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Changes in association of the *Xenopus* origin recognition complex with chromatin on licensing of replication origins

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SUMMARY

During late mitosis and early G_1, a series of proteins are assembled onto replication origins that results in them becoming 'licensed' for replication in the subsequent S phase. In *Xenopus* this first involves the assembly onto chromatin of the *Xenopus* origin recognition complex XORC, and then XCdc6, and finally the RLF-M component of the replication licensing system. In this paper we examine changes in the way that XORC associates with chromatin in the *Xenopus* cell-free system as origins become licensed. Restricting the quantity of XORC on chromatin reduced the extent of replication as expected if a single molecule of XORC is sufficient to specify a single replication origin. During metaphase, XOrc1 associated only weakly with chromatin. In early interphase, XOrc1 formed a strong complex with chromatin, as evidenced by its resistance to elution by 200 mM salt, and this state persisted when XCdc6 was assembled onto the chromatin. As a consequence of origins becoming licensed the association of XOrc1 and XCdc6 with chromatin was destabilised, and XOrc1 became susceptible to removal from chromatin by exposure to either high salt or high Cdk levels. At this stage the essential function for XORC and XCdc6 in DNA replication had already been fulfilled. Since high Cdk levels are required for the initiation of DNA replication, this 'licensing-dependent origin inactivation' may contribute to mechanisms that prevent re-licensing of replication origins once S phase has started.

Key words: DNA replication, Licensing factor, ORC, Cdc6, RLF

INTRODUCTION

In eukaryotes the initiation of replication forks occurs at multiple replication origins scattered throughout the genome, and is regulated so that origins fire no more than once during each S phase. Biochemical analysis of chromosome replication can be performed in cell-free extracts of *Xenopus* eggs that support complete chromosomal DNA replication in vitro. DNA added to this system is first assembled into functional nuclei, and then undergoes a single complete round of semiconservative replication (Blow and Laskey, 1986). Re-replication does not occur unless the extract is allowed to progress into mitosis, or unless the nuclear envelope is transiently permeabilised (Blow and Laskey, 1988). The precise duplication of chromosomal DNA in the *Xenopus* cell-free system is achieved by two distinct signals that are required for initiation to occur at any given replication origin (reviewed by Tada and Blow, 1998). The first signal, replication licensing factor (RLF), stably binds or 'licenses' replication origins by putting them into an initiation-competent state (Blow and Laskey, 1988); the second signal, S-phase promoting factor (SPF), induces licensed origins to initiate (Blow and Nurse, 1990; Fang and Newport, 1991; Strausfeld et al., 1996), and in doing so removes the licence. So long as the licensing signal and the initiation signal act sequentially, and do not act on DNA at the same time, the precise duplication of chromosomal DNA is achieved.

The licensing system became amenable to detailed analysis following the demonstration that certain protein kinase inhibitors, such as 6-dimethylaminopurine (6-DMAP) inhibit the RLF activation that normally occurs on exit from metaphase (Blow, 1993; Kubota and Takisawa, 1993). Using the rescue of replication in 6-DMAP-treated extracts as an assay for RLF activity, RLF has been shown to consist of two distinct components: RLF-M, comprising all 6 members of the MCM/P1 family (Chong et al., 1995; Kubota et al., 1995, 1997; Thömmes et al., 1997), and RLF-B, a currently unpurified activity that functions as RLF-M is loaded onto chromatin (Chong et al., 1995; Tada et al., 1999). On licensing, up to 20 copies of the RLF-M complex are loaded onto chromatin per replication origin (Mahbubani et al., 1997); they are then displaced as replication occurs.

Two other proteins, XORC and XCdc6, are also required on the chromatin before licensing can occur (Coleman et al., 1996; Rowles et al., 1996; Romanowski et al., 1996). XORC is the *Xenopus* homologue of the *Saccharomyces cerevisiae* origin recognition complex, which was originally identified by its ability to specifically bind to yeast origins of replication (Bell and Stillman, 1992). When sperm nuclei are added to *Xenopus*

egg extracts, XORC is rapidly loaded onto chromatin, which becomes saturated with approximately 1 copy of XOrc1 per 10-15 kb DNA (Rowles et al., 1996). Since this corresponds to estimates of the average spacing between replication origins in the *Xenopus* early embryo (Mahbubani et al., 1992; Hyrien and Méchali, 1993), this suggests that the binding of a single molecule of XORC may be sufficient to specify a single replication origin. XCdc6 is the *Xenopus* homologue of *S. cerevisiae* Cdc6 and *Schizosaccharomyces pombe* cdc18 proteins, both of which have been implicated in preventing re-replication of DNA in a single cell cycle (Diffley et al., 1994; Nishitani and Nurse, 1995; Liang and Stillman, 1997). XCdc6 is only loaded onto chromatin already containing XORC (Coleman et al., 1996). After licensing is complete, XCdc6 disappears from the chromatin, and may become distributed to other components of the nucleus (Coleman et al., 1996; Hua and Newport, 1998). Although XOrc1 remains bound to chromatin during S phase, levels decline during G_2 (Rowles et al., 1996). This is particularly surprising given that the amount of DNA (and the number of potential origins) has doubled during S phase, and suggests that the interaction of XORC with chromatin weakens during later stages of the cell cycle.

