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Liliana Krasinska, Daniel Fisher. Replication initiation complex formation in the absence of nuclear function in *Xenopus*.. Nucleic Acids Research, 2009, 37 (7), pp.2238-2248. <10.1093/nar/gkp081>. <hal-00368674>

HAL Id: hal-00368674

<https://hal.science/hal-00368674v1>

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Replication initiation complex formation in the absence of nuclear function in *Xenopus*

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Received January 12, 2009; Revised January 28, 2009; Accepted January 29, 2009

ABSTRACT

In this article, we study how intercalation-induced changes in chromatin and DNA topology affect chromosomal DNA replication using *Xenopus* egg extracts. Unexpectedly, intercalation by ethidium or doxorubicin prevents formation of a functional nucleus: although nucleosome formation occurs, DNA decondensation is arrested, membranous vesicles accumulate around DNA but do not fuse to form a nuclear membrane, active transport is abolished and lamins are found on chromatin, but do not assemble into a lamina. DNA replication is inhibited at the stage of initiation complex activation, as shown by molecular combing of DNA and by the absence of checkpoint activation. Replication of single-stranded DNA is not prevented. Surprisingly, in spite of the absence of nuclear function, DNA-replication proteins of pre-replication and initiation complexes are loaded onto chromatin. This is a general phenomenon as initiation complexes could also be seen without ethidium in membrane-depleted extracts which do not form nuclei. These results suggest that DNA or chromatin topology is required for generation of a functional nucleus, and activation, but not formation, of initiation complexes.

INTRODUCTION

Initiation of DNA replication in eukaryotic cells can be divided into steps of licensing to form pre-replication complexes (pre-RC), their cyclin-dependent kinase (Cdk)-dependent conversion to pre-initiation complexes (pre-IC) which unwind DNA, and DNA polymerase loading and elongation (1). It requires a functional nucleus to concentrate replication promoting factors (2,3). However, replication of single-stranded DNA does not involve these steps and can occur without a nuclear membrane (4) indicating that unwinding of the double helix is a rate-limiting step in DNA replication. To date, the requirements for a nucleus and for Cdk activity have not been uncoupled.

Chromosomal DNA is highly organized, forming supercoils around nucleosomes, but the consequences of this organization for replication initiation are not well understood. We hypothesized that intercalating agents, which release supercoiling, disrupt the double helix, and relax chromatin, might promote DNA unwinding, and thus specifically affect requirements for replication initiation. Alternatively, intercalation might influence binding of essential factors—ORC, for example, preferentially binds negatively supercoiled DNA (5). Understanding direct effects of intercalation-induced alterations in DNA and chromatin topology on DNA replication is important, since many cancer chemotherapeutic agents can intercalate DNA but may have multiple mechanisms of action, including inhibition of transcription and direct induction of DNA damage. The latter seems to be important for chemotherapeutic properties, since ethidium, a pure intercalator, has no therapeutic use (6).

Ethidium exerts concentration-dependent effects on DNA topology such as unwinding of the double helix and induction of positive supercoiling (7); it also induces relaxation of chromatin structure, displaces histone H1 and increases nucleosome mobility (8). It is not known how these changes affect the sequential steps of replication-competent chromatin formation, pre-RC or pre-IC assembly, initiation and elongation. Therefore, in this study, we revisited the effects of intercalation on DNA replication in a regulated model system, that of *Xenopus* egg extracts (4,9). We find evidence that formation of a functional nucleus depends on higher order chromatin structure, but loading of replication complexes does not, and occurs independently of nuclear function.

MATERIALS AND METHODS

***Xenopus* egg extract, replication assays, chromatin isolation and DNA combing**

Extracts were prepared and replication reactions performed as previously described (4,10). Where indicated, drugs at 1:100 dilution or solvent alone (DMSO for echinomycin; water for doxorubicin and ethidium bromide) were added. For membrane formation and functional experiments, DHCC at 1:10 000 or GST-NLS-GFP

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construct at 1:100 (gift from N. Morin) were added. Chromatin purification and molecular combing were performed as described (10,11). Where used to induce a checkpoint response, 50-mer poly dA-poly dT (12) nucleotides were annealed and added at 50 ng/ μ l. Recombinant GST-geminin, a gift of Malik Lutzmann and Marcel Méchali, was used as described (13).

Micrococcal nuclease digestion

Sperm nuclei (at 2800 μ l) were incubated in either interphase egg extract (IEE) or extract buffer alone (20 μ l), with or without ethidium bromide at concentrations indicated, for 20 min. CaCl_2 (3 mM final) and MNase (50 U) were added and samples incubated for 10 min., diluted in 100 μ l Proteinase K (500 μ g/ml)/20 mM Tris pH 8.0/30 mM EDTA/1% SDS, and incubated at 37°C for 1 h. DNA was purified by phenol-chloroform extraction and ethanol precipitation. DNA was electrophoresed on a 10% polyacrylamide gel and stained with SYBR-Gold.

Immunofluorescence

Immunofluorescence was performed as described (10) using AlexaFluor secondary antibodies according to manufacturer's instructions (Molecular Probes). For DHCC and NLS-GFP analysis, images were deconvoluted (Huygens, Science Volume Imaging).

Antibodies

Antibodies used were: polyclonals: XcyclinE (gift from C. Bonne-Andrea); XCdc45 (gift from H. Takisawa); RCC1, XLamin B3 (gift from N. Morin); histone H3 and phospho-histone H2A.X (Cell Signaling); phospho MCM2 (S108) (Bethyl Laboratories); phospho ATM/R substrate (Cell Signaling); geminin, Cdt1, Cdc6, RPA, (gifts from Marcel Méchali); monoclonals: MCM7, PCNA, pol ϵ (Labvision); PSTAIR (Sigma); phospho-ATM (S1981) (Rockland).

RESULTS

High concentrations of ethidium inhibit initiation of DNA replication

Using interphase egg extracts, we analysed the effects of ethidium, and doxorubicin, a cancer chemotherapy agent which intercalates DNA, on replication of sperm nuclei chromosomal DNA. To correlate effects with their intercalating properties, we designed an intercalation assay based on displacement of SYBR-Green from DNA (Supplementary Data). In this assay, doxorubicin showed similar intercalation to ethidium (Supplementary Figure S1). We reproducibly found that 100 μ M ethidium blocks DNA replication, whereas concentrations of 50–75 μ M, surprisingly, only slowed DNA replication, dose-dependently (Figure 1A), and 25 μ M even had a slight stimulatory effect, perhaps due to promotion of DNA unwinding. In a previous study (14), it was found that 50 μ M ethidium had little or no effect on replication in egg extracts. We find that the dose-dependence of ethidium is affected by RNA present in extracts, as somewhat

lower concentrations of ethidium will block replication if extracts are pre-treated with RNase A to prevent RNA from sequestering ethidium (Supplementary Figure S2A), yet 10 μ M ethidium still stimulates replication rate in RNase-treated extracts (Supplementary Figure S2B). Thus, slight differences between extracts at 50 μ M ethidium are probably due to competition between stimulation due to unwinding and inhibition, the balance of which depends on titration of ethidium by variable amounts of RNA in the preparations.

