The EMBO Journal vol.10 no.4 pp.971 - 980, 1991

Stepwise assembly of chromatin during DNA replication *in vitro*

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Communicated by J.D.Watson

A cell free system that supports replication-dependent chromatin assembly has been used to determine the mechanism of histone deposition during DNA replication. CAF-I, a human cell nuclear factor, promotes chromatin assembly on replicating SV40 DNA in the presence of a crude cytosol replication extract. Biochemical fractionation of the cytosol extract has allowed separation of the chromatin assembly reaction into two steps. During the first step, CAF-I targets the deposition of newly synthesized histones H3 and H4 to the replicating DNA. This reaction is dependent upon and coupled with DNA replication, and utilizes the newly synthesized forms of histones H3 and H4, which unlike bulk histone found in chromatin, do not bind to DNA by themselves. The H3/H4-replicated DNA complex is a stable intermediate which exhibits a micrococcal nuclease resistant structure and can be isolated by sucrose gradient sedimentation. In the second step, this replicated precursor is converted to mature chromatin by the addition of histones H2A and H2B in a reaction that can occur after DNA replication. The requirement for CAF-I in at least the first step of the reaction suggests a level of cellular control for this fundamental process.

Key words: chromatin/DNA replication/histones/simian virus 40

Introduction

The replication of eukaryotic chromosomes requires not only the duplication of the DNA, but also accurate reproduction of the associated chromatin structures. Many studies indicate that specific chromatin structures can influence the transcriptional capability of the cell (reviewed by van Holde, 1988) and in certain cases, these chromatin structures are propagated during DNA replication (Weintraub, 1979; Groudine and Weintraub, 1982). Since DNA replication disrupts the structure of chromatin, it provides an opportunity to either change or maintain specific structures and hence, influence the transcriptional state of the cell. Very little is known, however, about the molecular mechanisms involved in propagating and changing chromatin structures. Understanding how chromosomal proteins are deposited during DNA replication will provide insights into this fundamental process.

How are the histones, the primary organizers of chromatin, assembled on newly replicated DNA? The nucleosome core particle, the basic subunit of chromatin, consists of two copies of each of the four core histones, H2A, H2B, H3 and H4, organized into an octameric structure. Electron microscopic analyses of replicating cellular (McKnight and Miller, 1977) and viral (Cremisi *et al.*, 1978; Seidman *et al.*, 1978) chromosomes indicate that nucleosomal structures are assembled on replicating DNA immediately after passage of the replication fork. It appears, however, that the structure of newly synthesized chromatin is somehow different from that of mature chromatin. Nascent chromatin is more sensitive than mature chromatin to micrococcal nuclease digestion, but over time, normal nuclease sensitivity is recovered (Seale, 1975, 1978; Hildebrand and Walters, 1976; Levy and Jakob, 1978; Klempnauer *et al.*, 1980; Cusick *et al.*, 1981, 1983). The biochemical events that accompany this maturation process, however, are not well understood.

To study the mechanism of histone deposition during DNA replication, investigators have relied on *in vivo* studies which follow the fate of newly synthesized histones that are incorporated into chromatin during DNA replication. These studies have yielded an incomplete and somewhat confusing picture, and to date a single mechanism for histone deposition is still not generally accepted (Svaren and Chalkley, 1990). Early studies, which monitored the incorporation of pulse-labeled histones, indicated a sequential mechanism for histone deposition where newly synthesized histones H3 and H4 deposited first on newly replicated DNA, followed by deposition of new histones H2A and H2B (Worcel *et al.*, 1978; Cremisi and Yaniv, 1980). Subsequent studies, performed nearly a decade later, demonstrated that nascent chromatin consisted of nucleosomes containing newly synthesized histones H3 and H4 and mostly old histones H2A and H2B (Jackson, 1987, 1988). These more recent observations have compromised the interpretation of the earlier studies because the presence of old (and therefore unlabeled in pulse-chase experiments) histones H2A and H2B in newly replicated chromatin would have gone undetected. In addition, although most studies agree that newly synthesized histones H3 and H4 deposit exclusively on newly replicated DNA (Cremisi *et al.*, 1977; Worcel *et al.*, 1978; Senshu *et al.*, 1978; Cremisi and Yaniv, 1980; Jackson and Chalkley, 1981a,b, 1985), the mechanism that targets histones H3 and H4 to the replicating DNA remains unknown.

To address the mechanism of chromatin assembly during DNA replication, we have developed an *in vitro* system which closely mimics the *in vivo* process. Cytosol extracts derived from human cells support complete and authentic replication of SV40 origin-containing plasmid DNA in the presence of purified SV40 T antigen (for review see Stillman, 1989; Challberg and Kelly, 1989). Addition of chromatin assembly factor I (CAF-I), a multisubunit histone binding protein isolated from nuclei of human cells, promotes chromatin assembly during DNA replication (Stillman, 1986;

Smith and Stillman, 1989). This *in vitro* reaction resembles the *in vivo* process in at least two important ways. First, the cytosol histones used in the assembly reaction correspond to the newly synthesized histone pool and hence are the direct precursors for chromatin assembly *in vivo*. Second, chromatin assembly in this reaction is dependent upon and coupled with DNA replication and therefore is likely to reflect the events that occur during DNA replication *in vivo*, when *de novo* chromatin assembly occurs.

Biochemical fractionation has allowed the separation of the chromatin assembly reaction into two steps. During the first step of the reaction, CAF-I deposits newly synthesized histones H3 and H4 on the DNA in a reaction that discriminates between replicated and unreplicated DNA. This replicated precursor then serves as the template for the deposition of either old or new histones H2A and H2B. We present a model for the stepwise assembly of chromatin and discuss possible mechanisms for targeting histone deposition to replicating DNA.

Results

Biochemical fractionation of the histones in the cytosol extract

In the SV40 *in vitro* replication system, the cytosol extract provides all of the cellular proteins required for DNA replication as well as the four core histones needed for chromatin assembly. To identify other chromatin assembly factors and to investigate the mechanism of histone deposition during DNA replication, we attempted to separate the histones from the replication components through biochemical fractionation (Figure 1A). The cytosol extract was divided into two fractions by phosphocellulose chromatography; a flow-through fraction at 0.6 M NaCl ('0.6M') and a bound fraction, eluted with 2 M NaCl ('2M'). Based upon previous studies we predicted that under these conditions the replication components would flow through the column (Tsurimoto and Stillman, 1989), whereas the histones, relatively tight DNA-binding proteins, should remain bound. This fractionated system was tested for its ability to support replication-dependent chromatin assembly.