In this paper we explore changes in the way that XORC binds to chromatin as licensing occurs. We show that XORC can associate with chromatin in at least two distinct forms: a tight binding that is seen during early interphase before licensing has occurred, and a weaker interaction that is seen after licensing. On licensed chromatin (but not on unlicensed chromatin) XOrc1 is susceptible to removal from the chromatin by exposure to metaphase extract containing high levels of cyclin B kinase. The destabilisation of XORC that occurs as a consequence of origin licensing may therefore contribute to mechanisms that prevent re-replication of DNA in a single cell cycle.

MATERIALS AND METHODS

Preparation of egg extracts

Metaphase-arrested *Xenopus* egg extracts were prepared as described (Chong et al., 1997). For replication assays they were supplemented with 100 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase and [α-^{32}P]dATP, and were then released into interphase with 0.3 mM $CaCl_2$. Licensing factor-defective '6-DMAP extracts' were prepared in a similar way, except 3 mM 6-dimethylaminopurine was added prior to $CaCl_2$ release (Blow, 1993; Chong et al., 1997). Immunodepletion of interphase extracts with antibodies raised against XOrc1 (Rowles et al., 1996), XCdc6 (Coleman et al., 1996) or XMcm3 (Madine et al., 1995) or with antibodies from non-immune rabbit serum, was performed as described (Chong et al., 1997). Control extracts were diluted with an equivalent amount of buffer. Once immunodepletion was complete, extracts were snap frozen in liquid nitrogen in 10 μl aliquots for future use.

Chromatin templates

Demembranated *Xenopus* sperm nuclei were prepared as described (Chong et al., 1997). They were assembled into chromatin by incubating them for 15 minutes at 23°C in untreated or 6-DMAP-treated extracts at 20,000-35,000 nuclei/μl (50-100 ng DNA/μl, which corresponds to the capacity of the extract to license sperm nuclei; Mahbubani et al., 1997), or in immunodepleted extracts at 10,000-13,000 nuclei/μl (25-30 ng DNA/μl, the concentration being lower to compensate for the approximate 3-fold dilution that occurs as a consequence of immunodepletion). The extract was then diluted 10-fold in nuclear isolation buffer (Chong et al., 1997) (NIB: 50 mM KCl, 50 mM Hepes KOH, pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM spermine 3HCl, 0.15 mM spermine 4HCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) supplemented with 2.5 mM Mg-ATP and 0.01% Triton X-100, and then underlayered with the same buffer containing 15% sucrose. For the salt-wash experiments, the NIB was also supplemented with additional KCl, both in the component for extract dilution as well as in the sucrose cushion. The chromatin was then pelleted at 1,500 ***g*** in a swinging bucket centrifuge for 5 minutes at 4°C. The diluted extract and the top part of the cushion were carefully removed, and the chromatin pellet then resuspended in either NIB or LFB1 (40 mM Hepes-KOH, 20 mM K_2HPO_4/KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 10% sucrose, 1 μg/ml leupeptin, pepstatin and aprotinin, 0.5 mM PMSF, pH 8.0) supplemented with 2.5 mM Mg-ATP and 0.01% Triton X-100 for immediate use. Aliquots of pelleted chromatin were either used for replication assays, or were subjected to immunoblotting by standard techniques using 10% SDS-PAGE and ECL visualisation (Amersham).

Replication assays

DNA synthesis was measured by incorporation of [α-^{32}P]dATP into acid insoluble material as described (Chong et al., 1997), assuming a dATP pool in the extract of 50 μM (Blow and Laskey, 1986). Licensing assays were performed by measuring DNA synthesis in 6-DMAP-treated extract as described (Chong et al., 1995, 1997). Final DNA concentrations in these assays were kept in the range 1-10 ng DNA/μl extract. All incubations were performed at 23°C. GST-p21$^{Cip1/Waf1}$ was prepared as described (Strausfeld et al., 1994).

RESULTS

XORC requirement for replication

We have previously shown that in *Xenopus* egg extract, sperm chromatin becomes saturated with approximately one XOrc1 per replication origin, suggesting that the binding of a single molecule of XORC may be sufficient to specify a single replication origin (Rowles et al., 1996). We tested this by controlling the quantity of XORC bound to chromatin, and then measuring the subsequent extent of DNA replication (Fig. 1). To assemble different quantities of XORC onto chromatin without restricting the assembly of any other independent factor, sperm nuclei were incubated with differing proportions of XOrc1-depleted (XOrc1$^-$) and non-immune-depleted (NI$^-$) extracts. Chromatin was then isolated and the amount of chromatin-bound XOrc1 was quantified by immunoblotting (Fig. 1A). Consistent with our previous results, XOrc1 saturated the chromatin with about 1 XOrc1 molecule for every 10 kb of DNA (~20 ng XOrc1/μg DNA; Fig. 1C). The different chromatin samples were then transferred to XOrc1-depleted extract, and the amount of DNA synthesis occurring over a further 140 minute incubation was measured (Fig. 1B). Before DNA replication occurs in *Xenopus* extracts, there is a lag period of 30-90 minutes whilst the DNA is first assembled into an intact interphase nucleus. Immunodepleted extracts typically have an increased lag time, and replication rates in the XOrc1-depleted extract were close to maximal 90-140 minutes after chromatin addition (data not shown). Fig. 1B shows that when less XOrc1 was present on the chromatin, less replication was seen after 140 minutes in the XOrc1-depleted extract. When the quantity of XOrc1 on the chromatin was plotted against the amount of replication in the XOrc1-depleted extract, a positive correlation was revealed, consistent with the quantity of XOrc1 on the chromatin directly determining the number of replication origins that are used (Fig. 1C).

