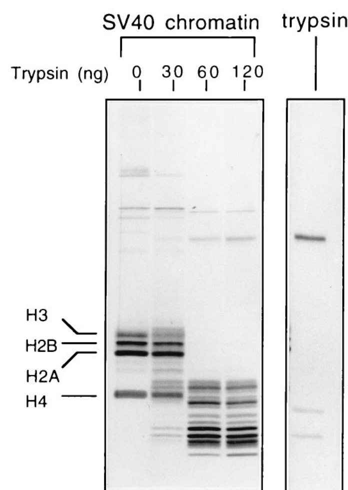


FIG. 1. Removal of the amino-terminal histone domains by trypsin. Salt-treated SV40 minichromosomes were incubated for 10 min with the amounts of trypsin indicated. Polypeptides were investigated by denaturing polyacrylamide gel electrophoresis (29) and visualized by staining with silver (52) (left). Trypsin alone was electrophoresed under identical conditions (right).

are most probably the proteolytic products of the nonhistone proteins remaining on salt-treated SV40 minichromosomes.

We investigated the chromatin templates by comparative micrococcal nuclease digestion experiments. Using control minichromosomes as the substrate, we obtained the familiar ladder of monomeric, dimeric, trimeric, and higher-order DNA repeats after short incubation times and DNA fragments of about 160 bp after longer incubation times (Fig. 2). In contrast, trypsinized minichromosomes yielded shorter final digestion products, about 145 bp in length (Fig. 2). Thus, limited nuclease digestion shows that trypsinized nucleosomes protect smaller DNA segments against nuclease attack.

For further characterization, we centrifuged control and trypsinized minichromosomes in parallel through sucrose gradients (in 50 mM NaCl) and found that trypsinized minichromosomes had an apparent sedimentation coefficient of only 39S, compared with 50S for control minichromosomes (Fig. 3A). We examined both chromatin preparations by electron microscopy to determine whether the reduced sedimentation rate was due to a loss of nucleosomes caused by trypsin treatment. We found in several independent experiments no significant differences between the numbers of nucleosomes on control and on trypsinized minichromosomes (Fig. 3B and C). We conclude that the reduced sedimentation coefficient of trypsinized minichromosomes is due to a more open conformation compared with control minichromosomes.

To investigate the arrangement of nucleosomes on SV40 chromatin, we determined the center-to-center distances of nucleosomes (1a) in electron micrographs of control and trypsinized minichromosomes carrying equal numbers of nucleosomes. As shown in Fig. 3D, the distributions of internucleosomal spacer lengths are quite similar in the two preparations. This result excludes a significant sliding of nucleosomes (5) as a consequence of trypsin treatment.

Replication of trypsinized minichromosomes. For the experiments to be reported below, it was important to determine the accessibility of the viral origin of replication for replication factors. To address this point, we took advantage of the fact that a single *Bgl*I restriction site is located in the SV40 origin sequence. We found that similar fractions of control and of

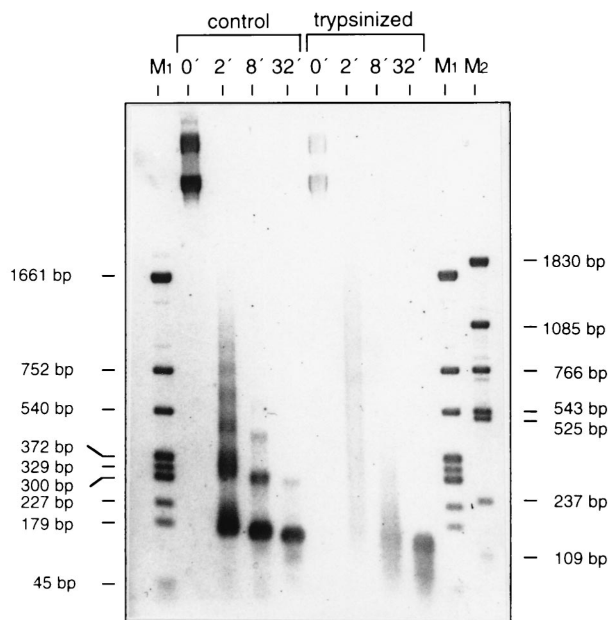


FIG. 2. Conformation of minichromosomes as determined by micrococcal nuclease digestion. Control minichromosomes (8 μg) and trypsinized minichromosomes (3 μg) were incubated with 8 and 3 U of micrococcal nuclease, respectively. Equal aliquots were removed at the indicated times and used for DNA extraction, analyzed by agarose gel electrophoresis, and stained with ethidium bromide. M1 and M2, marker DNA fragments as indicated.

trypsinized minichromosomes are linearized by *Bgl*I restriction (Fig. 4A) and conclude that molecules with open origins are equally abundant in the two preparations.

We also investigated whether minichromosomes function as templates for in vitro replication. For this purpose, equal amounts of control and trypsinized minichromosomes were added to in vitro replication mixtures containing the viral initiator protein (T antigen) and an unfractionated cytosolic extract from proliferating HeLa cells as a source for other replication functions (31, 44). Incubation with deoxynucleotides, including [α-^{32}P]dATP, was performed for 120 min under previously described conditions (17, 27). The DNA was extracted after incubation and investigated by agarose gel electrophoresis and autoradiography. Both control and trypsinized minichromosomes replicated well in the in vitro system and gave rise to very similar replication products (Fig. 4B, left two lanes), although trypsinized minichromosomes appeared to replicate more efficiently than control minichromosomes (see below). As usually found in experiments of this kind, a considerable fraction of the replication products had the electrophoretic migration rate of high-molecular-weight DNA (Fig. 4B) composed of replicative intermediates, catenated DNA (47), and aberrant forms of replicating DNA which arise after artifactual breakage of replication forks (42). Another fraction of replicated molecules corresponded to mature closed circular form I and open circular form II DNA. Most mature replication products were supercoiled, suggesting that the replication of minichromosomes was accompanied by a formation of nucleosomes. This can be concluded because supercoils are formed when histones are removed from topologically constrained chromatin, and it is known that one supercoil in deproteinized DNA corresponds to one nucleosome originally present in chromatin (12).

