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Mitotic Remodeling of the Replicon and Chromosome Structure

Jean-Marc Lemaitre,[1] Etienne Danis,[1] Philippe Pasero,[2] Yegor Vassetzky,[1] and Marcel Méchali[1,*]
[1]Genome Dynamics and Development
[2]Maintenance of Genome Integrity during DNA Replication
Institute of Human Genetics, CNRS, 141 rue de la Cardonille, 34396 Montpellier, France
*Contact: mechali@igh.cnrs.fr
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SUMMARY

Animal cloning by nuclear-transfer experiments frequently fails due to the inability of transplanted nuclei to support normal embryonic development. We show here that the formation of mitotic chromosomes in the egg context is crucial for adapting differentiated nuclei for early development. Differentiated erythrocyte nuclei replicate inefficiently in *Xenopus* eggs but do so as rapidly as sperm nuclei if a prior single mitosis is permitted. This mitotic remodeling involves a topoisomerase II-dependent shortening of chromatin loop domains and an increased recruitment of replication initiation factors onto chromatin, leading to a short interorigin spacing characteristic of early developmental stages. It also occurs within each early embryonic cell cycle and dominantly regulates initiation of DNA replication for the subsequent S phase. These results indicate that mitotic conditioning is crucial to reset the chromatin structure of differentiated adult donor cells for embryonic DNA replication and suggest that it is an important step in nuclear cloning.

INTRODUCTION

Nuclear transfer is a powerful method that can be used to produce cloned animals and to obtain new sources of multipotential cells from differentiated tissues. By transplanting nuclei from differentiated amphibian or mammalian cells into enucleated eggs, blastula or blastocyst embryos can be obtained, which can develop into entire animals or be used to form a wide range of tissues and cell types (Gurdon et al., 2003). The potential ability to deliver supplies of multipotential cells, which hold great promise for cell-based therapies for numerous disorders, makes nuclear transfer an appealing alternative to the difficult practice of directly isolating natural stem cells from normal adult tissues (McKay, 2000).

Despite its many advantages, however, nuclear transplantation is often inefficient due to the difficulty involved in completely reprogramming differentiated adult nuclei for the events of early development. Indeed, it is known that the ability of the egg to reset the epigenetic marks of adult donor cells is essential for the efficiency of nuclear cloning. Identifying the specific epigenetic properties of differentiated cell nuclei that must be reset before development can begin anew, and how such resetting can be efficiently achieved, thus represents a challenge of major biological and medical significance.

Various methods have been identified that can enhance the efficiency of nuclear transplantation. In amphibians, for example, cloning efficiency is substantially improved by serial nuclear transfers. This consists of transferring a nucleus from a differentiated donor cell to an enucleated egg, allowing the cell to undergo several divisions, and then using the daughter nuclei as donors for a second nuclear-transfer experiment (Gurdon, 1962). Injections of nuclei into maturing oocytes instead of eggs (DiBerardino and Hoffner, 1983) led to the hypothesis that components of maturing oocytes may enable the injected nucleus to respond to DNA synthesis-inducing factors in activated eggs (Leonard et al., 1982).

One possible factor contributing to the low efficiency of cloning experiments is that the chromosome organization of differentiated adult nuclei may not be well adapted for DNA replication. DNA replication occurs at several hundred foci within the nuclei of proliferating cells, with origins that appear to be synchronously set up prior to entry into S phase (Jackson, 1990) These foci are stable throughout S phase and can persist across successive divisions (see Berezney et al., 2000 for review). When most chromatin is removed by high salt extraction or LIS detergent, replication foci and several DNA replication proteins remain in a residual nuclear structure (Berezney et al., 1995; Hozak et al., 1993; Nakayasu and Berezney, 1989; Neri et al., 1992). This underlying nuclear organization has not yet been fully biochemically characterized but is known to consist of repeated DNA loop domains anchored to a residual skeleton (Cook and Brazell, 1975, 1976; Paulson and Laemmli, 1977). The higher-order chromatin associated with the nuclear matrix has long been suggested to play a role in organizing the genome for

replication (Pardoll and Vogelstein, 1980; Vogelstein et al., 1980), and the fact that DNA replication intermediates associate with the matrix has been exploited for their purification. It has also permitted the localization of DNA replication origins in eukaryotes (Dijkwel et al., 1991; Hyrien and Mechali, 1993).

Here we have investigated the factors that control the ability of differentiated adult cell nuclei to participate in early developmental events when transplanted into eggs or egg extracts. In particular, we show that mitosis is crucial for resetting the nuclear organization of differentiated nuclei and for adapting them for the accelerated DNA replication of early embryos. Both in metaphase-arrested *Xenopus* egg extracts and at mitosis of early embryonic cycles, the formation of mitotic chromosomes is a necessary step in organizing DNA for subsequent replication. Incubating differentiated adult nuclei in a mitotic extract shortens the average size of replicons and chromatin loop domains to those typical of endogenous chromatin present during early development. Molecular DNA combing demonstrates that a single mitosis is both necessary and sufficient to reset interorigin spacing. This reprogramming of replicon organization is topoisomerase II dependent and results in an increased recruitment of replication factors to origins that is not simply a function of the amount of available prereplication-complex proteins. Finally, we show that an equivalent remodeling of the chromatin occurs at mitosis of each cell cycle during early development. These results can explain how the egg is able to remodel differentiated nuclei and why cloning experiments by nuclear transfer of differentiated nuclei have such a high failure rate.

RESULTS

Reprogramming Differentiated Nuclei for DNA Replication Requires Passage through Mitosis

When sperm nuclei are introduced into *Xenopus* interphase egg extracts, they replicate almost immediately and with an efficiency of close to 100%, similar to what happens in vivo following fertilization (Blow and Laskey, 1986). In contrast, erythrocyte nuclei replicate inefficiently (Leno and Laskey, 1991; Lu et al., 1999). Both human and *Xenopus* eggs are normally blocked at the stage of the second meiotic division with condensed chromosomes at the metaphase stage (Tunquist and Maller, 2003), and fertilization induces a calcium pulse that triggers the end of mitosis and the onset of embryonic cleavage. However, when differentiated nuclei are transplanted into eggs, microinjection induces an immediate exit from mitosis and thereby prevents differentiated nuclei from undergoing mitotic chromosome condensation prior to passage into postmitotic cell-cycle phases.

We asked whether passage through mitosis might be a prerequisite for reprogramming the nucleus for rapid DNA replication. The experimental procedure outlined in Figure 1A was used. Erythrocyte nuclei were either permeabilized and directly incubated in *Xenopus* S phase extracts or incubated in a mitotic egg extract before activation in S phase. We observed that permeabilized erythrocyte nuclei replicate less efficiently in S phase egg extracts than do permeabilized sperm nuclei (Figure 1B). However, when permeabilized erythrocyte nuclei were first incubated in an M phase extract prepared from eggs blocked at the second meiotic metaphase by EGTA (Murray, 1991) prior to S phase induction using $CaCl_2$, replication occurred as rapidly and efficiently as in sperm nuclei (Figure 1B). We confirmed that chromosomes were formed before the Ca^{2+} was added in each experiment (see below and data not shown). In other words, the formation of chromosomes by an initial exposure to mitotic conditions made erythrocyte nuclei as competent for DNA replication as sperm chromatin. As previously seen with sperm chromatin (Adachi and Laemmli, 1992), erythrocyte DNA replication occurred in foci colocalizing with RPA (Figure 1C); we observed an increased number of such foci when erythrocyte chromatin was allowed to first pass through mitosis.

M Phase Extract Conditioning Increases Number of Replication Origins

While DNA replication initiates at origins spaced every 10 to 20 kbp during early *Xenopus* development, permitting a high rate of replication (Hyrien and Mechali, 1993; Walter and Newport, 1997), in most dividing somatic cells, the replicon size ranges from 50 to 300 kbp (Berezney et al., 2000). To address whether mitotic remodeling of erythrocyte nuclei affects replicon size, we analyzed the spacing of origins by DNA combing (Michalet et al., 1997). In this method, DNA molecules are stretched uniformly, providing an accurate determination of origin density along the DNA (Pasero et al., 2002). Erythrocyte nuclei were incubated in egg extracts in the presence of BrdUTP, which labels initiation sites, and a low concentration of aphidicolin, which permits initiation but slows elongation dramatically (Walter and Newport, 2000; Wu et al., 1997; data not shown). Chromosomal DNA was purified and combed on silanized glasses, BrdU incorporation was detected using anti-BrdU antibodies, and DNA fibers were counterstained with an anti-guanosine antibody. Figure 2A shows that sperm nuclei had an average spacing of 23.4 kbp between replication origins, while the spacing for erythrocyte nuclear chromatin incubated in S phase egg extract ranged from 30 to 230 kbp (Figure 2B). Seventy-seven percent of the replicons were smaller than 30 kbp in the *Xenopus* nuclei, whereas ninety-seven percent of the replicons were larger than 30 kbp in the erythrocyte nuclei. We conclude that the slow replication observed in erythrocyte nuclei that had been exposed to S phase extract was due to a low frequency of replication initiation within the genome.