Cdk activity has been found to be required up until the point where DNA is unwound at the replication origin, and Cdk activity is not required for replication of single-stranded DNA. Because 25 μ M ethidium does not inhibit replication, but probably promotes at least a degree of unwinding, we wished to see whether or not it could bypass the requirement for Cdk activity. This is not the case since Cdk2 inhibition by Nu6102 slows replication even in the presence of 25 μ M ethidium (Supplementary Figure S3), or indeed any concentration of ethidium (data not shown), and consequently still reduces chromatin loading of replication initiation complexes in the presence of ethidium (Supplementary Figure S3).

At 100 μ M, doxorubicin blocked DNA replication (Figure 1A), whereas a weaker intercalator, fascaplysin (which is also a Cdk4 inhibitor) only reduced DNA replication rate (Supplementary Figures S1 and S4). The chemotherapy agents cisplatin and oxaliplatin do not displace SYBR-Green in our assay, and at 100 μ M only have a minor effect on DNA replication in egg extracts (Supplementary Figures S1 and S4).

We confirmed that high ethidium concentrations inhibit replication by analysing biotin-dUTP incorporation into replicating DNA by immunofluorescence (Figure 1B). At 45 mins, whereas control nuclei stained strongly for biotin-dUTP, no incorporation was seen in 100 μ M ethidium-treated extracts. Interestingly, DNA did not fully decondense in the presence of 100 μ M ethidium, the chromatin losing its initial corkscrew shape but remaining elongated (see below). Nevertheless, decondensation is not required for DNA replication in *Xenopus* egg extracts (15).

Although ethidium can inhibit polymerase- α *in vitro* (16,17), we surmised that it may also affect prior steps of replication. Addition of 100 μ M ethidium slowed, but did not abolish, replication of M13 single-stranded DNA in extracts (Figure 1C), suggesting that it can indeed hinder progression of the polymerase complex, but, in conditions where origin activation is not required, it does not abrogate DNA replication. Next, we added 100 μ M ethidium only after 60 min of incubation of DNA with extracts, to allow prior assembly of pre-RC complexes (see also Figure 5). DNA replication was still strongly inhibited (Figure 1D), suggesting that ethidium still acts after pre-RC have formed. To test whether or not replication initiates, we analysed replication of single DNA molecules by 'molecular combing' (11). This allows visualization of replication origin firing at high resolution. Whereas in the control extract DNA replication initiated and progressed, as judged by prolonged stretches of Br-dUTP incorporation on DNA fibres, we observed no Br-dUTP

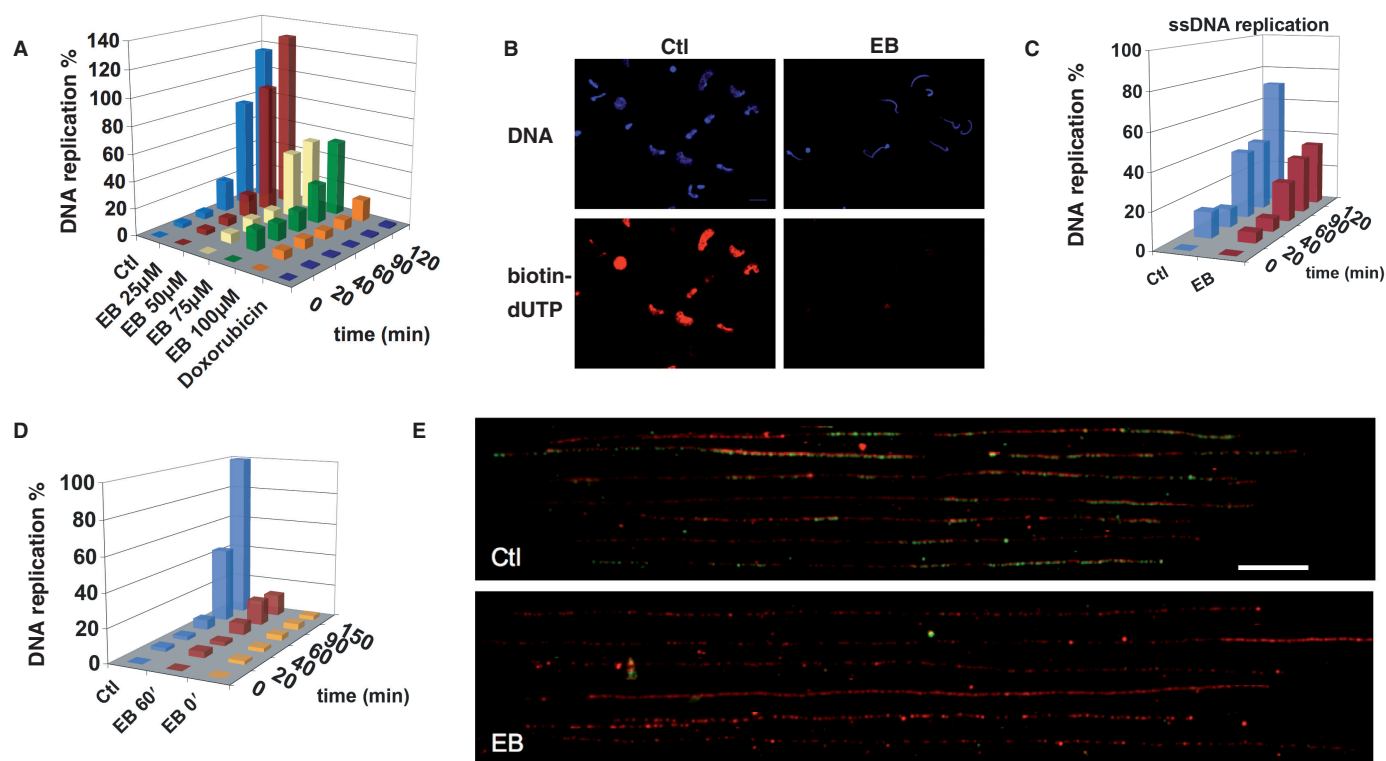


Figure 1. Intercalation inhibits initiation of chromosomal DNA replication. (A) Replication time-courses in the control extract or in the presence of ethidium bromide or doxorubicin at indicated concentrations. (B) Replication was assessed by immunofluorescence of incorporated biotin-dUTP in the absence (Ctl) or presence of 100 μ M ethidium (EB), the 45 min time-point is shown. (C) Replication time-course of ssDNA (M13 bacteriophage) in the absence or presence of 100 μ M ethidium bromide. (D) Replication time-course of dsDNA in the absence or presence of 100 μ M ethidium, added either at the beginning (EB 0') or at 60 min. (EB 60'). (E) Sperm nuclei were incubated in the presence of Br-dUTP without (Ctl) or with 100 μ M ethidium bromide (EB). At 75 min DNA was combed and single fibre analysis was performed. Images represent examples of DNA fibres; ssDNA in red, Br-dUTP in green, bar 10 μ m.