As also shown in Fig. 4B (right two lanes), *Bgl*I-restricted normal and trypsin-treated minichromosomes did not replicate. This is an important control for several reasons. First, the experiment shows that minichromosomes with closed origin regions did not participate in replication and implies that nucleosomes lacking the amino-terminal histone domains blocked the access of replication proteins to the origin as efficiently as normal nucleosomes. Second, the experiment excludes the possibility of extended DNA repair synthesis in this system. This conclusion was also supported by earlier experiments in which unrestricted control minichromosomes were incubated under replication conditions, but in the absence of T antigen. Without T antigen, no nucleotides were incorporated into SV40 chromatin (27).

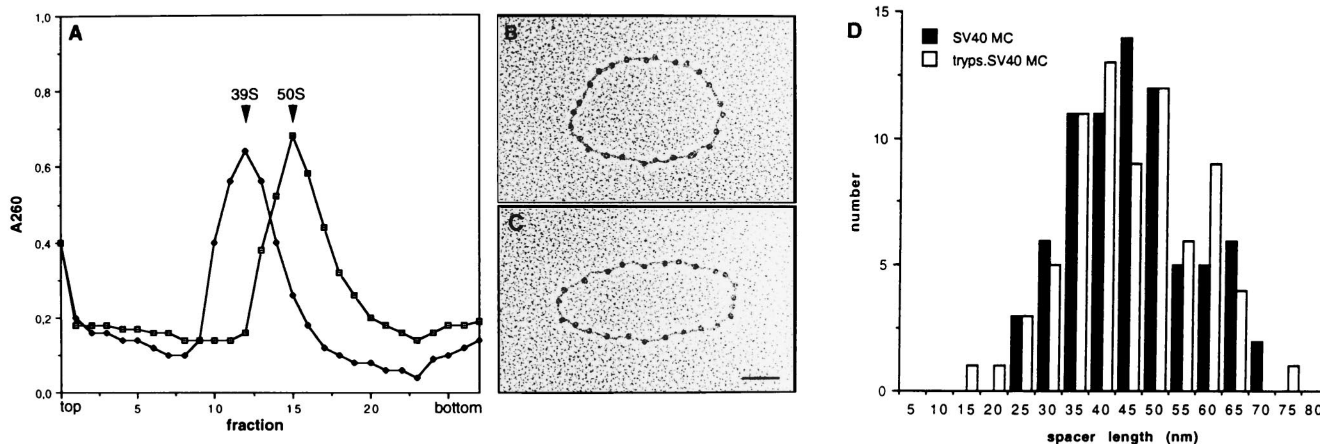


FIG. 3. Conformation of trypsinized minichromosomes. (A) Sedimentation rates. Control (squares) and trypsinized (diamonds) minichromosomes were sedimented through parallel 5 to 30% sucrose gradients made up in 50 mM NaCl–10 mM Tris-HCl–1 mM EDTA (pH 7.5). (B and C) Electron microscopy. Control (B) and trypsinized (C) minichromosomes were fixed in glutaraldehyde and spread by the BAC technique (49) for electron microscopic examination (bar, 200 nm). We counted 30 molecules from each preparation and determined averages of 24.4 (± 2.8) nucleosomes for the control and 22.3 (± 1.7) nucleosomes after treatment with trypsin. (D) Spacing of nucleosomes. We determined the distances between the centers of neighboring nucleosomes by using magnified (200,000-fold) positives of control (B) and trypsinized (tryps.) (C) minichromosomes (MC) carrying equal numbers of nucleosomes. Methods of length determination have been described in detail before (36, 51). Note that spacer lengths are operationally defined. Spacers, as measured in electron micrographs of chromatin, include linker DNA and nucleosomal DNA partially uncoiled from histone octamers during BAC spreading (14, 49). The estimated average size of linker DNA in minichromosomes with 25 nucleosomes is 45 bp, assuming 165 bp of DNA per nucleosome (total length of SV40 DNA, 5,243 bp).

