Analysis of mammalian origin specification in ORC-depleted *Xenopus* egg extracts

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Abstract

Background: ***Xenopus* egg extracts initiate replication specifically at the Chinese Hamster Ovary (CHO) cell dihydrofolate reductase (DHFR) origin with CHO G1-phase nuclei as a substrate, providing that these nuclei have intact nuclear envelopes and are isolated from cells that have passed through a distinct transition (origin decision point; ODP) early in G1-phase. With intact pre-ODP nuclei, or with post-ODP nuclei that have permeabilized nuclear envelopes, replication initiates efficiently but, at apparently random sites. We have investigated whether the *Xenopus* embryonic origin recognition complex (XORC) influences origin specification in this system.**

Results: ***Xenopus* egg extracts were immunodepleted of XORC, eliminating their ability to assemble pre-initiation complexes. These extracts were deficient in the replication of CHO metaphase chromosomes but supported efficient DNA replication within both pre- and post-ODP hamster G1-phase nuclei, even after permeabilization and extraction of soluble nuclear proteins. XORC-depleted extracts initiated replication specifically at the DHFR origin with intact post-ODP nuclei but still initiated at apparently random sites with intact pre-ODP nuclei or permeabilized post-ODP nuclei.**

Conclusions: ***Xenopus* embryonic ORC is clearly not required for random origin site selection in *Xenopus* egg extracts. We conclude that a modification of Chinese Hamster chromatin takes place shortly after metaphase that complements a lack of XORC activity. This modification most likely represents an interaction of mammalian ORC with chromatin that is required for replication but, that is not sufficient for origin specification.**

Introduction

In most double stranded DNA replication systems, the initiation step is regulated by the recognition of a specific replicator sequence (replication origin) by an initiator protein that facilitates the unwinding of adjacent DNA and recruits additional factors that are required for DNA synthesis (Stillman 1993; Baker & Bell 1998). In *Saccharomyces cerevisiae*, chromosomal origins consist of specific DNA sequences that serve as the binding sites for a complex of six proteins, termed the origin recognition complex (ORC). The ORC remains bound to the replicator throughout the cell cycle and serves as the 'landing pad' for essential initiation factors (e.g. Cdc6 and Mcm proteins) that associate with the ORC shortly after mitosis to form a pre-initiation complex (Aparicio *et al*. 1997; Stillman 1996; Dutta & Bell 1997; Tanaka *et al*. 1997; Gilbert 1998). Similar to previously characterized initiator proteins, the ORC contains a DNA-dependent ATPase activity that requires origin binding (Klemm *et al*. 1997). Furthermore, at least one *S. cerevisiae* origin initiates replication within an 18-bp region immediately adjacent to the ORC binding site (Bielinsky & Gerbi 1998). Taken together, these results strongly suggest that ORC is the eukaryotic initiator protein, directing the sites of initiation of replication by promoting a local distortion of the DNA double helix.

Although ORC has been shown to have highly conserved counterparts in a variety of species, from *S. pombe* to humans (Gavin *et al*. 1995), its role in the replication of metazoan genomes is poorly understood,

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largely because metazoan replicator sequences have not been identified (Gilbert 1998). In mammalian cells, replication initiates within defined segments of chromosomes (initiation loci). Some of these initiation loci have been mapped to within several kb (Aladjem *et al*. 1995; Kumar *et al*. 1996). Other initiation loci appear to encompass several tens of kb, within which replication initiates at multiple, closely spaced sites (Kalejta *et al*. 1996; Kobayashi *et al*. 1998; Wang *et al*. 1998). Despite the complexity of mammalian origins, the prevailing hypothesis is that a specific ORC–replicator interaction will eventually be discovered (Dutta & Bell 1997; Baker & Bell 1998). A major challenge to the replicator:initiator paradigm in metazoa is the observation that replication begins within random DNA sequences in *Xenopus* and *Drosophila* preblastula embryos and their cell-free extracts (Hyrien *et al*. 1995; Mahbubani *et al*. 1992; Hyrien & Mechali 1993; Shinomiya & Ina 1993). Nonetheless, the immunodepletion of *Xenopus* ORC (XORC) from *Xenopus* egg extracts has been shown to prevent the ability of these extracts to load *Xenopus* Cdc6 and Mcm proteins on to *Xenopus* sperm chromatin and to inhibit the initiation of DNA replication (Carpenter *et al*. 1996; Coleman *et al*. 1996; Romanowski *et al*. 1996; Rowles *et al*. 1996; Walter & Newport 1997). One hypothesis to account for these observations is that high concentrations of XORC in embryos may allow lower affinity nonspecific DNA binding, or that *Xenopus* embryos may express a unique form of ORC that is capable of binding to many DNA sequences. Obviously, the extent to which a specific ORC–origin interaction dictates the sites of initiation in metazoa is critical if we are to understand the regulation of metazoan replication.

Recently, it was shown that *Xenopus* egg extracts can initiate replication specifically at the Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR) origin locus, providing the substrate is introduced in the form of intact nuclei prepared from cells that have passed through a distinct G1-phase regulatory point—the Origin Decision Point (ODP) (Gilbert *et al*. 1995; Lawlis *et al*. 1996; Wu & Gilbert 1996; Wu & Gilbert 1997; Wu *et al*. 1997; Dimitrova & Gilbert 1998; Wu *et al*. 1998). With either intact pre-ODP nuclei or post-ODP nuclei that have damaged nuclear envelopes, replication initiates within apparently random DNA sequences (Dimitrova & Gilbert 1998; Gilbert *et al*. 1995). The ability to elicit different patterns of initiation by manipulating the substrate, coupled with an ability to immunodeplete XORC proteins from *Xenopus* egg extracts, provided the opportunity of evaluating the role of *Xenopus* embryonic ORC in establishing the pattern of initiation of replication within CHO nuclei. We found that intact pre- and post-ODP nuclei, as well as permeabilized post-ODP nuclei replicated efficiently in ORC-depleted extracts. By contrast, CHO metaphase chromosomes did not bind Mcm proteins and replicated poorly in these same extracts. Thus, an XORC-complementing activity associates with CHO cell chromatin after metaphase but prior to the ODP. Furthermore, the pattern of initiation sites within pre- and post-ODP nuclei was not influenced by the absence of XORC. The simplest interpretation of these results is that mammalian ORC, like *Xenopus* ORC, can direct the initiation of replication to many different chromosomal sites. Additional events taking place in CHO nuclei at the ODP are required to focus initiation to specific replicator sequences.

