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Nucleosome assembly in mammalian cell extracts before and after DNA replication

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Protein-free DNA in a cytosolic extract supplemented with SV40 large T-antigen (T-Ag), is assembled into chromatin structure when nuclear extract is added. This assembly was monitored by topoisomer formation, micrococcal nuclease digestion and psoralen crosslinking of the DNA. Plasmids containing SV40 sequences (ori$^-$ and ori$^+$) were assembled into chromatin with similar efficiencies whether T-Ag was present or not. Approximately 50–80% of the number of nucleosomes *in vivo* could be assembled *in vitro*; however, the kinetics of assembly differed on replicated and unreplicated molecules. In replicative intermediates, nucleosomes were observed on both the pre-replicated and post-replicated portions. We conclude that the extent of nucleosome assembly in mammalian cell extracts is not dependent upon DNA replication, in contrast to previous suggestions. However, the highly sensitive psoralen assay revealed that DNA replication appears to facilitate precise folding of DNA in the nucleosome.

Key words: chromatin assembly/DNA replication/psoralen crosslinking/SV40

Introduction

DNA replication and chromatin assembly in eukaryotic cells are coordinated events. The replication fork transiently disrupts the chromatin fiber and the newly replicated DNA is organized into chromatin as rapidly as double-stranded DNA becomes available (DePamphilis and Bradley, 1986; Sogo *et al.*, 1986; Cusick *et al.*, 1989). A series of events must occur after DNA replication before the structure of chromatin becomes that of bulk chromatin. Assembly of the newly synthesized histones, redistribution of the pre-existing, parental nucleosomes on the two daughter molecules (Cusick *et al.*, 1984; Sogo *et al.*, 1986; Jackson, 1987, 1990; Seidman *et al.*, 1979; Handeli *et al.*, 1989) and the deposition of histone H1 are steps required for the chromatin maturation process (Cusick *et al.*, 1989). An appropriate *in vitro* system to study the relationship between DNA replication and chromatin assembly is provided by simian virus 40 (SV40), which associates with cellular histones to form a minichromosome indistinguishable from host chromatin (DePamphilis and Bradley, 1986).

In recent years, subcellular systems which allow both initiation and continuation of SV40 DNA replication *in vitro* using either SV40 origin-containing plasmids or SV40 minichromosomes as templates have been developed. They have greatly improved our understanding of the molecular processes involved in eukaryotic DNA replication (reviewed in DePamphilis, 1987; Kelly, 1988; Challberg and Kelly, 1989; Stillman, 1989); These systems rely on crude cytoplasmic extracts prepared either from transformed human cell lines, namely HeLa or 293 cells, supplemented with high amounts of purified SV40 large T-antigen (T-Ag) (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985) or crude cellular extracts prepared from SV40 infected monkey cells (CV-1), the natural host for SV40 (Li and Kelly, 1984; Yamaguchi and DePamphilis, 1986; Decker *et al.*, 1987). All of the *in vitro* replication systems mimic most of the known properties of SV40 DNA replication *in vivo* except that under such experimental conditions, no chromatin is formed and replication products formed in the presence of cytosolic extract supplemented with T-Ag are mostly relaxed (form II) DNA molecules.

Addition of nuclear extracts from mammalian cells to the cytosolic extracts leads to chromatin assembly on protein-free DNA templates. It has been sugested that this assembly is dependent on DNA replication (Stillman, 1986), reflecting the assembly of correctly spaced nucleosomes *in vivo* (Smith and Stillman, 1989). It has been proposed that chromatin assembly follows at least a two step maturation process promoted by the nuclear extract, where the first step is replication-dependent and the second step occurs only after replication has been completed (Fotedar and Roberts, 1989).

Alternatively, cell-free extracts derived from *Xenopus* eggs or oocytes have been reported to assemble chromatin, containing correctly spaced nucleosomes, independently of DNA replication (Laskey *et al.*, 1977; Dilworth *et al.*, 1987; Almouzni and Mechali, 1988b; Rhodes and Laskey, 1989; Shimamura *et al.*, 1989). In this case, no functional origin of replication is required for quantitative packaging of input DNA into chromatin. Moreover, plasmid DNA injected into frog (Ryoji and Worcel, 1985) or mouse oocytes (Martinez-Salas *et al.*, 1989) is assembled into chromatin in a process requiring ATP but independent of DNA replication.

In an effort to elucidate the process of chromatin assembly in cell-free systems derived from cultured mammalian cells further, we have analyzed chromatin assembly in unreplicated and post-replicative molecules, and to a limited extent, in replicating intermediates. We observe that (i) the template plasmid DNA undergoes a rapid process of relaxation followed by supercoiling, mediated by assembly of nucleosomes, (ii) both unreplicated and post-replicative DNA molecules are packed into chromatin with similar efficiencies suggesting that replication and chromatin assembly are not coupled and (iii) although DNA replication *in vitro* does not influence the extent of chromatin assembly, it appears to facilitate precise DNA folding in the nucleosome.

Results

In vitro replication with extracts from SV40 infected CV-1 and uninfected human 293 cells

Addition of a nuclear extract to reaction mixtures containing a cytosolic extract (S100) and T-Ag leads to assembly of chromatin on protein-free DNA templates (Stillman, 1986). In this paper we call such extracts nucleo-cytosolic replication extracts. We describe the process of chromatin assembly and its relationship to DNA replication by comparing extracts from two different sources and using several DNA templates. Nucleo-cytosolic extracts were prepared either from SV40 infected CV-1 cells or from uninfected human 293 cells (see Materials and methods). Protein analysis of the extracts shows that the core histones were provided almost exclusively by the nuclear extracts (estimated concentration: 250–350 ng/reaction for extracts of 293 cells), whereas the cytosolic extracts do not contain detectable amounts of histones (see Figure 1 for results with 293 cells; data not shown for CV-1 cells). We used constructs of the plasmid vector pML-1 (Figure 2a), containing either the SV40 origin, pSVori (2643 bp), or deletions in this region, pSVoriΔ6 (2638 bp) and pSV 21 (2535 bp) for DNA templates (mainly form I).

As previously reported, pSVori DNA replicates efficiently in extracts from both cell types (for 293 cells, see Figure 2b, lanes 3, 6; for SV40 infected CV-1 extracts, see Guo *et al.*, 1989), shown by the appearance of a *Dpn*I resistant band on the autoradiograph (Peden *et al.*, 1980). Note that a second broad and smearing band, sensitive to *Dpn*I, is visible (Figure 2b, lane 4), which corresponds to replicative intermediates. During incubation of pSVori with S100 and T-Ag (in the absence of nuclear extract), ~30% of the template DNA is replicated (135 pmol dNTPs incorporation for the standard assay as described in Materials and methods) in agreement with previous reports (Li and Kelly, 1985; Stillman and Gluzman, 1985). The template DNA (form I) is relaxed by endogenous nucleases and remains mostly relaxed with only a few topoisomer bands occurring during the reaction (Figure 2b, lane 1). The pSVoriΔ6 and the pSV21 templates are unable to replicate in these extracts (for pSVoriΔ6, note the absence of a *Dpn*I resistant band in Figure 2b, lane 9; data not shown for pSV21), indicating that DNA replication in these extracts is dependent on a functional SV40 origin. DNA replication with 293 extracts is also dependent on T-Ag (note the absence of a *Dpn*I resistant band in Figure 2b, lane 12). The labeled DNA in the lanes of undigested (−) or *Eco*RI digested (E) DNA with the 293 extracts might arise partially from repair synthesis (1.5 pmol dNTP), because after *Dpn*I digestion of the pSVoriΔ6 DNA (Figure 2b, lane 9) or pSVori DNA incubated without T-Ag (Figure 2b, lane 12), there remains no resistant (i.e. replicated) DNA. Therefore, repair synthesis occurs preferentially on supercoiled molecules. This has not been investigated further.

Addition of nuclear extract to the reactions leads to a 20-fold reduction of the replication efficiency (7 pmol for the standard assay as described in Materials and methods).

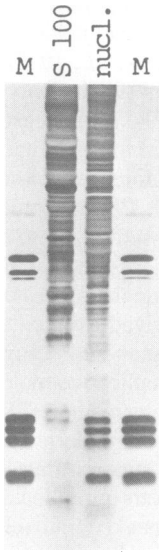

Fig. 1. Protein analysis of S100 and nuclear extract from human 293 cells. 30 μg of protein each of a S100 and a nuclear extract were subjected to 15% SDS–PAGE together with 1.6 μg of rat-liver histones (M). Proteins were visualized by silver-staining.