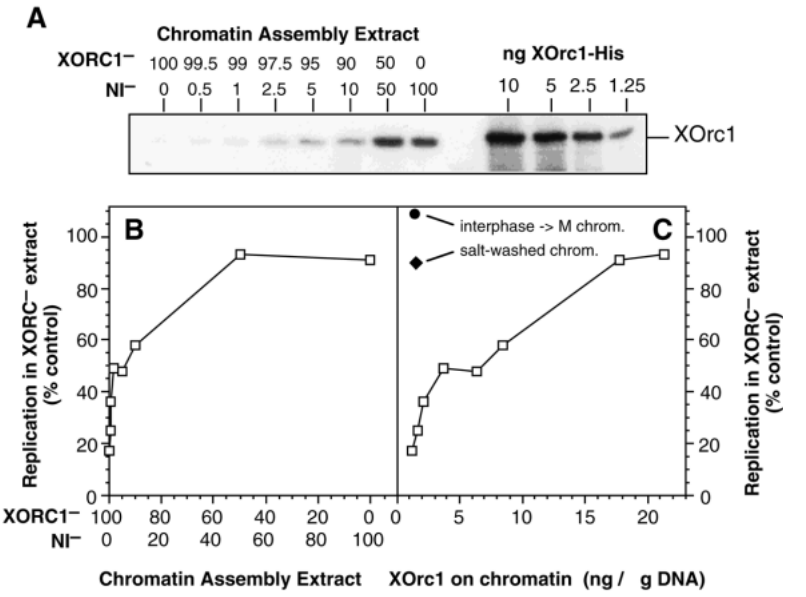

Fig. 1. Effect on replication of limiting XORC. *Xenopus* sperm nuclei were incubated for 15 minutes in different mixtures of extract depleted with antibodies against XOrc1 ('XORC1⁻') or non-immune antibodies ('NI⁻'). Chromatin was isolated and then either (A) subjected to SDS-PAGE alongside known quantities of recombinant XOrc1 and immunoblotted with antibodies to XOrc1; or (B) incubated for 140 minutes with [α-^{32}P]dATP in XOrc1-depleted extract and the total amount of DNA synthesis determined. DNA synthesis is expressed as a percentage of that obtained in extract immunodepleted with non-immune antibodies. (C) The quantities of XOrc1 present on the chromatin samples in A were determined by immunoblotting, and are plotted against the replication values shown in B (open squares). Also shown is the 250 mM salt-washed chromatin from Fig. 4 (diamond) and interphase chromatin incubated in metaphase extract from Fig. 6 (circle).

Changes in XORC association with chromatin

We next investigated whether the interaction between XORC and chromatin changed during the early part of the cell cycle when functional origins are assembled. Extracts were prepared from unactivated *Xenopus* eggs, which are naturally arrested in meiotic metaphase II (Fig. 2, 'metaphase'), and aliquots were supplemented with 0.3 mM $CaCl_2$ to mimic the fertilisation signal and release them into interphase of the first cell cycle (Fig. 2, 'interphase'). Following release into interphase, aliquots were immunodepleted with antibodies specific for XOrc1 (which pull down the entire XORC complex; Rowles et al., 1996; Romanowski et al., 1996), XCdc6, or XMcm3 (which pull down the entire RLF-M complex, Thömmes et al., 1997). Sperm nuclei were briefly incubated in these extracts, and were then isolated in buffers containing either low salt (~100 mM; 'L') or high salt (~250 mM; 'H'), and then blotted for the presence of chromatin-bound XOrc1, XCdc6 or XMcm3. In metaphase extract, where replication licensing does not occur, no XCdc6 or XMcm3 was observed on chromatin. Only low levels of XOrc1 were seen on the metaphase chromatin under low salt conditions, and this was completely removed by exposure to high salt. In XOrc1-depleted interphase extracts, neither XOrc1, XCdc6 nor XMcm3 were associated with chromatin (Fig. 2, XOrc1⁻). In XCdc6-depleted interphase extracts, XOrc1, but not XCdc6 or XMcm3, was associated with chromatin (Fig. 2, XCdc6⁻). In XMcm3-depleted extracts, both XOrc1 and XCdc6 were associated with chromatin. These results are consistent with previous reports showing that XCdc6 binding to chromatin is dependent on XORC, and that the binding of the MCM/P1 proteins to chromatin is dependent on the presence of both XORC and XCdc6 (Coleman et al., 1996; Rowles et al., 1996; Romanowski et al., 1996).

In XCdc6- or XMcm3-depleted extract, before licensing had occurred, the association of XOrc1 with chromatin was completely resistant to elution by high salt (Fig. 2, compare samples L and H). When replication licensing occurs, XMcm3 and the other MCM/P1 proteins are loaded onto chromatin (Chong et al., 1995; Kubota et al., 1995, 1997; Thömmes et al., 1997). The XMcm3 loaded onto licensed chromatin was resistant to elution from chromatin by exposure to high salt (Fig. 2, 'interphase'). Unexpectedly, the behaviour of XOrc1 and XCdc6 changed dramatically once licensing occurred: XOrc1 remained bound to chromatin but became sensitive to removal by exposure to high salt, whilst the level of XCdc6 on chromatin dropped to almost background levels. These results demonstrate that a significant change in the way XORC and XCdc6 associate with chromatin occurs as a consequence of the chromatin becoming licensed for replication.