The SV40 replication reaction consists of the cytosol extract, T antigen, SV40 origin-containing plasmid DNA and nucleoside triphosphates including [α-^{32}P]dATP. Topoisomerases I and II, found in the cytosol in limiting amounts, were also added to the reaction. When the DNA products of this reaction were isolated and analyzed by agarose gel electrophoresis, autoradiography of the replicated DNA revealed predominantly relaxed monomer circle DNA (Figure 1B, lane 1). When purified CAF-I was included in the reaction, the replicated DNA was assembled into chromatin. This was visualized, after deproteinization, as supercoiled DNA (Figure 1B, lane 2). A comparison of the replicated DNA (autoradiograph, Figure 1B, left panel) with the total input DNA (ethidium bromide stained gel, Figure 1B, right panel), demonstrated that while all of the replicated DNA was assembled into chromatin, the input, unreplicated DNA remained relaxed, indicating the preferential assembly of chromatin on replicating DNA. Similar to the cytosol extract, the 0.6M flow-through supported DNA replication (Figure 1C, lane 1). In contrast, however, addition of CAF-I to the 0.6M flow-through did not result in chromatin assembly (Figure 1C, lane 2), demonstrating that while the 0.6M extract contained all the components required for DNA replication, it had been depleted of one or more factors needed for chromatin assembly. These factors were in the 2M step fraction, since addition of this extract restored chromatin assembly activity (Figure 1C, lane 4).

We considered it likely that, because of their ability to bind tightly to DNA, the histones might be retained on the phosphocellulose column at 0.6 M NaCl. To determine the fate of the cytosolic histones during the fractionation scheme described in Figure 1, the proteins in the extracts were subjected to analysis by two-dimensional gel electrophoresis. To detect the very small amounts of histones contained in the cytosol, fractionated extracts were prepared from cells

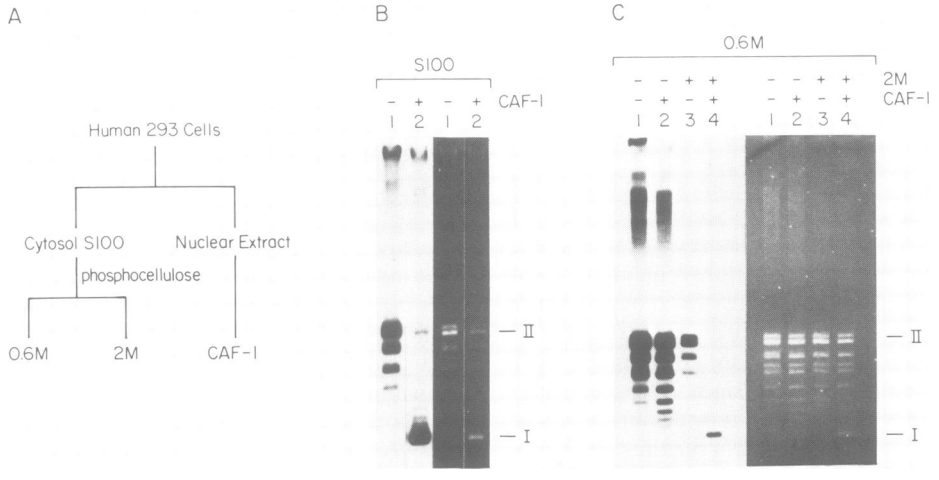

Fig. 1. Biochemical fractionation and reconstitution of the chromatin assembly reaction. (**A**) Fractionation scheme. Human 293 cells were fractionated into a cytosol and nuclear extract. CAF-I was purified from the nuclear extract (see Materials and methods). The cytosol replication extract was separated into two components, the 0.6M flow-through and the 2M step fraction. (**B**) and (**C**) DNA replication reactions containing pSV011, SV40 T antigen, topoisomerases I and II, nucleoside triphosphates including [α-^{32}P]dATP, the cytosol S100 extract (B) or the 0.6M flow-through fraction (C) and the indicated components were incubated at 37°C for 60 min. The DNA was then isolated and subjected to electrophoresis in 1% agarose gels. (B) and (C) Left panel shows the autoradiographs and right panel shows the ethidium bromide-stained gels. The positions of form I and form II marker DNAs are indicated at right.

that were metabolically pulse-labeled with [^{14}C]lysine and [^{14}C]arginine. As shown in Figure 2B, the unfractionated cytosol extract (S100) contained the four core histones: H2A, H2B, H3 (H3.1, H3.2, H3.3) and H4 (modified form). Note that the specific activity of ^{14}C-label for histones H2A and H2B is significantly lower than for histones H3 and H4 (Smith and Stillman, 1989). Analysis of the fractionated extracts demonstrated that the 0.6M flow-through contained histones H3 and H4 (Figure 2C), but was depleted of histones H2A and H2B, which were in the 2M step fraction (Figure 2D). The relatively low affinity of histones H3 and H4 for phosphocellulose was unexpected, considering the strong DNA-binding properties ascribed to these histones. However, under identical conditions, histones H3 and H4

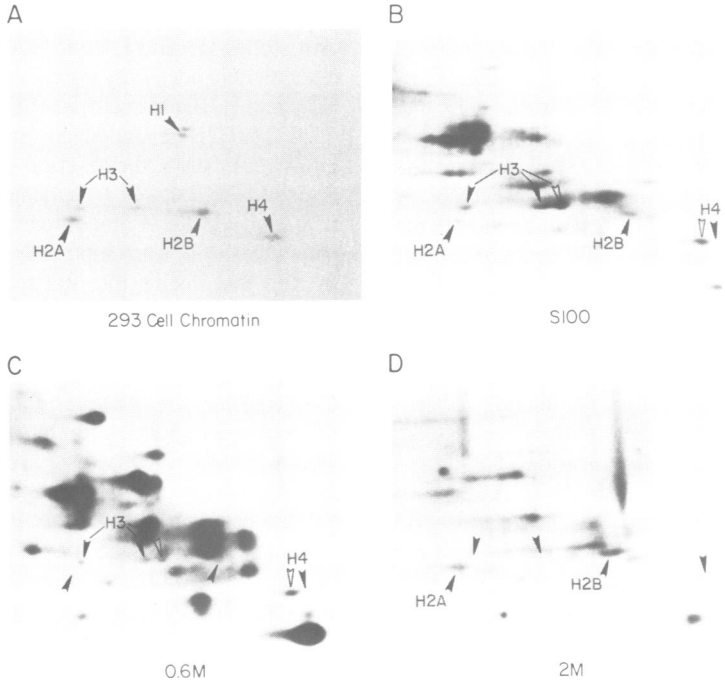

Fig. 2. Two-dimensional gel analysis of the histones contained in ^{14}C-labeled fractionated cell extracts. Fractionated extracts were prepared from cells that had been labeled for 60 min with [^{14}C]lysine and [^{14}C]arginine and were analyzed by two-dimensional gel electrophoresis as described in Materials and methods. TAU−polyacrylamide gels were used for the first dimension (horizontal direction) and SDS−polyacrylamide gels were used for the second dimension (vertical direction). (**A**) Unlabeled marker histones isolated from 293 cell chromatin. The proteins were visualized by staining with Coomassie brilliant blue. (**B**), (**C**) and (**D**) ^{14}C-labeled histones contained in the fractionated cell extracts. The proteins were visualized by autoradiography. The migration positions of unlabeled bulk histones isolated from chromatin are indicated by filled in arrows and identified in (A). The histone species contained only in the fractionated cell extracts but not in bulk chromatin, are shown by open arrows; H3.3 migrates more rapidly in the first dimension than H3.1 and H3.2 and modified H4 migrates more slowly in the first dimension than the unmodified form of H4.