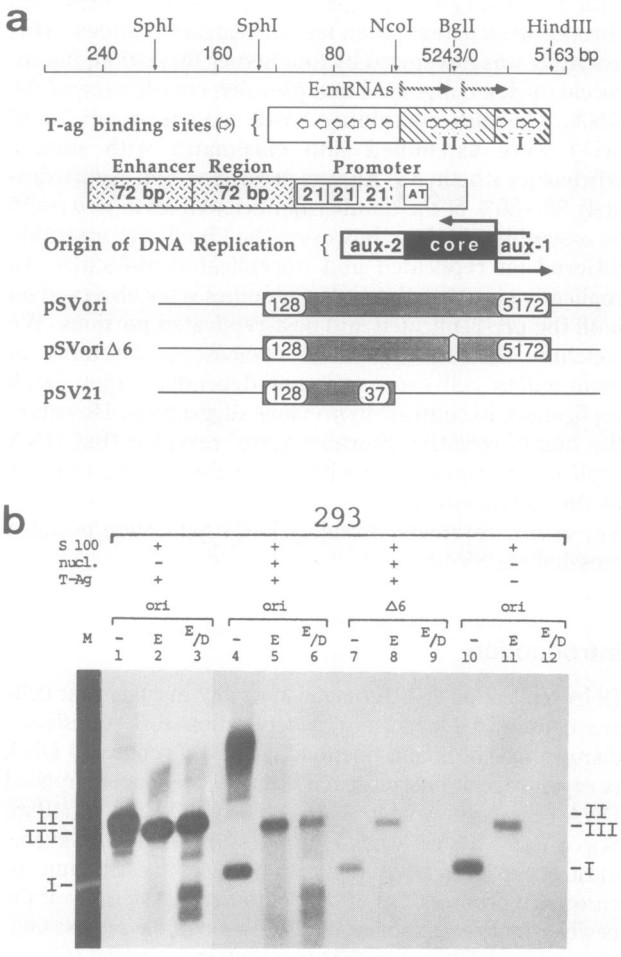

Fig. 2. *In vitro* replication assay with different DNA templates.
a: Map of the SV40 origin region and the plasmids carrying SV40 ori-components (for further description see Guo *et al.*, 1989).
b: Autoradiograph showing DNA replication assays: pSVori and pSVoriΔ6 DNA were incubated for 2 h at 37°C in the absence (−) or presence (+) of cytosolic (S100) and nuclear extracts from 293 cells or supplemented with purified T-Ag. The purified DNA was subjected to electrophoresis either undigested (−), *Eco*RI (E) or *Eco*RI and *Dpn*I (E/D) digested. I, II, III denote the positions of form I (covalently closed circular), form II (relaxed circular) and form III (linear) DNA, respectively.

We detect a *Dpn*I resistant band on the autoradiograph only and not in the ethidium bromide stained gel (data not shown), indicating that only a small proportion (1% or less) of the pSVori DNA is replicated. However, under these conditions, both newly synthesized [^{32}P]DNA (Figure 2b, lane 4) and bulk DNA (ethidium bromide stained, data not shown) are converted into form I, indicating that the DNA becomes complexed with proteins or might even be packed into nucleosomes (Germond *et al.*, 1975). Non-replicating pSVoriΔ6 DNA also appears as form I DNA (ethidium bromide stained; data not shown, but see Figure 3).

Thus, these results indicate that the conversion of form II DNA into form I DNA occurs independently of DNA replication.

Chromatin assembly in replication extracts as analyzed by topoisomer formation

Supercoiled DNA is obtained after treatment of minichromosomes with topoisomerase I and deproteinization. The number of supercoils present corresponds closely to the number of nucleosomes bound to the DNA during the relaxation reaction (Germond *et al.*, 1975; Barsoum and Berg, 1985). We incubated pSVori DNA for 1 h in both nuclear and cytosolic extracts of 293 cells (Figure 3a) or in cytosolic extract alone (Figure 3b), and then treated the products with an excess of topoisomerase I in order to remove all non-constrained supercoils. Incubation for 1 h in a cytosolic extract, which promotes replication of pSVori (see Figure 2b, lane 3) but contains no histone proteins (see Figure 1), and treatment with topoisomerase I yields form II DNA and some topoisomers, but no supercoiled form I band is seen (Figure 3b, lane 3). As a control, when the incubation was done in the absence of cell extracts, only some topoisomers were resolved as a consequence of the interaction between pSVori and T-Ag (data not shown, but for details see Gutierrez *et al.*, 1990, Figure 5). In the samples incubated with nuclear and cytosolic extracts, analysis of the purified DNA revealed the presence of several bands between form I and form II, which we interpret as a ladder of topoisomers (Wang, 1985). The band of form I DNA is quite prominent (Figure 3a, lane 4), suggesting that significant packing of the DNA with proteins had occurred in these extracts. When samples were incubated for 2 min instead of 1 h, topoisomerase I relaxed most of the supercoiled input DNA (Figure 3a, lane 3) but no form I DNA could be detected, probably because the incubation time was too short for the packing of the DNA with proteins. An aliquot of the sample incubated for 1 h in the replication reaction was treated with *Dpn*I instead of topoisomerase I (Figure 3a, lane 5). The fact that *Dpn*I resistant material is undetectable in the ethidium bromide stained gel indicates that the majority of the input DNA in Figure 3a, lane 4, is not replicated; therefore the packing of the DNA in these extracts as revealed by the topoisomerase I assay appears to be independent of the DNA replication mediated by these extracts.

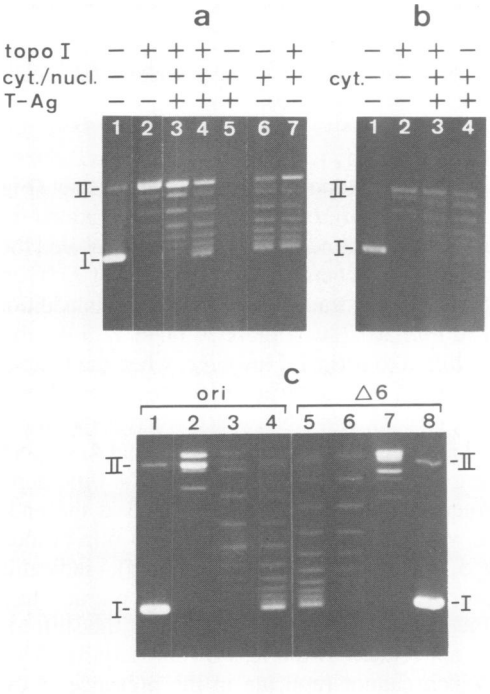

Fig. 3. Chromatin assembly in unreplicated molecules.
a and b: Topoisomerase I assay. pSVori DNA (300 ng) was incubated either in nucleo-cytosolic extracts (a: lanes 3, 4, 5, 6, 7) or in cytosolic extract (b: lanes 3, 4) from 293 cells. Immunopurified T-Ag was added as indicated (+). Incubations were performed for 1 h at 37°C, except for the sample 'a' in lane 3, which was incubated for 2 min at 37°C. After incubation, the purified DNA of one aliquot (a: lane 5) was digested with *Dpn*I. The protein-free input DNA (a: lane 1, b: lane 1) was treated for 5 or 30 min at 37°C with 16 U topoisomerase I (a: lane 2, b: lane 2, respectively). The reconstituted molecules from samples a: lanes 3, 4, 6, 7 were treated for 5 min, from samples b: lanes 3, 4 for 30 min with 16 U of topoisomerase I. The reaction products were isolated by protease digestion, phenol extraction and ethanol precipitation and then subjected to agarose gel electrophoresis. The gel was stained with ethidium bromide.
c: Supercoiling assay with relaxed template DNAs. pSVori (1) and pSVoriΔ6 (8) DNA templates were relaxed by purified topoisomerase I (1 U/200 ng DNA) for 30 min at 37°C and purified. Relaxed template DNAs (pSVori lane 2, pSVoriΔ6 lane 7) were incubated for 2 h at 37°C in either cytosolic extract (lane 3, pSVori; lane 6, pSVoriΔ6) or in nucleo-cytosolic extracts (lane 4, pSVori; lane 5, pSVoriΔ6). Reaction products were purified (compare Figure 3a,b) and subjected to 1% gel electrophoresis.

Experiments were also performed without T-Ag (Figure 3a, lanes 6, 7). Although no replication takes place in the absence of T-Ag (see Figure 2b, lane 12), after incubation for 1 h and subsequent treatment with or without topoisomerase I, we obtained the same ladder of topoisomers with a prominent supercoiled form I band (Figure 3a, lanes 6 and 7) as described above for the experiment in the presence of T-Ag. That a similar distribution of topoisomers is seen in the presence and absence of topoisomerase (Figure 3a, lanes 6 and 7) suggests that the extracts used contain a topoisomerase activity (see also Figure 3b, lanes 3 and 4 and Figure 2b, lane 1).

