Chromatin assembly on replicating DNA *in vitro*

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ABSTRACT

Replicating single-stranded DNA is preferentially assembled into chromatin in *Xenopus* egg extracts relative to non-replicating double-stranded DNA. We have examined the molecular basis of this phenomenon. Single-stranded DNA itself is not a favored template for nucleosome assembly in comparison to double-stranded DNA. Complementary strand synthesis is required for the rapid assembly of nucleosomes. We present evidence that the assembly of chromatin on replicating DNA is a two step phenomenon. The first step involves the replication of DNA and the assembly of an intermediate structure, the second step involves the sequestration of histones H2A/H2B onto DNA. Histones H2A/H2B are preferentially sequestered onto replicated DNA in comparison to non-replicated DNA incubated in the extract.

INTRODUCTION

In vivo the process of chromatin assembly is coupled to DNA replication (1, 2). A role for the replication process itself in promoting chromatin assembly has been suggested from *in vitro* experiments using mammalian (3, 4) and *Xenopus* cell-free extracts (5). Although assembly factors have been identified in both mammalian and *Xenopus* extracts (6, 7), how histones are added to DNA and why replicating DNA is preferentially assembled into nucleosomes is not resolved.

We have begun to examine the molecular mechanisms responsible for the assembly of replicating DNA into chromatin in *Xenopus* extracts (5). Single-stranded DNA incubated in *Xenopus* extracts will serve as a template for complementary strand synthesis, nucleosome assembly occurs concomitant with DNA synthesis on the replicating template. This is a rapid process compared to the assembly into nucleosomes of non-replicating doubled-stranded DNA in the same extract. Our approach has been to examine two possible explanations for this phenomenon. First, core histones or the enzyme complexes involved in chromatin assembly might preferentially associate with the single-stranded DNA used as a template for replication in the *Xenopus* system, as compared to non-replicating double-stranded DNA. Second, the rate of chromatin assembly might be inversely related to the concentration of double-stranded DNA present in the extract (8). With a single-stranded DNA template, double-stranded DNA appears as replication progresses and is quickly assembled into nucleosomes (5). At any one time, very little 'free' double-stranded DNA would be present on the replicating template, as compared to DNA added in double-stranded form to the extract. As multiple enzyme complexes may be involved in chromatin assembly, there could be a problem nucleating these macromolecules onto a single segment of double-stranded DNA, especially when double stranded DNA is in excess. Therefore, interaction between components of the chromatin assembly apparatus might be promoted through binding to the short regions of free double-stranded DNA on a replicating template (i.e., a local concentration effect). We find that neither of these explanations can simply explain the advantage a single-stranded DNA template possesses for the assembly of newly synthesized double-stranded DNA into nucleosomes.

Finally, we asked whether the preferential assembly of replicating DNA into chromatin was due to selective association with all of the histones or whether a subset of histones preferentially associate with the replicating template. This latter possibility was recently suggested by Fotedar and Roberts (9) using the mammalian *in vitro* chromatin assembly system. Our results confirm this prediction and suggest that the preferential chromatin assembly on replicating single-stranded DNA in the *Xenopus* extract is due to the formation of an intermediate in nucleosome assembly which directs the selective sequestration of histones H2A/H2B. This process is dependent on the enzyme-DNA complexes mediating complementary DNA synthesis on single stranded DNA.

MATERIALS AND METHODS

Preparation of the X. laevis egg extract and oocyte nuclear extract

Unfertilized eggs were obtained from *X. laevis* frogs, and extracts were prepared as previously described (5, 10). Briefly, dejellied eggs were disrupted by direct centrifugation (12 000 g for 30 min) at 4°C in a modified extraction medium (20 mM HEPES pH 7.5, 70 mM potassium chloride, 1 mM dithiothreitol (DTT), 5% sucrose, 10 μg/ml leupeptin). The supernatant was recentrifuged at 150 000 g for 60 min. The final supernatant was

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stored in small aliquots at −80°C. *Xenopus laevis* oocyte nuclear extract was prepared as previously described (11). Ovaries were removed from adult frogs, fragments of ovary were swollen for 3 hours in 5mM Tris HCl (pH 7.8) and 10mM $MgCl_2$. Individual fragments of ovary were transferred to ice cold 'J' buffer (70mM NH_4Cl, 7mM $MgCl_2$, 0.1mM EDTA, 2.5mM DDT, 10% (v/v) glycerol, 10mM HEPES (pH 7.4) and 2% polyvinyl pyrrolidone). The oocytes were broken using two pairs of forceps. Nuclei were taken up into a pipette tip. Five hundred nuclei were collected into a microfuge tube in a total volume of 1ml. The nuclei were disrupted by brief vortexing. The lysate was centrifuged at 10,000 ×g for 1 min at 4°C to pellet nuclear debris.

Preparation of DNA

DNA from bacteriophage M13mp18 was prepared from phage purified by CsCl buoyant density centrifugation. Double-stranded form I M13 DNA was isolated and prepared as described (12). Radiolabeled double-stranded form I M13 DNA was prepared by incubating single-stranded M13 in the egg extract in the presence of [α-^{32}P]dATP (10−20μCi) and purifying the replicated DNA by deproteinization and ethanol precipitation. Radiolabeled single-stranded M13 DNA was obtained by labeling *in vivo* as previously described (5).