signal in the 100 μ M ethidium-treated sample (Figure 1E), corresponding to the complete inhibition of replication observed in the replication assay (Figure 1A, B and D). This result confirms that ethidium prevents initiation of DNA replication. To confirm that the effect is due to its interaction with DNA, we pre-treated sperm chromatin with ethidium and then re-isolated the chromatin by centrifugation through a sucrose cushion to remove ethidium. In these experiments, chromatin pre-treated with ethidium is delayed in replication, although replication is not entirely prevented, probably because the equilibrium between DNA-bound and free (or RNA-bound) ethidium will change upon introduction into the egg extract (Supplementary Figure S5).

We then asked whether ethidium leads to checkpoint activation. In *Xenopus* egg extracts, uncoupling MCM-dependent DNA unwinding from polymerase activity activates an intra-S phase, caffeine-sensitive checkpoint (18). If ethidium acts only by slowing replication fork progression, this should trigger the checkpoint response, inhibiting further origin firing. As a control we used a low concentration of aphidicolin, which slows replication forks and thus triggers checkpoint activation but does not prevent polymerase activity.

Aphidicolin reduced the replication rate by 75%, and this was rescued by the addition of caffeine (Figure 2A). As expected, caffeine also slightly stimulated replication

without aphidicolin, since there is a basal level of checkpoint activation in the absence of DNA damage (19–21), which is probably required to prevent onset of mitosis while replication is ongoing. One hundred micromolar ethidium abolished replication, but caffeine did not overcome this block, suggesting that ethidium does not inhibit replication by provoking a checkpoint response (Figure 2A). We verified this notion by analyzing different checkpoint response markers. MCM2 phosphorylation on serine-92 [equivalent to serine-108 in human MCM2 and recognized by a monoclonal anti-phosphoserine-108 (22)] occurs in response to replication blocks or DNA damage in *Xenopus* egg extracts (23). Although present during normal DNA replication due to basal checkpoint activity (see also Supplementary Figure S6), MCM2 phosphorylation was stimulated by induction of the checkpoint by damaging DNA with bleomycin or mimicking double-strand breaks with poly-dA/poly-dT oligonucleotides (22), as well as by aphidicolin (Figure 2B). In the latter case, this increase was suppressed by caffeine (Figure 2B, lanes Aphi and A + C, respectively). However, even at these high nuclear concentrations (5600 μ M), at which extracts more readily undergo checkpoint responses, ethidium did not stimulate MCM2 phosphorylation (Figure 2B, lane EB). Similarly, two other checkpoint markers: ATM phosphorylation at a residue equivalent to serine 1981 (22), and phosphorylation of ATM/ATR