We also used form II DNAs from pSVori and pSVoriΔ6 as templates. The almost completely relaxed molecules were obtained by treating the DNA with purified topoisomerase I (Figure 3c, lanes 2 and 7). After incubation with cytosolic extract and T-Ag, the number of topoisomers increases but practically no form I DNA is formed (lanes 3 and 6). However, when the incubation was performed in nucleo-cytosolic extracts supplemented with T-Ag, a prominent band corresponding to form I DNA appears for both DNA templates used (Figure 3c, lanes 4 and 5). The formation of such structures occurs independently of DNA replication (pSVoriΔ6 does not replicate) and is also independent of the topology of the input DNA (supercoiled in Figure 3a, 3b and relaxed in Figure 3c).

In order to test whether chromatin assembly occurs with

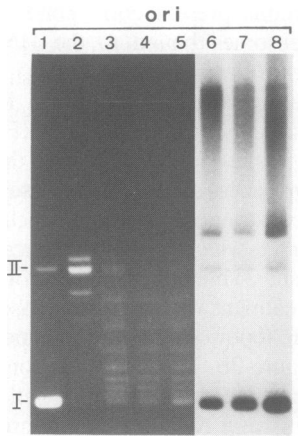

Fig. 4. Kinetics of supercoiling of unreplicated and post-replicative molecules. Relaxed pSVori DNA (lane 2, see Figure 3) was incubated for 30 min (lanes 3, 6), 1 h (lanes 4, 7) and 2 h (lanes 5, 8) in nucleo-cytosolic extract supplemented with T-Ag. Purified reaction products were subjected to 1% gel electrophoresis. The ethidium bromide stained gel (lanes 1, 2, 3, 4 and 5) and the autoradiograph (lanes 6, 7 and 8) are shown.

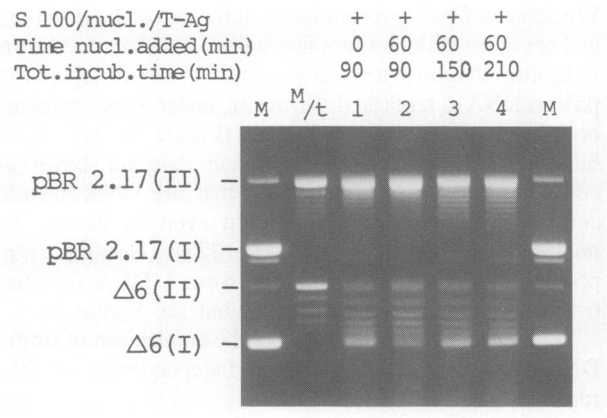

Fig. 5. Influence on DNA supercoiling of incubation time after addition of nuclear extract. pSVoriΔ6 DNA (150 ng) was incubated for 90 min with S100, nuclear extract and T-Ag (lane 1) or preincubated for 1 h with S100 and T-Ag with subsequent addition of nuclear extract. Reactions were further incubated for 30 min (lane 2), 90 min (lane 3) and 150 min (lane 4). pBR 2.17 DNA was added after this time and incubated for an additional 5 min at 37°C. The input DNA (M) was then relaxed for 5 min at 37°C with 10 U topoisomerase I (M/t). Reaction products were isolated by protease digestion, phenol extraction and ethanol precipitation and then subjected to agarose gel electrophoresis.

different kinetics on newly replicated DNA compared with unreplicated molecules, we repeated the same kind of experiment (form II template DNA, nucleo-cytosolic extract, T-Ag) but in the presence of ^{32}P-labeled precursors. After 30 min incubation, only a faint band of the total DNA is detected as form I (Figure 4, lane 3), which becomes more prominent after 1 h (Figure 4, lane 4) or 2 h (Figure 4, lane 5) incubation. In contrast, in the corresponding autoradiograph, form I DNA is already the almost exclusively labeled band after 30 min incubation (together with replicative intermediates, Figure 4, lane 6). This result clearly indicates that the replicated DNA assembles faster into chromatin compared to the unreplicated input molecules (see also Almouzni and Mechali, 1988a).

The fact that a large amount of supercoiling is obtained in the experiments with cytosolic extract supplemented with nuclear extract (Figure 3a, lane 4) which contains significant amounts of histones (see Figure 1), but not in the experiments with cytosolic extract alone (Figure 3b, lanes 3 and 4), confirms that the histones in the nuclear extract associate with the input DNA to form chromatin.

Kinetics of the supercoiling reaction

It has been suggested that chromatin assembly occurs only on replicating DNA molecules (Stillman, 1986; Smith and Stillman, 1989; Fotedar and Roberts, 1989). Preincubation of ori$^+$ DNA with S100 and T-Ag and subsequent addition of nuclear extract prevented supercoiling of the DNA. We have repeated these experiments with pSVoriΔ6. After incubation under different conditions (see below), form I pBR 2.17 DNA was added as an internal control for the topoisomerase I activity and incubation was continued for another 5 min. pBR 2.17 DNA does not contain the SV40 origin core sequence and therefore no specific interaction with SV40 T-Ag is to be expected (Gruss *et al.*, 1984). The purified DNA samples were then analyzed by gel electrophoresis (Figure 5). After incubation for 90 min with nucleo-cytosolic extracts and T-Ag, most of the pSVoriΔ6 DNA is shifted to form I (Figure 5, lane 1). However, the pBR 2.17 (form I) which was added at the end of the reaction appeared to be mostly relaxed. This DNA was relaxed either by the endogenous topoisomerases in the extract (Figure 5, lane 1, upper band) or by exogenously added topoisomerase I (data not shown). When we preincubated the DNA with cytosolic extract and T-Ag for 60 min at 37°C and then added the nuclear extract and incubated for an additional 30, 90 or 150 min at 37°C (Figure 5, lanes 2, 3 4), the DNA is again shifted to form I. However, when the reaction with nuclear extract was allowed to proceed for 30 min only (Figure 5, lane 2), less DNA was converted into form I than observed after 90 and 150 min (lanes 3 and 4, respectively), indicating that the shift to form I DNA is time-dependent. The protein-free pBR 2.17 DNA added at the end of the reaction was readily relaxed within the 5 min of incubation (Figure 5, lanes 2, 3, 4, upper band), indicating that topoisomerase activity remained in the extract during the entire incubation. This demonstrates that the shift to form I DNA is not related to the loss of this activity. Therefore, DNA which cannot replicate in the presence of cytosolic extracts and T-Ag is shifted to form I in a time-dependent manner upon addition of nuclear extract.

Analysis of unreplicated and replicated assembled chromatin by micrococcal nuclease digestion

In order to test whether the conversion of the non-replicating pSVoriΔ6 into form I DNA reflects assembly into chromatin upon incubation in nucleo-cytosolic extracts, the reaction products were digested with micrococcal nuclease. pSVoriΔ6 was incubated for 2 h as described in the previous section. Assembled protein−DNA complexes were treated with micrococcal nuclease and the purified DNA digestion products were fractionated by agarose gel electrophoresis, blotted and hybridized to nick-translated pSVoriΔ6 DNA (Figure 6). As expected, control pSVoriΔ6 DNA was quickly degraded into small fragments which were not retained in the gel. In contrast, the smallest digestion fragments obtained from the assembled products were ~150 bp and they were detectable at the earliest digestion

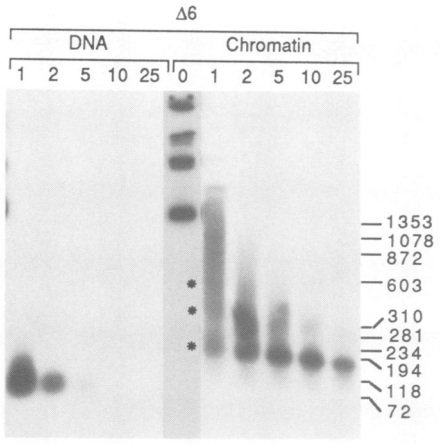

Fig. 6. Micrococcal nuclease digestion of *in vitro* reconstituted pSVoriΔ6. pSVoriΔ6 DNA was incubated for 2 h at 37°C in nucleo-cytosolic extracts, supplemented with T-Ag. Assembled mini-chromosomes were separated on a Sepharose CL-4B column and the fractions containing protein−DNA complexes were adjusted to 3 mM $CaCl_2$. Micrococcal nuclease digestion was performed for 0, 1, 2, 5, 10 and 25 min at 22°C with 30 U (pSVoriΔ6 chromatin) or 6 U (protein-free pSVoriΔ6 DNA) micrococcal nuclease. DNA was purified, subjected to 1.5% agarose gel electrophoresis and, after Southern transfer, hybridized to nick-translated pSVoriΔ6 DNA. Asterisks correspond to monomeric, dimeric and trimeric forms (from bottom to top). A φX174/*Hae*III digest was used as DNA size markers (right hand side).

time. Moreover, a characteristic repeating pattern of ~150 bp was observed. Both the size (150−180 bp) of the most promiment band and the presence of the ladder suggest chromatin assembly of the non-replicating pSVoriΔ6. The fact that the nucleosomal bands were superimposed over a smear (especially at short digestion times) indicates that chromatin assembly is either incomplete and/or that the inter-nucleosomal spacing is irregular (see next paragraph). Indistinguishable results were obtained (data not shown) when the incubation was done with pSVori (replicated molecules).