DNA synthesis and chromatin assembly in the egg extract

Unless otherwise specified, our standard reaction mixtures contained 10−20μg DNA/ml egg extract supplemented with 3 mM ATP and 5mM $MgCl_2$. All reactions contained an ATP regenerating system of 40mM creatine phosphate, and 1μg creatine phosphokinase. Supplementation of the egg extract with Mg^{2+}/ATP was carried out before DNA was added to the extract. DNA synthesis was followed by the addition of 10−20 μCi of [α-^{32}P]dATP to the reaction. Aliquots were taken at various times during incubation at 22°C and either transferred to Whatman GF/C filters and processed for the counting of acid-insoluble material as previously described (13), or processed for digestion by micrococcal nuclease. When subjected to electrophoresis, the samples were deproteinized by proteinase K (500 μg/ml) followed by phenol extraction and ethanol precipitation. Topoisomers were resolved on 1% agarose gels containing 40mM Tris, 20mM sodium acetate, 2mM EDTA, pH 8.3. In order to resolve highly supercoiled DNA, gels containing 30μg/ml chloroquine were used (14).

Preparation of histones and reconstitution of nucleosomes with purified histones

Chicken erythrocyte histones H2A/H2B and H3/H4 were prepared by chromatography on hydroxyapatite (15), and dialyzed into 10mM Tris-HCl, pH 7.5, 1mM Na_3 EDTA, 0.25mM phenylmethanesulphonyl fluoride. These purified histones were reconstituted onto single and double-stranded DNA by salt/urea dialysis.

RESULTS

Single stranded DNA is not a preferred substrate for nucleosome assembly

We examined whether proteins involved in chromatin assembly might prefer to associate with single-stranded relative to double-stranded DNA in the *Xenopus* egg extract. A fixed volume of the egg extract was incubated with increasing masses of double-stranded (pUC 9) or single-stranded DNA (M13 mp 19) for three hours in the presence of aphidicolin, an inhibitor of DNA synthesis (17). This inhibitor does not affect either transcription or chromatin assembly in the egg extract (not shown). The residual capacity of the extract to assemble chromatin was then measured by introducing a small amount of radiolabelled double-stranded DNA and measuring the change in linking number of this closed circular template. Each nucleosome changes the linking number (supercoiling) of the template by −1, after relaxation of the linker DNA by topoisomerases in the extract, therefore chromatin assembly correlates with the number of supercoils present after deproteinization (18).

The most efficient chromatin assembly is obtained in the absence of any other DNA (Figure 1, Control). Double-stranded DNA efficiently titrates the capacity of the extract to assemble chromatin (Figure 1, lanes 2−4; see ref. 5). In contrast pre-incubation with a much larger mass of single-stranded DNA is required to inhibit chromatin assembly on the labelled DNA (Figure 1, lanes 5−8). The 4−5 fold excess of single-stranded DNA over double-stranded DNA required to prevent chromatin assembly to the same extent, suggests that the molecules facilitating chromatin assembly associate with single-stranded DNA weakly in comparison to double-stranded DNA in the absence of replication. Addition of purified core histones restores supercoiling to the extract titrated with either double- or single-

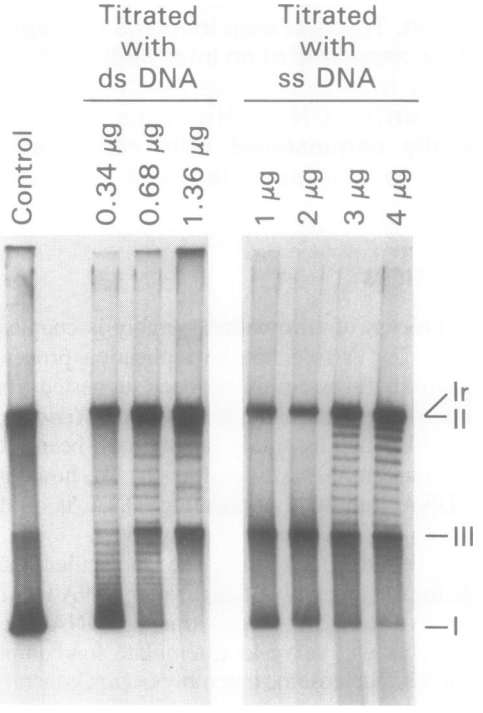

Figure 1. Titration of chromatin assembly on double-stranded DNA by preincubation with single-stranded or double-stranded DNA in the presence of aphidicolin. For each reaction 10μl of egg extract was preincubated for three hours with various masses of DNA as indicated, in the presence of 20μg/ml aphidicolin. Either no DNA was added to the reaction (Control) or double-stranded circular DNA (pUC9; ds DNA) or single-stranded circular DNA (M13mp19; ss DNA) was used to titrate proteins or activities required for chromatin assembly. After this preincubation period, 100ng of radiolabelled double-stranded (M13 mp 19) DNA was added and the reaction incubated for a further four hours. The DNA was deproteinized and resolved on a 1% agarose gel before autoradiography. The positions of supercoiled double-stranded DNA (I), linear ds DNA (III), nicked closed circular ds DNA (II) and relaxed closed circular ds DNA (Ir) are indicated.