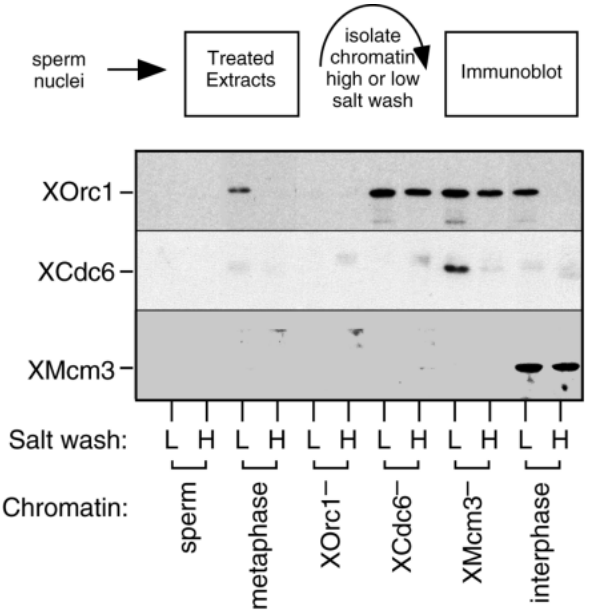

Fig. 2. Removal of XOrc1 by exposure to high salt at different stages of origin assembly. *Xenopus* sperm nuclei were incubated for 15 minutes in interphase extracts previously immunodepleted with antibodies against XOrc1, XCdc6, or XMcm3 or in undepleted interphase or metaphase extract. Untreated sperm nuclei were used as control. Chromatin was isolated through nuclear isolation buffer containing either 50 mM KCl (L) or 200 mM KCl (H), and was then immunoblotted for XOrc1, XCdc6 or XMcm3.

Fig. 3A shows that the XORC assembled onto chromatin in XCdc6-depleted and XMcm3-depleted extract, which is resistant to removal by high salt, can still function to support replication licensing. Chromatin was assembled in immunodepleted extract, isolated and then incubated in XOrc1-depleted extract to determine whether it contained functional XORC. Only if the chromatin contained functional XORC can the chromatin become licensed for replication, since XORC is required for licensing to occur (Rowles et al., 1996). Chromatin assembled in either XCdc6-depleted extract or XMcm3-depleted extract (which both contain XOrc1, as shown in Fig. 2) was efficiently licensed by a second incubation in XOrc1-depleted extract, demonstrating that they both contained functional XORC (Fig. 3A).

The XMcm3 loaded onto chromatin in untreated interphase extract resisted elution by high salt even though the XOrc1 and XCdc6 were removed (Fig. 2, 'interphase'). Fig. 3B shows that this XMcm3 was fully functional, since the high salt-treated chromatin was replicated equally efficiently in untreated interphase extract and 6-DMAP-treated extract (which is unable to license chromatin and load MCM/P1 proteins onto it, Chong et al., 1995). In contrast, when unlicensed '6-DMAP chromatin' was isolated under low or high salt conditions, it remained unlicensed and unable to replicate in 6-DMAP extract (Fig. 3B, right-hand panel). This suggests that although XORC is required for the assembly of MCM/P1 proteins onto chromatin, it is not required for their continued association with chromatin. Fig. 3C shows that although both XOrc1 and XOrc2 could be removed from licensed chromatin by exposure to high salt, they were resistant to elution from unlicensed chromatin assembled in 6-DMAP extract. This is consistent with the results shown in Fig. 2 and supports the idea that XORC becomes destabilised on chromatin as a consequence of replication licensing.

No further role for XORC and XCdc6 once licensing is complete

By using high salt treatment to remove XOrc1 and XCdc6 from licensed chromatin without removing functional XMcm3, we could test whether XOrc1 and XCdc6 have any other role in replication after origin licensing is complete. Sperm nuclei were licensed by a brief incubation in interphase egg extract, and the chromatin was isolated in buffers containing increasing concentrations of KCl. Fig. 4A shows that although virtually all the XOrc1, XOrc2 and XCdc6 could be eluted from the chromatin in 200 mM KCl, >50% of the XMcm3 remained chromatin-bound up to 300 mM KCl (Fig. 4A). XOrc2 was eluted at a slightly lower salt concentrations than XOrc1, suggesting that the XORC complex may be destabilised under these conditions. However, treatment with these different salt concentrations had no effect on the ability of these chromatin templates to replicate in XOrc1-depleted extract (Fig. 4B). Even when interphase chromatin was treated with 300 mM salt so that it contained virtually no detectable XOrc1 or 2 (Fig. 4A), it still remained capable of replicating in a XOrc1-depleted extract at close to 100% efficiency (Fig. 4B). In contrast, the replication of untreated sperm nuclei (which contains neither XORC nor MCM/P1 proteins) underwent <5% replication in the XOrc1-depleted extract. The replication of salt washed chromatin in XOrc1-depleted extract shown in Fig. 4B cannot be due to residual XORC remaining on the chromatin since comparison with Fig. 1 shows that even small reductions in the quantity of