Analysis of chromatin assembly in replication extracts with the psoralen assay

Upon psoralen crosslinking of chromatin (Hanson *et al.*, 1976; Cech *et al.*, 1977; Sogo *et al.*, 1984; Conconi *et al.*, 1984) crosslinking occurs predominantly in the linker region between nucleosomes but not in the nucleosomal DNA. Spreading of the deproteinized DNA under denaturing conditions then allows visualization of the DNA of individual nucleosomes as rows of single-stranded bubbles (ss-bubbles) with a size of about 140 to 160 nucleotides.

Unreplicated chromatin. We investigated the process of putative chromatin assembly in nucleo-cytosolic replication extracts from SV40-infected CV-1 cells or from human 293 cells. Since the appearance of the molecules from the extracts of both cell sources was indistinguishable, the experiments from different nucleo-cytosolic extracts are not discussed separately. After incubation of the DNA in extracts for 120 min, the samples were photoreacted with psoralen and analyzed by electron microscopy. DNA from the endogenous SV40 chromatin (Figure 7a, arrows, twice the size of the exogenously added DNA templates), present in SV40 infected CV-1 extracts was organized in ss-bubbles, exactly as described previously (Sogo *et al.*, 1986). It is well established that the ss-bubbles correspond to DNA that is inaccessible to psoralen because of the presence of a nucleosome (see also data below). However, the pSVori molecules, which were added as protein-free DNA to the nucleo-cytosolic extract, showed a heterogenous bubble pattern. While some of these molecules consisted almost completely of ss-bubbles, other molecules had only very few such bubbles [Figure 7a arrowheads; the thick molecule showing no bubbles, which is indicated by an asterisk, corresponds to covalently closed form I DNA (Sogo *et al.*, 1986)].

Since we cannot distingiush between unreplicated and post-replicative molecules using pSVori (although from the data of Figures 2 and 3, the proportion of post-replicative molecules must be very low), we also tested pSVoriΔ6 and pSV21 (Figure 7b, c), which cannot be replicated (see Figure 2b). Both plasmids were organized in ss-bubbles with a heterogeneous pattern similar to that observed with pSVori DNA. These bubbles disappear when the crosslinking is performed in the presence of 1.2 M NaCl or heparin (500 μg/ml) (data not shown; see Ohlenbusch *et al.*, 1967; Stockley and Thomas, 1979; Sogo *et al.*, 1984; Lucchini *et al.*, 1987), which indicates nucleosomal packing. Moreover, after incubation of DNA with nucleo-cytosolic extracts, the sample was divided into two aliquots. One of them was immediately psoralen crosslinked, whereas the other was fractionated on a Sepharose column to reduce the excess of unbound protein in the fractions containing the assembled molecules. Fractions containing most of the DNA−protein complexes were then psoralen crosslinked. DNA analysis of both crosslinked aliquots by electron microscopy showed that the average number of ss-bubbles remained unaltered, suggesting that the excess of unbound protein does not change the crosslinking pattern. These experiments confirm our data obtained with the topoisomerase I assay (see Figures 3, 4 and 5), where we have shown that unreplicated DNA is shifted to form I DNA after incubation in nucleo-cytosolic extracts. The results also agree with beaded structures observed by direct visualization of the nucleoprotein complexes by electron microscopy (data not shown). This chromatin assembly process can clearly occur in the absence of DNA replication.

Plasmid pSVori was also incubated for 30 (Figure 8a) and 120 min (Figure 8b) in cytoplasmic extracts from 293 cells. In contrast to the reactions with nucleo-cytosolic extracts, the DNA appeared rather homogeneously crosslinked with only a few, small ss-bubbles. Both bidirectionally replicating molecules (Figure 8a, arrow) and rolling circle-type replicating molecules (sigma structures, Figure 8b) had the same appearance. The size of these small bubbles was, on average, 82 ± 16 nucleotides. They are considered not to be derived from nucleosomes (see Sogo *et al.*, 1984). Since cytosolic extracts induce only minor supercoiling in the topoisomerase I assay described above (Figure 3b, lane 3 and 4), we interpret this result as an indication that these small bubbles originate mostly from protein−DNA interactions not related to chromatin structure. This interpretation is consistent with the lack of histones in the cytosolic extracts (see Figure 1) and with previous observations that cytosolic extracts are not capable of chromatin assembly (Smith and Stillman, 1989).

The observations described so far indicate that in nucleo-cytosolic extracts from mammalian cells, chromatin assembly

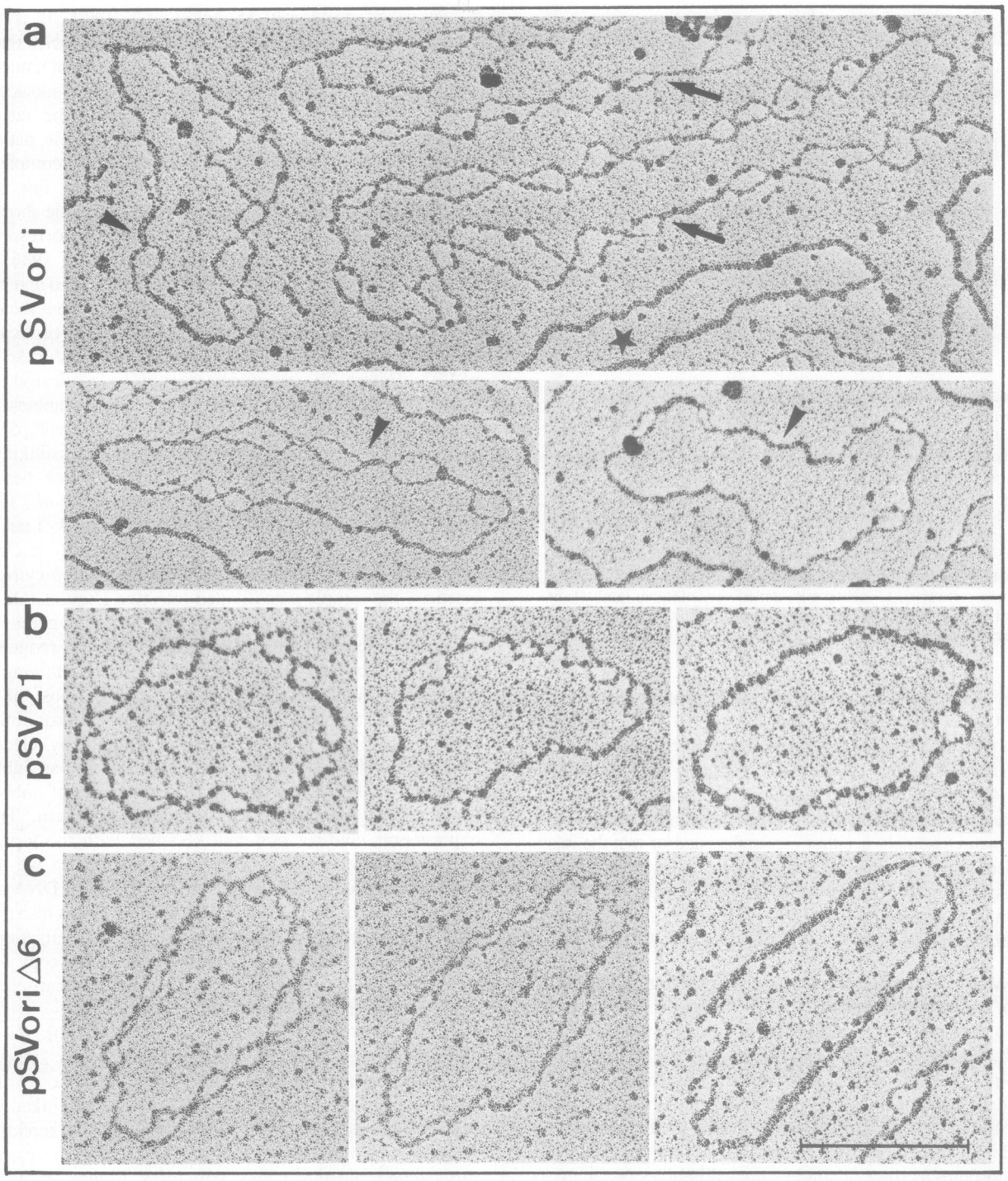

Fig. 7. Chromatin assembly. pSVori (**a**), and pSV21 DNA (**b**) were incubated for 2 h at 37°C with nucleo-cytosolic extracts from SV40 infected CV-1 cells; pSVoriΔ6 DNA (**c**) was incubated for 2 h at 37°C with nucleo-cytosolic extracts from 293 cells. The samples were photoreacted with 4,5′,8-trimethylpsoralen, deproteinized, relaxed with DNase I and spread for electron microscopy under denaturing conditions. The relaxed molecules show bubbles consisting of single-stranded (ss) DNA with a size of ~140 nucleotides for the pSVori (a), pSV21 (b) and pSVoriΔ6 (c) and ~160 nucleotides for the endogenous SV40 chromatin (arrow) (see also Figure 10). The SV40 molecules are twice the size of the pSVori,, pSVoriΔ6 and pSV21 constructions used as templates. The bar represents 500 nucleotides.