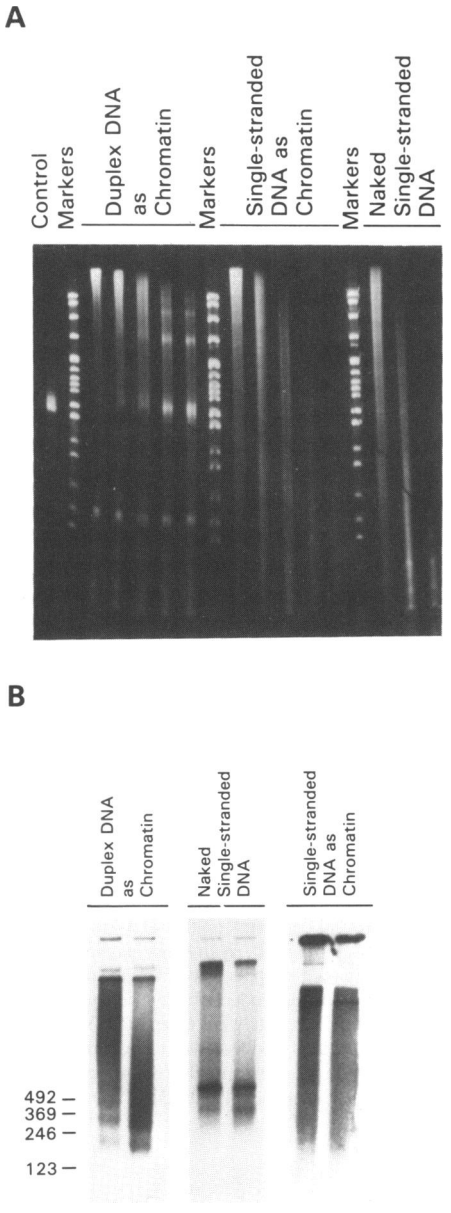

Figure 2. 'Nucleosome-like' particles do not form with high efficiency on single-stranded DNA. A. Purified core histones were complexed with double- or single-stranded DNA as indicated. Deposition of histones onto DNA was confirmed by non-denaturing gel electrophoresis of the nucleoprotein complexes (see Figure 3). The chromatin samples (20μg DNA per ml) were digested with micrococcal nuclease (0.085 units per μg DNA) for increasing periods of time (2, 5, 10, 20 and 60 min) in a buffer containing 2mM $CaCl_2$, 35mM NaCl, 10mM Tris HCl pH 7.5; 1mM Na_3 EDTA, 1mM β mercaptoethanol. Naked DNA was also digested and samples treated with micrococcal nuclease for 2, 5 and 10 min are shown. The deproteinized products of digestion were resolved on a denaturing 10% polyacrylamide gel before staining with ethidium bromide and photography. For reference, DNA isolated from chicken erythrocyte nucleosome core particles (Control) and a Msp I digest of pBR322 (Markers) are shown. The band at the bottom of the gel in all of the lanes using duplex DNA is due to tRNA contamination of the duplex M13 preparation. B. Radiolabelled single stranded DNA (M13mp19; 100ng) was incubated in *Xenopus* egg extract (10μl) for 3 hours either with aphidicolin (20μg/ml) ('Single stranded DNA as Chromatin') or without the inhibitor ('Duplex DNA as Chromatin'). At the end of this time the two samples were digested with micrococcal nuclease for 3 or 5 minutes before deproteinization, resolution on a 1% agarose gel and autoradiography. Positions of size markers multiples of 123bp duplex DNA (BRL) run on the same gel are indicated. As an additional control, naked radiolabelled single-stranded DNA in extraction buffer (plus 1mM $CaCl_2$) was digested for 15 or 30 seconds before ethanol precipitation and resolution on the same gel ('Naked Single-stranded DNA').

stranded DNA, suggesting that core histones are the limiting components for chromatin assembly (not shown, but see Figure 6). Control experiments demonstrated that single-stranded DNA is stable in the extract in the presence of aphidicolin (not shown, but see Figure 3). Without replication occurring in the extract, core histones do not therefore associate more stably with single-stranded DNA than with double-stranded DNA. This result need not imply that core histones do not bind tightly to single-stranded DNA. For example core histones might be prevented from interacting with single-stranded DNA because of an excess of single-stranded DNA binding proteins in the extract. Our next experiments attempt to clarify this issue by using purified components to examine the interaction of single-stranded DNA with histones. We also looked for any detectable protein-nucleic acid interaction with single-stranded DNA in the egg extract.

The formation of 'nucleosome-like' structures on single-stranded DNA has been reported (19, 20). Formation of such structures is one possible explanation for the inhibition of chromatin assembly following titration of histones in the egg extract by single-stranded DNA. We attempted to assemble nucleosomes or nucleosome-like structures with purified core histones and single- or double-stranded M13 DNA using the salt-urea dialysis method for nucleosome reconstitution (16). Although nucleosomes were clearly formed on double-stranded DNA as revealed by micrococcal nuclease digestion of the reconstitute, no discrete protected DNA fragments were observed on digestion of single-stranded DNA complexed with histone (Figure 2A). Non-denaturing gel electrophoresis revealed that a stable complex of histones and single-stranded DNA had been formed (Figure 3). Control experiments indicated that the single-stranded DNA was not nicked or degraded in the course of this experiment (not shown).