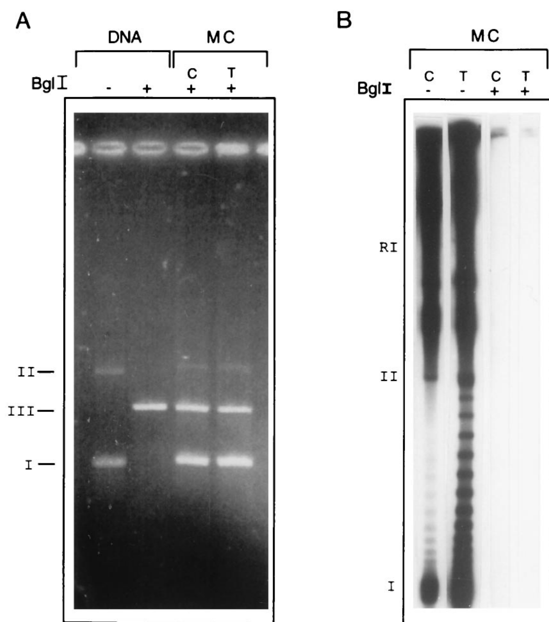


FIG. 4. Accessibility and function of the origin in control and trypsinized minichromosomes. (A) Accessibility. Protein-free SV40 DNA was completely linearized by an excess of *Bgl*I (left two lanes). Under the same conditions, only about a quarter of control and of trypsinized minichromosomal (MC) DNA was attacked by *Bgl*I (right two lanes). We show the results of an agarose gel electrophoresis after staining with ethidium bromide. (B) Function. Equal amounts of control (C) and trypsinized (T) minichromosomes were used as replication templates under standard in vitro replication conditions. DNA was extracted and investigated by agarose gel electrophoresis and autoradiography (left two lanes). The experiment was exactly performed as described above except that *Bgl*I-restricted control and trypsinized minichromosomes were used as templates for replication (right two lanes). RI, replicative intermediate DNA; I, superhelical form I DNA; II, relaxed and open circular form II DNA.

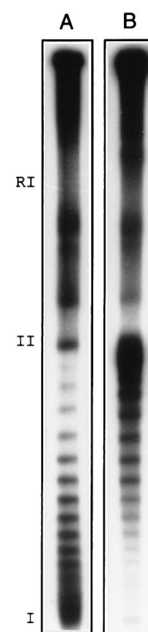


FIG. 5. Replicated minichromosomal DNA, but not replicated protein-free DNA, is highly supercoiled. Trypsinized minichromosomes (A) and SV40 DNA (B) were incubated for 2 h under standard replication conditions. The DNA was extracted and analyzed by agarose gel electrophoresis and autoradiography.

We conclude that trypsinized minichromosomes serve well as templates for in vitro replication and that replication products carry bound nucleosomes. We wanted to demonstrate that these nucleosomes originate from the parental template and are not assembled de novo from soluble histones present in the cytosolic extract (41). We therefore performed replication experiments in parallel with trypsinized minichromosomes and protein-free SV40 DNA. As shown in Fig. 5, replication of trypsinized minichromosomes yielded highly superhelical DNA, while the replication of protein-free DNA produced mainly circular DNA characterized by a few supercoils only. As previously discussed, supercoils in replicated protein-free DNA are mainly due to thermal fluctuations at the time of ring closure (27). The results are in agreement with earlier experiments which had shown that the experimental conditions allow a transfer of parental nucleosomes to replicated DNA but not the assembly of new nucleosomes from soluble histones. One reason for this is that CAF1, the chromatin assembly factor promoting the replicative assembly of new nucleosomes, is not present in the cytosolic extract used as source for replication enzymes (26, 41, 42).

As an independent assay to demonstrate the presence of nucleosomes in replicated chromatin, we performed digestion experiments using in vitro replicated chromatin as the substrate for micrococcal nuclease. In contrast to the experiment in Fig. 2 (control), we cannot expect to obtain a regular ladder of DNA fragments because parental nucleosomes are randomly dispersed to replicated DNA. Consequently, replication products contain an average of only half the maximal number of nucleosomes (27). In addition, the substrates for nuclease digestion are not only mature replication products but also replicative intermediate DNA structures with uncompletely assembled nucleosomes (17).

As shown in Fig. 6, replicated chromatin was more resistant against micrococcal nuclease than replicated protein-free DNA. Protein-free DNA was degraded to oligonucleotides after incubation for 2 min, while replicated minichromosomes yielded larger DNA fragments even after considerable longer incubation (Fig. 6). The nuclease-resistant DNA fragments appeared in two size classes. The digestion products of control minichromosomes are about 160 and 50 to 80 bp in size (Fig. 6). On the basis of earlier detailed analyses of Gruss et al. (17), we assume that the longer DNA fragments correspond to unit-length nucleosomal DNA, while the smaller fragments probably originated from histone H3-H4 tetramers on replicating molecules. The digestion products of replicated trypsin-treated minichromosomes were slightly smaller than the products in the control reaction (Fig. 6), as expected since the trypsinized nucleosomes appear to protect smaller sections of DNA against nuclease attack (Fig. 2).

Trypsin-treated minichromosomes replicate faster than control minichromosomes. In many independent experiments, we consistently observed that trypsinized minichromosomes are significantly more efficient as templates than untreated control minichromosomes (Fig. 4). To investigate this point in more detail, we performed experiments in which equal amounts of control and trypsinized minichromosomes were incubated under identical conditions in parallel replication mixtures. Equal aliquots were removed at increasing incubation times and analyzed by agarose gel electrophoresis (Fig. 7). The data show that during replication of trypsinized minichromosomes, labeled nucleotides were incorporated at higher rates into replicative intermediate DNA as well as into mature form I and form II DNA compared with control minichromosomes. For example, after 30 min of incubation, a substantial amount of