Results

An XORC-complementing activity associates with Chinese hamster chromatin shortly after metaphase

Xenopus egg extracts were immunodepleted of >99% of the 63 kd subunit of ORC, XORC2, by incubation with protein A beads coated with affinity purified anti-XORC2 antibodies (Fig. 1). Control ('Mock') depletions were carried out with nonimmune rabbit IgG bound to protein A beads. Immunodepletion of XORC2 also removed >95% of the 115 kDa subunit XORC1 from the extract (Fig. 1A,B), consistent with the previous finding that XORC2 exists as a complex with other members of the ORC (Romanowski *et al*. 1996; Rowles *et al*. 1996). Also consistent with previous results (Carpenter *et al*. 1996; Coleman *et al*. 1996; Romanowski *et al*. 1996; Rowles *et al*. 1996; Walter & Newport 1997), the immunodepletion of XORC complexes from *Xenopus* egg extracts prevented the assembly of pre-initiation complex protein XMcm3 on to chromatin (Fig. 1C), and severely impaired the ability of these extracts to support the replication of *Xenopus* sperm chromatin (Fig. 2A).

Immunofluorescence studies of XORC localization in *Xenopus* egg extracts (Coleman *et al*. 1996), *Xenopus* somatic tissue culture cells (Romanowski *et al*. 1996), and human HeLa cells (P. Romanowski, personal communication), have indicated that ORC is displaced from chromatin, uniquely during metaphase. Similar studies in *Drosophila* Schneider L2 cells were consistent with a de-stabilization of the ORC–chromatin interaction during metaphase (Pak *et al*. 1997). Hence,

we reasoned that replication of CHO metaphase chromosomes in *Xenopus* egg extracts might require the presence of XORC. CHO (CHOC 400) cells were synchronized in metaphase to near homogeneity (> 95%) by selective detachment after brief nocodozole exposure (Wu *et al*. 1997). These cells were permeabilized with digitonin and the soluble proteins were washed away. These permeabilized cells were introduced into Mock- and XORC-depleted *Xenopus* egg extracts, where CHO metaphase chromosomes were decondensed and assembled into nuclei by the extract, as previously described (Lawlis *et al*. 1996). As shown in Fig. 1C, XMcm3 was efficiently loaded on to CHO metaphase chromatin in Mock depleted extracts, but not in XORC-depleted extracts. The percentage of input genomic DNA replicated was then evaluated by acid precipitation. As shown in Fig. 2B, the ability of ORC-depleted extracts to replicate CHO metaphase chromosomes was severely reduced as compared to mock-depleted extracts. Thus, CHO metaphase chromosomes, like *Xenopus* sperm chromatin, require XORC to assemble pre-initiation complexes and replicate in *Xenopus* egg extract.

Previous experiments have shown that permeabilized HeLa G1-phase nuclei can replicate in XORC-depleted extracts (Romanowski *et al*. 1996). However, the efficiency of this XORC-independent replication relative to mock depleted extracts was not investigated, nor was there a means to determine whether these nuclei were in the pre- or post-ODP stage of G1-phase. To determine whether pre- and post-ODP CHOC 400 nuclei are competent for DNA replication in XORC-depleted extracts, metaphase synchronized CHOC 400 cells were released into G1-phase for either 1.5 h (pre-ODP) or 5 h (post-ODP). Intact nuclei were prepared from these cells by digitonin permeabilization and introduced into mock-and XORC-depleted extracts. To verify that these nuclei did not accumulate significant amounts of the residual (< 1%) XORC2 remaining in depleted extracts, aliquots of these nuclei were removed, washed, and the presence of XORC2 was detected by Western blotting. No detectable XORC2 was found within the nuclei incubated in XORC-depleted extracts, whereas XORC2 was efficiently imported into mammalian nuclei in mock--depleted extracts (Fig. 1D). The percentage of input genomic DNA replicated was then evaluated by acid precipitation, as in Fig. 2A,B. As seen in Fig. 2C,D, the absence of XORC had little or no effect on the efficiency of replication within either intact pre- or post-ODP nuclei.

To specifically compare the relative amounts of semi-conservative DNA replication observed in mock-and XORC-depleted extracts, density substitution experiments were performed. The four substrates described in Fig. 2A–D were incubated for 2 h in mock- and XORC-depleted extracts supplemented with 5′-bromodeoxyuridine (BrdU) triphosphate and [α-^{32}P]dATP. DNA from equal numbers of nuclei was then purified and subjected to neutral pH density gradient centrifugation to separate BrdU substituted (HL) from unsubstituted (LL) DNA. Results (Fig. 2E–H) revealed that the total amount of HL DNA synthesized in XORC-depleted extracts with sperm and CHO metaphase chromatin as a substrate was a small fraction of that synthesized in mock--depleted extracts, even less than that calculated from acid precipitation results. By contrast, the amount of HL DNA synthesized with pre- and post-ODP nuclei was nearly equal in mock- vs. ORC-depleted extracts. Thus, pre-ODP nuclei have already completed a step that can fully complement the lack of XORC.