We asked whether the association of histones or any other component of the chromatin assembly apparatus with single-stranded DNA could be detected in the egg extract. The addition of radiolabelled single- or double-stranded DNA to the egg extract with or without aphidicolin leads to the formation of nucleosomes on the replicated DNA (Figure 2B, Duplex DNA as chromatin), but to no discrete pattern of protection on single-stranded DNA following nuclease digestion (Figure 2B, Single-stranded DNA as chromatin). Cleavage of naked single-stranded DNA with micrococcal nuclease yields a few resistant DNA fragments on agarose gel electrophoresis (Figure 2B, Naked single-stranded DNA). This protection is presumeably due to stem-loop structures formed in M13 DNA (21). The absence of this pattern on digestion following addition of the DNA to the extract suggests that these structures are altered following incubation in the extract. The association of purified histones with single-stranded DNA following dialysis does not provide any advantage over naked single-stranded DNA, for replication and chromatin assembly on addition to the egg extract. However, unlike naked single-stranded DNA, single-stranded DNA complexed to histones does not inhibit chromatin assembly on double-stranded DNA (not shown) presumably because there is less free DNA available to sequester histones. Furthermore the histones complexed to single-stranded DNA do not promote chromatin assembly on double-stranded DNA (not shown).

We conclude that histones can be stably sequestered onto single-stranded DNA, but that this DNA is not efficiently assembled into nucleosome-like structures. Furthermore, pre-formed complexes of histones and single-stranded DNA are not a better substrate than naked single-stranded DNA for the assembly of chromatin. The components of the chromatin assembly apparatus

do not appear to associate more stably with single-stranded DNA than with double-stranded DNA. Selective assembly of replicating single-stranded DNA into chromatin in the extract must be related to the enzyme complexes mediating complementary DNA strand synthesis and is not an innate property of single-stranded DNA.

The DNA concentration dependence of the rate of chromatin assembly

There is very little free double-stranded DNA at any instant of time during the synthesis of the complementary strand on a single-stranded DNA template in the *Xenopus* egg extract (5). Chromatin assembly might be facilitated if the large excess of macromolecules recognizing naked double-stranded DNA could only interact with this nascent template. We have previously noted that the rate of nucleosome formation on a segment of double-stranded DNA is increased as the concentration of DNA is reduced in the egg extract (8). Supercoiling of DNA alone is only an approximate measure of the rate of chromatin assembly, this conclusion is also dependent on experiments measuring the rate at which DNA is protected from micrococcal nuclease digestion. Double-stranded DNA at low concentration (2μg/ml) is supercoiled more rapidly and efficiently than at high concentration (10μg/ml). At low DNA concentrations, the endogenous histone pool is still in excess relative to the template in the extract. However, high concentrations of replicating single-stranded DNA (10μg/ml) are assembled into chromatin even more rapidly than low concentrations of double-stranded DNA. At low concentrations of single-stranded DNA, supercoiling is apparently inhibited whereas replication efficiency is unaffected. Under these conditions a complex is formed that will not migrate into the gel matrix (Figure 4, lanes 3 and 4). We do not understand the origin of this complex. This DNA is assembled into chromatin as revealed by micrococcal nuclease digestion (not shown) suggesting that the origin of the complex is not due to single-stranded DNA binding proteins complexing the DNA and perhaps preventing the completion of replication. Double-stranded DNA concentration clearly contributes to changing the kinetics of

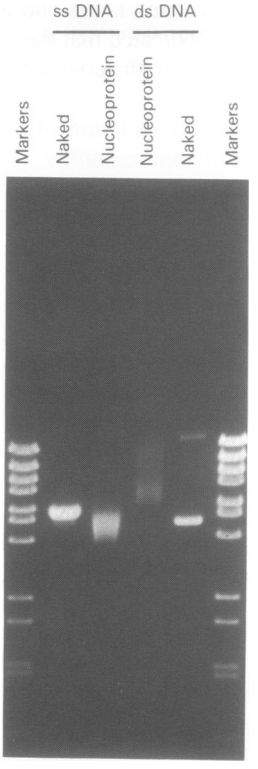

Figure 3. Non denaturing gel electrophoresis of histone-DNA complex. Purified chicken erythrocyte core histones and purified double- stranded or single-stranded M13 DNA were complexed during step dialysis from high to low salt concentration (16) before resolution on a non denaturing (0.7%) agarose gel in TAE (12). Free double- and single-stranded DNA were resolved for reference, as was a Bst EII digest of λ DNA. Note that single-stranded DNA and relaxed circular DNA molecules have an increased mobility when complexed with histones relative to free DNA in this assay. In contrast, the mobility of free supercoiled DNA is reduced following the association of histones.