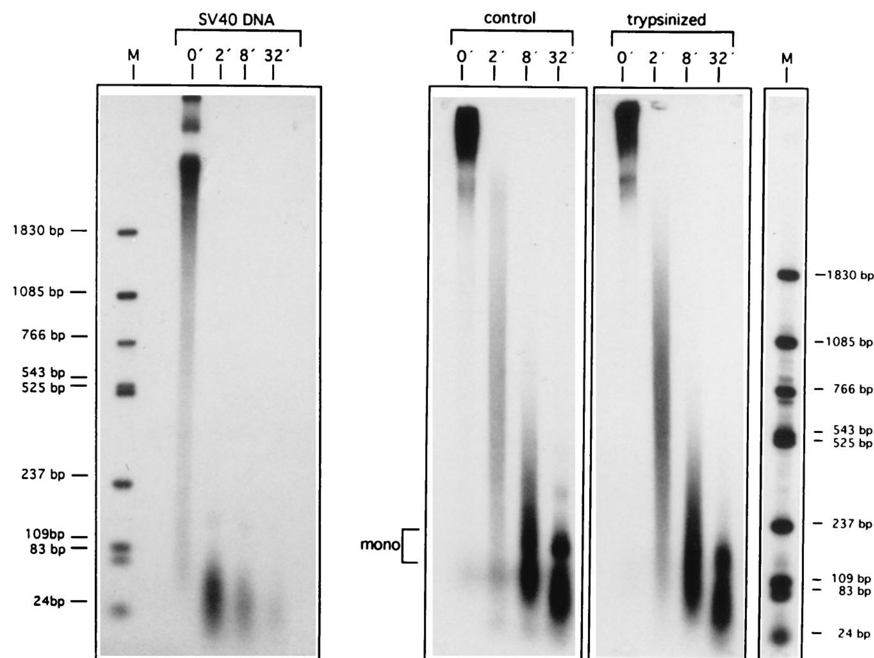


FIG. 6. Replicated minichromosomal DNA is partially protected against nuclease attack. SV40 DNA and control and trypsinized minichromosomes were replicated in parallel assay mixtures under standard conditions. Micrococcal nuclease (5 U) and $CaCl_2$ (final concentration, 3 mM) were then added to initiate nucleolytic degradation. Equal aliquots were removed for DNA extraction and agarose gel (1.5%) electrophoresis. Marker DNA fragments (M) were labeled by a fill-in reaction using the Klenow DNA polymerase fragment. We show the results after autoradiography. Times are indicated in minutes.

nucleotides was already incorporated into replicating trypsinized minichromosomes, while the replication of control minichromosomal templates had just started. Similarly, with trypsin-treated minichromosomes as templates, replicated mature form I DNA became clearly visible at 45 min of incubation and rapidly increased thereafter. In contrast, with control minichromosomes, comparable amounts of mature progeny DNA molecules were detected only after 60 min of incubation and thereafter increased at a reduced rate compared with the trypsin-treated chromatin template (Fig. 7). The results in Fig. 7 were supported by a parallel experiment in which replicated DNA was precipitated in 10% trichloroacetic acid to determine total incorporated nucleotides. We found that about 220 pmol of nucleotides was incorporated into replicating trypsinized minichromosomes after 90 min of incubation, compared with about 100 pmol of nucleotides incorporated into control minichromosomes.

The results in Fig. 7 could be due to a more efficient initiation or to a higher rate of replicative fork movement on trypsinized minichromosomes. This point was investigated in more detail by dissecting replicating minichromosomal DNA with restriction endonucleases which separate the origin-containing SV40 segment from flanking DNA segments and the termination region (Fig. 8A). For comparisons, we performed an identical experiment in a parallel assay mixture using protein-free SV40 DNA as the replication template.

Autoradiograms of restriction fragments show that nucleotides were incorporated first into the origin DNA fragment and then, after a short delay, into both flanking DNA segments and finally into the termination region (Fig. 8B). The order of nucleotide incorporation was the same in all three templates investigated, and the fact that both flanking segments (a and b in Fig. 8) were simultaneously labeled showed that protein-free DNA as well as minichromosomal DNA replicated in a bidirectional manner. However, as expected from the data in Fig.

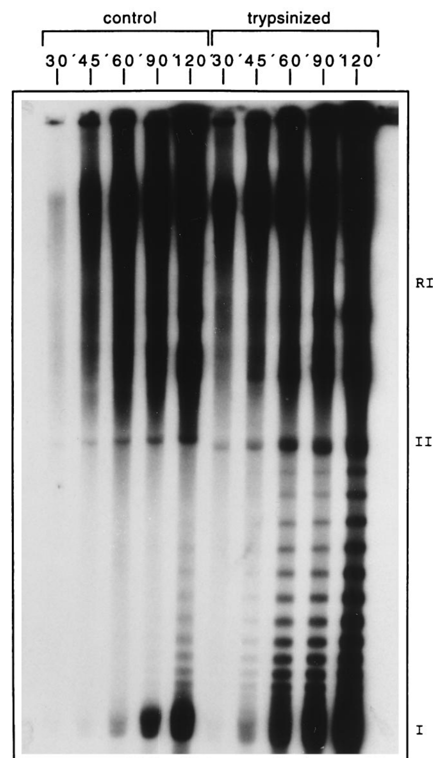


FIG. 7. Rates of chromatin replication. Trypsinized and control minichromosomes were incubated in parallel reaction mixtures under in vitro replication conditions with [α-^{32}P]dATP. At the times (minutes) indicated, equal aliquots were removed from the reactions and used for DNA extraction. Isolated DNA was investigated by agarose gel electrophoresis and autoradiography. RI, replicative intermediates; II, relaxed and open circular DNA; I, superhelical form I DNA.