When erythrocyte nuclei were first exposed to M phase extract before entry into S phase, however, the spacing of origins was shortened to 24.9 kbp, similar to sperm nuclei (Figure 2C). The proportion of replicons larger than 30 kbp was dramatically decreased, with 74% of the replicons being in the 10–30 kbp range. Finally, incubation of sperm nuclei in an M phase extract prior to S phase had no effect on origin spacing (Figure 2D). We conclude that prior conditioning of erythrocyte nuclei in M phase extract set an origin spacing similar to those of sperm chromatin upon entry into S phase.

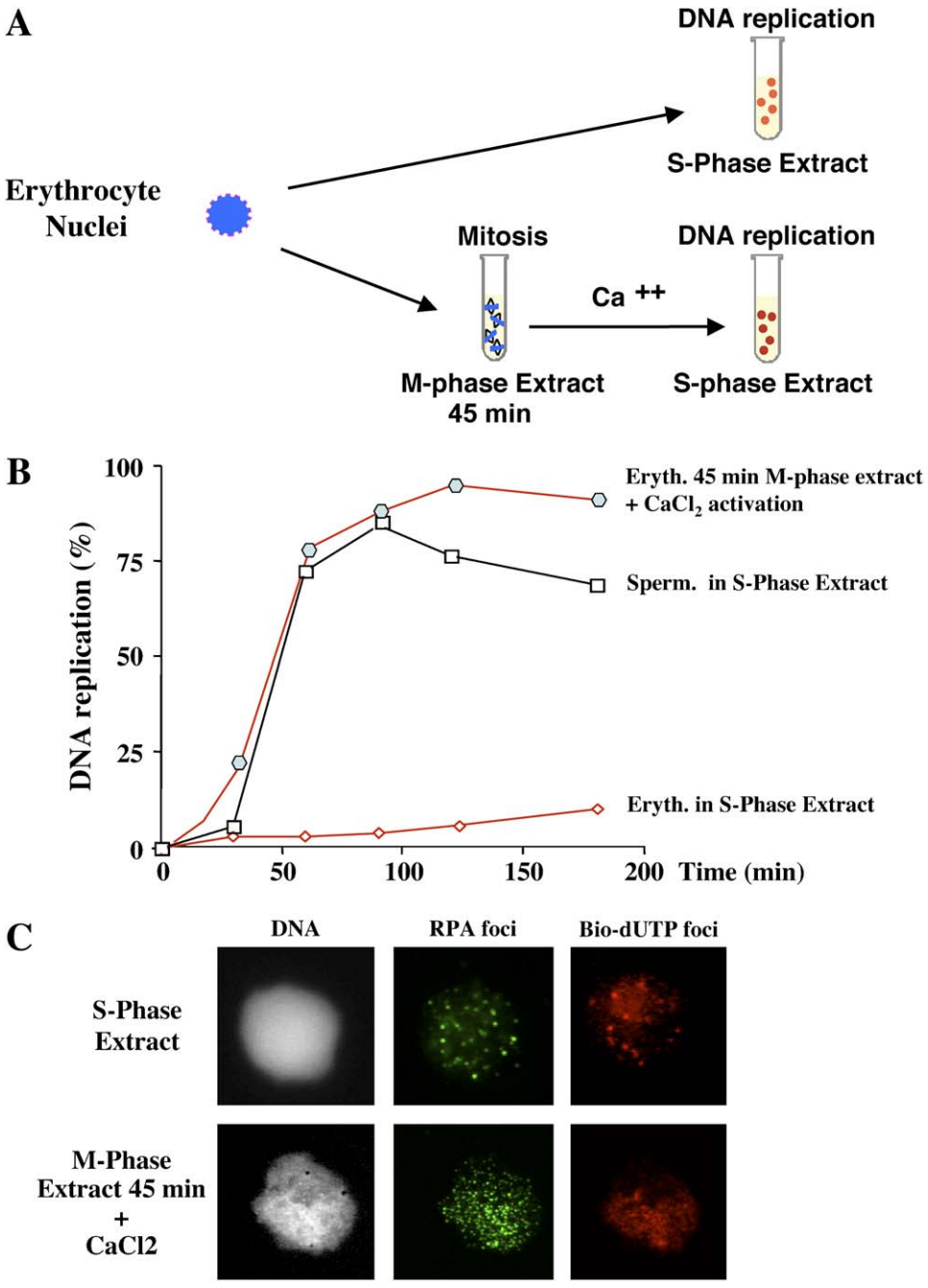


Figure 1. Exposure to M Phase Conditions Makes Erythrocyte Nuclei Fully Competent for DNA Replication

(A) Scheme of the experimental procedure.

(B) Permeabilized erythrocyte nuclei were incubated in S phase or M phase extract for 45 min before $CaCl_2$ activation to trigger S phase. Five microliter samples were taken at different times. DNA replication was monitored by TCA precipitation of [^{32}P]αdCTP incorporated into DNA and expressed as the percentage of replicated DNA compared to the total input DNA. Sperm nuclei incubated in S phase extract were used as a control. Our other independent experiments show that permeabilization was not necessary when erythrocyte nuclei are incubated in M phase extracts.

(C) Replication initiation foci were analyzed in erythrocyte nuclei by incorporating biotin-16-dUTP in the presence of 5 μg/ml aphidicolin, as indicated. DNA was stained with Hoechst 33258. The RPA antibody was revealed with an anti-mouse FITC (green), and biotin-16-dUTP was revealed with streptavidin Texas red (red).

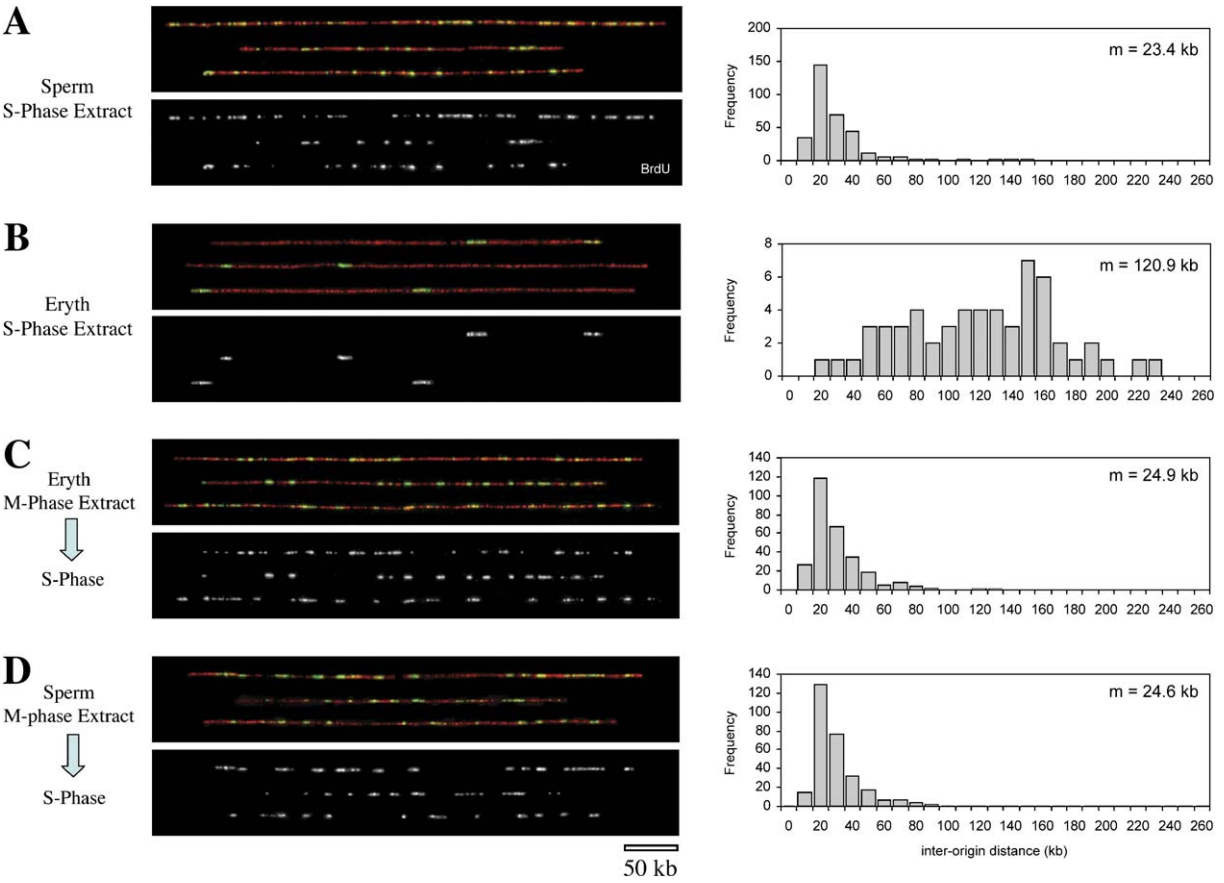


Figure 2. Single-Molecule Analysis of the Interorigin Spacing by Molecular Combing

Sperm nuclei (A) and permeabilized erythrocyte nuclei (B) were incubated for 75 min in S phase extract supplemented with 5 μg/ml aphidicolin and 40 μM BrdUTP. Erythrocyte nuclei (C) and sperm nuclei (D) were first incubated in M phase extract for 45 min before $CaCl_2$ activation and addition of aphidicolin. Fibers were combed on silanized coverslips, and the center-to-center distances between adjacent BrdU tracks were measured. The center-to-center distance between BrdU tracks is indicated in kbp. Lower panel, BrdU; upper panel, merge BrdU (green)/DNA (red).