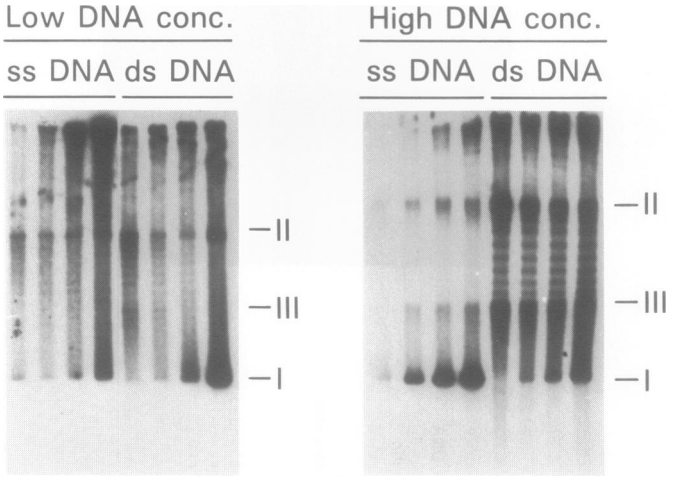

Figure 4. The effect of DNA concentration on the kinetics of chromatin assembly. DNA was incubated in 20μl egg extract either at a low concentration (2μg/ml) or at a high concentration (10μg/ml). With single-stranded DNA, [α-^{32}P]dATP (10μCi) was added to follow the appearance of replicated DNA. With double-stranded DNA, the deproteinized product of a replication reaction in the presence of [α-^{32}P]dATP was used. Aliquots were taken after 30 min, 1 hour, 2 hours and 4 hours, and the DNA analyzed from left to right on 1% agarose gels after deproteinization. The positions of supercoiled double-stranded DNA (I), linear ds DNA (III) and nicked closed circular ds DNA (II) are indicated.

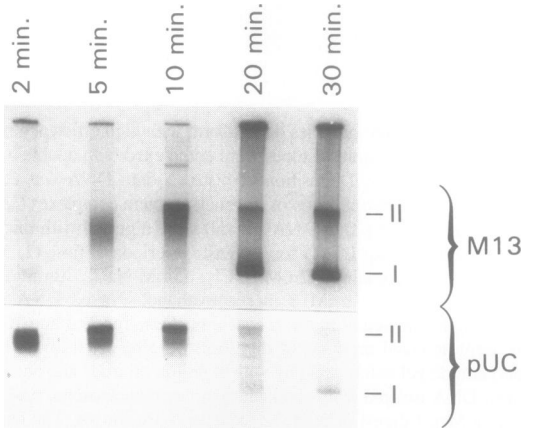

Figure 5. Replicating single-stranded DNA at high concentration is assembled into chromatin more rapidly than double stranded DNA at low concentration in the same mixture. Mixtures of single-stranded DNA (M13, mp19 10μg/ml) and nick-translated double-stranded DNA (pUC, 2μg/ml) were incubated in 20μl egg extract plus [α -^{32}P]dATP (20μCi) for the times indicated. The DNA was analyzed on 1% agarose gels after deproteinization. The positions of supercoiled double-stranded DNA (I) and nicked circular ds DNA (II) are indicated.

chromatin assembly. However, there is still a significant advantage for single-stranded DNA (at high concentration) over double-stranded DNA (at low concentration) under the optimal chromatin assembly conditions for the two templates.

We confirmed the advantage of single-stranded over double-stranded DNA for chromatin assembly directly by incubating a low concentration of nick translated double-stranded DNA with a high concentration of replicating single-stranded DNA in the same reaction mixture (Figure 5). In this experiment, the early time points show the completion of replication of the single-stranded DNA, note that the incorporation of [α ^{32}P]dATP increases. Even though the nascent DNA is associated with nucleosomes the template is not supercoiled because ligation of duplex DNA regions is not complete (5). On completion of ligation, a fully supercoiled DNA molecule appears because of the presence of nucleosomes, compare the 10 and 20 minute time points. Although we caution that factors such as the presence of abundant DNA binding proteins other than histones in the extracts may prevent an accurate determination of free DNA concentration, we do conclude that DNA concentration effects are unlikely to simply explain the preferential deposition of nucleosomes on a replicating template.

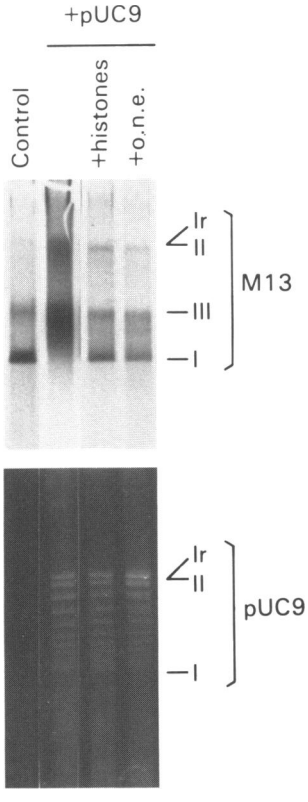

Figure 6. Replicated DNA is preferentially assembled into chromatin. *Xenopus* egg extract (20μl) was preincubated with double-stranded DNA (pUC9; 200μg/ml) for 2 hours. Then single-stranded DNA (M13mp19; 10μg/ml) was incubated in the extract for 2 hours in the presence of [α-^{32}P]dATP (10μCi). After this period of replication, either J buffer (14μl per 20μl egg extract), lane 2; or all four core histones (500ng in 14μl J buffer) lane 3 or oocyte nuclear extract (14μl) were added, lane 4. After a further one hour incubation, the DNA was deproteinized and resolved on a 1% agarose gel. As a control, single- stranded DNA (M13mp19; 10μg/ml) was incubated in the extract without the pre-addition of pUC9. The replicated radiolabelled product was also resolved on the gel (Control). The gel was dried, radioautographed to examine supercoiling of the replicating DNA (upper panel, M13) and then stained with ethidium bromide to examine supercoiling of the pUC9 DNA (lower panel, pUC9). The positions of supercoiled double-stranded DNA (I), linear ds DNA (III), nicked closed circular ds DNA (II) and relaxed closed circular ds DNA (Ir) are indicated. In order to resolve the double-stranded pUC9 DNA clearly this gel is not run quite long enough to give a clear resolution of the intermediate topoisomers in the M13 DNA.