can take place independently of DNA replication. Cytosolic extracts alone do not promote assembly of such structures.

Replicating and post-replicative molecules. To characterize the relationship between *in vitro* chromatin assembly and DNA replication, we analyzed replicating and post-replicative molecules from nucleo-cytoplasmic extracts from both cell sources. In replicative molecules, ss-bubbles were found on daughter strands (Figure 9d) and also on parental strands (Figure 9a, b). However, most of the early (up to 70% replicated) replicating molecules (Figure 9a–c) contained only a few ss-bubbles. The frequency of ss-bubbles increased considerably on the daughter strands of late replicating intermediates (Figure 9d). This may indicate that

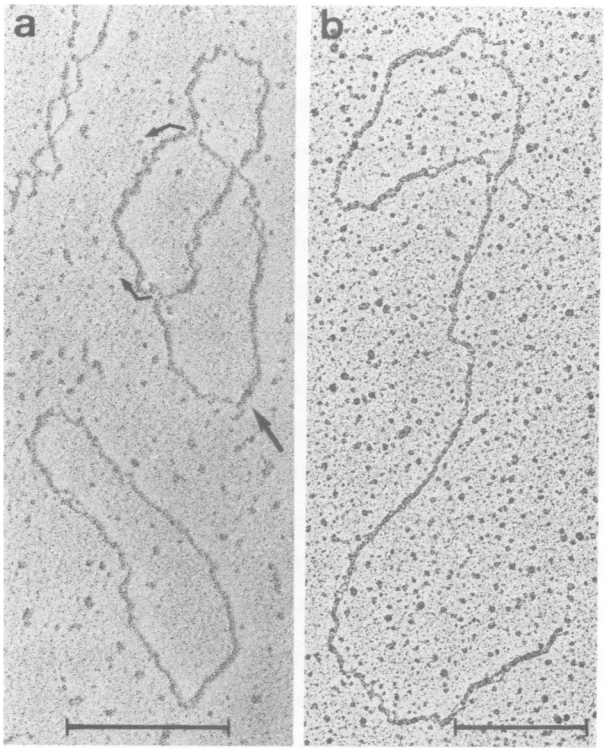

Fig. 8. Analysis of replicating molecules in cytosolic extracts. Reaction mixtures containing pSVori DNA, 293 cytosolic extract and purified T-Ag were incubated for 30 (**a**) and 120 min (**b**) at 37°C and photoreacted with trimethylpsoralen. The purified DNA was prepared for electron microscopy as described in Figure 7. The bar represents 500 nucleotides. The statistical analysis of the replicating molecules revealed that after 30 min, most (80%) of the molecules were so-called θ structures (bidirectional replication); a second type of branched molecules (σ structures, rolling circle replication), consisting of the circular pSVori genome and a single tail, was observed with a low frequency (20%). Length measurements of the tail showed a variable length, but the majority of the tails was smaller than the circular genome. In contrast to this, after incubation for 2 h, 85% of replicating molecules were σ structures with a tail up to 20 times longer than the circular molecules. These results indicate, in agreement with previous results shown for COS cells (Li and Kelly, 1984), that DNA replication proceeds bidirectionally only at early times of incubation in cytosolic extracts (either from uninfected CV-1 or human 293 cells, data not shown) and then switches to rolling circle replication.

the preferred template for initiation of DNA replication in this system is protein-free DNA or DNA with only a few nucleosomes.

For the analysis of post-replicative molecules, pSVori was incubated for 1 h in nucleo-cytosolic extracts, photoreacted with psoralen, purified and digested with *Dpn*I. An aliquot of the reaction was analyzed by electrophoresis. In agreement with the data shown in Figure 2b, a *Dpn*I resistant, form I DNA band was only seen on the autoradiograph, but not after ethidium bromide staining of the gel (data not shown). Therefore, the post-replicative molecules represent only a minor fraction of the total DNA. In the electron microscopy study, we selected for the circular molecules which represent the *Dpn*I resistant, and therefore post-replicative, molecules. Again, a heterogeneous bubble pattern was observed in these molecules. Some molecules were almost completely organized in ss-bubbles (Figure 9e), but we also found molecules with only some or few denatured regions (Figure 9f).

Table I. Number of single-stranded bubbles in unreplicated and post-replicative chromatin

Nucleo-cytosol extract	Time	Unreplicated		
		pSV ori* 2643 bp	Δ 6 2638 bp	'21' 2535 bp
SV40 infected CV1 cells	20 min	5 ± 3 (27 ± 4)	n.d.	n.d.
SV40 infected CVI cells	120 min	5 ± 2 (24 ± 2)	6 ± 2 (27 ± 3)	5 ± 2 (26 ± 2)
293 cells	30 min	10 ± 2 (26 ± 1)	11 ± 2 (27 ± 2)	n.d.
293 cells	120 min	10 ± 1 (26 ± 1)	10 ± 1 (24 ± 1)	n.d.
		Post-replicative		
293 cells	60 min	8 ± 1		

*Mainly unreplicated molecules with ~1% replicating and post-replicative molecules.
Numbers in brackets correspond to number of single-stranded bubbles in SV40 chromatin formed *in vivo*.

Quantitative analysis of the electron micrographs

The average number of assembled nucleosomes. For each molecule, the ratio of the sum of the length of all single-stranded sections and the total contour length, i.e. the R-value, was determined (Sogo *et al.*, 1986). This value, together with the average size of the ss-bubbles, is a measure of the nucleosome density on a given section of chromatin. To eliminate the length of single-stranded regions originating from incomplete crosslinking of protein−DNA interactions not related to chromatin structure, we also determined the R-value from a mixed population of molecules incubated in a cytoplasmic 293 extract (see Figure 8). This background value (0.18) was subtracted from the R-values of the molecules from the nucleo-cytosolic extracts.

In the experiments with extracts from SV40 infected CV-1 cells, the R-ratio (0.8) of the endogenous SV40 chromatin was used as an internal control for the spreading procedure. For the experiments with the 293 cell extracts, SV40 DNA which had been crosslinked in SV40 chromatin was mixed into the spreading mixture as a standard. The expected number of nucleosomes for SV40 chromatin consisting of a mixed population of 80% completely nucleosome-packed molecules and 20% molecules with a nucleosome-free gap around the origin (Varshavsky *et al.*, 1978, 1979; Scott and Wigmore, 1978; Sundin and Varshavsky, 1979; Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980; Robinson and Hallick, 1982) is 26.5 (Sogo *et al.*, 1986). In all our experiments, the calculated number of nucleosomes on the SV40 chromatin fits very well with this known value (Table I, lower numbers in brackets).