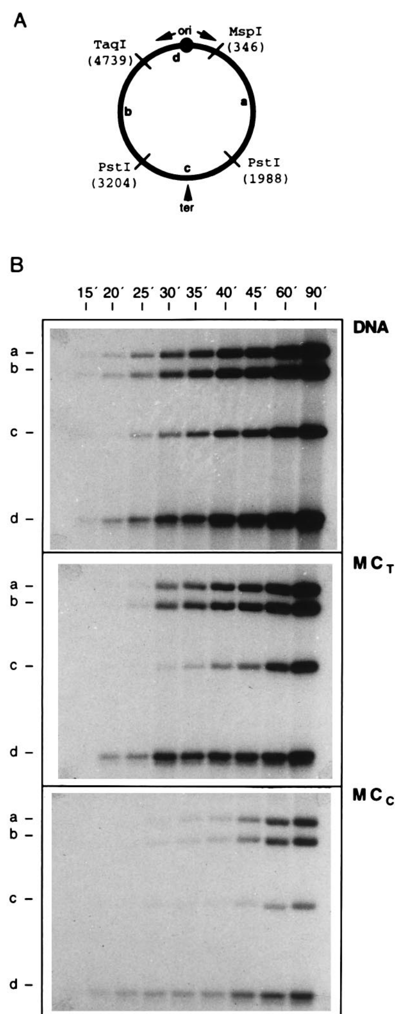


FIG. 8. Restriction analysis of replicating protein-free SV40 DNA and of minichromosomal DNA. (A) Restriction map showing restriction sites on circular SV40. Numbers refer to the SV40 nucleotide coordinates. The positions of the origin (ori) of bidirectional replication and the termination region (ter) are indicated. Restriction fragments are labeled a to d according to their lengths: d, origin fragment; a and b, flanking fragments; c, terminal fragments. (B) Protein-free SV40 DNA, trypsin-treated minichromosomes (MC_T), and control minichromosomes (MC_C) were replicated under standard conditions in parallel assay mixtures. Aliquots were removed at the indicated times (minutes) and used for the isolation of DNA. Extracted DNA was incubated with restriction nucleases *Msp*I, *Pst*I, and *Taq*I. The resulting DNA fragments were analyzed by agarose gel electrophoresis and autoradiography.

7, there are significant differences between the three templates with respect to the kinetics of nucleotide incorporation.

For a better evaluation, we determined the relative intensities of the autoradiographic signals by laser scanning densitometry and plotted the values as a function of replication times (Fig. 9). The data show that nucleotides were incorporated during the first 30 min with similar kinetics into the origin fragment of protein-free DNA and into the origin fragment of replicating trypsinized minichromosomes. However, the incorporation of nucleotides into the origin fragment of control minichromosomes is much slower (Fig. 9A). Interestingly, the origin fragment of protein-free DNA, but not of minichromosomal DNA, was replicated a second time beginning at 35 min of incubation (Fig. 9A). This result is consistent with an earlier report showing that already replicated protein-free DNA is able to rereplicate in vitro for at least one additional cycle, whereas replicated minichromosomes do not rereplicate under the same in vitro conditions (28).

Scanning data also show that nucleotides were incorporated with very similar kinetics into the two flanking segments of replicating protein-free DNA and of replicating trypsinized minichromosomes (Fig. 9B). Since initiation was delayed on control minichromosomal templates, nucleotides began to be incorporated at later times into the flanking DNA segments, but, in addition, the rate of nucleotide incorporation into flanking DNA segments was reduced compared with trypsin-treated minichromosomal templates or protein-free DNA.

Our conclusion is that the rate of replication fork movement is higher in replicating trypsinized than in control minichromosomes. This conclusion is consistent with the data shown in Fig. 9C, where we compare the times required for replication forks to reach the termination fragment. It is evident that termination is most efficient with protein-free DNA templates (nucleotide incorporation into the termination fragment could be detected after 25 min of incubation), while termination is clearly delayed for both minichromosomal templates. Thus, termination could be a time-limiting step in minichromosome replication, but in the present context, it is more important to note that the time required for the termination step was significantly shorter in trypsinized than in control minichromosomes. In summary, our results indicate that the observed increase in template efficiency after trypsinization of minichromosomes is most likely due to higher rates of replication fork movement.

Amino-terminal histone domains are not required for a transfer of parental nucleosomes to replicated DNA. The high replication efficiency of trypsinized minichromosomes cannot be explained by a substantial loss of nucleosomes during replication because experiments show that the replication products of trypsinized minichromosomes are organized as chromatin (Fig. 4 and 5) and that nucleosomes protect replicated DNA fragments of about 140 bp just as do nucleosomes in the corresponding minichromosomal templates (Fig. 6).

The question arises of whether trypsinized minichromosomes transfer their nucleosomes as efficiently as control minichromosomes to replicated DNA, or whether a fraction of trypsinized parental nucleosomes is lost during replication. The resolution of one-dimensional gel electrophoresis (as in Fig. 7) is insufficient for a quantitative investigation of this point. We therefore performed two-dimensional (2D) gel electrophoresis whereby electrophoresis in the second dimension is carried out in the presence of the intercalating drug chloroquine. Under these conditions, unconstrained linear or open circular DNA molecules migrate similar distances in both dimensions and appear on a diagonal, whereas topologically fixed circular DNA changes its electrophoretic migration rate depending on the number of supercoils present and the amount of intercalating chloroquine. Thus, closed circular DNA is displayed on 2D gels as an arc of DNA topoisomers (34). As shown in Fig. 10A, trypsinized minichromosomes replicated to produce progeny molecules which acquired an average of 12 to 14 supercoils after deproteinization. This result excludes a substantial loss of trypsinized nucleosomes during replication.