Histones preferentially associate with DNA replicated in the egg extract relative to double-stranded DNA incubated in the extract for the same time period

Previous work has established that the addition of a nuclear extract to a mixture of double-stranded DNA replicated in a cytosolic extract and of non-replicated double-stranded DNA added to the cytosolic extract will lead to the preferential assembly of the previously replicated DNA into nucleosomes (7, 9). The double-stranded DNA replicated in the extract is believed to be associated with proteins that facilitate chromatin assembly. We have reproduced this observation in the *Xenopus* egg extract (Figure 6). Core histones and other chromatin proteins present in the egg extract were sequestered onto double-stranded DNA (pUC9) in a 2 hour incubation (5, 22). Single-stranded DNA was then added to the core-histone depleted extract and replication allowed to occur for 2 hours. After this time either a mixture of all four purified core histones (Figure 6, + histones) or a nuclear extract of *Xenopus* oocytes (Figure 6, + o.n.e.) were added to the reaction and the mixture incubated for a further hour. Both purified histones and the nuclear extract promoted supercoiling of the replicated DNA (Figure 6, upper panel), but had no measurable effect on the supercoiling of the double- stranded DNA initially used to sequester endogenous core histones (Figure 6, lower panel). We next asked whether all four core histones were necessary for this effect, or whether histones H3/H4 or histones H2A/H2B alone would suffice.

Our approach to this problem was again to titrate the chromatin assembly capacity of the egg extract with double-stranded DNA (pUC9). Template DNA was then added to the extract, either radiolabelled double-stranded DNA or single-stranded DNA plus [α ^{32}P] dATP. This DNA was incubated in the extract for 4 hours. The single-stranded DNA was replicated but not fully

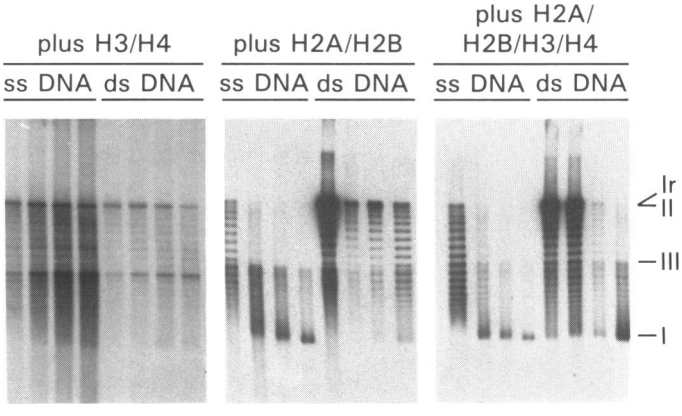

Figure 7. Histones preferentially associate with replicated DNA. *Xenopus* egg extract (20μl) was preincubated with double-stranded DNA (pUC9; 200μg/ml) for two hours. Then template DNA, either radiolabelled double stranded DNA (M13mp19; 10μg/ml) or single stranded DNA (M13mp19; 10μg/ml) with [α-^{32}P]dATP (10μCi) was added for a further 4 hours of incubation. To the reactions were added either histones H3/H4 (4μg in 14μl J buffer); histones H2A/H2B, (4μg in 14μl J buffer) or all four histones (8μg in 14μl J buffer). Samples were taken at zero time (just after addition of histones or extract), after 30 minutes, 1 hour and 3 hours. The DNA was deproteinized and resolved on a 1% agarose gel, before the gel was dried and radioautographed. The positions of supercoiled double- stranded DNA (I); linear ds DNA (III), nicked closed circular ds DNA (II) and relaxed closed circular ds DNA (Ir) are indicated.

supercoiled because of the prior depletion of chromatin proteins. The reactions were then complemented with either histones H3/H4, histones H2A/H2B or all four of the core histones (Figure 7). After various times, the DNA was deproteinized and topoisomers were resolved to assess the degree of supercoiling, and hence chromatin assembly.

Addition of histones H3/H4 does not lead to any change in supercoiling with either replicated (ss DNA) or non replicated DNA (ds DNA) (Figure 7). This result is surprising because histones H3/H4 alone are able to supercoil DNA (23, 24). We presume that the lack of effect of histones H3/H4 on supercoiling is because the template is already saturated with these proteins (see later). In contrast, the addition of all four core histones, or just H2A/H2B, leads to a significant increase in DNA supercoiling for both replicated single- and double-stranded DNA. This analysis reveals that the addition of histones H2A/H2B to the reaction mixture will lead to preferential supercoiling of the replicated DNA. Histones H2A/H2B do not form nucleosomes and contribute to DNA supercoiling unless histones H3/H4 are present (23, 24). We therefore attribute the partial supercoiling of the replicated DNA before exogenous histones are added, to the prior association of histones H3/H4 with the template. The pre-incubation with pUC9 removed all of the available histones H2A/H2B but left some histones H3/H4. An intermediate complex is assembled that facilitates the deposition of histones H2A/H2B onto the replicated DNA. We suggest that the explanation for the selective supercoiling of replicated DNA on addition of nuclear extracts (7, 9; Figure 7) is that histones H2A/H2B preferentially associate with replicating DNA. We next examined whether what the nature of this putative intermediate that directed the selective sequestration of histones H2A/H2B onto replicated DNA might be.