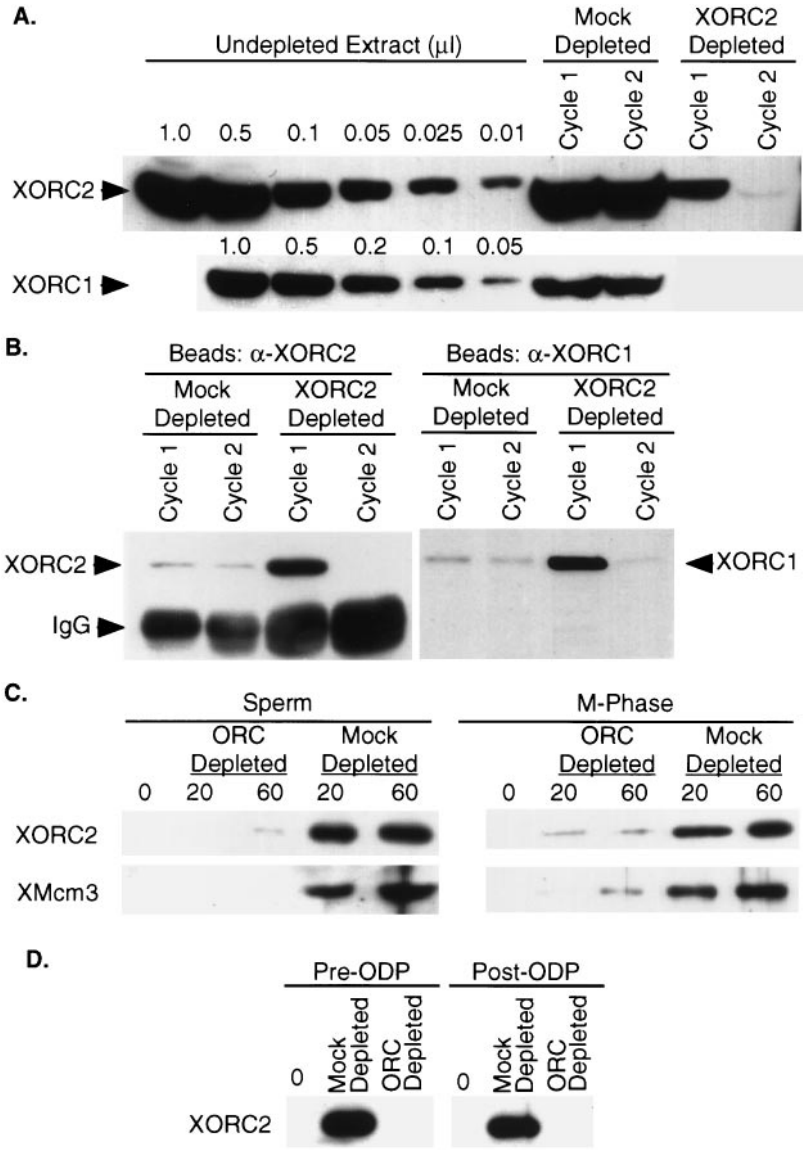
The results shown in Fig. 2 suggest that an XORC-complementing activity is present within CHO G1-phase nuclei prior to the ODP or that this activity has modified CHO chromatin in such a way that it no longer requires XORC activity. To address whether the mammalian ORC-activity is a stable property of pre-ODP chromatin, or whether it represents a soluble activity within pre-ODP nuclei, soluble nuclear proteins were extracted from pre- and post-ODP nuclei by prolonged exposure of these nuclei to high concentrations of digitonin (Wu & Gilbert 1997; Wu *et al*. 1997; Dimitrova & Gilbert 1998). In Fig. 3A & B, the efficiency of replication of permeabilized nuclei was evaluated in XORC- and mock-depleted extracts. Consistent with previous results in undepleted extracts (Dimitrova & Gilbert 1998), mock-depleted extracts replicated DNA within digitonin permeabilized nuclei as efficiently as intact nuclei (compare to Fig. 2). XORC-depleted extracts supported $\approx$ 70% of the replication observed in mock-depleted extracts. Importantly, this moderate reduction in replication rate in XORC-depleted extracts was similar with both pre- and post-ODP nuclei.

Figure 3C & D summarizes the dependence of various substrates on XORC proteins for replication in *Xenopus* egg extracts. In Fig. 3C the maximum rate of replication (percentage of input DNA replicated per minute from 60 to 120 min) was calculated from the data shown in Fig. 2 and Fig. 3A & B. Figure 3D expresses the relative rates of replication in ORC- vs. mock-depleted extracts. Both *Xenopus* sperm chromatin

and CHO metaphase chromosomes required the presence of XORC for efficient replication, whereas the initiation of replication within pre-formed mammalian nuclei was largely independent of *Xenopus* ORC proteins. These results indicate that a mammalian activity stably modifies chromatin prior to the ODP such that it can be replicated in *Xenopus* extracts lacking XORC activity. This activity may be a modification of ORC's association with chromatin that has been shown to occur late in mitosis in *Xenopus* (Romanowski *et al*. 1996), Drosophila (Pak *et al*. 1997), and human cells (P. Romanowski, personal communication).

XORC-depleted extracts initiate replication within pre-ODP nuclei without preference for the DHFR origin locus

As discussed above (see Introduction), random origin selection in *Xenopus* egg extracts could result from either high concentrations of XORC or a unique form of ORC present only in rapid cleavage embryos. Alternatively, mammalian ORC and *Xenopus* embryonic ORC may perform analogous roles in both systems, being required for the assembly of pre-initiation complexes but not sufficient to specify particular origin sequences. Since pre- and post-ODP



nuclei replicate efficiently in XORC-depleted extracts (Fig. 2), we were in an excellent position to distinguish between these two possibilities. If XORC proteins are responsible for the random initiation pattern within pre-ODP nuclei, then their removal, forcing initiation to rely on hamster ORC, should result in a more specific initiation pattern. Similarly, if XORC contributes a certain level of nonspecific initiation in the replication of post-ODP nuclei, then the removal of XORC2 should increase the preference for the DHFR origin locus over and above that which is observed in mock-depleted extracts.

To address this question, replication initiation sites were mapped at the DHFR locus when pre- and post-ODP nuclei were introduced into mock- and ORC-depleted extracts. Since origin mapping experiments are typically performed at 25 000 nuclei/μL extract (origin-mapping at the concentration of nuclei shown in Fig. 2 would require prohibitively large amounts of ORC-depleted extract), we first examined the efficiency of DNA replication with nuclei at this concentration (Fig. 4A,B). Consistent with previous results in undepleted extracts (Dimitrova & Gilbert 1998), replication in mock-depleted extracts was less efficient at higher concentrations of nuclei but, was still similar in both pre- and post-ODP nuclei. A modest reduction in replication rate was observed in ORC- vs. Mock-depleted extracts. This difference could either be due to a contribution of XORC to the replication of nuclei at this high concentration, or to some other replication factor that partially co-depletes with ORC and becomes limiting at high concentrations of nuclei. In any case, the replication of both pre- and post-ODP nuclei were equally effected by the absence of XORC, and replication proceeded at 60–70% of the rate in mock-depleted extracts, which was sufficient for origin mapping.