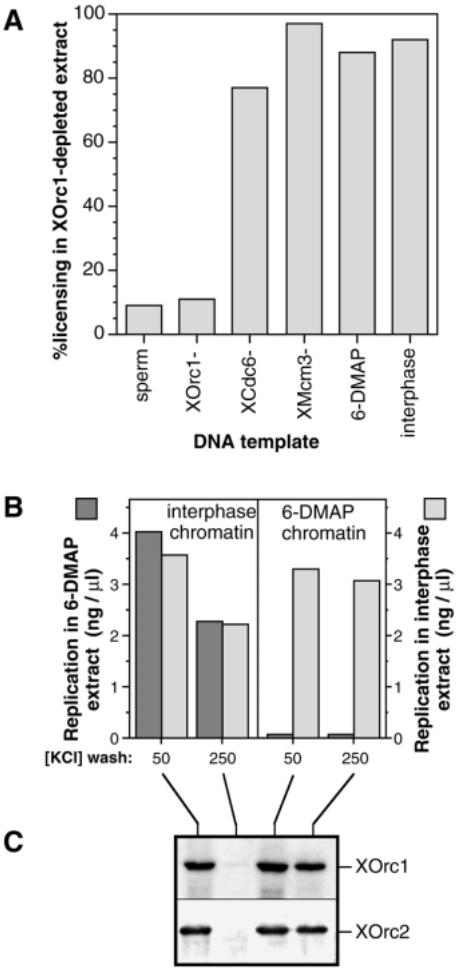

Fig. 3. Activity of chromatin-bound XORC during assembly of licensed origins. (A) *Xenopus* sperm nuclei were incubated for 15 minutes in interphase *Xenopus* extracts previously immunodepleted with antibodies against XOrc1, XCdc6, XMcm3 or in 6-DMAP-treated extract, or in control extract. Chromatin was isolated and then re-incubated for a further 15 minutes in a second aliquot of XOrc1-depleted extract, before being transferred to 6-DMAP treated extract and incubated for a further 90 minutes in the presence of [α-^{32}P]dATP, to assess the degree of licensing. In order to control for varying chromatin recoveries, the extent of licensing was expressed as the percentage of that seen when the template was licensed in control extract. (B,C) Chromatin was prepared by incubating *Xenopus* sperm nuclei for 15 minutes either in control extract (for interphase chromatin,) or in 6-DMAP-treated extract (for 6-DMAP chromatin), and was then washed in Nuclear Isolation Buffer containing either 50 mM KCl or 250 mM KCl. Aliquots of chromatin were either (B) incubated in untreated interphase extract or 6-DMAP treated extract and the extent of replication over 90 minutes measured, or (C) immunoblotted for the presence of XOrc1 and XOrc2.

XOrc1 on the chromatin should lead to proportionate decreases in the extent of replication observed. The experiments shown in Figs 1 and 4 were performed under identical conditions in order to make them directly comparable. The 250 mM salt-washed chromatin sample from Fig. 4B has also been quantified and included in Fig. 1C (diamond symbol): if this chromatin sample had not been previously licensed, the quantity of XOrc1 on it should only give background levels of replication, instead of the

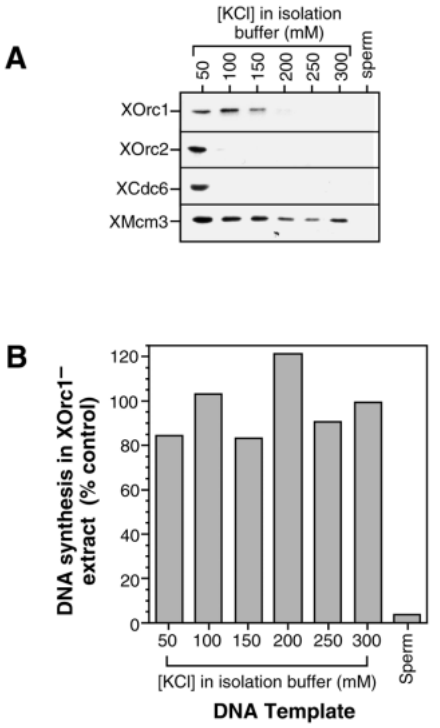

Fig. 4. Removal of XORC and XCdc6 by high-salt treatment. *Xenopus* sperm nuclei were incubated for 15 minutes in untreated interphase *Xenopus* extract. Chromatin was then isolated in buffers containing increasing concentrations of KCl. (A) Chromatin was immunoblotted for XOrc1, XOrc2, XCdc6 and XMcm3. Untreated *Xenopus* sperm were also blotted as a control. (B) Chromatin was transferred to XOrc1-depleted extract. Untreated sperm nuclei were used as control. Following incubation in the presence of [α-^{32}P]dATP for 140 minutes, the total amount of DNA synthesised was determined. DNA synthesis is expressed as a percentage of that obtained in extract immunodepleted using non-immune antibodies.