Mitotic Remodeling Is Not Due to Global Changes in Nucleosome Organization or to Histone Acetylation Levels

Although the above results could suggest a superior ability of M phase extract to assemble proteins of the prereplication complex, we doubted this possibility for two reasons. First, M phase extracts do not contain higher levels of prereplication-complex proteins than do interphase egg extracts (data not shown; see also Figure S1A in the Supplemental Data available with this article online). Second, several of the proteins do not bind to chromatin during mitosis, including ORC, CDC6, Cdt1, RPA, and MCMs (Figure S1B).

An alternative explanation is that building mitotic chromosomal structures is sufficient to reset the nuclear organization of erythrocyte nuclei for DNA replication in the next cell cycle. To test this possibility, we first examined nucleosome assembly and spacing in the nuclei. As shown in Figure S1C, the global nucleosome organization was similar regardless of whether the nuclei were added directly to the S phase extract or were first incubated in an M phase extract.

We also investigated whether the level of histone acetylation could account for our results, particularly in view of the recent suggestion that acetylation may contribute to the specification of replication origins (Aggarwal and Calvi, 2004; Danis et al., 2004). Histone acetylation is determined by the equilibrium between the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), both of which are present in *Xenopus* oocytes (Ryan et al., 1999; Wade et al., 1999). This equilibrium can be modified in favor of acetylation by either inhibiting deacetylases with trichostatin (TSA) or activating acetylases with CTPB. Conversely, histone deacetylation can be promoted by inhibiting histone acetylases with anacardic acid (AA) (Balasubramanyam et al., 2003). Figure 3A shows that favoring acetylation (TSA or CTPB) had no significant effect on the replication rate of sperm chromatin incubated in S phase extract. These results indicated either that histone acetylation is not necessary for DNA replication of sperm nuclei or that the level of acetylation present in the extract is sufficient to allow a maximum rate of replication. Figure 3B shows that the latter possibility is more

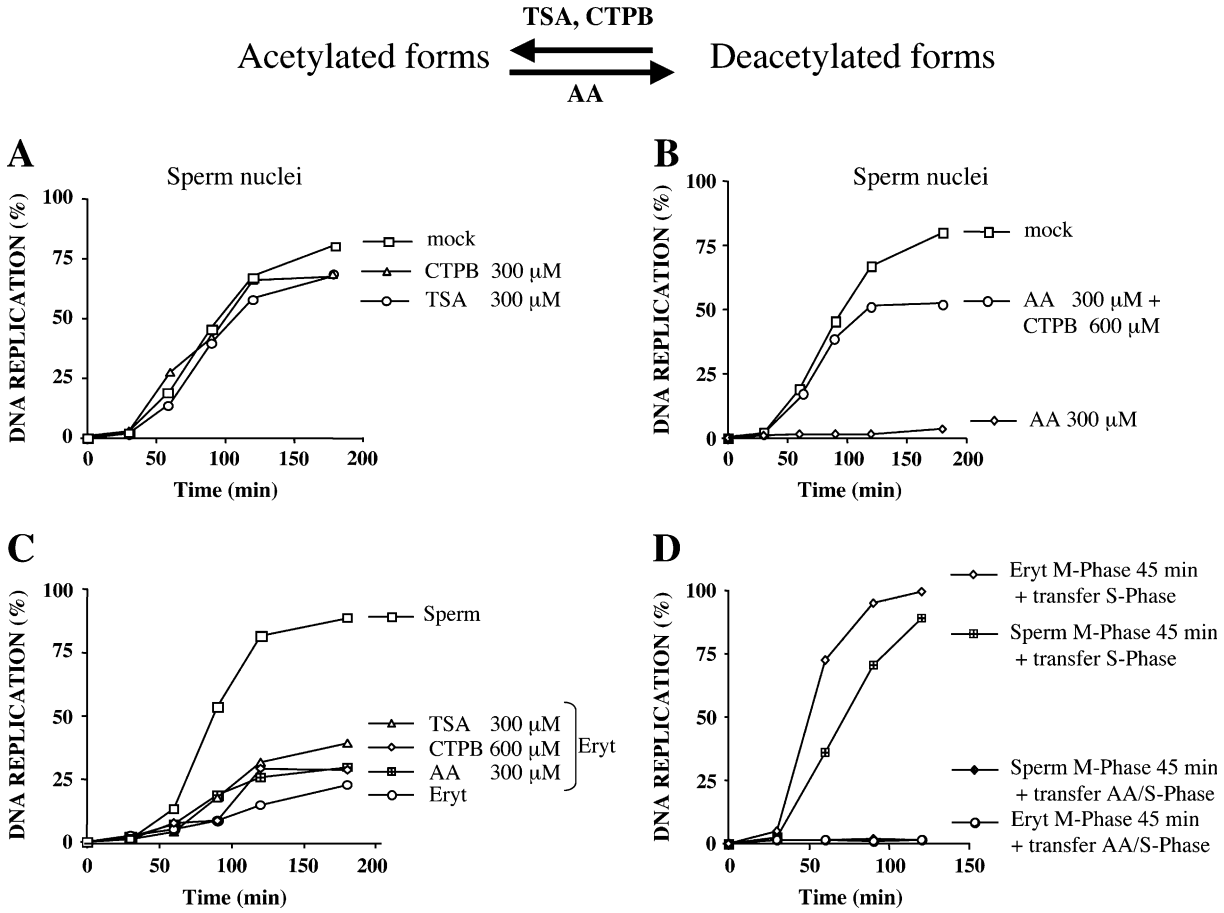


Figure 3. Histone Acetylation Does Not Induce Erythrocyte Nuclei Remodeling

(A and B) Sperm nuclei were incubated in S phase extract in the presence of histone acetylation activators (300 μM TSA and 300 μM CTPB) (A) or an inhibitor (300 μM AA) (B). A prior incubation of 600 μM CTPB was necessary to prevent inhibition of DNA replication by 300 μM AA (B).

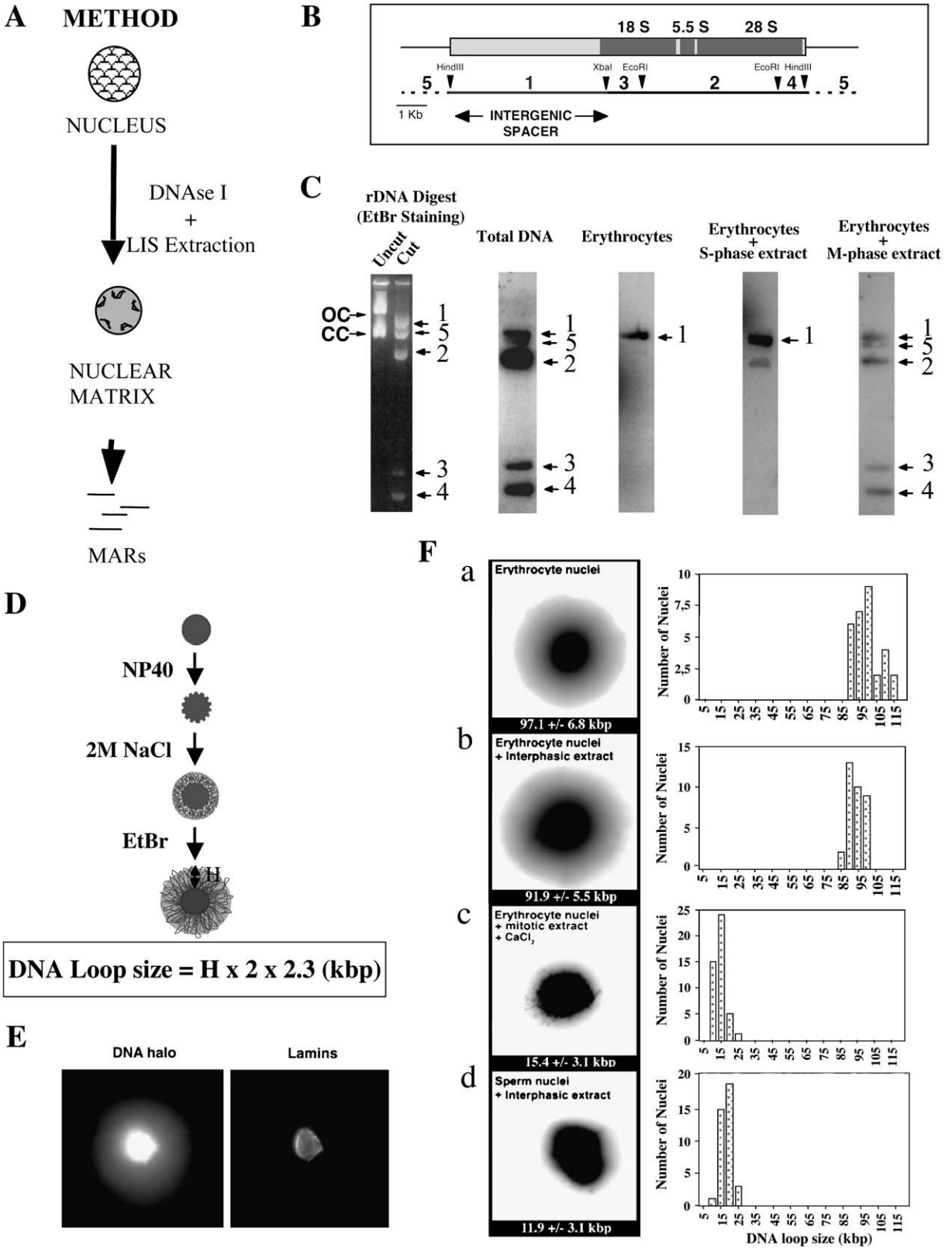
(C and D) Permeabilized erythrocyte nuclei were incubated in S phase extract containing either 300 μM TSA, 600 μM CTPB, or 300 μM AA (C). Sperm nuclei incubated in S phase extract were used as a control (D). Sperm and erythrocyte chromatin were transferred from a 45 min incubation in M phase extract to S phase extract containing 300 μM AA. Five microliter samples were taken at different times, and DNA replication was monitored by TCA precipitation of [^{32}P]αdCTP incorporated into DNA.