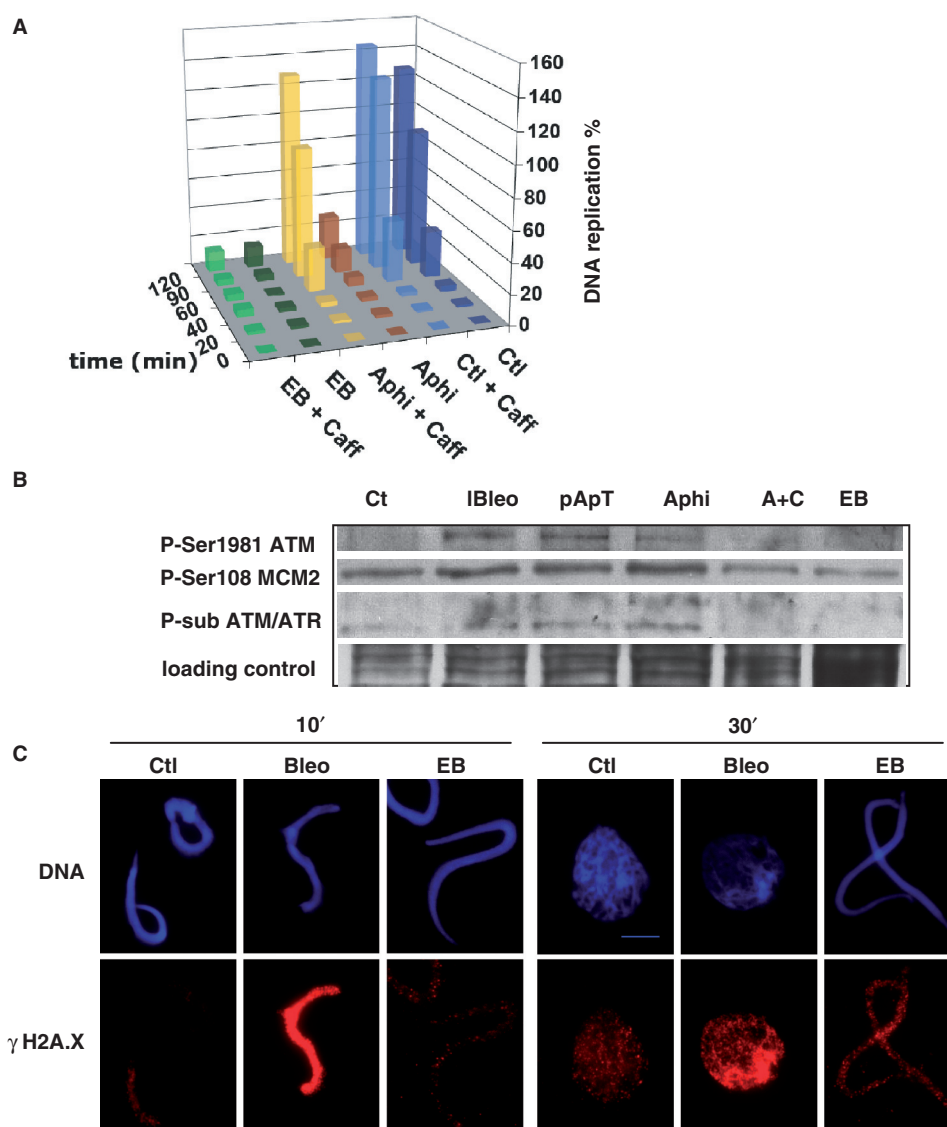


Figure 2. Ethidium does not activate the intra-S phase checkpoint. (A) Replication time-courses in the control extract (Ctl) or in the presence of 100 μ M ethidium bromide (EB), 2 μ M aphidicolin (Aphi), with or without 5 mM caffeine (Caff). (B) Western blots of nuclei purified at 60 min from control extracts (Ctl), or extracts containing 0.5 μ M bleomycin (Bleo), 50 ng/ μ l double-stranded poly-dA/poly-dT oligonucleotides (pApT), 100 μ M aphidicolin (Aphi), 100 μ M aphidicolin and 5 mM caffeine (A + C), or 100 μ M ethidium bromide (EB). Non-specific bands are shown as loading control. (C) Analysis of H2A.X phosphorylation in 0.5 μ M bleomycin (Bleo) or 100 μ M ethidium bromide (EB) treated extract by immunofluorescence, at indicated time-points (bar, 10 μ m).

consensus sites, although induced by triggering or mimicking DNA damage and by aphidicolin, in a caffeine sensitive manner, were not affected by ethidium (Figure 2B). Finally, ethidium did not lead to accumulation of phosphorylated H2AX on chromatin, in contrast to bleomycin (Figure 2C: representative nuclei are shown). Taken together, we conclude that ethidium does not induce DNA damage nor does it disturb the unwinding/elongation equilibrium, and thus does not activate the intra-S-phase checkpoint.

Intercalation disrupts nuclear envelope and lamina assembly

Sperm chromatin decondensation in egg extracts occurs in two stages: a rapid phase, in which histones are loaded (24), and a slower, nuclear membrane-dependent

phase (25). One hundred micromolar ethidium did not prevent the first phase of DNA decondensation; however, chromatin remained elongated even at 90 min (Figure 3A and B). One hundred percent of nuclei showed this phenotype. To confirm that nucleosome formation takes place, we used micrococcal nuclease (MNase) to digest linker DNA. Without incubation in the extract, DNA was completely degraded by MNase, whereas after incubating nuclei in extract with up to 25 μ M ethidium, a typical nucleosome pattern could be seen (Figure 3C). With increasing ethidium concentration, the pattern became more diffuse, as expected (8). We conclude that nucleosomes can form at low ethidium concentrations, but when saturating, ethidium disrupts DNA supercoiling around the core particle.

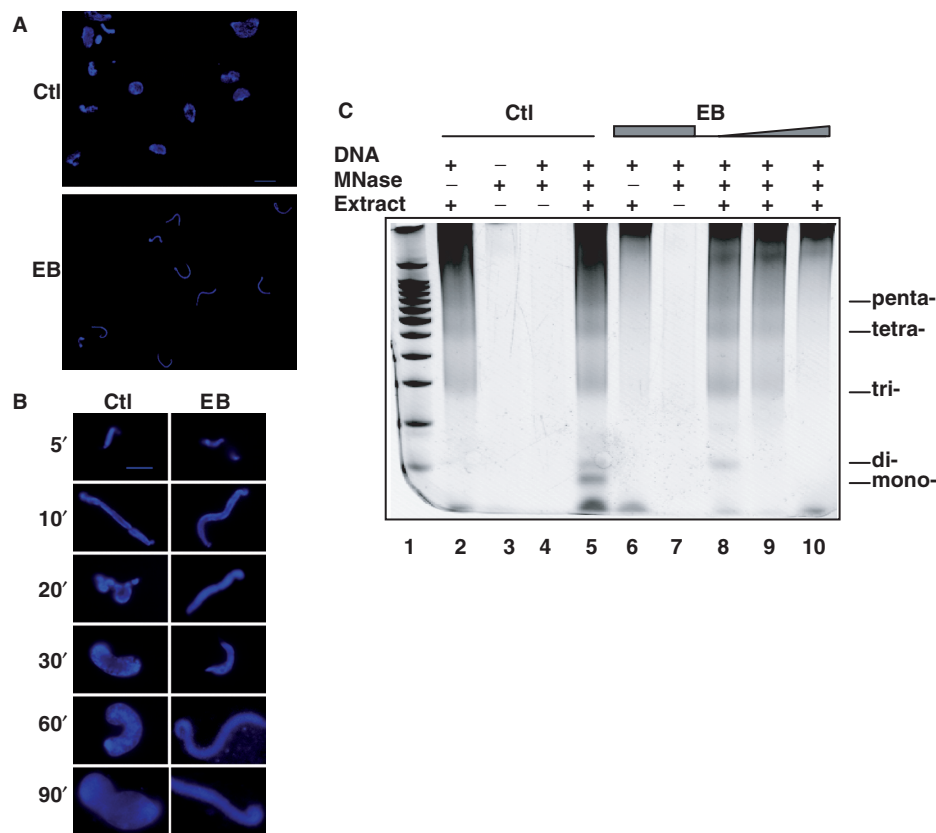


Figure 3. Ethidium inhibits the second phase of chromatin decondensation. **(A)** Sperm nuclei were incubated for 60 min in control (Ctl) or 100 μ M ethidium (EB) treated extracts, and decondensation was monitored by staining DNA with Hoechst 33258. **(B)** Sperm decondensation in the absence (Ctl) or presence of 100 μ M ethidium (EB) was monitored by incubating sperm nuclei in IEE, isolating them and staining directly with Hoechst 33258, at indicated time-points. **(C)** Sperm nuclei (or without DNA, lane 3) were incubated in buffer alone (lanes 4 and 7) or in interphase egg extract, in the absence (lanes 2, 3, 4 and 5) or in presence of EB, at 100 μ M (lanes 6, 7 and 10), 50 μ M (lane 9) or 25 μ M (lane 8), without (lanes 2 and 6) or with micrococcal nuclease, for 10 min. Nucleosome assembly was monitored by gel electrophoresis; lane 1:100 bp DNA ladder.