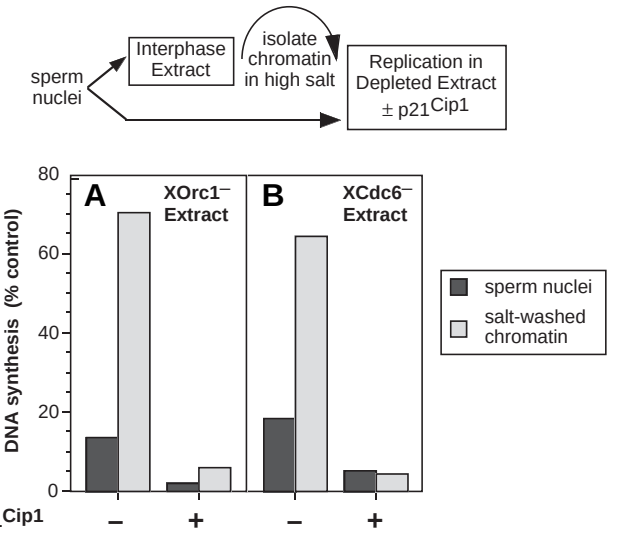

Fig. 5. Replication of high salt-washed chromatin in XOrc1- and XCdc6-depleted extracts is sensitive to p21^{Cip1}. *Xenopus* sperm nuclei were incubated for 15 minutes in untreated interphase *Xenopus* extract, and chromatin was then isolated in nuclear isolation buffer containing 250 mM KCl. This high salt-washed chromatin, or untreated sperm nuclei, were incubated for 140 minutes with [α-^{32}P]dATP plus or minus p21^{Cip1} in extract immunodepleted with antibodies to (A) XOrc1, or (B) XCdc6. DNA synthesis is expressed as a percentage of that obtained in extract immunodepleted using non-immune antibodies.

virtually maximal replication observed. Taken together, these results suggest that once licensing has taken place and RLF-M has been loaded onto chromatin (a reaction dependent on XORC), replication can then proceed in the complete absence of XORC. XCdc6 is likely to behave in a similar manner, as it was washed off the chromatin by the high salt treatment (Fig. 4A) and cannot then re-associate with chromatin in the absence of XORC (Fig. 2; also see below).

Previous work using the *Xenopus* cell-free system has shown that Cdks are required late in G_1 to provide an activity called 'S-phase promoting factor' (SPF) that is required for the initiation of DNA replication (Blow and Nurse, 1990; Fang and Newport, 1991; Strausfeld et al., 1994). SPF acts after licensing is complete, just prior to the initiation of replication, and is inhibited by the Cdk inhibitor p21$^{Cip1/Waf1}$ (Strausfeld et al., 1994). Fig. 5 shows that the replication of the high-salt washed chromatin used in Fig. 4 is still dependent on SPF activity. Sperm nuclei were incubated for 15 minutes in interphase *Xenopus* egg extract, and the chromatin was then isolated in 250 mM KCl; this 'salt-washed chromatin' was then incubated in egg extract previously immunodepleted of either XOrc1 (Fig. 5A, light shading) or XCdc6 (Fig. 5B, light shading), plus or minus recombinant p21$^{Cip1/Waf1}$. The replication of untreated sperm nuclei is shown as control for the immunodepletion (dark shading). Consistent with the results shown above, the salt-washed chromatin was able to replicate in XOrc1-depleted extract (Fig. 5A), although it contained no detectable XOrc1 or 2 (Fig. 4A). However, replication of this chromatin was strongly inhibited by p21$^{Cip1/Waf1}$, showing that this chromatin still requires SPF activity before initiation can occur (Fig. 5A). Similarly, the salt-washed chromatin was capable of replicating in XCdc6-depleted extract (Fig. 5B), although it contained no detectable XCdc6 (Fig. 4A), and replication of chromatin was also inhibited by p21$^{Cip1/Waf1}$. These experiments therefore demonstrate that the essential roles of XORC and XCdc6 in replication are fulfilled prior to the completion of the SPF-dependent step.

Removal of XORC from chromatin by Cdks is facilitated by licensing

We next wanted to determine whether the weakened XOrc1 association with chromatin that occurs once licensing is complete and as demonstrated by sensitivity to salt, can be revealed by a more physiological treatment. Hua and Newport (1998) have previously shown that when licensed chromatin was exposed to high cyclin A kinase levels, XORC but not XMcm3 was removed from the chromatin. Since this behaviour is similar to the effect of high-salt treatment on licensed chromatin, we next investigated whether the removal of XOrc1 from chromatin on exposure to high Cdk levels was dependent on the chromatin having been licensed (Fig. 6). Instead of using recombinant cyclin A, we provided high Cdk levels by exposing chromatin to metaphase extract, as this is likely to represent a more physiological situation. When licensed interphase chromatin was incubated in metaphase extract, XOrc1, XOrc2 and XCdc6 were removed, but XMcm3 remained bound to the chromatin (Fig.