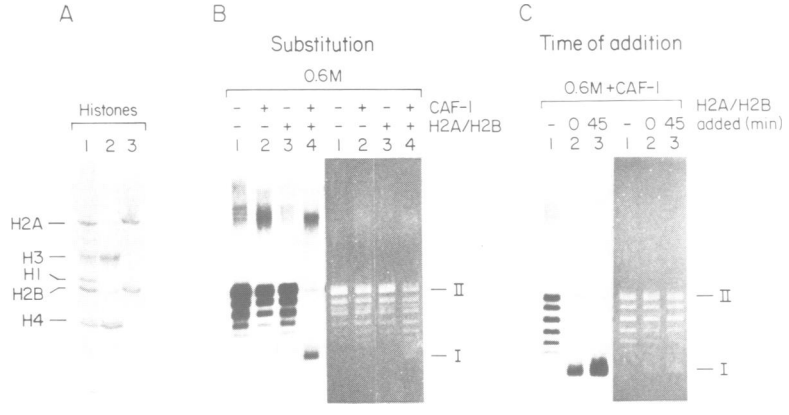

Fig. 3. Histones H2A and H2B purified from chromatin can substitute for the 2M step and can be added after DNA replication. (**A**) Chromatin was isolated from 293 cell nuclei (lane 1); histones H2A and H2B (lane 3) and histones H3 and H4 (lane 2) were eluted by sequential salt extraction. The proteins were subjected to electrophoresis in a TAU−polyacrylamide gel and visualized by staining with Coomassie brilliant blue.
(**B**) Substitution with histones H2A and H2B. DNA replication reactions containing the 0.6M flow-through fraction and the indicated components were incubated at 37°C for 60 min. (**C**) Time of addition of histones H2A and H2B. DNA replication reactions containing the 0.6M flow-through fraction and CAF-I were carried out in the absence of histones H2A and H2B (lane 1) or with H2A and H2B added at the start of the reaction (lane 2) or at 45 min (lane 3). Reactions were incubated at 37°C for a total time of 60 min. (B) and (C) The DNA products were isolated and subjected to electrophoresis in 1% agarose gels. Left panel shows the autoradiographs and right panel the ethidium bromide stained gels.

purified from chromatin (shown in Figure 3, lane 2), did bind to phosphocellulose (data not shown), indicating a difference between the newly synthesized histones H3 and H4 in the cytosol extract and the chromatin-bound forms of these histones.

The 2M extract contains histones H2A and H2B but does not provide any essential replication components. We therefore determined whether a purified fraction of histones H2A and H2B could substitute for the 2M extract. A purified fraction of histones H2A and H2B was isolated from 293 cell chromatin by column chromatography and sequential salt extraction (Figure 3A, lane 3). As shown in Figure 3B, lane 4, purified histones H2A and H2B substituted for the 2M extract. These results demonstrate that the only essential chromatin assembly components in the 2M extract are the histones H2A and H2B. Furthermore, either cytosol or chromatin bound histones H2A and H2B will function in the replication-dependent chromatin assembly reaction.

Separation of replication-dependent chromatin assembly into two steps

Since the chromatin assembly components histones H2A and H2B were separated from the essential replication factors, it was possible to determine whether these histones were required to be present during DNA replication. To address this question, the time of addition of histones H2A and H2B to the replication reaction was varied. As shown in Figure 3C, addition of histones H2A and H2B at the start of the reaction (lane 2) or 45 min after the start of DNA synthesis (lane 3) resulted in the same level of supercoiling when reactions were terminated at 60 min. Note that under the conditions described here, the vast majority of DNA synthesis occurs before 45 min (data not shown) and this completely replicated DNA is still supercoiled by subsequent addition of histones H2A and H2B. These results demonstrate that the H2A/H2B-dependent chromatin assembly of the replicated DNA can occur after DNA replication.

The experiments described above allowed separation of the chromatin assembly reaction into two steps. During the first step of the reaction, DNA was replicated in the presence of histones H3 and H4 and CAF-I. The second step of the reaction depended only upon the addition of purified histones H2A and H2B and occurred after DNA replication. Although this H2A/H2B-dependent chromatin assembly occurred after DNA replication, the reaction still maintained the ability to discriminate between replicated and unreplicated DNA (Figure 3C, lane 3; compare the replicated DNA, left panel, with the total input DNA, right panel). These data suggest that during the first step of this two step reaction, the replicated DNA is somehow marked for subsequent chromatin assembly.

CAF-I generates a micrococcal nuclease-resistant structure on replicating DNA

The first step of the two step reaction contained CAF-I, an essential component of the chromatin assembly reaction. To try to ascertain whether there is a difference between DNA replicated in the absence or presence of CAF-I, the products of the replication reactions were analyzed. We have already demonstrated that the DNA products of the two reactions

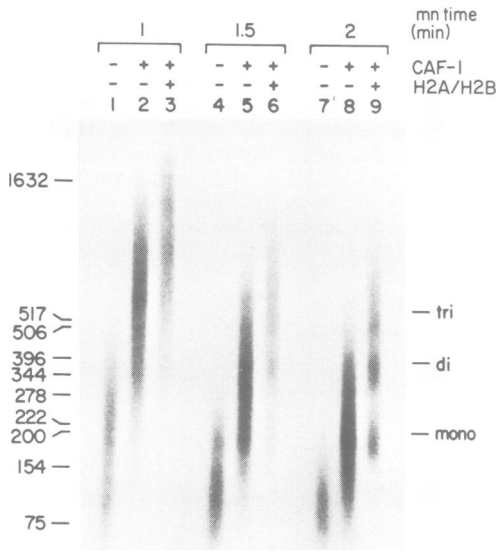

Fig. 4. Micrococcal nuclease digestion products of the replicated DNA. DNA replication reactions containing the 0.6M flow-through fraction were incubated at 37°C for a total time of 60 min in the absence of CAF-I (lanes 1, 4 and 7), the presence of CAF-I (lanes 2, 5 and 8), or in the presence of CAF-I with histones H2A and H2B included in the last 15 min of the reaction (lanes 3, 6 and 9). The reactions were then adjusted to 3 mM $CaCl_2$ and digested with micrococcal nuclease for the indicated times. The DNA was isolated, subjected to electrophoresis in 2% agarose, and autoradiographed. The length in base pairs of double-stranded DNA markers are indicated at left. The positions of the mono-, di- and tri-nucleosomes are indicated at right.