For a complete *in vitro* chromatin reconstitution, one would expect a maximum of 14 nucleosomes for all three DNA samples used (pSVori, pSVoriΔ6 and pSV21). This number was not reached under our experimental conditions (Table I). On average, 5−6 nucleosomes per molecule were found for extracts from SV40 infected CV-1 cells. This packing efficiency was reached after just 20 min of incubation. Using the extracts from 293 cells, however, ~10

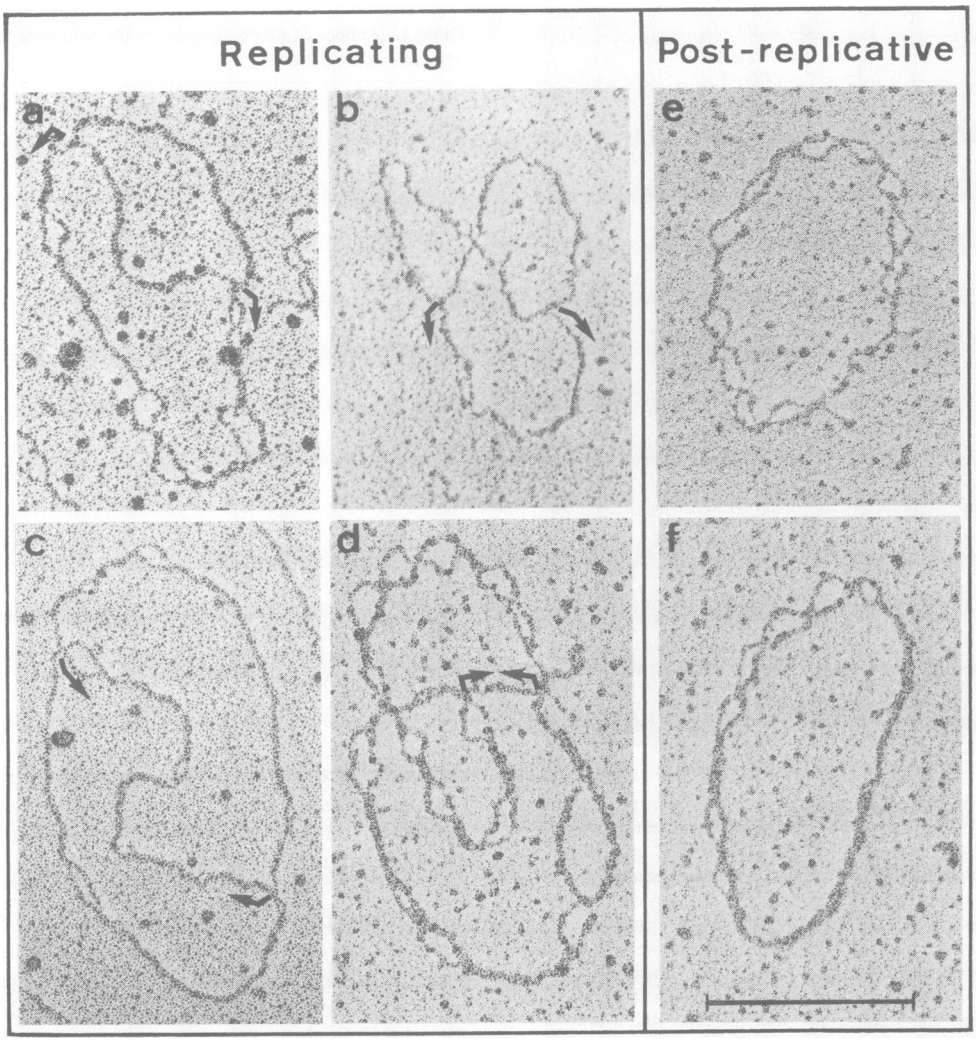

Fig. 9. Chromatin assembly with replicating and post-replicative molecules. pSVori molecules (a, b, c, d) were isolated after 20 (a,b) and 30 min (c,d) incubations at 37°C in SV40 infected CV-1 extracts (a,b) or human 293 extracts (c,d) and treated with trimethylpsoralen as described in Figure 7. The parental strands are marked by arrows. For the analysis of post-replicative pSVori molecules (e,f), the DNA was psoralen crosslinked and isolated after incubation for 1 h. The DNA was treated with *Dpn*I and only circular *Dpn*I resistant molecules were analyzed. The bar represents 500 nucleotides.

nucleosomes were obtained on average. This packing was reached after just 30 min of incubation.

The same statistical analysis was performed on the 25 replicative intermediates found in the set of experiments described here. The R-value determined for the newly synthesized strands of the early replicating molecules was found to be 0.15 (14 molecules) (for a mature pSVori mini-chromosome with 14 nucleosomes the R-value would be 0.78). This number increased to 0.47 on the newly synthesized strands of late replicating intermediates (11 molecules). The R-value for the parental strands was only calculated in the early replicating molecules and was found to be 0.17.

The size distribution of the nucleosomal bubbles. The standard in these experiments was the SV40 DNA which had been psoralen crosslinked either in the CV-1 extracts or in purified SV40 chromatin as described in the previous section. The size distribution of the nucleosomal bubbles in these molecules is shown in the histogram in Figure 10a. The average size of 156 ± 41 nucleotides is in good agreement with our previous data (Sogo *et al.*, 1986) and with the known size of the DNA in the nucleosome (for review, see Igo-Kemenes *et al.*, 1982). As an additional control, H1-depleted soluble rat liver chromatin was analyzed (Figure 10c). The histograms for the individual experiments shown in Table I did not differ significantly (not shown). The size distribution of the single-stranded bubbles in the molecules pSVoriΔ6 and pSV21 is similar to that of the pSVori molecules, which contain ~1% post-replicative strands. Therefore the data for all three constructions were combined (Figure 10b). Most of the bubbles had a length between 100–150 nucleotides and the histogram was clearly asymmetric and non-Gaussian. Figure 10d (continuous line) shows the distribution of the bubble sizes in post-replicative molecules. In contrast to the unreplicated molecules, this histogram is clearly Gaussian. The average size of the bubbles in the DNA of this chromatin is 147 ± 31 nucleotides. It is similar in size and distribution to that of SV40 or the H1-depleted rat liver chromatin. The size distribution of the ss-bubbles of the newly synthesized strands of late replicating molecules is also Gaussian. The average size of the ss-bubbles is 143 ± 46 nucleotides, which is very close in size and distribution to that described for post-

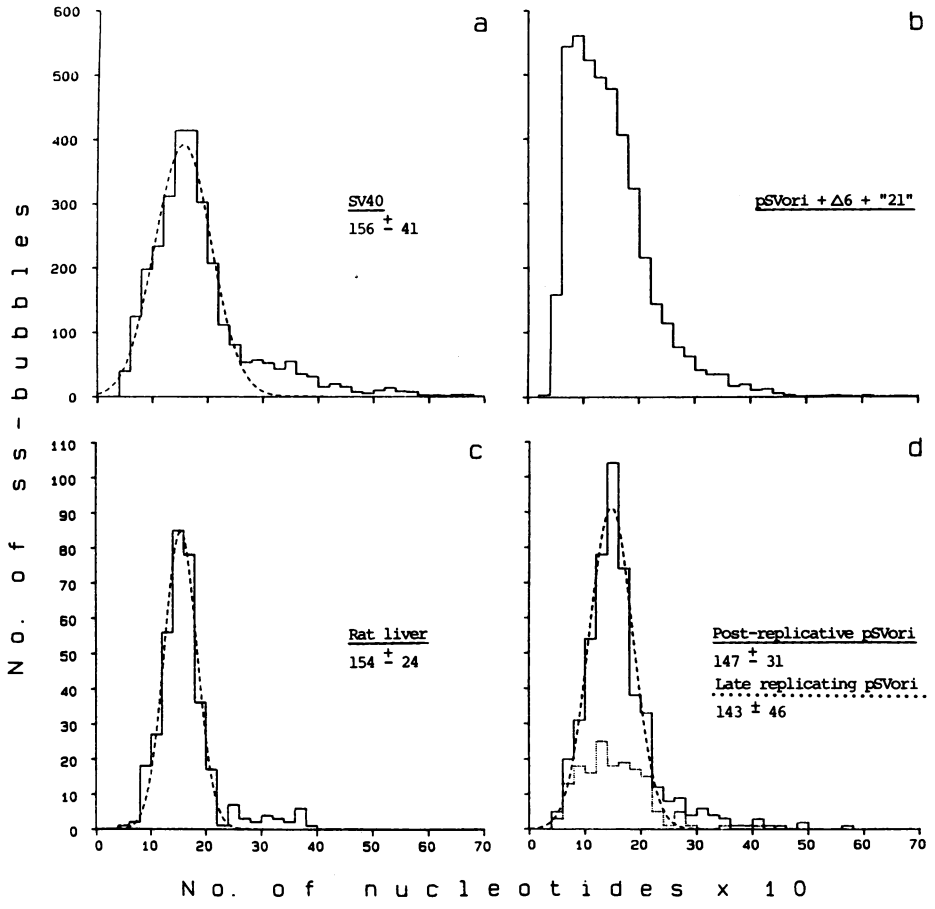

Fig. 10. Size distribution of single-stranded nucleosomal bubbles of *in vivo* and *in vitro* assembled chromatin. The length of the ss-bubbles of *in vivo* and *in vitro* assembled chromatin was compared for the following samples: (**a**) SV40 chromatin formed *in vivo* (2880 bubbles); (**b**) composition of the following eight histograms: pSVori reconstituted for 30 and 120 min either in SV40 infected CV-1 or 293 extracts, Δ6 reconstituted for 120 min in infected CV-1 extracts and 30 and 120 min in 293 extracts, pSV21 incubated for 120 min in infected CV-1 extracts (in total: 4299 bubbles); (**c**) rat liver DNA, psoralen crosslinked in soluble H1 depleted chromatin (349 bubbles); (**d**) pSVori post-replicating molecules (continuous line) after incubation for 1 h with 293 extracts and digestion with *DpnI* (493 bubbles) and newly synthesized strands from pSVori late replicating intermediates (dotted line, 161 bubbles). All histograms were fitted with a linear superimposition of a Gaussian distribution (dotted line, Koller *et al.*, 1978).

replicative molecules (Figure 10d, dotted line). Due to the low proportion (25 molecules analyzed in our experiments) of early replicative intermediates (which is even the case *in vivo*, see Oudet *et al.*, 1989), the size distribution of the ss-bubbles on the parental strand could not be analyzed statistically for comparison with the bubble sizes of post-replicative strands (note, that in the histogram of Figure 10b >4000 bubbles are included).