As the second stage of DNA decondensation did not occur, we investigated assembly and function of the nuclear envelope. By 90 min, control nuclei were surrounded by a continuous and functional membrane, as judged by accumulation of nuclear localization signal (NLS) coupled to green fluorescent protein (GFP) (Figure 4A). In contrast, ethidium dose-dependently inhibited nuclear membrane formation, which was blocked at 100 μ M (Figure 4A and data not shown). Membranous vesicles were distinguishable around DNA by 10 min (not shown), but 100 μ M ethidium inhibited their fusion, even at late time-points (Figure 4A, enlarged). NLS-GFP accumulation was also blocked. Ethidium had no effect on membrane structure or function if added after nuclear membrane formation (Supplementary Figure S7). Thus, it almost certainly disrupts nuclear envelope assembly through the effect it exerts on DNA. As similar results were previously found with the intercalating agent daunomycin (26), we asked whether other intercalating agents reproduce this phenotype. We thus examined the effects of echinomycin, a *bis*-intercalating quinoxaline which removes negative supercoils (27), but does not displace SYBR-Green from DNA in our assay (Supplementary Figure 1), and

doxorubicin. Echinomycin inhibited DNA replication in a dose dependent manner (Figure 4B) and DNA decondensation, as previously shown (28). However, a transport-competent nuclear envelope could form (Figure 4C). On the other hand, the phenotype induced by doxorubicin resembled that of ethidium; the drug inhibited DNA replication, DNA did not fully decondense, and no functional membrane was formed (Figures 1A and 4C). Since ethidium and doxorubicin have similar effects on DNA topology despite their different structures (29), we infer that formation of a functional nucleus depends on a particular DNA or chromatin topology.

Lamins are implicated in both nuclear envelope formation and DNA replication (30), and they associate with chromatin early in the process of nuclear formation, providing a 'bridge' for binding and fusion of membranous vesicles. Establishment of active nuclear transport then allows intranuclear concentration of lamins, which form a filamentous network, the lamina. In the presence of 100 μ M ethidium, we observed inhibition of lamina assembly, as deduced from visualization of lamin B3. At 100 μ M ethidium, instead of the typical rim of lamina staining surrounding the chromatin, lamins remained in a

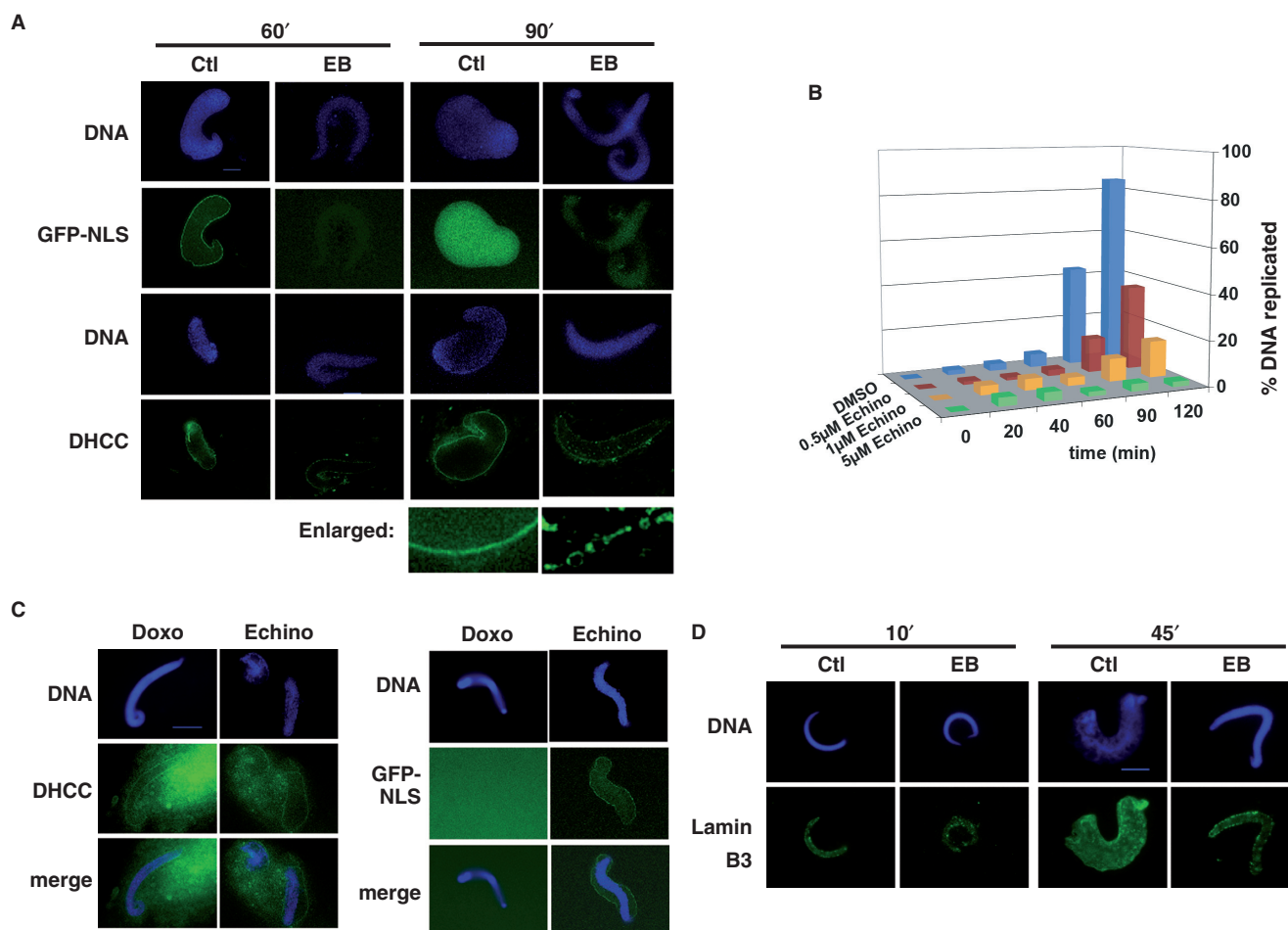


Figure 4. Intercalation disrupts nuclear membrane and lamina assembly. (A) Sperm nuclei were incubated in IEE with or without 100 μ M ethidium bromide, in the presence of the lipid die DHCC, to monitor nuclear membrane assembly, or NLS-GFP, to assess its competence for active transport. Enlargements of nuclear membrane are shown. (B) Replication time-courses of extract treated with increasing concentrations of echinomycin. (C) Nuclear membrane formation and function in the presence of 5 μ M echinomycin (Echino) or 100 μ M doxorubicin (Doxo) was monitored at 90 min by immunofluorescence, by staining with DHCC, and the ability of nuclei to concentrate NLS-GFP. (D) Assessment of lamina formation by immunofluorescence staining with antibodies against Lamin B3 in nuclei incubated in control (Ctl) or 100 μ M ethidium-treated extract (EB), at indicated time-points. Bars, 10 μ m. In this figure, for clarity, only individual nuclei are shown, but all nuclei showed this phenotype.

punctuate pattern (Figure 4D). Thus, lamins can associate with chromatin, but do not form a lamina. Lower concentrations of ethidium did not interfere with lamina formation (Supplementary Figure S8). There is therefore a strong correlation between the ethidium concentration which blocks DNA replication and that which blocks formation of both a functional nuclear membrane and lamina.