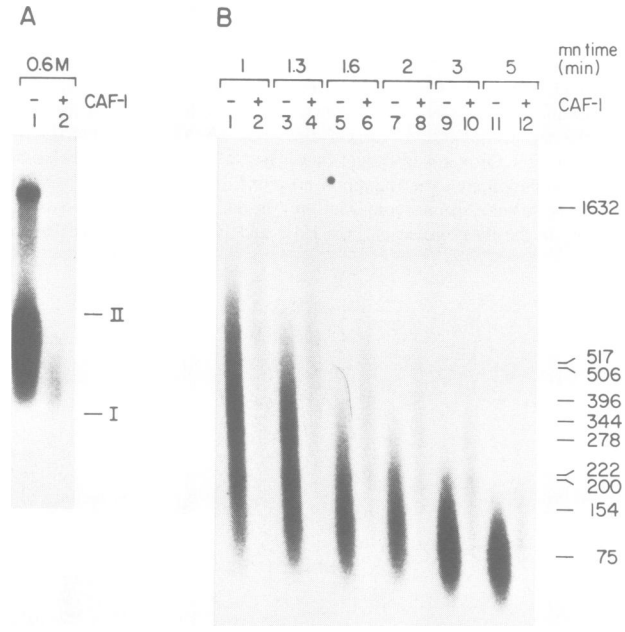

Fig. 5. The chromatin precursor is generated during DNA replication. DNA replication reactions containing the 0.6M flow-through fraction were incubated in the absence (A, lane 1) and (B, lanes 1, 3, 5, 7, 9 and 11) or presence (A, lane 2) and (B, lanes 2, 4, 6, 8, 10, 12) of CAF-I at 37°C for 15 min. (**A**) The DNA was isolated, subjected to electrophoresis in 1% agarose and autoradiographed. (**B**) The reactions were adjusted to 3 mM $CaCl_2$ and digested with micrococcal nuclease for the indicated times. The DNA was isolated, subjected to electrophoresis in 2% agarose, and autoradiographed.

are similar in that they are relaxed monomer circle DNA, although a slight change in the distribution of topoisomers in the DNA replicated in the presence of CAF-I can sometimes be observed (see Figure 1C, compare lanes 1 and 2). To probe more finely the structure of the chromatin precursor, the products of the replication reactions were analyzed by micrococcal nuclease digestion.

For these experiments, three types of reactions were considered: DNA replicated in the absence of CAF-I, DNA replicated in the presence of CAF-I, or DNA replicated in the presence of CAF-I followed by addition of histones H2A and H2B. Histones H3 and H4 fractionate with the 0.6M replication extract (see Figure 2C) and, therefore, are contained in all three reactions. The DNA−protein products of these three replication reactions were digested with micrococcal nuclease for increasing times and the DNA products isolated and analyzed by agarose gel electrophoresis. The results of this analysis, presented in Figure 4, demonstrate that DNA replicated in the presence of CAF-I was more resistant to micrococcal nuclease than DNA replicated in the absence of CAF-I (Figure 4, compare lanes 1 and 2). Moreover, nuclease digestion of DNA replicated in the presence of CAF-I yielded a smear of DNA which migrated in the same region of the gel as the DNA isolated from mono- and di-nucleosomes (Figure 4, compare lanes 5 and 6). Therefore, DNA replicated in the presence of CAF-I, but in the absence of histones H2A and H2B, displayed a relatively nuclease-resistant structure which resembled mature chromatin but appeared more sensitive to nuclease digestion. Addition of histones H2A and H2B converted this precursor to mature chromatin containing correctly spaced nucleosomes (Figure 4, lane 9).

CAF-I assembles the chromatin precursor during DNA replication

To determine whether the nuclease-resistant structure was generated during DNA replication, in association with passage of the replication fork, DNA replication products from a very early time point in the replication reaction were digested with micrococcal nuclease. Although the standard time for the replication reaction was 60 min, for these experiments DNA was replicated in the absence or presence of CAF-I for only 15 min. As shown in Figure 5A, the replicated DNA products at this early time point did not appear as completed monomer circles, but rather, migrated as a smear between forms I and II which correspond to replicative intermediates (Tsurimoto *et al.*, 1990). In this experiment, DNA replication was greatly inhibited in the presence of CAF-I. While we have previously noted inhibition of DNA replication by CAF-I (Smith and Stillman, 1989), this inhibition appears to be exaggerated at this early time point. Nevertheless, the DNA−protein products from these replicative intermediates were digested with micrococcal nuclease and the DNA products isolated and analyzed by agarose gel electrophoresis. The results presented in Figure 5B demonstrate that even at this early time point, DNA replicated in the presence of CAF-I is more resistant to micrococcal nuclease digestion than DNA replicated in the absence of CAF-I. These data suggest that CAF-I can function during DNA replication to generate the chromatin precursor.

Sucrose gradient sedimentation of the chromatin precursor

We have shown above that DNA replicated in the presence of CAF-I and histones H3 and H4 displays a micrococcal nuclease-resistant structure that is more sensitive to nuclease digestion than mature chromatin. This observation, coupled with the presence of histones H3 and H4 in the 0.6M cytosol replication extract, suggested the possibility that the chromatin precursor contained histones H3 and H4. Velocity sedimentation behavior has previously been used as a means of analyzing the protein−DNA products of the DNA replication reactions; replicated DNA assembled into a mature chromatin structure containing all four core histones sediments much more rapidly through sucrose gradients than

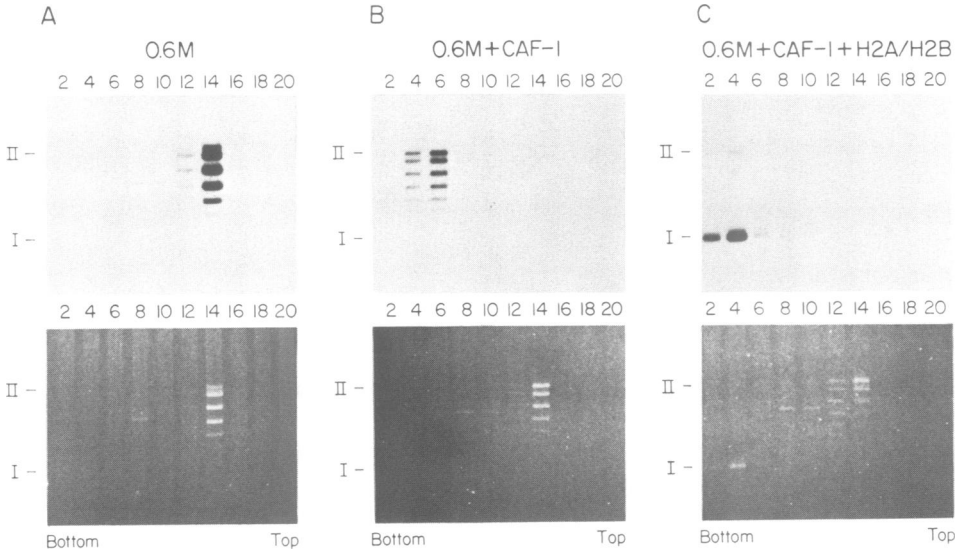

Fig. 6. Sucrose gradient sedimentation of the chromatin precursor. DNA replication reactions containing the 0.6M flow-through fraction were incubated at 37°C for a total time of 60 min in the absence of CAF-I (**A**), the presence of CAF-I (**B**), or the presence of CAF-I with H2A and H2B included in the last 15 min of the reaction (**C**). The reaction mixtures were then sedimented through 15−30% sucrose gradients and fractions collected. The DNA products contained in the even numbered fractions were isolated and subjected to electrophoresis in 1% agarose gels. Top panel shows the autoradiographs and the bottom panel the ethidium bromide stained gels. The direction of sedimentation was from right to left.