The sites of initiation of replication were then evaluated using the early labelled fragment hybridization (ELFH) assay (Fig. 4C & D). This assay evaluates the genomic positions of the earliest nascent DNA strands to be synthesized (Gilbert *et al*. 1995; Wu *et al*. 1997). Nuclei were incubated in extract supplemented with aphidicolin for 90 min. Aphidicolin blocks the processive elongation of replication forks, allowing replication bubbles to accumulate with forks arrested close to their sites of initiation (Gilbert *et al*. 1995). Aphidicolin was then washed away and nascent replication forks were briefly labelled with [α-^{32}P]dATP. The genomic distribution of these short, pulse labelled nascent strands was determined by hybridizing them to 18 unique DNA sequences derived from specific positions encompassing 120 kb of the DHFR locus. In Fig. 4C & D, the relative amounts of hybridization of these labelled strands are plotted vs. the map position of each DNA segment, giving a distribution of the relative frequency at which replication initiated near each probe. The results revealed that the specificity of initiation at the DHFR locus was unaltered by the absence of XORC2 in the extract. We conclude that an apparently random initiation of replication within pre-ODP nuclei does not require the *Xenopus* embryonic ORC but is most likely mediated by mammalian ORC.

Figure 1 Immunodepletion of XORC complexes from *Xenopus* egg extract. (A) *Xenopus* egg extracts were incubated with affinity purified polyclonal anti-XORC2 (XORC2 depletion) or Rabbit IgG (mock depletion) coated protein A Sepharose beads as described in Experimental procedures. XORC2-bound beads were removed and the resulting depleted extract ('Cycle 1') was subjected to a second round of depletion ('Cycle 2'). 1 μL of extract from each round of depletion was then analysed by Western blotting using an affinity purified polyclonal anti-XORC2 antibody (top panel). To assess the extent of depletion, serial dilutions of undepleted extract were included in the same gel. In parallel, aliquots of the same samples were immunoblotted against a monoclonal anti-ORC1 antibody to determine the extent of co-depletion of ORC subunits. In some depletions, detectable XORC1 (< 5%) remained after XORC2 depletion. Also, in some depletions, very little XORC2 was detectable after the first round of depletion. (B) Proteins were stripped from the beads after each round of the mock and XORC2 depletions and subjected to immunoblotting with either anti-XORC2 or anti-XORC1 antibodies. Since the amount of anti-XORC2 bound to the filter was revealed with an anti-rabbit IgG antibody, IgG from both mock and ORC-coated beads is detected; anti-ORC1 is revealed with an anti-mouse IgG antibody. Detection of XORC2 on beads after the second round of depletion varied depending upon the amount of XORC2 remaining after the first round of depletion. The small amount of XORC1 and XORC2 present in the mock bead preparation was also variable and is probably due to a small amount of extract that carries over with the washed beads. (C) Either digitonin permeabilized CHOC 400 metaphase cells (M-Phase) or *Xenopus* sperm chromatin (Sperm) were incubated at 1000 nuclei/μL in either ORC-depleted or mock-depleted *Xenopus* egg extract. After 0, 20 or 60 min, the chromatin was isolated and washed as described in Experimental procedures and then subjected to immunoblotting with antibodies specific for XORC2 or XMcm3. (D) Intact pre-and post-ODP nuclei were incubated in either mock-or ORC-depleted extracts. After 60 min, nuclei were washed and subjected to immunoblotting with anti-XORC2 antibody. In (C) and (D), 0 min indicates that no extract was added.

XORC-depleted extracts initiate replication within permeabilized post-ODP nuclei without preference for the DHFR origin locus

Permeabilization of post-ODP nuclei, prior to their introduction into *Xenopus* egg extract, prevents the ability of S-phase promoting factors in *Xenopus* egg extract from recognizing the DHFR origin (Gilbert *et al*. 1995; Dimitrova & Gilbert 1998). Since permeabilized nuclei replicate efficiently in ORC-depleted extracts (Fig. 3), it did not seem likely that the loss of origin specificity was due to a complete removal of soluble mammalian ORC. However, the presence of XORC accounted for ≈30% of the replication observed in mock depleted extracts. Hence, it remained possible that the permeabilization of nuclei in some way allows for the contribution of XORC to the observed pattern of replication. To evaluate the contribution of XORC to the pattern of initiation within permeabilized post-ODP nuclei, CHOC 400 cells were synchronized in the post-ODP stage of G1-phase and permeabilized nuclei prepared from these cells were introduced into *Xenopus* egg extracts at 25 000/μL (Fig. 5A). In mock-depleted extracts, permeabilized nuclei replicated at a similar rate as did intact nuclei at the same concentration (compare to Fig. 4). When these same nuclei were introduced into ORC-depleted extracts, replication proceeded at ≈50% of the rate observed in mock-depleted extracts. This loss of replicative capacity is slightly greater than that observed after either the 25-fold increase in concentration of nuclei (Fig. 4) or the permeabilization of nuclei (Fig. 3) alone, and probably represents an additive effect of these two substrate manipulations. Again, we cannot determine whether this results from a requirement for XORC, or for some other component that is partially co-depleted with XORC. Nonetheless, sufficient replication was observed to map the sites of initiation of replication at the DHFR locus (Fig. 5B). CHOC 400 cells were synchronized in the post-ODP stages of the G1-phase. Intact nuclei were prepared from an aliquot of these cells and introduced into undepleted *Xenopus*