Pre-RC and initiation complexes are present on chromatin despite the absence of a nuclear membrane and lamina

The nuclear envelope is a prerequisite for DNA replication (2), but is not required for loading of the MCM putative helicase complex (31). Given that 100 μ M ethidium prevented assembly of the nuclear envelope and lamina, we expected it to prevent pre-IC formation; we also asked whether it would affect pre-RC formation. As such, we analysed components of pre-RCs and initiation complexes by immunofluorescence. The pattern of staining of MCM7 was unaffected by 100 μ M ethidium (Figure 5A),

suggesting that pre-RC formation does not depend on higher order chromatin structure. However, to our surprise, at 100 μ M ethidium, although DNA replication does not take place, as confirmed by staining for biotin-dUTP incorporation, PCNA, RPA and polymerase ϵ were present on the chromatin, polymerase ϵ foci only reduced in number. We verified that the staining of these factors is specific as it was abrogated by the addition of recombinant geminin, which prevents MCM-loading and subsequent formation of initiation complexes. We confirmed these results by western blotting of chromatin fractions. All factors analysed, of both the pre-RC (MCMs, Cdc6, Cdt1 and Geminin), pre-IC (RPA, Cdc45 and Cut5) and initiation factors (PCNA) were loaded onto chromatin in the presence of 100 μ M ethidium (Figure 5B). We estimated levels of these proteins by densitometry scanning of western blots from independent experiments (Table 1). The results suggest that most replication proteins are at most only slightly reduced in quantity on chromatin, whereas some even appear to be more

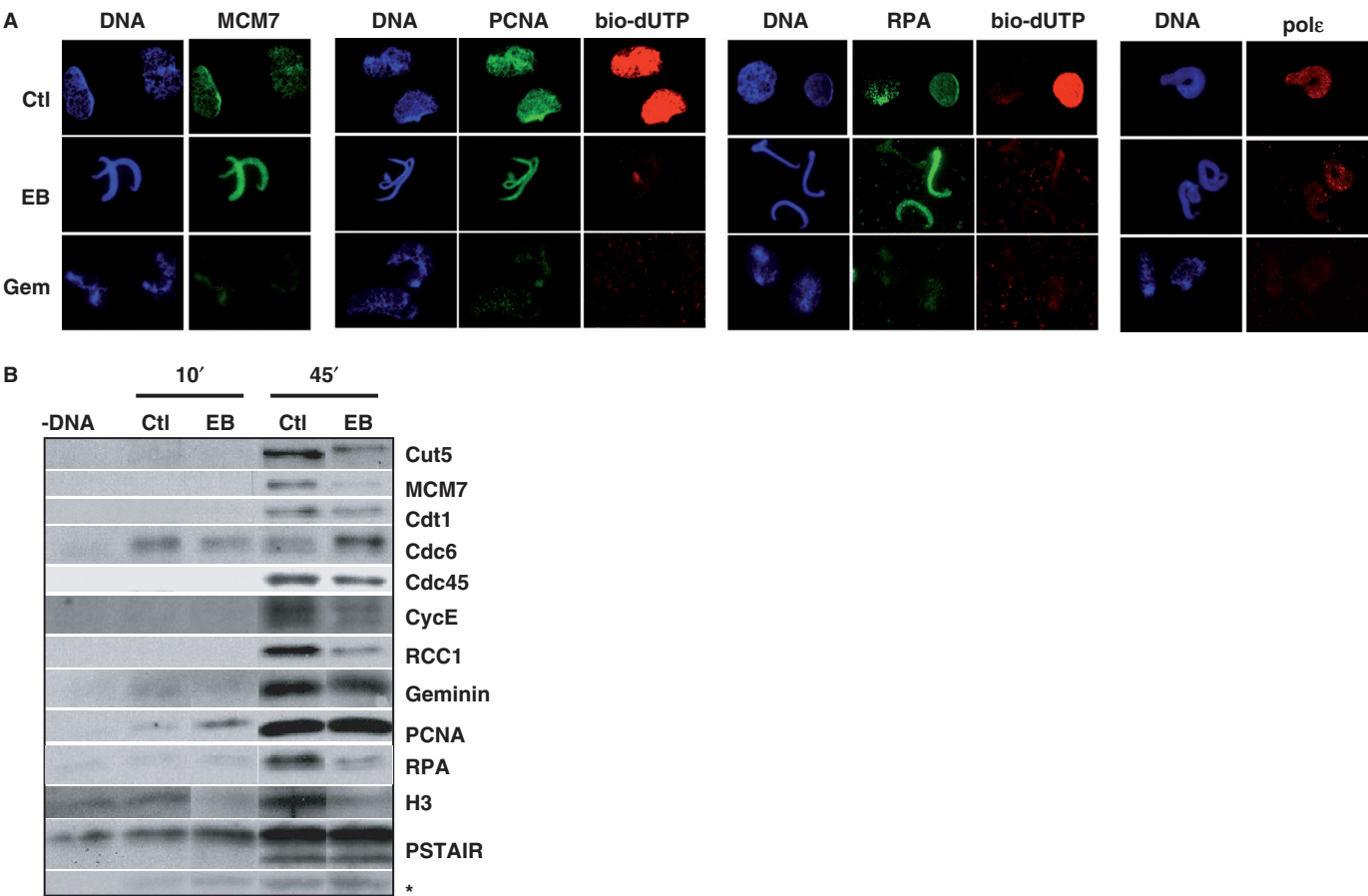


Figure 5. Pre-RC and initiation complexes are present on the chromatin, despite the absence of nuclear membrane and lamina, but replication does not initiate. **(A)** Assessment by immunofluorescence of the presence of pre-RC (MCM7) and initiation (PCNA, RPA and polymerase ϵ) complex proteins on the chromatin at 60 min in control (Ctl) extracts or extracts with 100 μ M ethidium bromide (EB) or 100 nM recombinant geminin (Gem). DNA replication was monitored by biotin-dUTP incorporation (bio-dUTP). **(B)** Chromatin was purified from replicating nuclei incubated in the absence (Ctl) or presence of 100 μ M ethidium (EB) at indicated time-points, and blotted with antibodies against proteins of the pre-RC, pre-IC, Cdk1/2 (PSTAIR, which recognizes Cdk1, upper band, and Cdk2, lower band), Cyclin E, RCC1 and histone H3; *bottom band, loading control (non-specific chromatin protein band recognized by Cdc6 antibody). For clarity, lanes are assembled electronically but all are from a single exposure of one gel.