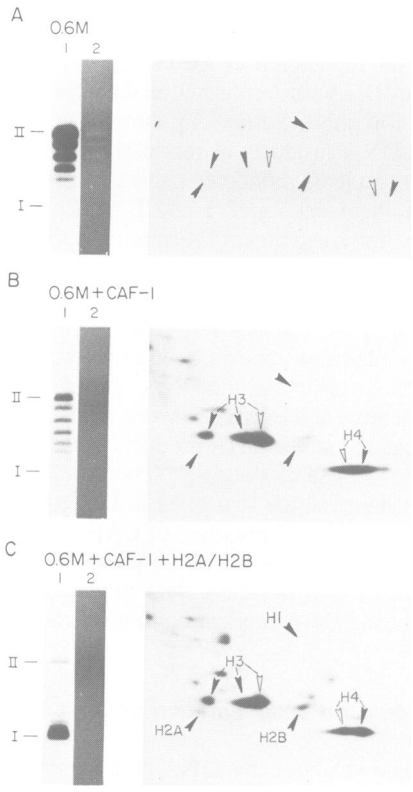

Fig. 7. Two-dimensional gel analysis of the histones contained in the chromatin precursor. DNA replication reactions containing the ^{3}H-labeled 0.6M flow-through fraction were incubated at 37°C for a total time of 60 min in the absence of CAF-I (**A**), the presence of CAF-I (**B**), or the presence of CAF-I with the ^{3}H-labeled 2M step fraction included in the last 15 min of the reaction (**C**). The reaction mixtures were then sedimented through 15–30% sucrose and fractions collected and analyzed exactly as described in Figure 6. The fractions from each sucrose gradient that contained the replicated DNA were then subjected to further analysis. Sucrose gradient fractions containing DNA replicated in the absence of CAF-I (A), DNA replicated in the presence of CAF-I (B), or DNA replicated in the presence of CAF-I followed by addition of the ^{3}H-labeled 2M extract (C), were each pooled separately. The DNA was isolated and subjected to electrophoresis in 1% agarose gels (left panels). Lane 1 shows the autoradiographs and lane 2 the ethidium bromide stained gels. The proteins were subjected to two-dimensional gel electrophoresis exactly as described in the legend to Figure 2 (right panels). The proteins were visualized by autoradiography.

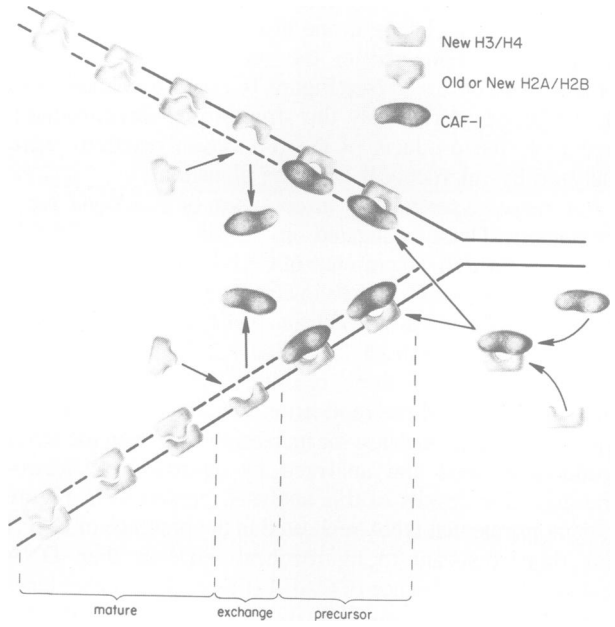

Fig. 8. Proposed model of CAF-I mediated chromatin assembly during DNA replication. CAF-I binds the newly synthesized histones H3 and H4 and deposits the complex on newly replicated DNA to generate the chromatin precursor. CAF-I is then exchanged for histones H2A and H2B (either the newly synthesized or chromatin-bound forms) to generate mature chromatin. The deposition of histones H2A and H2B constitutes part of the maturation process which may also include additional steps such as post-translational histone modification. The solid lines indicate the parental DNA strands and the dashed lines the newly synthesized DNA. The fate of parental histones is not addressed in this system.

unassembled, replicated DNA (Stillman, 1986; Smith and Stillman, 1989). We therefore analyzed the sedimentation behavior of the intermediate described above. If the chromatin precursor contains histones H3 and H4, then it should sediment more rapidly than DNA replicated in the absence of CAF-I. DNA replication reactions were performed in the absence of CAF-I, the presence of CAF-I, or in the presence of CAF-I followed by addition of histones H2A and H2B. The DNA–protein products from these reactions were then sedimented through sucrose gradients and the DNA products present in each fraction isolated and subjected to agarose gel electrophoresis (Figure 6).

The results presented in Figure 6 demonstrate that DNA replicated in the presence of CAF-I sedimented more rapidly through sucrose than DNA replicated in the absence of CAF-I (compare A and B, top panels). The sedimentation behavior of the chromatin precursor was similar to that of mature minichromosome (compare B and C, top panels) and thus may reflect assembly of the replicated DNA into a protein–DNA complex. Moreover, in the presence of CAF-I, while all of the replicated DNA exhibited this rapid sedimentation behavior, the input unreplicated DNA displayed a much slower sedimentation velocity (compare top and bottom panels, Figure 6B). Therefore, the presence of CAF-I in the replication reaction produced a rapidly sedimenting structure only on the replicated DNA.