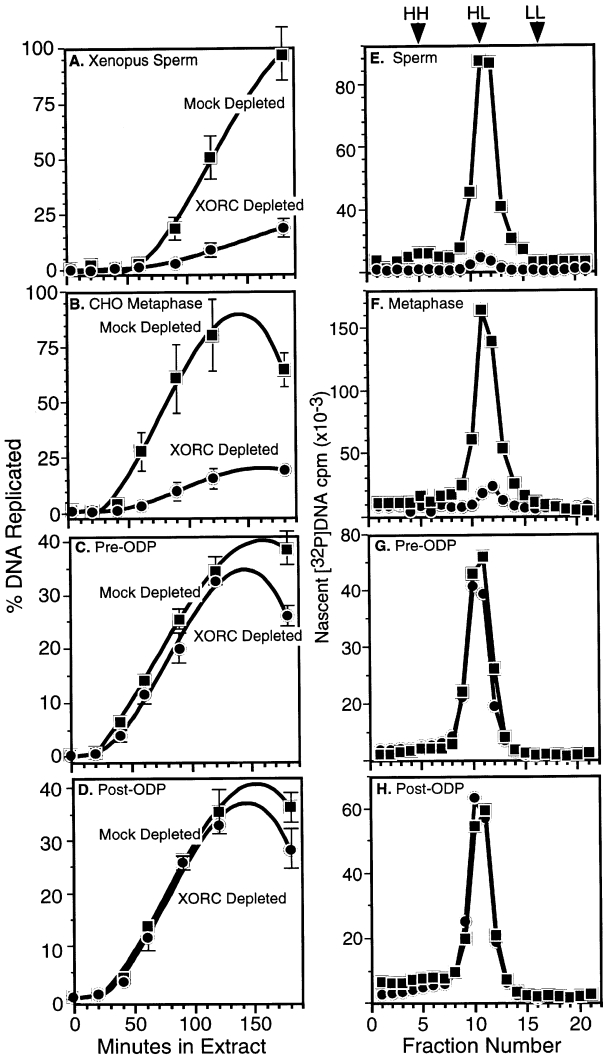


Figure 2 An XORC-complementing activity appears between metaphase and early G1-phase in CHOC 400 cells. Panels A–D: Demembranated *Xenopus* sperm nuclei (A) or digitonin permeabilized CHOC 400 cells synchronized in (B) Metaphase or (C) 1.5 h (pre-ODP) and (D) 5 h (post-ODP) thereafter were introduced into XORC- (●) or mock- (■) depleted *Xenopus* egg extracts supplemented with [α-^{32}P]dATP at 1000 nuclei/μL. At the indicated times, aliquots were removed and the percentage of input DNA replicated (e.g. 100% DNA synthesis would indicate one complete round of replication) was determined by acid precipitation, as described (Wu *et al*. 1997). Shown are the mean values for three experiments performed with independent preparations of extract and the standard error of the mean (± SEM). Panels E–F: Nuclei prepared as in A–D were incubated in a *Xenopus* egg extract supplemented with [α-^{32}P]dATP and BrdUTP for 2 h. Genomic DNA was isolated and subject to neutral Cs_2SO_4 density gradient centrifugation. Shown are the total c.p.m. present in each gradient fraction after incubation in XORC- (●) or mock-(■) depleted extracts. The relative amounts of semiconservative DNA synthesis in ORC-vs. Mock-depleted extracts (total c.p.m. in the four peak HL fractions for ORC/mock) for each sample are as follows: (E) Sperm, 6.6%; (F) metaphase, 15.7%; (G) Pre-ODP, 83%; (H) Post-ODP, 106%. The positions of the peaks for marker DNA centrifuged to equilibrium in a separate gradient in the same experiment are indicated at the top of the figure with arrowheads. Similar results were obtained in four independent experiments with separate extracts.

egg extracts to verify that these nuclei were derived from cells that had passed through the ODP transition and were capable of eliciting origin-specific initiation of replication. Permeabilized nuclei prepared from these same cells were introduced into mock- and XORC-depleted extracts. The results (Fig. 5B) revealed that replication initiated at apparently random sites, regardless of the presence or absence of XORC proteins. Thus, XORC does not contribute to the loss of origin specification that is observed when post-ODP nuclei are permeabilized. Rather, the act of permeabilization of post-ODP nuclei appears to disrupt some component of the nucleus that is required for origin specification without completely disrupting the competence of nuclei to initiate replication in the absence of XORC.

Discussion

The results reported here demonstrate that the replication initiation sites observed when G1-phase CHO nuclei are incubated in *Xenopus* egg extracts are independent of the ability of these extracts to assemble *de novo* pre-initiation complexes. With either intact or permeabilized G1-phase nuclei as a substrate, both the replication rate and the length of the lag period prior to the initiation of replication were nearly identical in XORC- and Mock-depleted extracts. Since the loading of Mcm on to metaphase chromatin, and the efficient replication of CHO metaphase chromosomes required XORC, these data strongly suggest that an interaction of mammalian ORC with chromatin shortly after metaphase complements the lack of

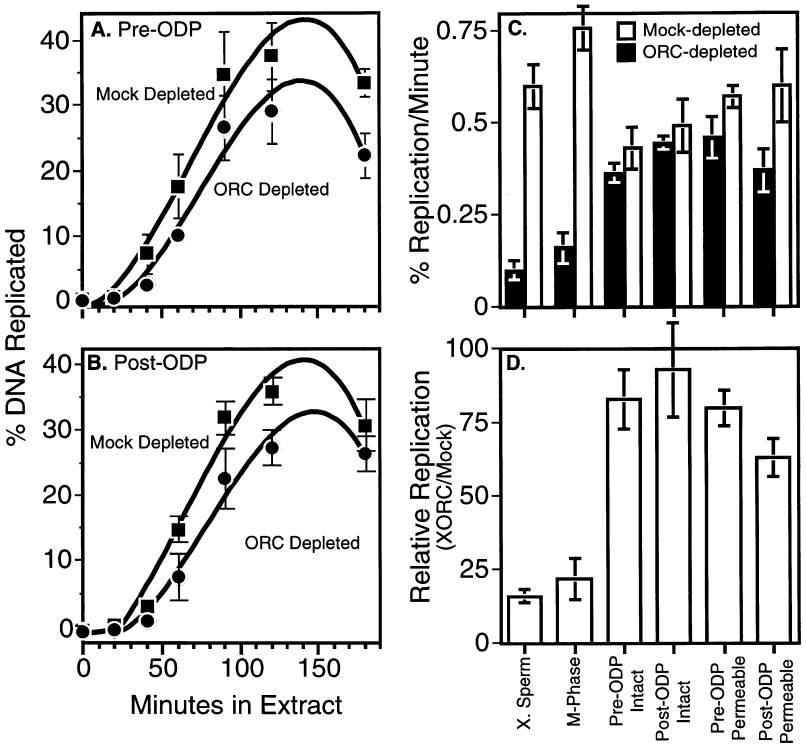


Figure 3 CHOC 400 chromatin is stably modified prior to the ODP. Panels A and B: CHOC 400 cells were synchronized in the pre-ODP (A) or post-ODP (B) stages of G1-phase and permeabilized nuclei were prepared as described in Experimental procedures. Permeabilized nuclei were introduced into XORC- (●) or mock- (■) depleted *Xenopus* egg extracts supplemented with [α-^{32}P]dATP at 1000 nuclei/μL and the percentage of input DNA replicated at the indicated times was determined as described in Fig. 2. Shown are the mean values for three experiments performed with independent preparations of extract ± SEM. (C) The percentage of input DNA replicated per minute between 60 and 120 min (period of peak DNA synthesis) was calculated from the data shown in previous figures for ORC- (filled bars) and mock- (open bars) depleted extracts. Shown are the mean of three independent batches of extract ± SEM. (D) The relative efficiencies of genomic DNA replication in ORC-vs. Mock-depleted extracts are expressed as a percentage of the replication rate ORC/mock. As can be seen, the values for sperm and metaphase chromatin in D are greater than those calculated from comparing the amounts of semiconservative DNA replication by density substitution (see Fig. 2) and therefore may overestimate the percentage of DNA replicated in ORC depleted extracts with these substrates.