Table 1. Estimation of replication factors loading onto chromatin in the presence of ethidium by densitometry quantitation of western blots

	MCM5	MCM7	Cdt1	Cdc6	RPA	PCNA	Cut5	Cdc45	H3	RCC1
Percentage loaded + EB	177	92	177	146	66	91	100	91	25	61

Results are expressed as percent abundance on chromatin in 100 μ M ethidium-treated extracts compared to control extracts. Western blots from 1 (H3, MCM5) or average from two (MCM7, RCC1, Cdt1, Cdc6, Cut5), three (Cdc45, RPA) or four (PCNA) independent experiments.

abundant in the presence of ethidium compared to controls. However, some variability might be due to variable loss during chromatin isolation. Taken together, we conclude that the nuclear membrane is not required for loading of these factors on chromatin *per se*, but may be necessary to provide their proper abundance or stability on chromatin, and possibly for their activation. Histone H3, albeit reduced, was also present, confirming that nucleosome formation occurs. Since nuclear envelope formation requires Ran GTPase-dependent binding and fusion of membranous vesicles (32), we also analysed the abundance on chromatin of the guanine nucleotide

exchange factor RCC1. RCC1, though present, was substantially decreased by 100 μ M ethidium (Figure 5B, Table 1). Interestingly, there were differences in the electrophoretic mobility of Cdc6 and Cut5 proteins in the presence of ethidium, implying that post-translational modifications are altered. This phenotype is not due to insufficient Cdk activity, since Cdk1, Cdk2 and cyclin E were present on chromatin, loading of pre-IC proteins, which is Cdk-dependent (33) still occurs, and adding excess recombinant Cdks had no effect on DNA replication in the presence of ethidium (data not shown). Furthermore, we have recently shown that, in *Xenopus*

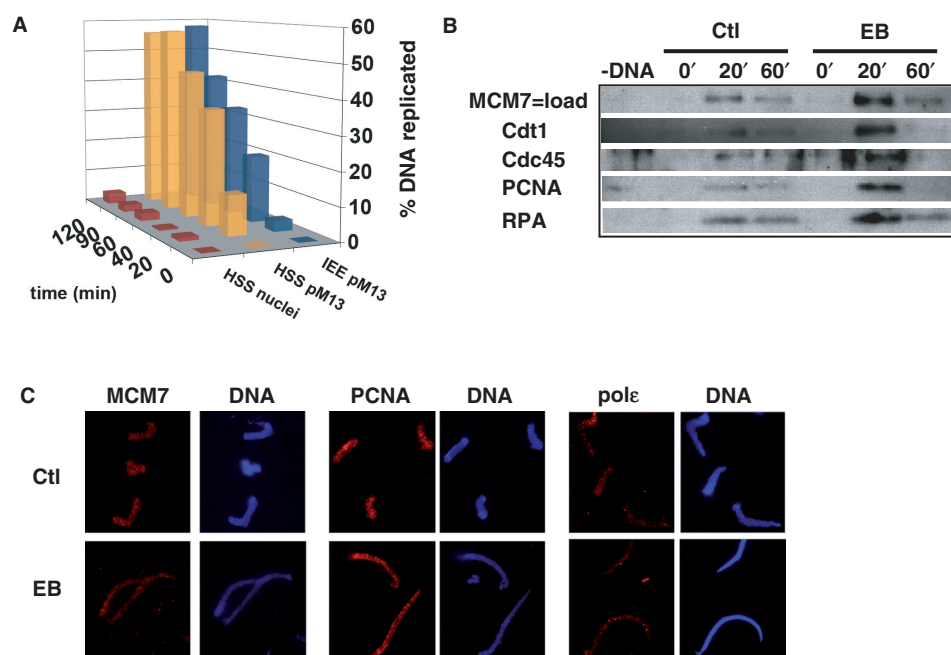


Figure 6. A nucleus is not required for pre-IC formation on chromatin. (A) Replication time-courses of single-stranded M13 DNA (pM13) or demembrated sperm nuclei (nuclei) in low speed interphase egg extracts (IEE) or high speed supernatants (HSS). (B) Chromatin was purified from control HSS (Ctl) or HSS containing 100 μ M ethidium (EB) after incubation with demembrated sperm nuclei for the indicated times, or without DNA (-DNA) and western blotted against the indicated proteins. In the 20 min time-point, more material was recovered as shown by increased MCM7 signal, but the ratio of pre-IC and initiation factors to pre-RC components remained the same. (C) Assessment by immunofluorescence of the presence of pre-RC (MCM7) and initiation (PCNA and polymerase ϵ) complex proteins on the chromatin at 60 min in control (Ctl) HSS extracts or HSS extracts with 100 μ M ethidium bromide (EB).

egg extracts, only very low Cdk activity is required to activate replication origins (10), implying that its concentration in the nucleus should not be required. Thus, despite the lack of nuclear membrane, pre-IC formation takes place, yet activation of this complex appears not to occur, as shown by the inhibition of DNA replication and lack of checkpoint response.

For replication complex formation, it is possible that a nucleus might be essential in normal experimental conditions, but becomes dispensable if DNA is unwound by ethidium. To test this hypothesis, we prevented nucleus formation by centrifuging egg extracts at high speed (about 200 000 $\times g$) to eliminate membrane components, before incubating with DNA. These high-speed supernatants cannot replicate chromosomal DNA but can replicate single-stranded (M13) DNA as efficiently as normal low speed extracts, as expected (Figure 6A). Efficient MCM loading onto chromatin does not require nuclear formation (31,33) which we confirm (Figure 6B) and thus is also useful as a loading control for recovery of chromatin. DNA does not decondense to form a nucleus, as expected (Figure 6C), although, interestingly, was more elongated in the presence of ethidium, presumably due to DNA unwinding. Nevertheless, replication-initiation complexes formed on chromatin in both the absence and presence of ethidium, as determined by western blotting of purified chromatin for Cdc45, PCNA and RPA (Figure 6B), and immunofluorescence for PCNA and polymerase ϵ (Figure 6C). Ethidium treatment did not increase the loading of initiation complexes with respect

to pre-replication complexes—if anything, in fact, it reduced the signal of initiation complex components at the 60 min timepoint (Figure 6B). We conclude that ethidium does not act to artificially promote formation of initiation complexes in the absence of nuclear function, and that membrane formation is not required *per se* to load components of the pre-initiation complex, but is necessary for their activation.