Evidence for the assembly of a pre-nucleosomal particle on DNA in Xenopus extracts

Replicated DNA under conditions of chromatin titration (Figure 6 and 7) is not extensively supercoiled. Digestion with micrococcal nuclease does not yield a discrete particle but does yield DNA fragments whose size distribution is that expected for micrococcal nuclease cleavage sites within a nucleosome and is similar to that of DNA reconstituted with histones H3/H4 (Figure 8, 16, 25). The addition of histones H2A/H2B to the titrated extract generated a discrete pause in micrococcal nuclease digestion, so that DNA fragments accumulated with the length expected for a complete nucleosome (145−160bp) (arrow in Figure 8). Supplementation of the replicated DNA with oocyte nuclear extract generated some nucleosomal length DNA, but the addition of histones H3/H4 was without effect. It should be noted that the extent of digestion with micrococcal nuclease is such that we would normally only recover monosome size particles.

We conclude that the addition of histones H2A/H2B is limiting

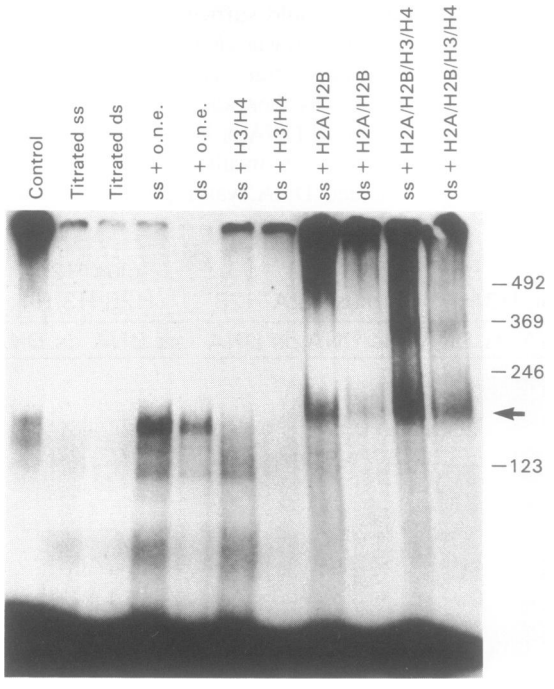

Figure 8. Micrococcal nuclease digestion of replicated and non-replicated DNA in Xenopus egg extract. This experiment utilized an protocol identical to that described in the legend to Figure 7. At the end of the incubation, after supplementation with the various components as described, the reactions were made to 3mM $CaCl_2$ and micrococcal nuclease was added (12−15 units) and digestion allowed to proceed for six minutes. At the end of this time the digestion products were deproteinized and resolved on a 4% acrylamide gel. Markers were multimers of 123bp purchased from BRL. The control reaction contained replicated single-stranded DNA assembled into chromatin without pre-titration of chromatin assembly by pUC9 (Control). (Radioactive material in the wells is probably to a failure to completely solubilize the sample).

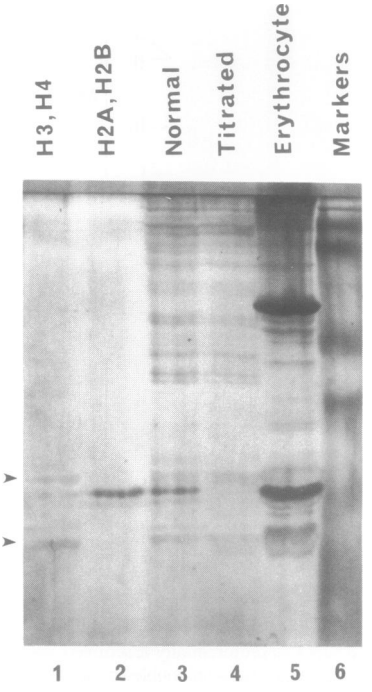

Figure 9. Histones H2A/H2B are deficient relative to histones H3/H4 on replicated DNA in Xenopus egg extracts when chromatin assembly has been titrated. This experiment used a protocol similar to that described in the legend to Figure 7 except the reaction was scaled up ten fold and double-stranded DNA bound to Sepharose was used to titrate the extract for chromatin assembly. At the end of the replication reaction, the nucleoprotein complex was resolved on a sucrose density gradient (27). Proteins present in the fraction containing DNA are resolved in lane 4 (Titrated). A control reaction using egg extract that had not been titrated is shown in lane 3 (Normal). Also shown are purified *Xenopus* histones H3/H4 (lane 1), histones H2A/H2B (lane 2), erythrocyte nuclei (lane 5) and Biorad low molecular weight markes (lane 6). The denatured proteins were resolved on an 18% SDS-polyacrylamide gel and stained using the Biorad silver-stain kit. Arrows indicate the position of purified histones H3/H4.

for the assembly of nucleosomes on DNA in the egg extract under these conditions. Therefore we can separate the chromatin assembly process into two steps. First, there is the deposition of histones H3/H4 onto DNA, (Figure 7). The second step is the binding of H2A/H2B to complete the nucleosome. This step occurs preferentially on replicated DNA.