XORC in *Xenopus* egg extracts. Since we have found that hamster Mcm proteins are also loaded on to chromatin during late mitosis (Dimitrova & Gilbert, submitted) it is likely that the replication of CHO nuclei in *Xenopus* egg extracts is mediated primarily by hamster pre-initiation complexes, with the extract simply providing a rich source of S-phase promoting factors. Furthermore, XORC was not required to establish either the random initiation pattern observed with intact pre-ODP and permeabilized post-ODP nuclei or the origin-specific initiation pattern observed with intact post-ODP nuclei. The simplest interpretation of these results is that the early G1-phase form of mammalian ORC can promote the initiation of DNA replication at many chromosomal sites. However, modifications taking place within CHO nuclei at the ODP, after the formation of pre-initiation complexes, regulate which of these sites will function as replication origins in the upcoming S-phase.

Our results are consistent with the recently reported finding that CHO cells can initiate replication at random sites in culture (Wu *et al*. 1998; Dimitrova & Gilbert, unpublished observations). Treatment of CHOC 400 cells shortly after mitosis with a protein kinase inhibitor was shown to arrest cells prior to the ODP. Transformation of these cells with SV40 abrogated this early G1-phase arrest point, resulting in

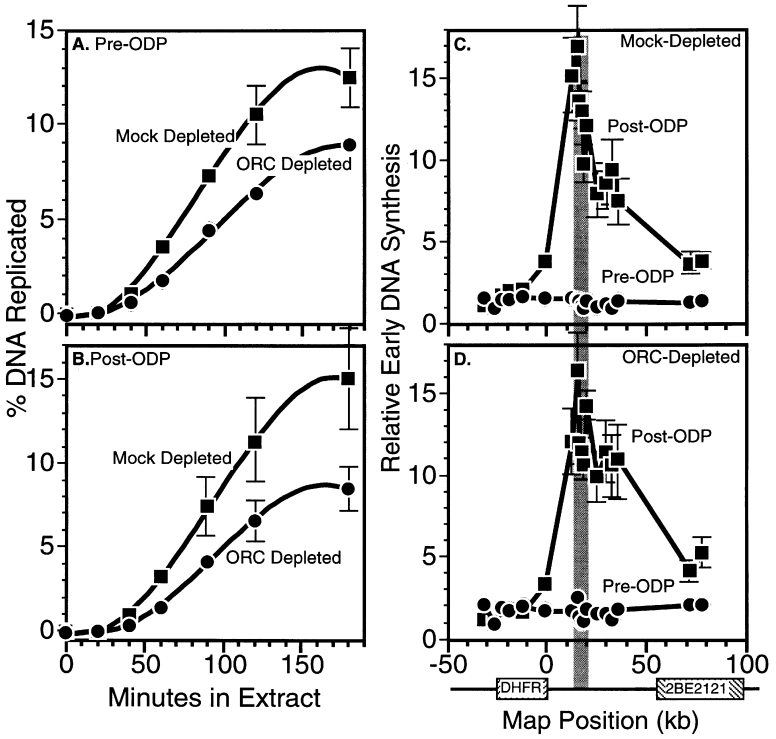


Figure 4 XORC does not influence the pattern of initiation within intact pre- and post-ODP nuclei. Panels A and B: CHOC 400 cells were synchronized in the pre-ODP (A) or post-ODP (B) stages of G1-phase metaphase. Intact nuclei were prepared and introduced into XORC2- (●) or mock-(■) depleted *Xenopus* egg extract supplemented with [α^{32}P]-dATP at 25 000 nuclei/μL, and the percentage of input DNA replicated was determined at the indicated times by acid precipitation, as in Fig. 2. Panels C and D: Intact nuclei from A and B were incubated at 25 000 nuclei/μL for 1.5 h in either XORC2- (●) or mock- (■) depleted *Xenopus* egg extract supplemented with aphidicolin. Nuclei were then washed free of aphidicolin and the earliest replicating nascent DNA chains were labelled briefly with [α-^{32}P]dATP, purified and hybridized to 18 unique probes distributed over a 120-kb region that includes the DHFR ori-β. The relative amounts of hybridization of these earliest labelled nascent DNA strands to each unique probe from the DHFR locus were obtained by phosphorimaging analysis and plotted vs. the map positions of each segment. The horizontal axis includes a diagram of the portion of the DHFR locus encompassed by these probes, including the positions of the DHFR and 2BE2121 genes (Hamlin & Dijkwel 1995). The vertical shaded line highlights the region of peak DNA synthesis described by other mapping techniques and encompasses at least two preferred sites of initiation (Kobayashi *et al*. 1998). Since the data are expressed as relative DNA synthesis values, it is the differences between the probes, not the area under the curve, that should be compared. Shown are the mean values for three experiments performed with independent preparations of extract ± SEM.

the entry of cells into S-phase in the presence of the kinase inhibitor and initiation of replication without specification of the DHFR origin. Since these prior experiments were performed in cultured cells, they also support the conclusion that mammalian ORC can mediate the initiation of replication at both origin and nonorigin sites. Hence, it is unnecessary to evoke the existence of a specialized *Xenopus* embryonic ORC to account for the random initiation pattern observed during the early stages of *Xenopus* development. It is also unlikely that the high concentration of XORC proteins in *Xenopus* embryos relaxes the sequence requirements for XORC binding. To the contrary, the results reported here suggest that an apparently random choice of initiation sites can take place in the absence of embryonic XORC, mediated by mammalian ORC present at physiological concentrations within intact pre-ODP CHO nuclei.