However, parental trypsinized nucleosomes may dissociate into solution during replication and then rebind to replicated DNA. In this case, the number of nucleosomes on replicated molecules should decrease in the presence of competing replicating protein-free DNA. This possibility was investigated by adding pSVori, a 2.6-kb plasmid carrying the SV40 origin (53), to a replication mixture with trypsinized minichromosomes. After incubation under standard replication conditions,

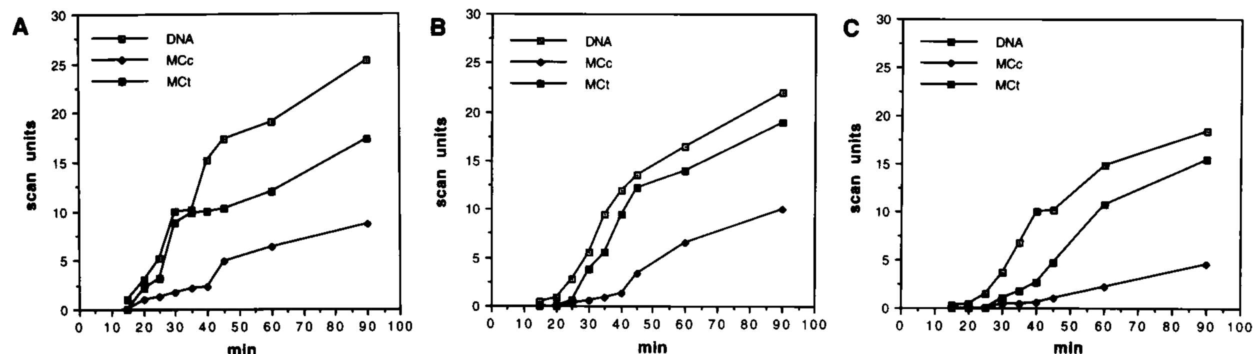


FIG. 9. Kinetics of nucleotide incorporation. The autoradiograms in Fig. 8 were analyzed by laser scanning densitometry. The results are expressed in arbitrary units and plotted as a function of incubation times. (A) Origin fragment (d in Fig. 8A); (B) flanking fragment (a in Fig. 8A; the scanning data for flanking fragment b are very similar and therefore not shown); (C) termination fragment (c in Fig. 8A). DNA, protein-free SV40 DNA; MCc, control minichromosomes; MCt, trypsin-treated minichromosomes.

replicated chromatin and replicated protein-free DNA were extracted and analyzed on one 2D gel (Fig. 10B). The data show that both trypsinized minichromosomes and protein-free pSVori replicated well in the same reaction mixture. More importantly, replicated minichromosomes contained 12 to 14 supercoils, exactly as found for the replication products synthesized in the absence of competing replicating DNA (compare Fig. 10A with Fig. 10B). Replicated protein-free DNA had a few positive and up to seven or eight negative supercoils ($\Delta LK = -7$ [Fig. 10B]). As previously discussed (27), these DNA topoisomers are mainly introduced by thermal fluctuations at the time of ring closure, but a very limited assembly of nucleosomes could be a possible explanation for the excess of negative over positive supercoils in replicated protein-free DNA. This could mean that a small number of nucleosomes were transmitted from replicating minichromosomes to replicating protein-free DNA. We excluded this possibility by showing in Fig. 10C the products of pSVori replicated in the absence of minichromosomes. The observed DNA topoisomer distribution was very similar to the distribution observed when plasmid DNA replicated together with minichromosomes in the same reaction mixture (compare Fig. 10C with Fig. 10B). Our conclusion is that trypsinized nucleosomes remained on minichromosomes during replication even in the presence of replicating competing plasmid DNA.

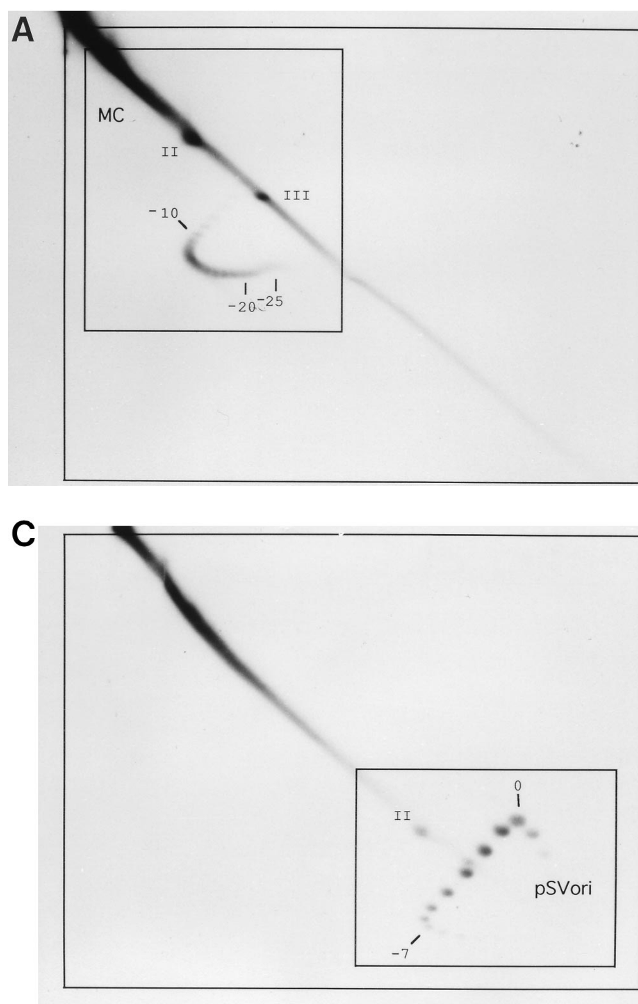
To confirm this conclusion, we performed several additional competition experiments with pSVori DNA in amounts higher than those used in the experiment of Fig. 10. We analyzed the replication products by one-dimensional agarose gel electrophoresis and could demonstrate that replicating trypsinized minichromosomes did not lose nucleosomes and replicating pSVori DNA did not gain nucleosomes when replicating in the same reaction mixture (not shown; see Fig. 10). However, analysis by gel electrophoresis provides information about closed circular DNA only, and the synthesis of mature replication products is severely inhibited when the relative amounts of competitor DNA exceed the amount of minichromosomes by factors of 2 or more (27). To determine the effects of higher competitor concentrations, we used the assay of Gruss et al. (17), who measured the formation of nucleosomes on replicating chromatin (including replicative intermediates) by using micrococcal nuclease digestion (Fig. 6). We found, in perfect agreement with published data (17), that competitor DNA in 5- to 10-fold excess over minichromosomes suppressed the assembly of nucleosomes on replicated DNA. But again, there was no detectable difference between the behaviors of control and trypsinized minichromosomes (data not shown). This result implies that the amino-terminal histone domains are not required for the transfer of parental nucleosomes to replicated DNA.