likely, as AA, which causes hypoacetylation of H3 and H4, strongly inhibited the replication of sperm nuclei in S phase extract. This inhibition could be reversed by the activator CTPB. These results clearly indicate that histone acetylation is required for the rapid replication seen during early development and that the steady-state level of acetylation activity in the egg is sufficient for a maximum rate of DNA replication.

As with sperm chromatin, the acetylation activators TSA and CTPB failed to significantly affect the replication rate of erythrocyte nuclei incubated in S phase extract (Figure 3C). Unlike with sperm chromatin, however, the acetylase inhibitor AA did not further inhibit the inefficient DNA replication of erythrocyte nuclei. Erythrocyte nuclei that had been remodeled in mitosis, however, were as sensitive as sperm nuclei to AA (Figure 3D). Together, these data suggest, first, that although acetylation is required for the rapid DNA replication characteristic of early S phases, its level is sufficient for a full rate of DNA replication and therefore cannot account for the slow DNA replication rate observed with erythrocyte nuclei in interphasic extracts. Second, they indicate that the organization of erythrocyte chromatin negatively regulates DNA replication in a dominant manner in S phase extracts unless mitotic chromosomes are first formed by incubation in an M phase extract.

Mitotic Reorganization of Erythrocyte Chromatin Domains

We next investigated global changes in the organization of erythocyte chromatin that could explain replicon remodeling. A transition from a random association with the nuclear matrix to a defined anchorage occurs during *Xenopus* development as chromatin domains become organized for transcription after the midblastula transition (Vassetzky et al., 2000). We hypothesized that the reverse could take place when erythrocyte nuclei are exposed to egg extracts. To test this, nuclei incubated in M phase extracts or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Erythrocyte nuclei
97.1 +/- 6.8 kbp
b
Erythrocyte nuclei
+ Interphasic extract
91.9 +/- 5.5 kbp
c
Erythrocyte nuclei
+ mitotic extract
+ CaCl2
15.4 +/- 3.1 kbp
d
Sperm nuclei
+ Interphasic extract
11.9 +/- 3.1 kbp
Number of Nuclei
DNA loop size (kbp)

S phase extracts or mock-incubated nuclei were treated with DNase I and then extracted with LIS, which removes histone and nonhistone proteins but preserves the attachment sites of DNA loops to the nuclear scaffold (Figure 4A). The DNA remaining on the matrix was isolated, labeled, and used as a probe to hybridize to agarose-gel-separated regions of the rDNA domain (Figures 4B and 4C). The gels were also probed with total *Xenopus* erythrocyte DNA (Figure 4C).

Each unit of the rDNA domain comprises a transcribed region and a nontranscribed spacer. A single band corresponding to the intergenic spacer was detected when matrix-associated erythrocyte DNA was used as a probe, whereas all rDNA bands were detected when total *Xenopus* erythrocyte DNA was used (Figure 4C). While exposing erythrocyte nuclei to S phase egg extract did not significantly alter the rDNA specificity, incubation in an M phase egg extract produced a randomization of the attachment sites, as all rDNA domains were detected with similar efficiency (Figure 4C). We obtained analogous results with a different method using oligonucleotide arrays (Figure S2).

To try to explain these results, we investigated whether mitosis affects the density of the loop attachment sites within the rDNA domain. The chromatin loop sizes were measured using the ''maximum fluorescent halo technique'' (Vogelstein et al., 1980). In this method, nuclei were first treated with high salt and were then briefly irradiated with UV in the presence of ethidium bromide, which causes extended DNA loops to form a fluorescent halo around the residual nuclear structure (Figure 4D and Experimental Procedures). The loop size was estimated based on the diameter of the fluorescent halo (Buongiorno-Nardelli et al., 1982; Vogelstein et al., 1980), which could be distinguished from the residual nucleoskeleton by immunolocalization of the nuclear lamina (Figure 4E). Figure 4Fa shows that erythrocyte nuclei have a mean loop size of 97.1 ± 6.8 kbp, similar to what is seen in other somatic cell nuclei (data not shown and Buongiorno-Nardelli et al., 1982; Vogelstein et al., 1980). While this size did not change when nuclei were permeabilized and incubated in S phase egg extracts (Figure 4Fb), exposure to an M phase egg extract prior to calcium activation caused the loop size to decrease to 15.4 ± 3.1 kbp (Figure 4Fc), close to the value of sperm nuclei or early embryonic nuclei in S phase (see Figure 4Fd and below).

We conclude that passage through M phase prior to S phase induction induces two kinds of rearrangements in erythrocyte nuclei. First, it reduces the loop size, consistent with a higher density of anchorage sites to the nuclear matrix. Second, it decreases the average spacing of replication origins in parallel proportions. In both cases, the organization of chromatin domains becomes similar to that of sperm nuclei entering S phase.

Topoisomerase II Is Involved in Replicon Resetting at M Phase

Since introducing sperm chromatin into mitotic egg extracts causes the chromosomes to condense (Lohka and Masui, 1983), we next investigated whether chromosome condensation, driven by topoisomerase II in *Xenopus* M phase egg extracts (Adachi et al., 1991; Wood and Earnshaw, 1990), is involved in replicon resetting in erythrocyte nuclei. While erythrocyte nuclei replicated efficiently when conditioned in the M phase extract for 45 min prior to S phase induction (Figure 1 and Figure 5A), replication was slow when they were introduced into an M phase extract and then immediately driven into S phase (Figure 5A). This showed that it is not the M-S transition that is critical for reorganizing the nuclei for rapid replication but rather the formation of mitotic metaphase chromosomes. Consistent with this, erythrocyte nuclei that were incubated for 45 min in M phase extract and then directly transferred to an S phase extract, but without Ca^{2+} activation, also replicated as efficiently as sperm nuclei (Figure S3).

It has previously been observed that chicken erythrocyte nuclei do not condense in topoisomerase II-depleted extracts (Adachi et al., 1991). Consistent with this, we found that the topoisomerase II-specific inhibitor ICRF 193 (Oestergaard et al., 2004; Sato et al., 1997) prevented chromosome condensation in erythrocyte or sperm nuclei incubated in an M phase extract (Figure 5B). When erythrocyte nuclei were incubated for 45 min in M phase extract containing ICRF 193, we also obtained a very low rate of replication (Figure 5A). We confirmed that the ICRF 193-containing extracts were still in mitosis by measuring H1 kinase activity (Figure S4). We also obtained similar results when erythrocytes were incubated in topoisomerase II-depleted M phase extracts (Figure S5). In contrast, topoisomerase II inhibition or depletion did not affect the replication of sperm chromatin

Figure 4. Mitosis-Induced Remodeling of Nuclear Organization

(A) Erythrocyte nuclei were incubated in either S phase or M phase egg extract or were mock incubated. The nuclear-matrix-associated DNA was purified using the LIS procedure, and the DNA fragments remaining on the matrix were ^{32}P labeled and used to probe specific regions of the rDNA domain.

(B) A plasmid containing the *Xenopus* rDNA domain was cut with HindIII, EcoRI, and XbaI to produce five fragments. Fragment 1 is the intergenic spacer element between the rDNA units; fragments 2, 3, and 4 are within the transcription unit; and Fragment 5 is the vector sequence.

(C) DNA fragments were separated by agarose-gel electrophoresis and stained with ethidium bromide or transferred to nylon membranes for hybridization. Total *Xenopus* DNA often partly hybridizes with one of the plasmid bands, as does the matrix-associated DNA from erythrocytes incubated in M phase extract, emphasizing the random nature of this fraction.

(D) Nuclei were recovered on coverslips and submitted to the maximum fluorescent halo technique (MFHT) for DNA loop-size measurements (Experimental Procedures).

(E) Immunostaining with anti-lamin antibody was also used to delimitate matrix and loop fractions, while ethidium bromide was used to stain DNA loops as in (D).

(F) The method was applied to both sperm nuclei and erythrocyte nuclei in interphase that had been previously incubated for 45 min in M phase egg extract. Histograms show individual loop-size measurements.