Our data suggest that hamster ORC is either displaced from chromatin or inactivated during metaphase. Metaphase chromatin could not recruit the binding of XMcm proteins and was replicated poorly when introduced into XORC depleted extracts (Fig. 1 and 2). Previous immunofluorescence data indicate that ORC is displaced from chromatin during metaphase in *Xenopus* egg extracts (Coleman *et al*. 1996), *Xenopus* tissue culture cells (Romanowski *et al*. 1996) and human HeLa cells (P. Romanowski, personal communication). However, others have reported that binding of *Drosophilia* ORC to metaphase chromatin can be observed using certain fixation conditions (Pak *et al*. 1997). All groups agree that ORC undergoes a modification shortly after metaphase that stabilizes its association with chromatin. Our data support and extend this general conclusion, demonstrating that, if mammalian ORC is bound to CHO metaphase chromatin, it is not active for eliciting the assembly of pre-initiation complexes but, acquires this activity shortly after metaphase.

With regard to the role of ORC in the specification of metazoan origins, the possibilities depend upon several as yet unconfirmed assumptions. The first of these assumptions is that ORC is directly required for DNA replication in metazoa. The observation that XORC-depleted extracts are deficient in the replication of *Xenopus* sperm chromatin (Carpenter *et al*. 1996; Coleman *et al*. 1996; Romanowski *et al*. 1996; Rowles *et al*. 1996; Walter & Newport 1997) and CHO metaphase chromatin (this report) does not prove a direct role for ORC in DNA replication. For example, *Xenopus* extracts depleted of lamin B3 proteins and nuclear pore protein p97 are also deficient in replication (Jenkins *et al*. 1993; Goldberg *et al*. 1995; Powers *et al*. 1995; Spann *et al*. 1997) even though, under the

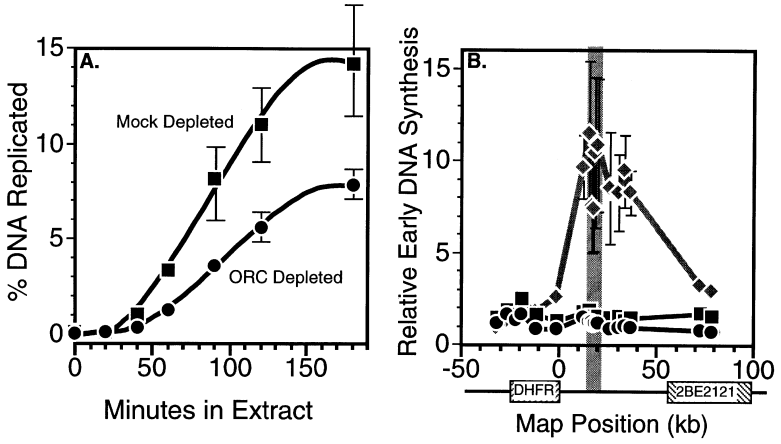


Figure 5 Loss of origin recognition upon nuclear envelope permeabilization is independent of XORC proteins. (A) CHOC 400 cells were synchronized in metaphase and released into G1-phase for 5 h (post-ODP) and digitonin permeabilized nuclei were prepared as in Fig. 3. Nuclei were introduced into either XORC2- (●) or mock- (■) depleted *Xenopus* egg extract supplemented with [α^{32}P]-dATP at 25 000 nuclei/μL, and the percentage of input DNA replicated was determined at the indicated times by acid precipitation, as in Figs 2–4. Shown are the mean values for three experiments performed with independent preparations of extract ± SEM. (B) Permeabilized post-ODP nuclei were incubated at 25 000 nuclei/μL for 1.5 h in either XORC2- (●) or mock- (■) depleted *Xenopus* egg extract supplemented with aphidicolin and the sites of initiation of replication at the DHFR locus were evaluated using the ELFH assay, as in Fig. 4. To verify that these nuclei were derived from post-ODP cells, intact nuclei prepared from the same cells were incubated in an undepleted extract, and the sites of initiation of replication were evaluated in parallel (◆). Shown are the mean values for two independent experiments ± SEM.

appropriate experimental conditions, replication in *Xenopus* egg extracts can proceed efficiently in the complete absence of a nuclear envelope (Walter *et al*. 1998). Furthermore, recent data suggest that ORC does not actually participate in the initiation step but that it is dispensable for replication after the assembly of Mcm proteins on to chromatin (Hua & Newport 1998; J. Blow, University of Dundee, personal communication). Finally, it is important to consider genetic and biochemical evidence for a role of ORC in silencing (Foss *et al*. 1993) and position effects (Pak *et al*. 1997; Huang *et al*. 1998), and that metazoan ORC complements the silencing but not the replication defect of *Saccharomyces cerevisiae* ORC mutants (Ehrenhofer-Murray *et al*. 1995). Thus, it remains a formal possibility that ORC is indirectly required for metazoan replication. Nevertheless, considering the compelling evidence for ORC as an initiator in *S. cerevisiae*, the remarkable conservation of ORC, Cdc6 and Mcm proteins, and the fact that the ordered assembly of pre-initiation complex proteins on to chromatin (ORC, cdc6, Mcm) is conserved from yeast to *Xenopus* (Newlon 1997), it seems likely that metazoan ORC plays a direct role in the assembly of pre-initiation complexes.

If we retain the assumption that replication initiation sites are dictated by ORC, how then can we explain its ability to elicit initiation at apparently random sites in pre-ODP nuclei, permeabilized post-ODP nuclei and in rapid early cleavage embryos such as *Xenopus* and *Drosophila*? Two hypotheses seem most plausible. One possibility is that the binding of ORC to specific sequences is delayed until after the ODP, and that what has been observed as association with pre-ODP chromatin is not actually sequence-specific DNA binding. A testable prediction from this model is that ORC must experience a modification at the ODP that increases it's affinity for specific DNA sequences. This modification should be reversible by nuclear permeabilization. A second possibility is that metazoan ORC binds to many DNA sequences and that some of these sites are inactivated at the ODP, potentially by changes in chromosome architecture that present only a subset of these sites to S-phase promoting factors (Lawlis *et al*. 1996). This model predicts that there should be many more DNA bound ORC molecules than there are origins. In either case, a single modification in the behaviour of metazoan vs. *S. cerevisiae* ORC, allowing for the binding of functional metazoan ORC to many DNA sequences, has the potential to reconcile otherwise paradoxical differences between these systems.