The change in size distribution of the nucleosomal bubbles between unreplicated and post-replicative molecules indicates that during DNA replication, a maturation of pre-replicatively assembled nucleosomes occurs.

Discussion

A number of cell-free systems using unfractionated cell lysates prepared from *Xenopus* eggs or oocytes (Laskey *et al.*, 1977; Glikin *et al.*, 1984) or from *Drosophila* embryos (Nelson *et al.*, 1979) have been described which assemble chromatin with the correct spacing of the nucleosomal repeat. ATP and Mg^{2+} appear to be required for correct nucleosome spacing (Almouzni and Mechali, 1988b). These systems assemble nucleosomes in the absence of DNA replication. Transfection of chimeric SV40 DNA into CV-1 or COS-1 monkey cells showed that the DNA is rapidly assembled into chromatin as revealed by the generation of a regular 190 bp repeat ladder after micrococcal nuclease digestion. DNA replication is not required for this assembly process (Cereghini and Yaniv, 1984). In contrast, experiments using a combination of a cytosolic and nuclear (i.e. a nucleo-cytosolic) extract from human 293 cells (Stillman, 1986; Fotedar and Roberts, 1989) or a cytosolic extract in combination with the purified chromatin assembly factor CAF-1 (Smith and Stillman, 1989), suggested that chromatin assembly occurs almost exclusively on replicating DNA molecules. Thus a number of contradictory reports and suggestions have appeared in the literature. Therefore, the question of whether DNA replication plays a decisive role in chromatin assembly is still open. Our approach to this problem was to investigate chromatin assembly in unreplicated and post-replicative, and to a limited extent in replicating, molecules of SV40 ori-containing plasmids using established mammalian cell-free DNA replication protocols.

Nicking of covalently closed, circular chromatin with topoisomerases and subsequent deproteinization showed that the number of superhelical turns in the deproteinized DNA corresponds roughly to the number of nucleosomes in the chromatin (Germond *et al.*, 1975). Therefore, since supercoiled (form I) input DNA is rapidly nicked in cell-free systems, supercoiling of the deproteinized DNA after

incubation in nucleo-cytosolic extracts and treatment with topoisomerase I has been considered to be a consequence of chromatin assembly (Stillman and Gluzman, 1985; Stillman, 1986; Smith and Stillman, 1989). With this assay we observed that unreplicated relaxed plasmid DNA [using plasmids defective for DNA replication (ori$^-$) and ori$^+$ plasmids in the absence of T-Ag] is converted into supercoiled DNA after incubation in nucleo-cytosolic extracts. That this supercoiling reflects nucleosome assembly is also suggested by the micrococcal nuclease digestion experiments with the non-replicating pSVoriΔ6. Predominantly relaxed DNA was observed after incubation with cytosolic extract and T-Ag alone. As suggested by the electron microscope analysis of the DNA crosslinked with psoralen in these extracts, only the DNA incubated in nucleo-cytosolic and not that incubated in cytosolic extracts, was assembled into chromatin. This indicates that the appearance of topoisomers, observed after incubation in cytosolic extract alone, might not necessarily mean chromatin assembly but could also arise by T-Ag helicase activity (Stahl *et al.*, 1986; Dodson *et al.*, 1987; Gutierrez *et al.*, 1990; C.Gruss, unpublished data).

Previous experiments had shown that preincubation of DNA in cytosolic extracts and T-Ag prior to addition of nuclear extract prevents supercoiling, i.e. chromatin assembly, of the DNA (Stillman, 1986; Smith and Stillman, 1989). We show here, with the non-replicating pSVoriΔ6 template DNA, that after preincubation with cytosolic extract and T-Ag followed by the addition of nuclear extract and topoisomerase I cleavage, the deproteinized DNA is shifted to form I. However this shift is time-dependent. Our results clearly reveal that the number of supercoils (i.e. nucleosomes) formed on ori$^-$ relative to ori$^+$ plasmids is similar.

When we analyzed our samples (pSVori and pSVoriΔ6) directly by electron microscopy after fixation with glutaraldehyde (Griffith, 1975; Griffith and Christiansen, 1977 Keller *et al.*, 1977), we obtained mostly highly compacted spheres of protein−DNA complexes (data not shown), even after keeping the samples on ice for 48 h (Stillman, 1986). Since the fraction of well-spread complexes may represent only a minor fraction of the whole population, such an analysis does not allow a reproducible and statistical measurement of the number of associated nucleosomes. Moreover, protein loss and artifacts during specimen preparation may occur. In contrast, the psoralen crosslinking technique for studying chromatin structure (Sogo *et al.*, 1984) allows a quantitative statistical analysis without selection of the molecules. It is well established that the single-stranded bubbles with a size of 130−150 nucleotides in length seen after denaturation of the deproteinized DNA correspond to nucleosomal DNA, whereas the crosslinked double-stranded regions correspond to the linker DNA (Hanson *et al.*, 1976; Cech *et al.*, 1977; Conconi *et al.*, 1984; Sogo *et al.*, 1984). The nucleosomal bubbles disappear when the crosslinking is performed in the presence of high ionic strength or heparin (DeBernadin *et al.*, 1986; Lucchini *et al.*, 1987).

Using extracts of 293 cells, no difference in the average number of associated nucleosomes between the three different plasmids used was observed. The maximal packing efficiency was reached after 1−2 h of incubation. However, with extracts from infected CV-1 cells, the final number of associated nucleosomes was significantly lower compared to extracts from 293 cells. The average number of nucleosomes given in Table I does not mean that every molecule is associated with a similar number of nucleosomes. The population of reconstituted molecules was heterogeneous (see also Bonne-Andrea *et al.*, 1990), ranging from molecules with only a few bubbles to molecules with a bubble density similar to that of the SV40 chromatin, which we used as a standard and control.

We observed that post-replicative molecules are organized in ss-bubbles. The average size and the clearly Gaussian size distribution of the ss-bubbles in post-replicative molecules are similar to the bubbles analyzed from H1-depleted, soluble rat liver chromatin and from SV40 minichromosomes. This suggests reconstitution of chromatin on these post-replicative molecules, which agrees in general with previous suggestions that chromatin assembly and DNA replication are coupled, for the extracts used (Stillman, 1986; Smith and Stillman, 1989). The analysis with two mutant DNA templates, pSVoriΔ6 and pSV21, which cannot replicate, revealed that these molecules are also associated with ss-bubbles of similar size. This ss-bubble pattern disappeared when the crosslinking was performed in the presence of 1.2 M NaCl or 500 μg/ml heparin. Micrococcal nuclease digestion and psoralen crosslinking after gel filtration are additional criteria supporting the presence of nucleosomes in the unreplicated input DNA. All these facts, together with the appearance of form I DNA (in topoisomerase I assays), demonstrate that chromatin assembly can occur not only on the newly synthesized DNA but also independently of the replication process in mammalian cell extracts.