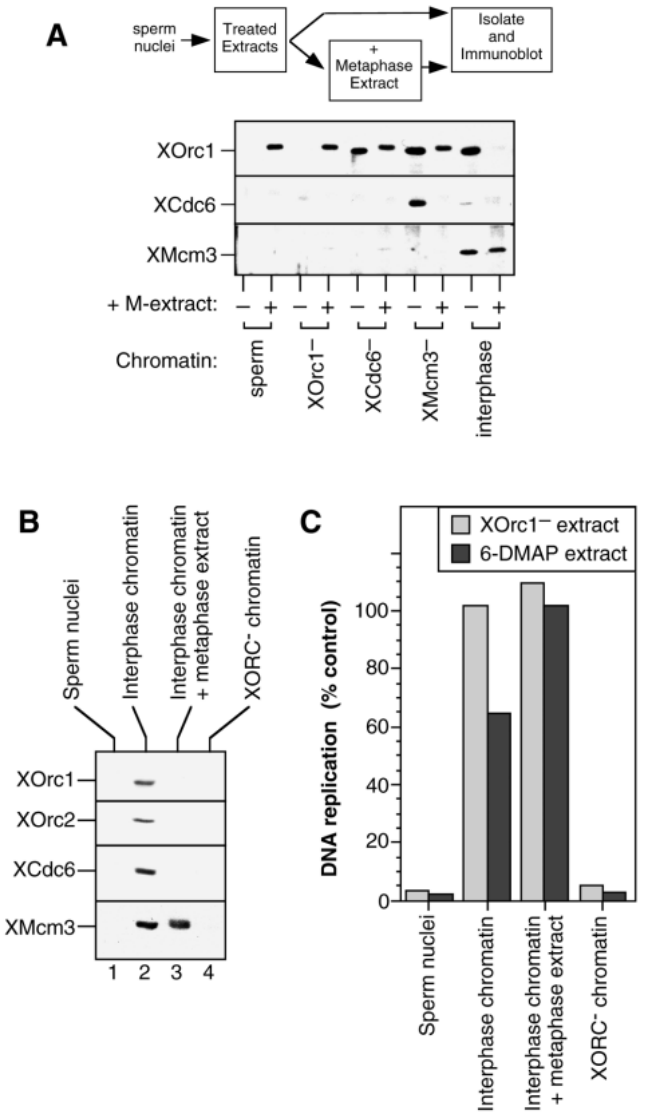

Fig. 6. Removal of XOrc1 by exposure to metaphase extract at different stages of origin assembly. *Xenopus* sperm nuclei were incubated for 15 minutes in interphase extracts previously immunodepleted with antibodies against XOrc1, XCdc6, or XMcm3 or in control interphase extract. Untreated sperm nuclei were used as control. Incubations were divided into two equal aliquots, and one aliquot was supplemented with 2 volumes of metaphase extract ('+ M-extract') for a further 15 minutes. (A,B) Chromatin was then isolated and immunoblotted for XOrc1, XOrc2, XCdc6 or XMcm3 as indicated. (C) The chromatin samples shown in B were incubated for 140 minutes with [α-^{32}P]dATP in XOrc1-depleted extract or in extract treated with 6-DMAP, after which the total amount of DNA synthesised was determined. DNA synthesis is expressed as a percentage of that obtained in extract immunodepleted with non-immune antibodies.

6A and B). Under these conditions, the chromatin remained licensed and competent to replicate in XOrc1-depleted extract (Fig. 6C). The replication of this chromatin in XOrc1-depleted extract cannot be due to residual XORC remaining on the chromatin since the experiment was performed under identical conditions to the experiment shown in Fig. 1, where reductions in the quantity of XOrc1 on the chromatin led to proportionate reductions in the extent of replication. The chromatin sample used in Fig. 6C has also been quantified and included in Fig. 1C (circle) to emphasise this point.

In contrast to the behaviour of licensed chromatin, when unlicensed chromatin (in the form of either naive sperm nuclei, or chromatin assembled in XOrc1-depleted, XCdc6-depleted or XMcm3-depleted extracts) was exposed to metaphase extract, XOrc1 was not removed from the chromatin. Therefore the ability of metaphase extract to remove XOrc1 from the chromatin is dependent on the chromatin having been licensed. Taken together with the results shown in Figs 2 and 3C, this demonstrates that prior to licensing the association of XORC with chromatin is relatively strong and is resistant to either high salt or high Cdks levels, but that as a consequence of licensing XORC becomes destabilised and can be removed from chromatin by either of these treatments.

DISCUSSION

The experiments described here reveal a complex series of events occurring at replication origins on chromatin as XORC, XCdc6 and RLF-M are loaded onto chromatin early in the cell cycle. We have shown that XOrc1 and XOrc2 (and presumably the rest of the XORC complex) binds to chromatin in at least two different states. The stability of these complexes may affect the way that replication is controlled during the cell division cycle.

Fig. 7 outlines the sequential assembly of different protein complexes onto *Xenopus* chromatin as it becomes licensed. The first step is the binding of XORC (Fig. 7A and B). Unlike its yeast counterpart (Diffley and Cocker, 1992; Liang and Stillman, 1997), XORC associates only weakly with chromatin during metaphase (Coleman et al., 1996; Romanowski et al., 1996; and this paper). No licensing occurs in metaphase since the RLF-B component of the licensing system is inactive (Mahbubani et al., 1997; Tada et al., 1999), and XCdc6 does not associate with chromatin. On exit from metaphase but prior to the completion of licensing, XOrc1 associates tightly with chromatin (Fig. 7B). On this unlicensed interphase chromatin, XOrc1 is resistant to elution by exposure to high salt or to metaphase extract. At this stage the chromatin becomes saturated at an average of about one XOrc1 molecule per 10-15 kb of DNA (Rowles et al., 1996), and if the quantity of XORC is restricted, the overall rate of subsequent replication drops. These results support the idea that the tight binding of a single XORC molecule during early interphase is sufficient to define a replication origin in the *Xenopus* system.