Experimental procedures

Cell culture and synchrony

CHOC 400 is a CHO cell derivative in which a 243-kb segment of DNA containing the DHFR gene has been amplified ≈ 500-fold by step-wise selection in methotrexate (Hamlin *et al*. 1994). Methods for the culture and mitotic synchronization of CHOC 400 cells have been described in detail (Wu *et al*. 1997).

Antibody production and immunodepletion

Rabbit polyclonal antibodies were raised against a six histidine-tagged XORC2 cDNA purified from baculovirus infected Sf9 insect cells as described (Carpenter *et al*. 1996). Anti-XORC2 antibodies were then affinity purified with bacterially expressed six histidine-XORC2 (baculovirus and pET9 bacterial expression vectors were both obtained from P. Carpenter and W. Dunphy), as described (Carpenter *et al*. 1996). This purified antibody preparation recognized a single polypeptide with an apparent molecular weight of 63 kDa in *Xenopus* egg extracts but does not recognize any polypeptide in extracts made from CHO cells.

For immunodepletions, Protein A Sepharose CL-4B (Pharmacia Biotech, 17-0780-01) was swelled in PBS overnight at 4 °C. Sepharose beads were then washed five times in PBS. 200 μg (as quantified by Bradford assay; Bradford 1976) of affinity purified XORC2 antibody or control rabbit IgG (Sigma) was mixed with 100 μL beads for 2 h at room temperature on a rotator. Sepharose resin was washed five times with 10 volumes of Immunodepletion Buffer (10 mM HEPES-KOH, pH = 7.5, 2.5 mM magnesium acetate, 50 mM potassium acetate, 250 mM sucrose, 1 mM DTT, 1 μg/mL each of leupeptin, chymostatin, pepstatin A and aprotinin) and as much immunodepletion buffer as possible was removed. Half the beads were added to 4 volumes of *Xenopus* egg extract, prepared as described (Wu *et al*. 1997) and supplemented with 250 μg/mL cycloheximide. After mixing for 45 min at 0 °C on a rotator, beads were removed by centrifuging the extract through a 20-μM spectra/mesh filter (Spectrum, Inc.) for 1 min at 16 000 *g* and 4 °C. Extract was then subjected to a second cycle of depletion with the remaining half of the anti-XORC2 coupled beads.

Evaluation of chromatin binding

Chromatin was isolated essentially as described (Rowles *et al*. 1996). Briefly, digitonin permeabilized CHOC 400 metaphase cells or *Xenopus* sperm chromatin were incubated at 1000/μL *Xenopus* egg extract. At various time points, 10 μL of extract was removed and diluted with 1 mL of NIB (50 mM KCl, 50 mM HEPES-KOH [pH 7.6], 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.5 mM spermine 4HCl, 0.15 mM spermidine 3HCl, 1 μg/mL each of leupeptin, pepstatin and aprotinin) supplemented with 0.1% NP40. This solution was underlaid with a 100-μL cushion of 15% sucrose in NIB and centrifuged at 6000 *g* in a swinging bucket rotor for 5 min at 4 °C. Pelleted chromatin was washed in

1 mL of NIB and centrifuged through a sucrose cushion a second time. Finally, the washed chromatin pellet was resuspended in 20 μL of 1 × SDS sample loading buffer and analysed by immunoblotting. To examine the nuclear import of XORC2, nuclei were incubated in mock or XORC depleted extracts for 60 min, washed twice in 1 mL cold transport buffer (Wu *et al*. 1997), and resuspended in 1 × SDS loading buffer for immunoblotting.

Analysis of DNA replication in *Xenopus* egg extracts

Intact nuclei were prepared by digitonin permeabilization of synchronized CHOC 400 cells as described (Wu *et al*. 1997). Metaphase cells were permeabilized by exactly the same method. Permeabilized nuclei were prepared similarly, incubating with twice the concentration of digitonin for 20 min. The methods for analysis of DNA synthesis by acid precipitation and for mapping the sites of initiation of replication using the Early Labelled Fragment Hybridization (ELFH) assay have been described in detail (Wu *et al*. 1997). Briefly, the ELFH assay is performed as follows. Nuclei were incubated for 90 min in a *Xenopus* egg extract supplemented with 100 μg/mL aphidicolin. Nuclei were then washed free of aphidicolin and the earliest replicating nascent DNA chains were labelled briefly with [α-^{32}P]dATP. 18 unique probes distributed over a 120-kb region that includes the DHFR ori-β were immobilized to nylon filters using an EVENFLOW slot blot apparatus (Laboratory Product Sales, Rochester, NY) and hybridized to the ^{32}P-labelled early replication intermediates. Relative c.p.m. were obtained by phosphorimaging analysis and adjusted for differences in probe size, deoxyadenine content, and hybridization efficiency by normalizing to the corresponding values for parallel hybridizations with labelled replication intermediates from exponentially growing cells. These values were then normalized by adjusting the lowest corrected value to 1.00. The hybridization probes used in this study have been previously described (Gilbert *et al*. 1995; Lawlis *et al*. 1996).

Analysis of DNA replication by gradient centrifugation

For density substitution experiments, 20 000 nuclei were incubated for 2 h in 20 μL *Xenopus* egg extract supplemented with 250 μM BrdUTP and 10 μCi [α-^{32}P]dATP. 4 μL samples were stopped by the addition of 1 mL ice cold hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$) and samples were centrifuged at 16 000 *g* for 5 min. Pellets were lysed in 300 μL stop C (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% sodium dodecyl sulphate) containing 200 μg/mL RNase A, incubated for 1 h at 56 °C, supplemented with 200 μg/mL Proteinase K and incubated for an additional hour. Samples were then extracted sequentially with one volume each phenol, phenol:chloroform:isoamyl alchohol (25:24:1) and chloroform. DNA was precipitated by adjusting to 30 mM sodium acetate and 70% ethanol and centrifuged at 16 000 *g* for 10 min. Pellets were washed twice with 70% ethanol and DNA was resuspended at 56 °C overnight in TE. Density gradient centrifugation was performed as described (Gilbert *et al*. 1995).

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