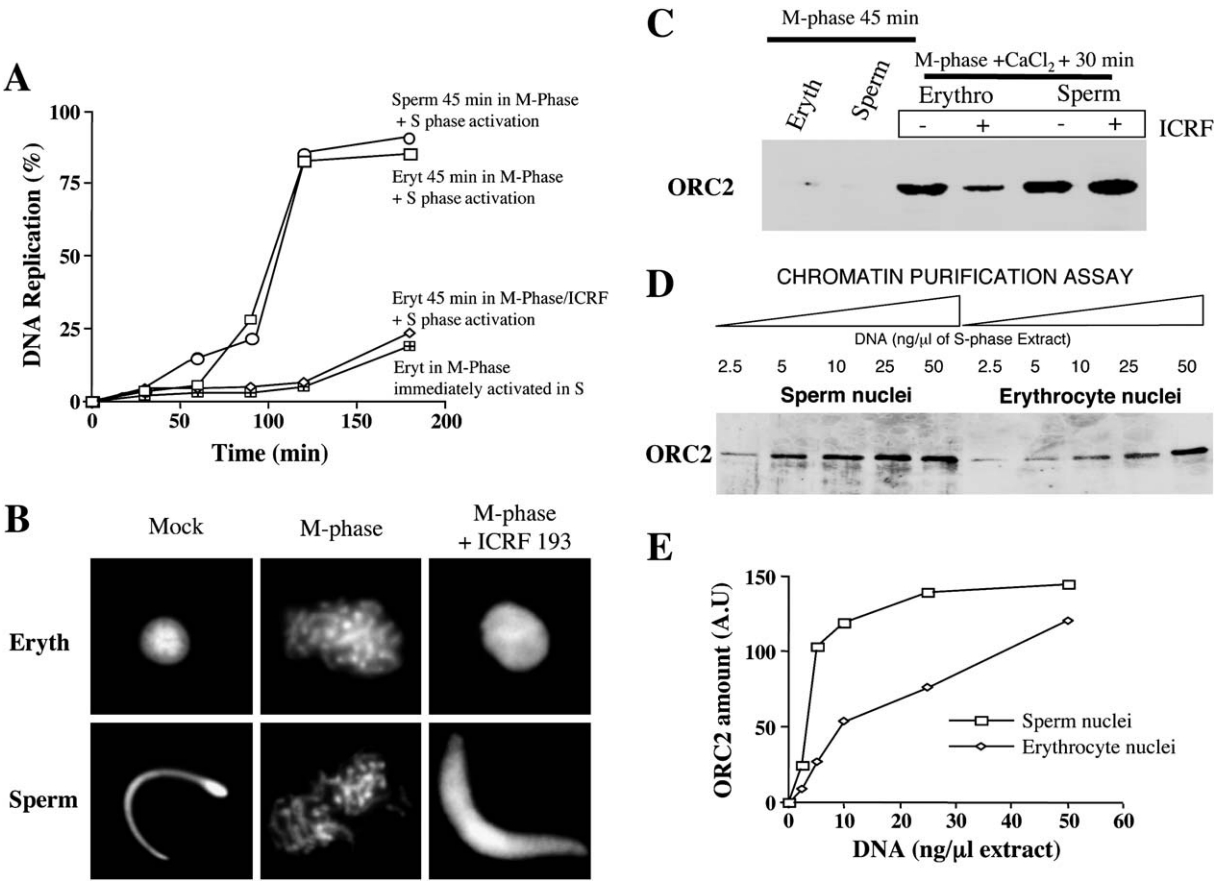

Figure 5. ORC Binding and DNA Replication Efficiency Depend on the Chromatin Context and Topoisomerase II Activity

(A) Erythrocyte chromatin was incubated either in M phase extract and immediately driven into S phase by Ca^{2+} or in M phase extract for 45 min before induction into S phase by Ca^{2+} in the absence or presence of ICRF 193. Control sperm chromatin in M phase extracts induced to enter S phase by calcium is shown. The amount of nuclei was 1000 nuclei/µl egg extract for sperm nuclei and 500 nuclei/µl egg extract for erythrocyte nuclei, to keep the chromatin concentration constant.

(B) Demembranated sperm nuclei or erythrocyte nuclei were incubated in a mitotic egg extract in the presence or absence of 50 µg/ml of the topoisomerase II inhibitor ICRF 193. DNA was stained with Hoechst dye for chromosome formation analysis.

(C) Chromatin was purified and proteins were analyzed by SDS gel electrophoresis as described in Experimental Procedures. Sperm nuclei (1000 nuclei/µl egg extract) or erythrocyte nuclei (500 nuclei/µl egg extract) were incubated in a mitotic egg extract induced to enter S phase with calcium in the presence or absence of ICRF 193. Chromatin was purified 30 min after calcium activation and analyzed by immunoblot with a *Xenopus* anti-ORC2 antibody.

(D) Erythrocyte nuclei and sperm nuclei were incubated for 30 min in S phase extract at various DNA concentrations. Chromatin was purified and analyzed by 10% SDS-PAGE.

(E) Quantitation from the immunoblot.

in interphase egg extracts, as previously published (Takasuga et al., 1995), although replication did decelerate in the final stages of DNA replication due to decatenation inhibition (Figure S5).

The Chromatin Source, Not ORC Protein Levels, Influences Initiation-Factor Recruitment

We next addressed the possibility that DNA topoisomerase II-dependent chromosomal organization controls the efficiency of ORC recruitment to chromatin. As shown in Figure 5C, ORC2 did not bind to erythrocyte or sperm chromatin incubated in M phase extracts, consistent with previous reports (Romanowski et al., 1996). When S phase entry was induced by Ca^{2+}, ORC2 recruitment onto M phase extract-treated erythrocyte chromatin was as efficient as it was onto sperm chromatin. ICRF 193 inhibition of topoisomerase II activity during mitotic remodeling, however, dramatically reduced the recruitment of ORC2 onto erythrocyte, but not sperm, chromatin for the subsequent S phase (Figure 5C). This indicates that the topoisomerase II-dependent mitotic remodeling of erythrocyte nuclei is required for the proper recruitment of ORC proteins in preparation for S phase.

It has been suggested that the absolute amount of replication factors in extracts can explain the observed high rates of DNA replication and that these factors are titrated by the

DNA that accumulates during the rapid divisions. This stoichiometric model was supported by the increased replicon size observed when nuclei concentrations are increased in *Xenopus* egg extracts (Walter and Newport, 1997). Our data, however, showed that the concentration of replication proteins could not explain the ability of M phase extract to program nuclei for rapid DNA replication. One limiting factor could be the ability of nuclei to recruit these proteins. Indeed, Figure 5D shows that ORC2 is titrated by high concentrations of sperm nuclei, as previously reported (Rowles et al., 1996), but also that the chromatin context dominantly influences the efficiency of this recruitment. With sperm chromatin, the titration curve of ORC in the egg extract reached a plateau at 25 ng of DNA, equivalent to the amount in a midblastula-stage embryo (Figures 5D and 5E). A similar titration curve was observed for MCM3, a subunit of the MCM helicase complex involved in the initiation of DNA replication (data not shown). In contrast, erythrocyte chromatin did not bind ORC2 as efficiently as sperm chromatin (Figures 5D and 5E), and the titration curve had not yet reached a plateau at 50 ng DNA. The recruitment of replication initiation factors thus is not only proportional to the amount of available chromatin but is also influenced by the chromatin's source.

Rearrangement of Chromatin Domains Occurs with Each Cell Cycle in Early *Xenopus* Development

In view of the above results, we investigated whether the same chromatin reorganization occurs during early development in vivo. Early *Xenopus* cell cycles consist of overlapping S and M phases, with no G1 or G2. S phase is initiated at the metaphase-anaphase transition, as individual chromosomes become surrounded by a nuclear membrane to form karyomeres, and before nuclei are reconstructed by the fusion of karyomeres at telophase (Figure 6A and Lemaitre et al., 1998; Montag et al., 1988). We isolated the nuclear matrix from either postreplicative, premitotic nuclei (pre-MBT, 32–64 cell embryos) or karyomeres subsequent to the metaphase-anaphase transition. Matrix-associated DNA was labeled and used to probe rDNA domain regions (as described above with erythrocyte nuclei; see Figure 4A). As shown in Figure 6B, while matrix-associated DNA from postreplicative nuclei was restricted to the rDNA intergenic spacer region, matrix-associated DNA from karyomeres was bound to all rDNA fragments, indicating random association with the matrix. These data provide in vivo confirmation that a major rearrangement of chromatin organization occurs at mitosis and show that this reorganization occurs with each early embryonic division. The data further suggest that the rapid DNA replication seen during early development involves the use of high numbers of closely spaced, random attachment points between the chromatin and the nuclear matrix.