The chromatin precursor is complexed with histones H3 and H4

The finding that the chromatin precursor formed in the presence of CAF-I sedimented rapidly through sucrose was consistent with its assembly into a protein–DNA complex containing histones H3 and H4. To test this hypothesis directly, the replicated DNA–protein complex was isolated and analyzed for its histone content. To allow detection of the very small amounts of histones assembled in these reactions, DNA replication reactions were performed using fractionated extracts prepared from cells that had been metabolically labeled with [^{3}H]lysine and [^{3}H]arginine. DNA was replicated with the ^{3}H-labeled 0.6M replication extract in the absence of CAF-I, in the presence of CAF-I, or in the presence of CAF-I, followed by addition of the ^{3}H-labeled 2M step fraction. Note that the 2M step is used here to provide ^{3}H-labeled histones H2A and H2B. The DNA–protein products were sedimented through sucrose gradients exactly as described in Figure 6. To analyze the

proteins complexed to the replicated DNA, the sucrose gradient fractions containing the slowly sedimenting replicated DNA (see Figure 6A, fraction 14), the rapidly sedimenting chromatin precursor (see Figure 6B, fractions 4–6), or the rapidly sedimenting mature chromatin (see Figure 6C, fractions 2–4) were subjected to two-dimensional protein gel analysis. The results presented in Figure 7 demonstrate that DNA replicated in the presence of CAF-I (the chromatin precursor) contained histones H3 and H4 (Figure 7B), whereas DNA replicated in the absence of CAF-I did not (Figure 7A). Addition of the 2M step fraction to the chromatin precursor resulted in the deposition of histones H2A and H2B (Figure 7C). Note that in the absence of CAF-I, the bulk of the histones in the cytosol extract did not sediment rapidly through sucrose gradients (data not shown; Smith and Stillman, 1989).

From these data we can draw two important conclusions. First, in the absence of CAF-I, histones H3 and H4 do not associate stably with DNA (replicating or non-replicating DNA) and second, in the presence of CAF-I, histones H3 and H4 form a stable complex with DNA in a reaction that discriminates between replicating and non-replicating DNA. Thus, CAF-I is required for the stable deposition of histones H3 and H4 on replicating DNA.

Finally, we note that in addition to the four core histones, the mature minichromosome contains two proteins which migrate in the second dimension with histone H1, but migrate in the first dimension with histone H3 (Figure 7C and Smith and Stillman, 1989). Further experiments will be required to determine whether these proteins are H1 related and if they are deposited specifically during the second step of the chromatin assembly reaction in association with or following H2A/H2B deposition.

Discussion

Model for sequential deposition of histones during DNA replication

Based upon our findings, we propose a model for stepwise assembly of chromatin during DNA replication (Figure 8). In this model, histones H3 and H4, synthesized in the cytoplasm during S phase, are transported to the nucleus whereupon CAF-I, a nuclear protein (S.Smith and B. Stillman, submitted for publication), binds the $(H3/H4)_2$ tetramer and targets this complex to replicating DNA. We have demonstrated that the deposition of newly synthesized histones H3 and H4 occurs exclusively on replicating DNA in close association with the passing replication fork. Moreover, the newly synthesized forms of histones H3 and H4 may be essential components of this reaction (discussed below). The finding that newly synthesized histones H3 and H4 deposit exclusively on replicating DNA is consistent with a large body of *in vivo* evidence (Cremisi *et al.*, 1977; Worcel *et al.*, 1978; Senshu *et al.*, 1978; Cremisi and Yaniv, 1980; Jackson and Chalkley, 1981a,b, 1985), demonstrating that in this regard the cell free system closely mimics the *in vivo* process, but adds a new level of complexity by introducing the requirement for the cellular factor CAF-I.

The chromatin precursor generated during the first step of this two step reaction can be isolated as a stable intermediate containing histones H3 and H4. We have demonstrated that this replicated precursor, like nascent chromatin found *in vivo*, is hypersensitive to nuclease digestion; this is consistent with the suggestion that newly synthesized chromatin contains only histones H3 and H4 (Worcel *et al.*, 1978; Cremisi and Yaniv, 1980). Moreover, our preliminary data indicate that in addition to histones H3 and H4, CAF-I is associated with the chromatin precursor; immunoblot analysis using monoclonal antibodies directed against CAF-I (Smith, 1990; S.Smith and B.Stillman, submitted for publication) indicate that CAF-I is contained in the sucrose gradient isolated chromatin precursor (data not shown). The model, therefore, depicts CAF-I as depositing along with histones H3 and H4 on the newly replicated DNA.

Next, as part of the chromatin maturation process, CAF-I exchanges with H2A/H2B dimers to generate nucleosomes and mature chromatin, perhaps through direct recognition of CAF-I by H2A and H2B. We have demonstrated that either the newly synthesized or chromatin-bound forms of histones H2A and H2B will deposit on the replicated precursor, indicating that unlike the first step, this exchange reaction does not require newly synthesized histones H2A and H2B. This is consistent with *in vivo* studies indicating that nascent nucleosomes contain newly synthesized histones H3 and H4 mixed with either old or new histones H2A and H2B (Jackson, 1987, 1988).

Newly synthesized histones H3 and H4 differ from the chromatin-bound forms

We have demonstrated that histones H3 and H4 present in the 0.6M extract do not stably associate with DNA in the absence of CAF-I (Figure 7A), a surprising result considering the strong DNA binding properties that have been described for these histones. However, most of what is known about histones H3 and H4 comes from studies using the chromatin bound forms of these proteins. The newly synthesized histones H3 and H4 contained in the cytosol replication extract (which are the precursors for *de novo* chromatin assembly *in vivo*) could differ from the chromatin-bound forms in several ways. First, the cytosolic histones may contain specific modifications not found in bulk chromatin. We have shown by two-dimensional gel analysis that the H4 in the cytosol extract migrates as the diacetylated form of this histone (Figure 2B; Smith and Stillman, 1989), a modification specifically associated with newly synthesized histone H4 found *in vivo* (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976; Bonner *et al.*, 1988). Second, newly synthesized histones H3 and H4 may be associated with other polypeptides not found in bulk chromatin, as is the case for the histones contained in the chromatin assembly extract derived from *Xenopus* eggs (Kleinschmidt and Franke, 1982; Dilworth *et al.*, 1987). In support of a difference between the newly synthesized and chromatin-bound forms of histones H3 and H4, we do find a change in their chromatographic behavior; under conditions where the cytosolic forms of histones H3 and H4 flow through phosphocellulose, histones H3 and H4 purified from chromatin bind quantitatively (data not shown). Thus, newly synthesized histones H3 and H4 have a reduced affinity for phosphocellulose, which could be due to histone modification and/or association with other polypeptides.

An important question is whether the newly synthesized forms of histones H3 and H4 are required for the replication dependent chromatin assembly reaction described here. Our preliminary experiments suggest that this may be the

case. Since the cytosol extract has not yet been depleted of histones H3 and H4, we are unable to perform a substitution experiment. We do find, however, that the addition of even very small amounts of histones H3 and H4 (purified from chromatin) to the 0.6M replication extract promotes supercoiling of both replicating and non-replicating DNA in the presence or absence of CAF-I (data not shown), suggesting that newly synthesized histones H3 and H4 are essential for the CAF-I-dependent discrimination between replicated and unreplicated DNA.