DISCUSSION

Structure of minichromosomes. SV40 minichromosomes, when extracted under low-salt conditions from infected cells, are dense nucleoprotein particles containing core histones in addition to linker histone H1 and numerous nonhistone chromatin proteins. Salt treatment of native minichromosomes induces the dissociation of the linker histone H1 and of many nonhistone proteins and converts the dense SV40 nucleoprotein into a more extended chromatin structure described as control minichromosomes in the present study (reviewed in reference 14).

Salt-treated minichromosomes carry 22 to 26 regularly spaced nucleosomes, and our nuclease digestion experiments (Fig. 2) and electron microscopy data (Fig. 3) provide no evidence for nucleosome sliding or other forms of rearrangements (5, 13). However, proteolytic removal of the amino-terminal basic domains (tails) induces a drastic conformational change, as demonstrated by a diminution of ca. 20% in sedimentation coefficient compared with the control. Electron microscopic examination excluded the possibility that the reduced sedimentation rate was due to a significant loss of nucleosomes following trypsinization. In fact, earlier experiments had shown that oligonucleosomes with proteolytically removed histone tails do not fold into compact chromatin structures (11) and possess reduced sedimentation coefficients compared with untreated controls (1a).

The flexible amino-terminal histone domains are not resolved in crystal structures of core nucleosomes and reach outside the two superhelical turns of DNA wrapped about the histone octamer (2). According to protein-DNA cross-linking data, the amino-terminal tails of histones H3 and H4 bind to DNA at about 1.5 helical turns from the dyad axis, whereas the amino-terminal domains of histones H2A and H2B are located more to the periphery of the nucleosomal core at distances of six to eight helical turns from the dyad axis (4, 10, 33). The amino-terminal domains of histones H2A and H2B may shield some of the negative charge of linker DNA and thus have a role in the folding of linker DNA in chromatin. The observed effects of limited proteolysis on chromatin structure support the notion that linker charge neutralization by the basic amino-



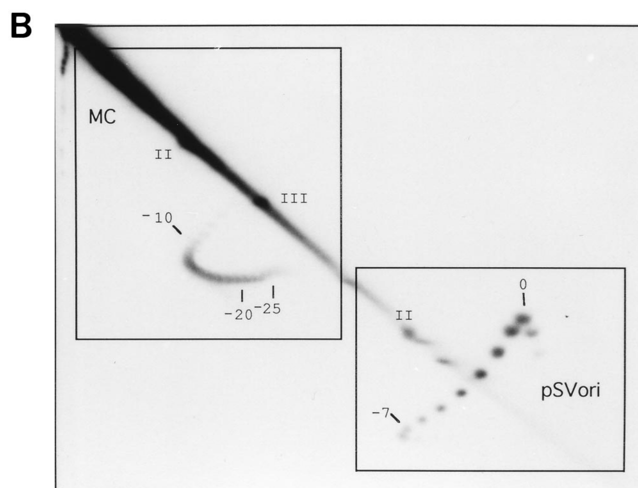


FIG. 10. Replicating protein-free DNA does not compete for the transfer of parental nucleosomes. (A) Five hundred nanograms of trypsinized minichromosomes (MC) was replicated as described in the legend to Fig. 4. The extracted DNA was investigated by 2D agarose gel electrophoresis: first dimension from top to bottom; second dimension from left to right in the presence of 0.5 μM chloroquine. II, open circular SV40 DNA; III, linear SV40 DNA. The position of closed circular DNA topoisomers is indicated by numbers referring to negative supercoils. (B) Five hundred nanograms of trypsinized minichromosomes (MC) and 1 μg of protein-free plasmid DNA (pSVori) were simultaneously replicated in the same replication mixture. Extracted DNAs were investigated on one 2D gel. (C) Control experiment; replication of 1 μg of protein-free plasmid DNA as analyzed by 2D agarose gel electrophoresis.

terminal histone tails contributes to the bulk of nucleosome-nucleosome interaction in compacted chromatin (18).

Replication efficiency. We observed that more DNA is synthesized with trypsinized minichromosomes as templates than with untreated control minichromosomes. We have excluded the possibility that a larger fraction of trypsinized minichromosomes participates in replication because only molecules with free origin regions are able to replicate in vitro, and such fractions are very similar in control and trypsinized chromatin (Fig. 4). The experimental data suggest instead that trypsinized minichromosomes are more efficient as templates than control minichromosomes.