According to this interpretation, early mitoses might produce short DNA loop sizes to prepare chromatin for subsequent S phase. The loop size was measured both on karyomeres (S phase entry) or fully reconstituted postreplicative nuclei from either cycloheximide-synchronized early embryos at the 32–64 cell stage (Gard et al., 1990; Lemaitre et al., 1998; Experimental Procedures) or from unsynchronized embryos at the 512–1024 cell stage (Figure S7). Figure 6C shows that karyomeres had a mean loop size of 17.3 ± 7.5 kbp, similar to that of erythrocyte nuclei following passage through mitosis (Figure 4F). This loop size also correlates with the replicon size previously measured in vivo during *Xenopus* early development (Hyrien and Mechali, 1993). The loop size increased to 59.7 ± 10.7 kbp in postreplicative early embryonic nuclei (Figure 6C and Figure S7), confirming the existence of a postreplicative remodeling process that increases the spacing between matrix attachment sites.

If mitotic resetting of chromatin to a short loop size were essential for high initiation rates, then postreplicative nuclei from early embryos that have not passed through mitosis would be incompetent for high rates of DNA replication. Indeed, although obtained from early embryos, postreplicative nuclei that were permeabilized and incubated in an S phase egg extract replicated slowly relative to sperm nuclei (Figure 7A) and similarly to erythrocyte nuclei (Figure 1B). Figure 7B shows an alkaline agarose gel of nascent DNA that was labeled during DNA replication. With sperm nuclei, strands grew to a value of around 9–10 kbp, followed by a shift to high molecular-weight values, as expected from the joining of replicated DNA from adjacent replicons. With postreplicative nuclei transfered to S phase, such a shift was not observed. DNA replication was less efficient (Figure 7A), as expected with more widely spaced replication origins, and a continuous stream of strands growing to larger sizes (>50 kbp) was observed throughout the reaction. Finally, as previously observed with erythrocyte nuclei (Figures 5D and 5E), recruitment of ORC to chromatin was severely diminished, confirming that the efficiency of DNA replication during early development is a matter of chromosome organization and not of the absolute amount of ORC (Figure S8).

We next addressed whether the increase in loop size was a postreplicative event or occurred during DNA replication. As shown in Figures 7C and 7D, loop size increased gradually throughout S phase (Figures 7C and 7D), and this S phase-dependent increase could be prevented with ICRF (data not shown). Finally, we addressed whether entry into mitosis can reset the large loop size of postreplicative early embryonic nuclei, as previously shown for erythrocyte nuclei. Figure 7C shows that the loop size in postreplicative embryonic nuclei introduced into M phase egg extracts was dramatically reduced to 11.2 ± 2.6 kbp, a value equivalent to that of sperm chromatin following incubation in mitotic extract or at the beginning of S phase (Figure 7). This mitotic remodeling of postreplicative nuclei was again blocked by the topoisomerase II inhibitor ICRF 193 (Figure 7C). We conclude that entry into mitosis resets the loop size of both postreplicative early embryonic nuclei and differentiated nuclei to a low value.

DISCUSSION

The Dynamic Organization of Nuclear Structures for DNA Replication during Early Development

Xenopus early development provides a good illustration of the plasticity of the nuclear structure for adapting to rapid

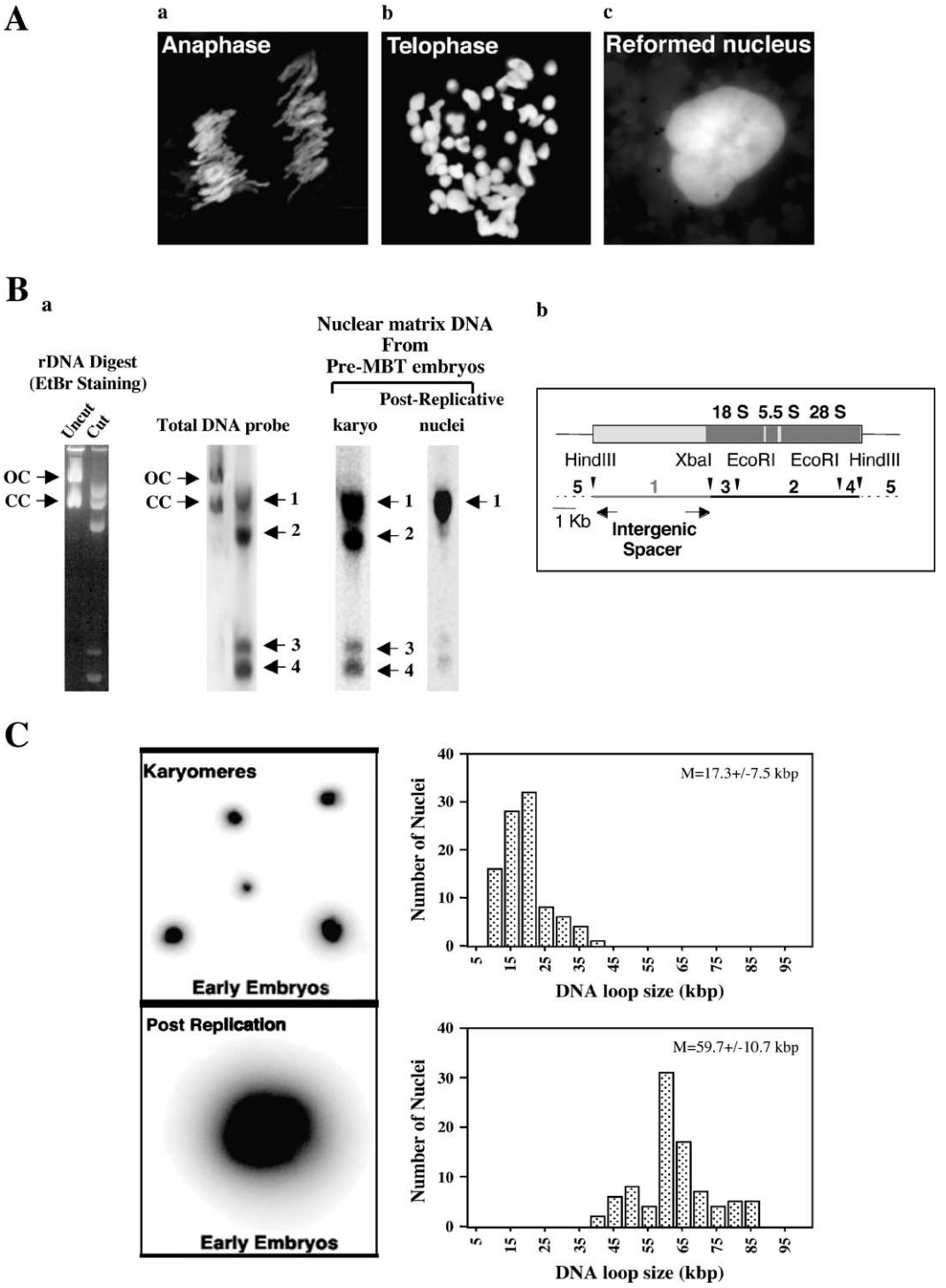

Figure 6. Cell-Cycle Remodeling of Chromatin Organization in Early *Xenopus* Embryos

(A) Karyomeres form at the anaphase-telophase transition and initiate DNA replication before nuclei are reconstructed (Lemaitre et al., 1998). DNA was stained with Hoechst 33258, and embryonic nuclei at anaphase (Aa), telophase (Ab), and after replication (Ac) are shown.

(B) Nuclei from early embryos were isolated and treated with DNase I. The nuclear matrix was prepared using the LIS procedure (Experimental Procedures), and the DNA fragments remaining on the matrix were ^{32}P labeled and used to probe for specific regions of the rDNA domain as in Figure 5. The fragments were separated by agarose-gel electrophoresis and stained with ethidium bromide or transferred to nylon membranes and hybridized with either total *Xenopus* DNA probe or with nuclear matrix DNA from karyomeres or early embryonic postreplicative nuclei.

(C) Nuclei were recovered on coverslips and submitted to the maximum fluorescent halo technique (MFHT).