In agreement with this suggestion is the recent demonstration by Gruss *et al.* (1990) that the addition of a crude nuclear extract (containing large amounts of histones) to the cytosol replication extract promotes supercoiling of both replicated and unreplicated DNA. These authors, however, interpret their results to indicate that nucleosome assembly is not coupled to DNA replication. We, on the other hand, suggest that their crude system which contains two populations of histones (the newly synthesized histones H3 and H4 and the chromatin derived histones) displays two disparate supercoiling reactions and that true chromatin assembly in their experiments only occurs during DNA replication. Their own data (shown in Figure 10 of Gruss *et al.*, 1990) demonstrate that the replication coupled supercoiling reaction produces DNA–protein complexes similar to nucleosome particles found in SV40 and host cell chromosomes *in vivo* and confirm our data reported here and elsewhere (Stillman, 1986; Smith and Stillman, 1989). In contrast, the DNA–protein complexes produced in the absence of DNA replication did not resemble the known structure of nucleosomes based upon the length of DNA associated with them. We therefore suggest that these complexes do not reflect the assembly of chromatin. It may well be that these DNA–protein complexes are similar to the DNA–protein complexes that we observe when we add chromatin-derived histones H3 and H4 to the reactions. It will be necessary to determine the exact nature of these replication-independent complexes before their significance is appreciated. Thus the conclusions by Gruss *et al.* (1990) that CAF-I and DNA replication do not play a decisive role in chromatin assembly do not seem to be warranted at this time.

This raises the question as to the role of DNA replication in nucleosome assembly. *In vivo*, the bulk of histone deposition into chromatin occurs during S phase of the cell cycle and is coupled to DNA replication. Our data, which reproduce this *in vitro*, indicate that the CAF-I-dependent coupling of chromatin assembly to DNA replication requires the exclusive use of the newly synthesized forms of histones H3 and H4 and the availability of these histones may be tightly controlled. It should also be noted that our results do not imply that chromatin assembly occurs exclusively during DNA replication *in vivo*. Clearly, cell free systems for the assembly of chromatin in the absence of DNA replication have been developed using *Xenopus* egg extracts (Dilworth and Dingwell, 1988) and it is easy to imagine many situations in which chromatin needs to be formed in non-replicating cells. In fact, recent studies using the *Xenopus* cell free system have demonstrated a sequential mechanism for histone deposition in the absence of DNA replication (Kleinschmidt *et al.*, 1990; Zucker and Worcel, 1990; Sapp and Worcel, 1990). A role for CAF-I in this *in vitro* chromatin assembly reaction and in the replication-independent assembly of chromatin *in vivo* remains to be determined. One interesting possibility is that the exchange of histones H2A and H2B observed in non-replicating cells (Jackson, 1987, 1988) requires CAF-I as an exchange factor. Finally, it should be noted that the role of CAF-I and DNA replication in the redistribution of old, chromosome-bound histones H3 and H4 after passage of the replication fork has not been addressed in these cell free systems.

CAF-I targets deposition of histones H3 and H4 to replicating DNA

The demonstration that in the absence of CAF-I, histones H3 and H4 have no affinity for DNA, points to a key role for the cellular factor (CAF-I) in targeting newly synthesized histones H3 and H4 to the replicating DNA. What is the mechanism which targets deposition of histones H3 and H4 to replicating DNA? Recently, Fotedar and Roberts (1989) have used a similar, but unfractionated cell free system of chromatin assembly to propose a multi-step pathway for replication coupled chromatin assembly. In this study the authors describe a DNA–protein particle which they suggest, although did not show, contains histones H3 and H4. Unlike the chromatin precursor described in our report, however, this particle was found on both replicated and unreplicated DNA and therefore does not reflect the replication-preferential nature of chromatin assembly. Since our results indicate that in the absence of CAF-I, histones H3 and H4 do not associate with the DNA (replicating or non-replicating DNA), we favor a model in which CAF-I binds to histones H3 and H4 and targets the complex to replicating DNA. CAF-I could modify histones H3 and H4 directly to somehow promote their deposition onto replicating DNA or a CAF-I–H3–H4 complex could deposit on replicating DNA. This latter possibility would be consistent with our preliminary findings (described above) that CAF-I is contained in the chromatin precursor. If CAF-I is responsible for localizing the complex to replicating DNA, it must do so through protein–protein interaction, since purified CAF-I does not bind DNA directly (Smith and Stillman, 1989). Such interactions could occur with one or more of the proteins associated with the replication fork. Since most of the proteins required for DNA replication in this cell free system have been identified and purified (see Stillman, 1989; Tsurimoto *et al.*, 1990), we can begin to test this hypothesis directly. Finally, we note that a two step nucleosome assembly mechanism was recently proposed, based upon studies using replicating single-stranded DNA and *Xenopus* egg extracts (Almouzni *et al.*, 1990).

Regulation of chromatin assembly in vivo

To investigate the mechanism of histone deposition during DNA replication we have used a cell free system which, by many criteria (Stillman, 1986; Smith and Stillman, 1989; this report), reproduces events that occur *in vivo*. We present evidence for a sequential mechanism of chromatin assembly during DNA replication; histones H3 and H4 deposit first on replicating DNA, followed by deposition of histones H2A and H2B. The demonstration that this reaction is catalyzed by a cellular protein (CAF-I) introduces a possible mechanism for the cellular control of this fundamental process. Interestingly, we have recently shown that CAF-I, a multisubunit protein, is phosphorylated on at least two subunits *in vivo* (Smith, 1990; S.Smith and B.Stillman,

submitted for publication), a modification which could affect the ability of CAF-I to bind histones. It will be important to determine whether this phosphorylation can affect the histone binding and assembly properties of CAF-I both *in vitro* and *in vivo*. Finally, it is possible that CAF-I could regulate access of non-histone proteins to the newly replicated DNA, thereby influencing the inheritance of chromatin states.

Materials and methods

Preparation of cytosol extracts and T antigen

The cytosol replication extracts were prepared from suspension cultures of human 293 cells as described previously (Stillman and Gluzman, 1985; Stillman, 1986). The radiolabeled cytosol replication extracts were prepared in the same way except that prior to harvesting, the cells were incubated in arginine-free and lysine-free media (MEM Joklik-Modified) supplemented with 5% dialyzed calf serum for 30 min at 37°C, followed by incubation in the same media containing 100 μCi/ml [^{14}C]lysine and 100 μCi/ml [^{14}C]arginine or 500 μCi/ml [^{3}H]lysine and 500 μCi [^{3}H]arginine for 60 min at 37°C. SV40 T antigen was obtained from recombinant baculovirus vector (941T)-infected *Spodotera frugiperda* insect cells as described (Lanford, 1988) and was purified by immunoaffinity chromatography according to Simanis and Lane (1985) and Stillman and Gluzman (1985).