One reason for this may be the more open chromatin structure induced by the proteolytic removal of the amino-terminal histone domains. Another reason for the increased template efficiency of trypsinized compared with control minichromosomes may relate to the structure of trypsinized nucleosomes. Although it is well established that the amino-terminal histone tails have little importance as far as nucleosome structure and stability are concerned, their removal by trypsinization has a dramatic effect on the melting of nucleosomal DNA (3). This implies that nucleosomal DNA may be more easily unwound in the absence of the amino-terminal tails. Consequently, trypsinized nucleosomes may be less formidable blocks on the path of the replication machine, allowing replication forks to migrate at higher rates.

Of course, proteolysis of histone tails does not normally occur in vivo, but experimental proteolysis may be considered to be an extreme case of charge reduction comparable to the reduction in charge balance that occurs in vivo by the acetylation of ε-amino groups in histone tails. In fact, recent experiments show that hyperacetylated viral minichromosomes function more efficiently as templates for replication than non-acetylated minichromosomes (1).

Replicative nucleosome transfer. SV40 minichromosomes with open origin regions function well as templates for chromatin replication in vitro. An important earlier finding was that the parental core nucleosomes remain bound to replicating DNA and do not dissociate into solution when challenged with an equal amount of replicating plasmid DNA as a nucleosome-free competitor (27, 37, 46). The problem is how DNA-histone contacts can be preserved when the replication machine moves along the nucleosomal DNA.

Two models have been proposed to explain the experimental results. One model assumes that nucleosome cores unfold by a disruption of histone-histone contacts within the octamer, while histone-DNA contacts remain when the DNA uncoils (7). We cannot formally exclude this model, but we think it unlikely given the extensive literature on nucleosome assembly and disassembly that favors the closing and opening of histone-DNA contacts as intermediate steps in chromatin formation (reviewed in reference 48).

A second model suggests a displacement of nucleosome cores during replication. Nucleosomes could be released in front of the advancing replication fork and would then immediately be recaptured on the emerging newly replicated DNA. This possibility was suggested (43) before it could be demonstrated that nucleosomes remain on replicating chromatin even in the presence of competing protein-free DNA. These results prompted a consideration of mechanisms by which parental nucleosomes could be directly transferred to replicated DNA.

A highly informative case of direct octamer transfer has been discovered in studies with phage SP6 RNA polymerase transcribing reconstituted chromatin in vitro (45). In this case, the histone octamer was shown to step around the transcribing RNA polymerase in a mechanism induced by the invading RNA polymerase. During this process, the DNA uncoils from the surface of the histone octamer to be immediately recaptured on the exposed octamer surface behind the RNA polymerase. As in replicating DNA (17), octamers are transferred to the transcribed template strand (in *cis*) in the presence of low concentrations of competitor DNA but to protein-free competitor DNA (in *trans*) at high competitor concentrations (45). The transcription studies of Studitsky et al. (45) provide a persuasive example for a direct nucleosome transfer, but it remains to be seen whether it can be applied as a model for replicative nucleosome transfer.

It has been shown in many studies in vivo and in vitro that the flanking histone H2A-H2B dimers dissociate from the tripartite nucleosome structure during replication, while the central histone H3-H4 tetramer remains bound to replicating DNA. The H3-H4 tetramer is therefore the unit that is directly transmitted from parental to newly replicated DNA. A complete histone octamer is assembled shortly after the transfer by an addition of histone H2A-H2B dimers to the DNA-bound H3-H4 tetramer (17, 21–23). Consistent with these findings, we have shown here that a substantial fraction of replicative intermediate chromatin is nucleolytically cleaved to give protected DNA fragments of 50 to 80 bp, indicating the presence of H3-H4 tetramers on replicating chromatin (17). Importantly, the amino-terminal tails of histones H3 and H4 are not required for their transfer from the prefork to a postfork position during replication. In fact, trypsinized nucleosomes behave in all aspects much like control nucleosomes. Our results exclude the possibility that the flexible basic tails interact with the first protein-free DNA regions emerging in their vicinity and then drag the histones to a postfork location. The results imply instead that an ordered opening of the inner contacts between the carboxy-terminal structured domains of histones H3 and H4 and replicating DNA must be followed by the closing of new contacts with postfork DNA. The process could be facilitated by positive supercoiling, believed to occur in front of replication forks (32). The uncoiling of nucleosomal DNA should be the same in control as in trypsinized chromatin because it is known that the conformations of histone-bound DNA are very similar in the central parts of untreated and trypsinized nucleosomes (9, 19).

The mode of replicative transfer of parental histone H3-H4 tetramers could in principle be related to the step-around mechanism of Studitsky et al. (45). However, at replication forks, histone complexes meet the large multiprotein replication machine (50), the leading part of which is most likely the T-antigen helicase, a hexameric complex of 500 kDa in molecular mass (51). The hexamer protects about 24 bp of the DNA double helix ahead of the fork and 10 to 20 bases of unwound DNA (51). Model studies have shown that T-antigen helicase alone is able to unwind nucleosomal DNA (36), but it has not been determined whether histones remain associated with unwound DNA. Thus, it remains a possibility that T-antigen helicase displaces the nucleosomes lying in its path while an additional and as yet unidentified component of the replication machine functions to channel histones from a prefork to a postfork position (25). The problem here is that this model does not account for the data of Bonne-Andrea et al. (7), who found that nucleosomes remain on DNA replicated by phage T4 enzymes, which never meet histones in their natural environment. It is therefore more likely that loops of replicating DNA reach around the replication complex to gain contact with the prefork histone H3-H4 tetramer. The loop cannot be larger than a few hundred base pairs (43), and it is presently not obvious how a loop of this size could bridge the large replication machine. Clearly, an understanding of the mechanism by which contacts are established between parental histones and new DNA requires more information about the topography of replication forks.

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