We wished to extend these observations by determining which proteins were associated with DNA when it is replicated under conditions of chromatin titration (Figure 6 and 7). In order to accomplish this we first titrated the chromatin assembly capacity of the extract with double-stranded DNA linked to Sepharose (26). The Sepharase linked chromatin was allowed to settle to the bottom of the microfuge tube and the supernatant was removed for use is all subsequent experiments. Single-stranded DNA was then added to the extract and allowed to replicate in the presence of [α ^{32}P]dATP. This replicated DNA was then fractionated as a nucleoprotein complex on sucrose density gradients (27). The isolated nucleoprotein complex retained the capacity to be further supercoiled on addition of histones H2A/H2B (not shown, Figures 6 and 7). Resolution of the proteins associated with this DNA as it is taken off the gradient demonstrates that the nucleoprotein complex is deficient in histones H2A/H2B (Figure 9, Titrated). Nucleoprotein complexes assembled in untreated extracts show histones H2A/H2B to be normally present (Figure 9, Normal). We conclude that histones H3/H4 are present on replicating DNA in titrated extracts when histones H2A/H2B are depleted.

DISCUSSION

The major conclusion from this work is that the molecular basis for selective chromatin assembly on replicating single-stranded DNA relative to non-replicating duplex DNA (Figures 4, 5, 6 and 7) is the formation of an intermediate complex that directs the preferential association of histones H2A/H2B with the replicating template. The chromatin assembly process responsible for the addition of histones H2A/H2B to DNA, discriminates between replicating DNA and non-replicating double-stranded DNA in the *Xenopus* egg extract, such that these histones are deposited selectively on a replicated template. Replicated DNA is always more supercoiled due to chromatin assembly than non-replicated DNA in *Xenopus* egg extracts and becomes even more highly supercoiled on subsequent addition of exogenous histones H2A/H2B (Figure 7). Our conclusions agree with a suggestion of Fotedar and Roberts, (9), that a discrete intermediate is assembled on replicating DNA (Figure 8). From previous studies examining the properties of a tetramer of histone H3/H4 (16, 23, 24, 28), we suggest that this is the intermediate observed in our experiments (Figures 8 and 9).

Nucleosome assembly in *Xenopus* has been suggested to proceed in two discrete steps (6, 29). Histones H3/H4 are deposited onto DNA in a reaction catalyzed by the molecular chaperone N1/N2. Histones H2A/H2B are then added to complete the nucleosome, via interaction with a second chaperone, nucleoplasmin. Our results suggest that the process of DNA replication facilitates the deposition of histones H2A/H2B, and hence their release from nucleoplasmin onto DNA. The apparent similarity between the chromatin assembly intermediates on replicating DNA in the *Xenopus* and mammalian systems (9) implies that similar molecular mechanisms may be involved (7).

We have examined two possible explanations for the preferential assembly of replicating single-stranded DNA into chromatin. Histones do not associate more stably with single-stranded DNA in comparison to double-stranded DNA (Figure 1). Moreover they do not form detectable nucleosome-like structures on circular single-stranded DNA (Figure 2). We have no evidence for the nucleosome-like structures detected by Palter *et al.*, (20). However it is possible that in their reconstitution experiments, these nucleosome-like structures form at such low levels that we would not detect them. It is certainly clear that histones bind to single-stranded DNA (Figure 3), and that most of them do not form nucleosome-like complexes.

The second possible explanation for preferential nucleosome assembly on replicating DNA is that the small amount of free double-stranded DNA on the replicating template would facilitate the assembly process by sequestering the large excess of proteins involved in chromatin assembly that preferentially interact with double-stranded DNA (Figure 1). This explanation may be true. However, a critical concentration of DNA is necessary for replicating single-stranded DNA to generate a closed circular DNA molecule showing preferential chromatin assembly (Figure 4). It has already been reported that for other biological processes a critical mass of DNA per egg equivalent is required (see refs 30, 31). Moreover, in mixtures of low concentrations of double-stranded DNA and high concentrations of single-stranded DNA, the replicating single-stranded DNA is still preferentially supercoiled, (Figure 6). This result also demonstrates that the slow supercoiling of the double-stranded template is not due to topological problems, since in Figure 6 the input template is nicked.

Our evidence that the chromatin assembly processes necessary for the addition of histones H2A/H2B are limiting in the *Xenopus* system has parallels with the assembly of chromatin *in vivo*. During S phase, histones H3/H4 are the first to be added to nascent DNA, followed by histones H2A/H2B (1, 2). Nascent DNA is also particularly sensitive to nuclease digestion (32, reviewed in ref 33) gradually maturing to a nuclease resistant state containing arrays of nucleosomes. The results *in vivo* could be explained if the addition of histones H2A/H2B to tetramers of histones H3/H4 already assembled onto the nascent DNA is rate limiting (Figures 8 and 9). Future experiments must determine the parallels between chromatin assembly on replicating single-stranded DNA and the assembly of chromatin in the nuclei of the *Xenopus* embryo *in vivo*. It will also be of interest to determine the transcriptional properties of chromatin at various stages in assembly on replicating DNA *in vitro* (34).

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