DISCUSSION

DNA replication in *Xenopus* egg extracts requires an intact nuclear membrane, whose role might be to concentrate replication-promoting factors, to exclude cytoplasmic inhibitors, or to organize replication ‘factories’ into a higher-order structure. Whether or not the nucleus plays a structural role is still debated, since the nuclear lamina is required for DNA replication (34), yet elimination of lamin B does not prevent efficient DNA replication if DNA is exposed to nucleoplasmic extracts which provide sufficient concentration of replication promoting factors in the absence of a nucleus (3). The accepted model is therefore that the role of the nucleus is to concentrate replication-promoting factors. One such factor might be Cdk2. Cdk2-cyclin E is the main Cdk2 complex in *Xenopus* egg extracts (10,35), cyclin E is actively imported and concentrated in the nucleus (36), and NLS-deficient cyclin E cannot restore replication efficiency of cyclin-E depleted extracts. Equally, nucleoplasmic extracts depleted of cyclin E or of Cdk2 do not support efficient

DNA replication (37). However, we recently found that although cyclin E and Cdk2 are important for efficient DNA replication, they are not indispensable since cyclin A-Cdk1 can and does promote DNA replication, even in the presence of Cdk2 (10). Cdk activity is in excess of that required for efficient DNA replication, as removal of most of Cdk2 and Cdk1 does not greatly affect replication rate, even though there are many fewer origins of replication activated. The reason appears to be that replication origins are grouped into clusters, and it is the frequency of clusters of replication origins which is determining for replication efficiency; cyclin E and Cdk2 more strongly affect the frequency of activation of clusters (10). Interestingly, cyclin A does not need to be imported into the nucleus in order to restore DNA replication inhibited by cyclin E depletion (36). In combination with the results shown here, this suggests that cytoplasmic cyclin A-Cdk1 complexes promote formation of replication-initiation complexes. At a molecular level, the only role to date found for Cdk activity is to promote the conversion of pre-replication complexes into pre-initiation complexes; indeed, in yeast, Cdk activity can be genetically bypassed by artificially promoting pre-IC complex formation (38,39). Cdc45 is then required for polymerase loading and DNA unwinding (1,40,41). Aphidicolin, which blocks DNA polymerase alpha activity, arrests DNA replication in extracts at a point at which Cdk activity is no longer required (42). It therefore appears that Cdk activity is required for forming and/or activating a helicase complex, and once accomplished, replication can proceed in the absence of Cdk activity. Moreover, single-stranded DNA replicates efficiently in the absence of a nucleus (4) confirming that the requirement for a nucleus is to separate the two strands of the double helix, independently of the mechanism involved. We find that, in the presence of ethidium, inhibition of Cdk2 reduces loading of pre-IC components onto chromatin, i.e. Cdk activity is still required. Although Cdk-dependent loading of pre-IC components occurs in the absence of a nucleus, these complexes do not initiate DNA replication, as shown by the absence of nucleotide incorporation. It remains possible that nuclear concentration of Cdk complexes might be required for activation of pre-formed replication initiation complexes although to date there is no known function for Cdk activity downstream of pre-IC formation, and by adding excess Cdk activity we do not rescue the replication block due to ethidium. We thus hypothesize that there is a nuclear requirement for activating initiation complexes. We also infer that the unwinding of the double helix due to intercalation is not equivalent to unwinding due to action of the pre-IC encoded replicative helicase: supercoils are removed but DNA does not become single-stranded. An alternative explanation, that the topology of ethidium-intercalated DNA directly prevents initiation, is unlikely, given that ethidium does not prevent replication of single-stranded DNA and that high-speed membrane-free extracts can load initiation factors onto the chromatin in the absence of ethidium, but cannot replicate DNA.

In spite of the fact that replication factors of both pre-IC and initiation complexes can be loaded onto

chromatin in the absence of a nucleus, there are probably quantitative differences in loading between different proteins. For example, we find that PCNA is present to essentially similar levels as in control samples, whereas RPA is reproducibly reduced in abundance on chromatin. One possibility is that since PCNA is a factor required for polymerase switching, whereas RPA is a single-stranded-binding protein which increases on chromatin during replication as more forks are activated, ethidium (or the consequent lack of nuclear function) blocks somewhere between these two points. Other more simple hypotheses are possible: PCNA, for example, forms a ring around double-stranded DNA whereas RPA binds to single-stranded DNA and might therefore be more strongly competed by ethidium (which can interact electrostatically with single-stranded DNA).

Our results unexpectedly suggest that DNA topology itself is important in determining nuclear structure and function. By modifying DNA topology using ethidium, which is not known to induce any chemical modifications of DNA nor inhibit any chromatin-modifying enzymes, we impede a sequence of events normally leading to the envelope and lamina assembly and subsequently allowing an intranuclear environment permissive for DNA replication. The inhibition of membrane formation, consequent to DNA intercalation, might be due to interference with the Ran GTPase pathway, as we show a reduced loading of the RCC1 factor onto the chromatin. Ran GTPase is required for binding and fusion of membranous vesicles in the process of envelope formation (43), along with lamins, which, containing DNA and vesicle-binding domains, provide a 'bridge' between the chromatin and the vesicles (30,34,43). It may be that if the GTP-gradient is below a necessary threshold, a quantitative phenotype (reduced RCC1 loading) is translated into a qualitative phenotype (absence of membrane fusion). Alternatively, since RCC1 docks to the chromatin via histones H2A/H2B, and this interaction stimulates its activity (44), distorted chromatin/nucleosome structure due to intercalation might not only be responsible for the observed defect in RCC1 loading but possibly also its activity. Equally, although lamins are present at the periphery of the DNA, their binding and interactions with chromatin or vesicles might be modified, either at the qualitative or quantitative level, thus hindering subsequent steps in nuclear membrane formation.

In conclusion, our study suggests that intercalating agents, including doxorubicin, a compound currently used in cancer treatment, can prevent initiation of DNA replication by affecting nuclear structure, which might contribute to their pharmacological effects.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

Thanks to Vincent Coulon and the DNA combing platform, and Nathalie Morin, Cathy Bonne-Andrea, Haruhiko Takisawa and Marcel Méchali for reagents (see 'Methods' section).

FUNDING

Institut National pour la Santé et la Recherche Médicale (Inserm Avenir programme grant to D.F. and CDD to L.K.). Funding for open access charge: Inserm Avenir grant.

Conflict of interest statement. None declared.

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