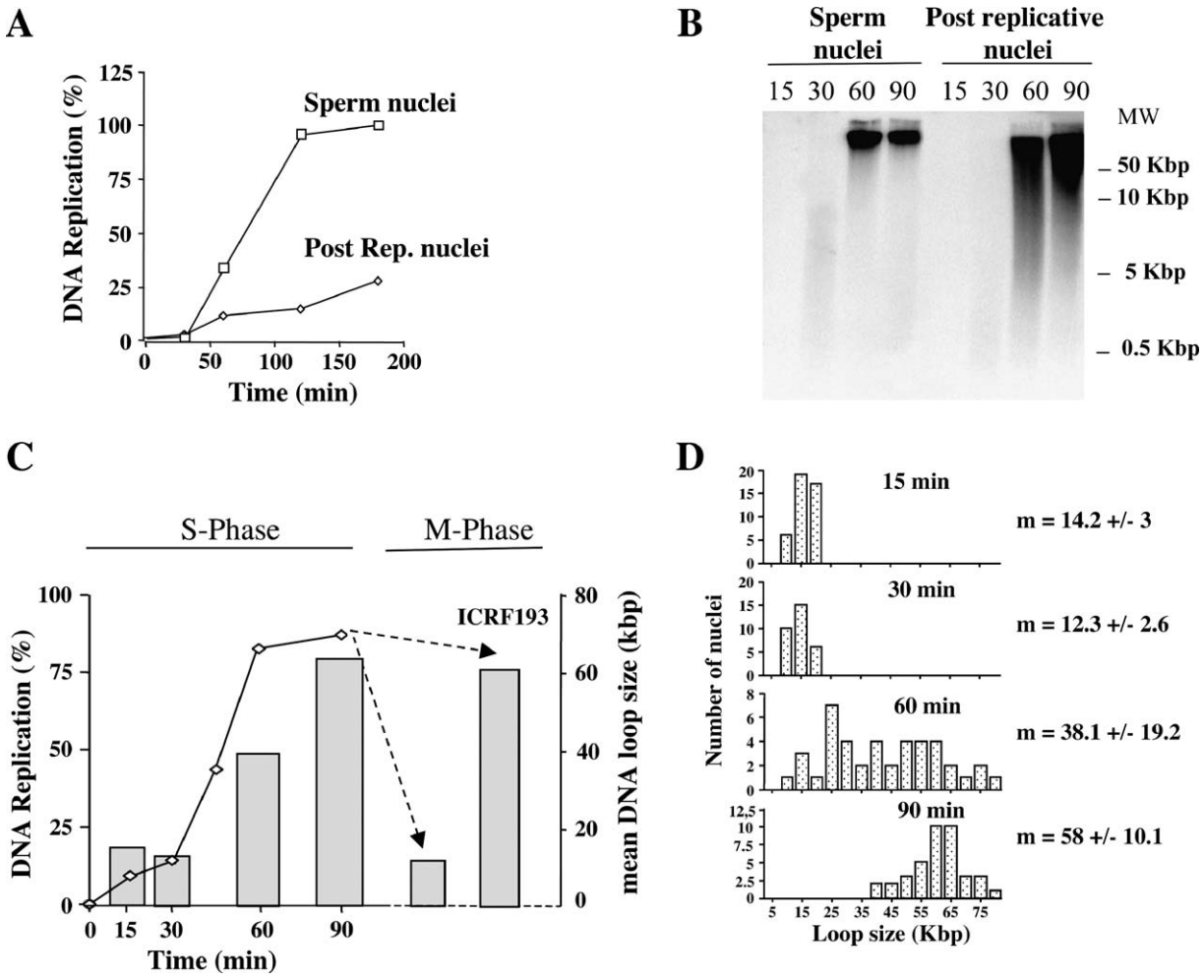

Figure 7. Mitotic Remodeling of Chromatin Loop Domains Occurs in S and M Phases

(A and B) Sperm nuclei or postreplicative nuclei were incubated in S phase extract in the presence of [^{32}P]αdCTP. Five microliter samples were taken at different times, DNA replication was quantitated by TCA precipitation (A), and the length of nascent DNA strands was analyzed by 0.8% agarose alkaline gel electrophoresis (B). The position of molecular-weight markers (MW) run in parallel is indicated.

(C) Sperm nuclei collected at different times during replication or following M phase were recovered on coverslips and submitted to MFHT for DNA loop-size measurements. Entry into mitosis was induced by incubating postreplicatve embryo nuclei in M phase extract in the presence or absence of ICRF 193.

(D) Distribution of loop sizes.

cell cycles (30 min) and DNA replication. Using cellular and biochemical techniques, we describe here specific features of early nuclear organization, including nonspecific anchorage of DNA to the nuclear matrix, short loops, and a close mean spacing between replication origins.

Our data, summarized in Figure 8, indicate that, at S phase entry in early development, nuclei are organized into short loops and replicons, allowing recruitment of a large amount of ORC protein. At this stage, DNA replication initiates nonspecifically (Hyrien et al., 1995). Loop size increases progressively during S phase, and mitosis reprograms nuclei so that they again include short loops and small replicons, enabling the rapid DNA replication of the early embryo. Topoisomerase II, which has been previously identified as a major component of the chromosomal scaffold or matrix (Berrios et al., 1985; Earnshaw et al., 1985; Gasser et al., 1986) and which is required at an early stage of chromosome condensation, is required for remodeling chromosomal loops and thus for the reprogramming of nuclei for rapid replication.

A probable second, apparently independent mechanism involves histone acetylation, as inhibition of histone acetylases decreases replication, and this inhibition can be rescued by histone acetylase activators. The steady-state endogeneous level of chromatin acetylation in early embryos, however, is sufficient for a maximum rate of sperm DNA replication. Our data cannot exclude the additional possibility that DNA replication factors are regulated by acetylation (Takei et al., 2001). In either case, acetylation appears to be required for the accelerated rate of DNA replication observed during early development.

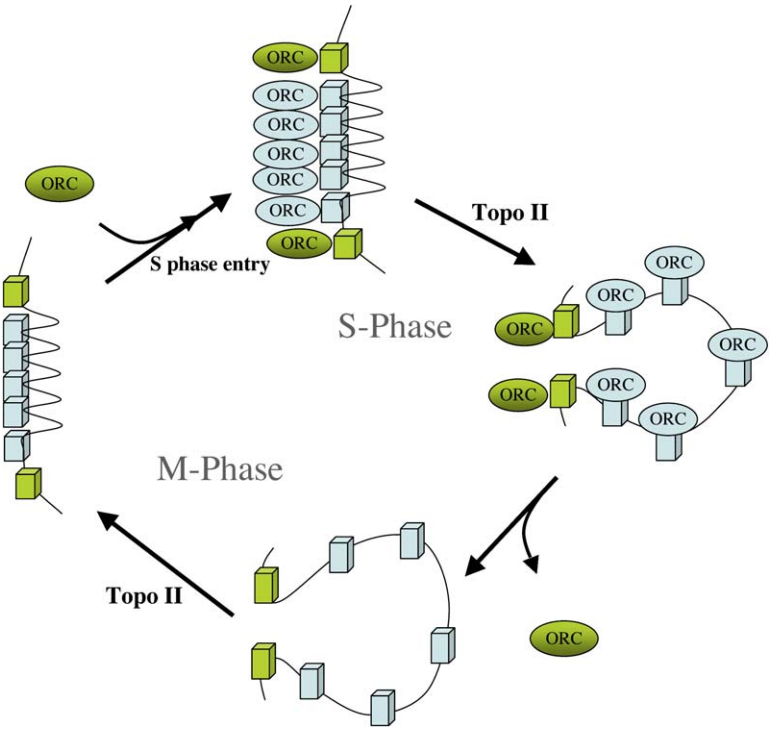

Figure 8. Cell-Cycle Remodeling of Chromatin Organization in the Early *Xenopus* Embryo

Reorganization of chromatin occurs with each cell cycle and is dependent on Topo II activity. During S phase, fusion of replicons leads to an increase in the mean DNA loop size. These large loops are remodeled into small loops at each mitosis and permit an increased binding of ORC for a higher rate of DNA replication. Border (green) and internal (blue) boxes represent both potential loop attachment sites and replication origins.

DNA Replication and Chromatin-Domain Organization

ORC binding to chromatin has been shown to be linked to origin spacing in *Xenopus* egg extracts (Rowles et al., 1999; Walter and Newport, 1997). As ORC is absent from mitotic chromatin (Romanowski et al., 1996; Figure 5C), our results indicate that mitotic remodeling occurs independently of pre-RC establishment. At the same time, mitotic remodeling, and the associated decrease in loop size, enhances the ability of chromatin to bind ORC. While chromatin bound ORC increases with the amount of sperm chromatin, at least until the MBT (4000 to 8000 cells), the increase is several times lower in postreplicative embryonic nuclei and erythrocyte nuclei. In these nuclei, decreased ORC binding occurs in striking conjunction with an increased DNA loop size, and inhibiting loop-size remodeling with ICRF 193 decreases ORC recruitment. A maximum number of ORC binding sites, corresponding to multiple matrix anchorage sites, might explain the high density of replication initiation sites prior to the MBT. While no strict DNA sequence specificity has been detected for ORC proteins in metazoans or in fission yeast, yeast ORC4 specifically binds to asymmetric AT-rich sequences (Chuang and Kelly, 1999), and *Xenopus* ORC preferentially associates with AT-rich regions (Kong et al., 2003). AT-rich regions are enriched at matrix-associated regions (MARs), and this may provide a basal nuclear architecture for DNA replication.

Chromosomal Architecture at Mitosis, Replicon Resetting, and Cloning

A major issue in nuclear-transfer experiments is how genetic or epigenetic marks within differentiated nuclei can be erased, as failure to do so decreases cloning efficiency. Cloning success is increased by serial transfer, in which donor nuclei are obtained from embryos that have passed through successive cell divisions following an initial transfer (Gurdon, 1962). Our data explain this observation and suggest that formation of chromosomes at mitosis is an important element in the genetic reprogramming that occurs in successful nuclear transplantation, permitting the restructuring of adult nuclei for rapid embryonic DNA replication.

One model that has been proposed to explain why *Xenopus* nuclear transplants often fail to develop invokes a cytoplasmic clock that imposes cell division every 30 min during early development (Hara et al., 1980). In terms of this model, nuclei from differentiated cells that have not been reprogrammed by the time mitosis begins will not be completely replicated, leading to abortive cleavage. Prior exposure to a mitotic egg extract, however, may allow them to keep up with the cell-cycle clock by allowing an increased number of replication origins.