However, in contrast to the size distribution of the ss-bubbles in the post-replicative molecules, the size distribution of the ss-bubbles of the unreplicated molecules is asymmetric and non-Gaussian. This qualitative difference is significant, indicating that part of the unreplicated nucleosomes are not yet correctly folded as '*in vivo*' chromatin. It might be possible that some of these structures contain only H3 and H4 (Fotedar and Roberts, 1989). This suggests that DNA replication has a maturation effect upon chromatin assembly or it might simply increase the speed of assembly. Alternatively, it could be that chromatin assembly follows two different pathways: replication independent and replication dependent. The replication independent mode appears to be a rather slow process where nucleosome assembly might occur with complete H3−H4:H2A−H2B octamers with an imperfect folding of the DNA while replication dependent assembly might use H3−H4 tetramers that later join H2A−H2B dimers in a much faster and more correct event. This latter hypothesis is supported by '*in vivo*' studies, showing that histones H3−H4 are randomly segregated to both sides of the replication fork as a tetramer which interacts with two H2A−H2B dimers to form a nucleosome (Jackson, 1990). It is important to realize that the data of the assembled, unreplicated chromatin collected by the psoralen technique and those obtained after digestion with micrococcal nuclease are practically the same in terms of the mean value calculated for the length of the mononucleosomal DNA (compare Figure 6 and Figure 10b). The difference seen with the psoralen technique arises from the qualitative difference in the size distribution. Whereas by using the psoralen technique all the single-stranded bubbles present in the intact molecules were taken into consideration, with the micrococcal nuclease assay, a selection in favour of small chromatin fragments, particularly of the monomer band, is performed. DNA fragments which are not perfectly folded into nucleosomes

might be degraded faster and would therefore not be detected in the gel (see Figure 6). Therefore we emphasize that the difference in the quality of the assembled unreplicated and post-replicative chromatin could only be detected by the psoralen crosslinking technique, which appears to be more accurate and more sensitive than the other techniques available such as micrococcal nuclease digestion, topoisomer counting and direct visualization of nucleoprotein complexes by electron microscopy.

Further evidence to suggest that DNA replication and chromatin assembly are not necessarily coupled in the extracts used comes from the analysis of replicating molecules. We found nucleosomes not only associated with daughter strands, as expected if chromatin assembly and DNA replication were coupled (Stillman, 1986; Smith and Stillman, 1989), but also on the parental strand. It has been suggested recently that the nuclear chromatin assembly factor CAF-1 might be associated with the replication machinery and thereby may promote nucleosome assembly on replicating DNA molecules (Smith and Stillman, 1989). We found that most of the replicating molecules contained only a few nucleosomes, except for the late replicating molecules. Therefore it seems unlikely that in the *in vitro* reconstitution system used, the chromatin assembly factor is directly associated with the replication machinery, at least in early replicating molecules. The reason for the low number of nucleosomes in early replicating molecules could be that: (i) initiation of replication may occur preferentially on molecules with few associated nucleosomes, because nucleosomes in the ori region might inhibit initiation of *in vitro* replication (Cheng and Kelly, 1989), and (ii) the rate of DNA replication might be faster than the rate of nucleosome formation on the newly synthesized daughter strands. It is known that the replication process slows down with accumulation of late replicative intermediates and that termination represents the rate-limiting step during SV40 DNA replication (for review see DePamphilis and Bradley, 1986). During the late stages of replication, protein rearrangement might allow correct chromatin folding. This is probably reflected by our findings showing that the number of nucleosomes present in late replicating molecules is clearly higher than the number determined for the early replicating forms. It can be postulated that topoisomerase I localized at or very near the replication fork (Aveman *et al.*, 1988) can act over a long distance, facilitating chromatin folding.

The separate analysis of chromatin assembly in unreplicated and post-replicative molecules has revealed that DNA replication *in vitro* has no significant influence on the amount of assembled nucleosomes, but it appears that replication facilitates the correct folding process by as yet unknown mechanisms.

Materials and methods

Plasmids

SV40 DNA fragments were cloned into pML-1 (2969 bp), a derivative of pBR322 lacking sequences that poison DNA replication in mammalian cells (Lusky and Botchan, 1981). pSVori (2643 bp) and pSVoriΔ6 (2637 bp), containing a 6 bp deletion at the *BglI* site of the SV40 origin, were as described (Yamaguchi and DePamphilis, 1986). pSV21 (2535 bp) was made by deleting the *NcoI*–*HindIII* fragment from pSVori (Gutierrez *et al.*, 1990). pBR 2.17 contains the mouse β-globin gene (4961 bp, H.Weber, personal communication). Supercoiled plasmid DNA was prepared from *E.coli* strain HB101 (Boyer and Roulland-Dussoix, 1969) by standard alkaline lysis (Birnboim, 1979) and two CsCl gradient centrifugations.

Preparation of cellular extracts and T-Ag

Cytosolic and nuclear extracts were prepared exactly as described either from SV40 infected CV-1 African green monkey kidney cells (Decker *et al.*, 1987; Guo *et al.*, 1989) or from human 293 cells (Stillman and Gluzman, 1985; Stillman, 1986). Protein concentrations were determined using the Biorad assay. Protein analysis was done on a 15% SDS–polyacrylamide gel (Laemmli, 1970). SV40 T-Ag was obtained from 293 cells infected with the recombinant Ad5SVR112 and was purified by immunoaffinity chromatography as described (Simanis and Lane, 1985).

DNA replication and chromatin reconstitution in vitro

In vitro DNA replication assays (60 μl) were carried out as previously described (Guo *et al.*, 1989). Reactions were performed in 30 mM HEPES–KOH (pH 7.8), 0.5 mM dithiothreitol, 7 mM Mg-acetate, 1 mM EGTA, 4 mM ATP, 80 μM each of CTP, GTP and UTP, 100 μM each of dGTP and dATP, 40 μM each of dCTP and dTTP, 5 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dTTP (3000 Ci/mmol, Amersham), 10 mM phosphoenolpyruvate and 0.6 μg pyruvate kinase. Each reaction contained 150 ng template DNA, and either 30 μl SV40 infected nucleo-cytosolic CV-1 cell extract (120–200 μg protein, including 20 μl cytosolic and 10 μl high salt nuclear extract) or 30–40 μl nucleo-cytosolic 293 cell extract (20–30 μl cytosolic S100 extract with 230 μg protein and 5–10 μl nuclear extract with 16 μg protein). Immunopurified T-Ag (1 μg) was added to reactions with human 293 extracts. To isolate replication products, reactions were stopped after 20–120 min of incubation by adding SDS to 0.7% and EDTA to 20 mM, treated with Proteinase K (500 μg/ml) for 1 h at 37°C and extracted with phenol. The aqueous phase was passed through a Sephadex G-50 spin column (Boehringer) to remove unincorporated [^{32}P]dNTPs and precipitated with ethanol. The deproteinized DNA was either linearized with *Eco*RI or digested with *Eco*RI and *Dpn*I. Both digested and undigested replication products were subjected to electrophoresis on 0.8% agarose gels in TAE buffer (0.04 M Tris–acetate, 0.002 M EDTA; Maniatis *et al.*, 1982). The gels were dried and autoradiographed.

To analyze the structure of chromatin reconstituted *in vitro*, after the appropriate times of incubation, 3 μl of 4,5′,8-trimethylpsoralen (200 μg/ml in 100% ethanol; HRI) were added three times to the reaction (60 μl) during a total irradiation time of 4 h, exactly as described by Sogo *et al.* (1984).

DNA extraction and spreading for electron microscopy

After psoralen crosslinking, the samples were deproteinized with Proteinase K (500 μg/ml) for 2 h at 50°C, phenol extracted and precipitated with ethanol. For visualizing well-spread bubbles in covalently closed circular molecules, the DNA was nicked with DNase I (0.5 μg/100 μl) for 1 h at 37°C in the presence of 45 μg/100 μl ethidium bromide. Cellular RNA was removed by RNase A and after Proteinase K treatment for another 30 min at 50°C, the DNA was precipitated with ethanol. To analyze post-replicative molecules, the psoralen crosslinked DNA was extensively digested with an excess of *Dpn*I. One aliquot was controlled on a 0.8% agarose gel and the other was analyzed by electron microscopy. Denaturation and spreading of the psoralen crosslinked DNA for electron microscopy was performed exactly as described by Sogo *et al.* (1984). H1 depleted rat liver chromatin was prepared according to Thoma *et al.* (1983). DNA contour length measurements were made with a Hewlett Packard digitizer on photographic prints.

Topoisomerase I assay

300 ng DNA or *in vitro* reconstituted chromatin (as indicated) were incubated in the replication mixture with 16 U topoisomerase I (Promega) for 5 or 30 min at 37°C. DNA was purified as described above.

Micrococcal nuclease digestion

After incubation of the DNA templates in nucleo-cytosolic extracts supplemented with T-Ag, the samples were adjusted to 3 mM $CaCl_2$ and digested with 30 U of micrococcal nuclease at 22°C for 0, 1, 2, 5, 10 and 25 min. The same amount of protein-free DNA was digested with 6 U of micrococcal nuclease under the same conditions. At the indicated time-points, the reactions were stopped with EDTA (20 mM) and SDS (0.5%). DNA extraction, electrophoresis in 1.5% agarose gels, Southern blotting onto Zeta-membranes (Bio-Rad), nick-translation and hybridization were done as described by Conconi *et al.* (1989).

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