Preparation of fractionated cytosol extracts

The cytosol replication extract derived from 16 l of 293 cells (8×10^9 cells) was adjusted to 0.6 M NaCl and loaded onto a 20 ml phosphocellulose column (2 × 6 cm) equilibrated in buffer A [25 mM Tris−HCl (pH 7.5), 1 mM EDTA, 0.01% NP-40, 10% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride] containing 0.6 M NaCl. The protein that flowed through the column was dialyzed against buffer A containing 50 mM NaCl and 20% sucrose (w/v), aliquoted, and stored at −70°C (0.6M flow-through fraction). The column was then washed with 40 ml of buffer A containing 0.6 M NaCl and the bound material eluted with 40 ml of buffer A containing 2 M NaCl. The eluted protein was concentrated in volume ~8-fold by ultrafiltration (Centricon), aliquoted, and stored at −70°C (2M step fraction).

Preparation of nuclear extracts, CAF-I and histones

Nuclei were isolated from 293 cells and extracted with 0.4 M NaCl as described previously (Smith and Stillman, 1989). The residual nuclear material was collected by centrifugation and used for the preparation of the histones (see below). CAF-I was purified from the supernatant by ammonium sulfate precipitation and fractionation through five chromatographic steps (Smith and Stillman, 1989). The highly purified Mono Q fraction was aliquoted and stored at −70°C (CAF-I). For the preparation of the histones, a chromatin extract was prepared from the residual nuclear material. The nuclear pellet was washed twice with 60 ml of buffer containing 25 mM NaCl and 8 mM EDTA (pH 8.0) and collected by centrifugation. The chromatin was prepared and fractionated by hydroxylapatite according to the procedure of Simon and Felsenfeld (1979). The fractions containing the purified histone pairs H2A/H2B and H3/H4 were dialyzed against buffer A containing 100 mM NaCl and 20% sucrose (w/v), aliquoted, and stored at −70°C. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

DNA replication reactions

SV40 DNA replication *in vitro* was assayed under standard conditions similar to those described previously (Stillman, 1986). Each reaction contained 4 μg/ml of plasmid DNA pSV011 [which contains the SV40 origin from *Hin*dIII (nucleotide no. 5171) to *Sph*I (nucleotide no. 128) in pUC18 and is 2.9 kb in size], 22 μg/ml purified SV40 T antigen, 3.2 mg/ml 293 cell cytosol extract or 6.4 mg/ml 0.6M flow-through fraction, 4 μg/ml purified topoisomerase I and 1.8 μg/ml purified topoisomerase II. Topoisomerases I and II were purified from calf thymus nuclear extract by slight modifications of published procedures (Liu and Miller, 1982; Schomburg and Grosse, 1986). The replication reactions contained the following additional components as indicated: 21 μg/ml purified CAF-I, 80 μg/ml 2M step fraction or 32 μg/ml H2A/H2B. Reactions were incubated at 37°C for 60 min unless otherwise indicated. The 8:1 ratio of H2A/H2B to DNA (weight:weight, i.e. a 4:1 ratio of each histone to DNA) was required to achieve complete supercoiling of the replicated DNA. It should be noted, however, that supercoiling could be observed at lower H2A/H2B ratios (i.e. 4:1 and 2:1, data not shown).

Replication product analysis

Reaction mixtures were digested first with ribonuclease A (20 μg/ml) for 15 min at 37°C in the presence of 10 mM EDTA and 0.5% SDS and then with 1 mg/ml of pronase for 1 h at 37°C. The samples were then extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and subjected to agarose gel electrophoresis in Tris−borate−EDTA buffer (Maniatis *et al.*, 1982) at ~2 V/cm. The gels were stained with ethidium bromide (1 μg/ml), then dried and autoradiographed.

Micrococcal nuclease digestions

After the DNA replication reaction, the mixture was adjusted to 3 mM $CaCl_2$ and 1 μl (15 U) of micrococcal nuclease (Worthington) was added to the reaction for various times at 30°C. All reactions were terminated by addition of EDTA to 10 mM. The DNA was isolated as described above and separated from unincorporated nucleoside triphosphates by spin dialysis (Maniatis *et al.*, 1982) with Sephadex G-50 in a 1 ml syringe. The DNA was subjected to electrophoresis through 2% agarose as described above. In order to load similar amounts of replicated DNA on the gel, in each case only 50% of the samples of DNA replicated in the absence of CAF-I was subjected to agarose gel electrophoresis.

Sucrose gradient sedimentation

Replication reaction mixtures were layered onto preformed 15−30% sucrose gradients in 10 mM Tris−HCl (pH 7.5), 1 mM EDTA, 0.25% NP-40, 0.15 M NaCl in Beckman SW41 centrifuge tubes. Samples were subjected to centrifugation at 30 000 r.p.m. for 16 h and fractions were collected from the bottom. The position of the minichromosome was determined by subjecting a sample of the sucrose gradient fractions to electrophoresis in agarose gels.

Histone analysis

Analysis of the proteins contained in the minichromosome was carried out as described previously (Smith and Stillman, 1989) according to the method of Shimamura *et al.* (1988). Sucrose gradient fractions were combined and layered over a 1 ml cushion of 30% sucrose (for the rapidly sedimenting products) or 15% sucrose (for the slower sedimenting products) and pelleted in a Beckman SW60 rotor at 30 000 r.p.m. for 16 h. The pellet was resuspended in H_2O and mixed with an equal volume of a solution containing 0.4 M HCl, 0.5 mg/ml protamine sulfate, 8 M urea and 0.02% pyronine Y. Proteins were subjected to two-dimensional gel electrophoresis. For the first dimension, TAU tube gels were run essentially as described (Alfageme *et al.*, 1974) using a 15% acrylamide gel containing 6 M urea and 0.37% Triton X-100. After electrophoresis the gels were equilibrated in 60 mM Tris (pH 6.8), 0.5% β-mercaptoethanol and placed over an 18% SDS−polyacrylamide gel and subjected to electrophoresis as described by Laemmli (1970). The positions of the marker histones were determined by co-electrophoresis of core histones isolated from 293 cell chromatin. After electrophoresis proteins were visualized by staining with Coomassie brilliant blue followed by fluorography where appropriate; gels were treated with H_2O for 20 min, 1 M sodium salicylate for 30 min, dried and autoradiographed. The histone pairs isolated from 293 cell chromatin were analyzed by electrophoresis through a TAU-15% polyacrylamide gel containing 6 M urea and 0.37% Triton X-100. Proteins were visualized by staining with Coomassie brilliant blue.

Acknowledgements

We would like to thank John Diffley for valuable discussion throughout the course of this work. We gratefully acknowledge Winship Herr, Mike Mathews and John Diffley for critically reading the manuscript. We also thank Jim Duffy and Phil Renna for the photography and preparation of the figures. This work was supported by a grant from the National Institutes of Health (CA 13106).

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Received on October 22, 1990; revised on January 11, 1991