Our data suggest that it is metaphase, and not the metaphase-anaphase transition, that resets the replicon organization. In classical nuclear transfer, when a somatic donor nucleus is introduced into an egg, the egg is simultaneously activated, triggering an immediate exit from metaphase of the second division and thereby preventing mitotic chromosomes from forming. Significantly, when erythrocyte nuclei are introduced into M phase extracts and immediately driven into S phase, they fail to replicate efficiently. We found that such nuclei can only be remodeled and efficiently participate in subsequent DNA replication when they are placed in a mitotic environment for 45 min prior to activation

(Figure 1B). Several lines of evidence indicate that the crucial parameter controlling this phenomenon is the organization of metaphase mitotic chromosomes, not the concentration of replication factors. First, we found that the stoichiometry between replication initiation factors and available chromatin cannot explain the observed rates of ORC recruitment and DNA replication. Second, M phase induces a global change in the chromosomal architecture, leading to a dramatic shortening of chromosome loop size. Third, topoisomerase II, which is involved in regulating chromosomal architecture and has been identified at the base of the loop domains (Adachi et al., 1989; Earnshaw et al., 1985; Gasser et al., 1986; Iarovaia et al., 1995), is necessary for ORC recruitment as well as the shortening of both loop size and interorigin spacing.

Our data may also explain observations showing that the specification of DNA replication origins occurs between the middle and end of G1 phase (Wu and Gilbert, 1996). During early *Xenopus* development, S phase and M phase occur successively without G1. Following the chromatin reprogramming that occurs in mitosis, this lack of G1 may prevent specific origins from being established, resulting in S phase without specified origins. This required mitotic reprogramming of the replicon and chromosome structure can also explain the observed benefit of the use of half-cleaved embryos as a donor source in animal cloning by serial transfer (Gurdon and Laskey, 1970; Gurdon et al., 1975). Specifically, as such nuclei were presumably exposed to a mitotic context in a first unsuccessful cleavage, they may have been reprogrammed through the mechanism described here. The mechanism also provides an explanation for recent improvements in human cloning methods in which a 2 hr incubation between nuclear injection and egg activation increases the cloning efficiency (Hwang et al., 2005).

Because the sperm and egg nuclei replicate before karyogamy, we expect that they must be organized for rapid DNA replication by the time they fuse, before the first mitotic division. Indeed, before fertilization occurs, the egg nucleus is blocked at metaphase and is therefore already organized into mitotic chromosomes. In addition, we observed that sperm nuclei were organized into short loops before fertilization, indicating that both the male and female pronuclei are already set for rapid replication at fertilization. These observations underscore the fact that it is unfertilized eggs, but not activated or fertilized eggs, that are capable of conditioning differentiated nuclei for development.

EXPERIMENTAL PROCEDURES

Xenopus Egg Extracts and Early Embryos

S phase and M phase low-speed (LS) extracts were prepared according to protocols described in detail by Menut et al. (1999) and available at www.igh.cnrs.fr/equip/mechali/. Embryos were grown in 0.1× Barth's medium as described (Lemaitre et al., 1995). To obtain karyomeres, perfectly synchronized embryos were selected at each division over the first four divisions. Embryos were then taken at the fifth division when the furrow appears. Subsequent divisions give rise to metasynchronous divisions in the embryos. G2-like synchronized embryos were obtained by 45 min incubation in 0.1× Barth's medium containing 150 μg/ml cycloheximide between the 32- and 64-cell stages and between the 512- and 1024-cell stages. Embryos were dejellied with 2% cysteine HCl (pH 7.9) and homogenized through a 1 ml Gilson tip before being centrifuged at 4°C for 10 min at 10,000 × g. Under these conditions, the endogenous embryonic nuclei (karyomere or reformed nuclei) remain in the supernatant (Lemaitre et al., 1998).

Replication Reactions

Demembranated sperm nuclei were prepared and used as described in Menut et al. (1999), and erythrocyte nuclei were purified from *Xenopus* blood as described. Nuclei were incubated in S phase (1000 nuclei/μl) or M phase (CSF) extracts that were activated with 1 mM $CaCl_2$. DNA synthesis was measured by [^{32}P]αdCTP incorporation as previously described (Menut et al., 1999).

Purification and Analysis of Chromatin Fractions

Fifty microliter samples were diluted with 5 volumes of extract buffer (XB: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM KOH-HEPES [pH 7.7], 50 mM sucrose) and pelleted by centrifugation at 7500 × g for 12 min through a 0.7 M sucrose cushion. Nuclear pellets were resuspended in XB and 0.3% Triton X-100 and incubated for 5 min on ice. After a further 5000 × g centrifugation for 5 min, chromatin pellets were recovered and adjusted in Laemmli buffer.

Antibodies

Lamins were visualized with the 687A7 antibody (Firmbach-Kraft and Stick, 1995). The anti-RPA34-specific monoclonal antibody (324A.1) was used as described (Francon et al., 2004). The rabbit polyclonal antibody against Cdc6 was produced as described (Lemaitre et al., 2002). Antibodies against Cdt1 and MCM4 were obtained by four injections of corresponding recombinant proteins. Other antibodies were generous gifts from J. Walter (ORC2) and D. Bogenhagen (Topo II).

Immunocytochemistry

Extracts containing nuclei were diluted 10-fold in 100 mM KCl, 50 mM sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM HEPES (pH 7.6), and nuclei were purified through a 0.7 M sucrose cushion. Alternatively, samples were directly fixed with an equal volume of XB containing 4% formaldehyde and 1 μg/ml Hoechst 33258. Rehydration was done in PBS. Isolated nuclei were incubated for 1 hr at room temperature in PBS, 2% BSA, 0.1% Tween 20 to block nonspecific interactions. Incubation with specific antibodies was carried out overnight at 4°C in PBS, 2% BSA. After several washes, the second FITC-conjugated or Texas red-conjugated streptavidin was added following instructions from the manufacturers. To reveal biotin-dUTP, FITC- or Texas red-conjugated streptavidin was mixed with the second antibody at the appropriate dilution. DNA was stained with 1 μg/ml Hoechst 33258.

DNA Combing

Nuclei embedded in agarose plugs (800 ng DNA/plug) were stained with YOYO-1 (Molecular Probes) and resuspended in 50 mM MES (pH 5.7) (150 ng/ml) after digestion of the plugs with agarase (Roche). DNA combing was performed as described (Michalet et al., 1997). Combed DNA fibers were denatured for 30 min with 1 N NaOH, and BrdU was detected with a rat monoclonal antibody (Sera Lab) and a secondary antibody coupled to Alexa 488 (Molecular Probes). DNA molecules were counterstained as previously described (Versini et al., 2003) with an anti-guanosine antibody (Argene) and an anti-mouse IgG coupled to Alexa 546 (Molecular Probes). Center-to-center distances between BrdU tracks were measured with MetaMorph (Universal Imaging Corp.) using adenovirus DNA molecules as a size standard (1 pixel = 680 bp).

Loop-Size Measurement

Maximum fluorescent halo radius (MFHR) was determined by treating nonfixed nuclei on coverslips. They were first dipped for 1 min in ice-cold NP40 buffer (0.5% NP40, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 50 mM HEPES [pH 7.8]) and then sequentially dipped for 30 s in a solution

containing 0.2 mM $MgCl_2$, 10 mM Tris (pH 7.4) with 0.5 M, 1 M, 1.5 M, 2 M NaCl. They were then incubated in 100 μg/ml ethidium bromide, 2 M NaCl and exposed for 1 min to short-wave UV light before observation by fluorescence (Buongiorno-Nardelli et al., 1982). Halo and matrix diameters were estimated using a micrometrics slide. DNA loop size was calculated, taking into account that the loop size is twice the MFHR. The length of linear DNA was calculated using the correspondence of 1 μm to 2.3 kbp.

Analysis of DNA Loop-Attachment Sites

Nuclear matrices were prepared by treating isolated nuclei with restriction endonucleases or DNase I followed by extraction with either lithium 3,5-diiodosalicylate (LIS) or 2 M NaCl, essentially as described in Gasser and Laemmli (1986) and Vassetzky et al. (2000). Nuclei were digested with 100 μg/ml DNase I for 3 hr at 0ºC in 100 mM NaCl, 25 mM KCl, 10 mM Tris-HCl (pH 7.5), 0.25 mM spermidine, 1 mM $CaCl_2$. The digestion was followed by a stabilization step, the addition of $CuCl_2$ to a final concentration of 1 mM, and incubation for 10 min at 4ºC. The nuclei were extracted with 5 volumes of LIS extraction buffer containing 10 mM Tris-HCl (pH 7.5), 0.25 mM spermidine, 2 mM EDTA-KOH (pH 7.5), 0.1% digitonin, and 25 mM LIS for 5 min at room temperature. The histone-depleted nuclear matrices were recovered by centrifugation at 2500 × g for 20 min at room temperature, and the nuclear-matrix pellet was washed three times in 20 mM Tris-HCl (pH 7.5), 0.25 mM spermidine, 0.05 mM spermine, 100 mM NaCl, and 0.1% digitonin. The size range of the nuclear-matrix-attached DNA was 400–1500 bp.

Supplemental Data

Supplemental Data include Supplemental References and eight figures and can be found with this article online at http://www.cell.com/cgi/content/full/123/5/787/DC1/.

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