The next step involves the binding of XCdc6 to XORC-containing chromatin (Coleman et al., 1996) (Fig. 7C). Although a direct demonstration is lacking, the binding of XCdc6 to chromatin is plausibly mediated by XCdc6 binding directly to XORC, since we show that XCdc6 is washed off chromatin at approximately the same salt concentrations as XORC is removed. Consistent with this interpretation, direct interactions between ORC and Cdc6 have been demonstrated in a number of other cell types (Grallert and Nurse, 1996; Saha et al., 1998). The binding of XCdc6 does not apparently change the strength of the binding of XOrc1 to chromatin.

The third step, corresponding to the origin becoming functionally licensed, involves the assembly of the RLF-M complex of MCM/P1 proteins onto chromatin that contains

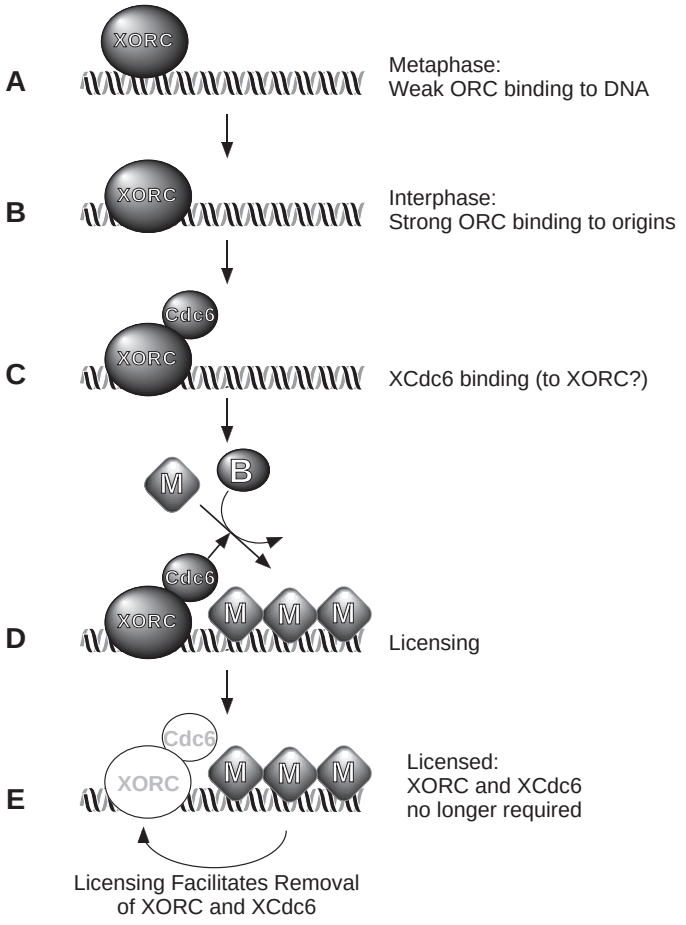

Fig. 7. Proposed steps in the assembly of licensed origins. A short section of DNA is shown at different stages during the assembly of a functional licensed origin. (A) In metaphase, XORC binds only weakly to chromatin and can be eluted with 200 mM salt. (B) On exit from metaphase, XORC binding to chromatin becomes resistant to salt elution. (C) XCdc6 then associates with the origin, possibly by binding directly to XORC. (D) Licensing of the origin by RLF-B and RLF-M results in multiple copies of RLF-M being assembled onto the chromatin at sites distinct from XORC and XCdc6. (E) As a consequence of licensing, the binding of XORC is destabilised so it can be removed by exposure to high salt or high Cdk levels. Once licensing has occurred, XORC and XCdc6 have fulfilled their essential functions in DNA replication, and can be removed from the chromatin without comprising the licensed state of the origin.

both XORC and XCdc6 (Fig. 7D) (Chong et al., 1995; Kubota et al., 1995; Coleman et al., 1996; Rowles et al., 1996; Romanowski et al., 1996). In addition to XORC and XCdc6, the licensing reaction requires both RLF-M and RLF-B components of the licensing system, along with hydrolysable ATP (Chong et al., 1995; Tada et al., 1999). Once licensing has occurred, the association of XOrc1 and XCdc6 with chromatin changes dramatically. The quantity of XCdc6 bound to licensed chromatin drops significantly (see also Hua and Newport, 1998). XOrc1 remains bound to licensed chromatin, but it changes to a form where it can be removed from chromatin by exposure to high salt or by exposure to metaphase extract. We propose that the process by which XORC becomes destabilised once licensing is complete should be termed ‘licensing-dependent origin inactivation’. The biochemical mechanism that mediates licensing-dependent origin inactivation is currently unclear, but it plausibly involves some direct interaction between XORC and the MCM/P1 proteins that occurs as origins become licensed.

Although XORC saturates sperm chromatin with one copy bound for each 10-15 kb DNA, up to 10-20 copies of XMcm3 can be loaded onto this amount of chromatin (Mahbubani et al., 1997). A similar excess of Mcm3 over replication origins has been reported in human cells (Burkhart et al., 1995) and budding yeast (Lei et al., 1996). This suggests that RLF-M does not bind directly to XORC and XCdc6, but instead binds to chromatin elsewhere, plausibly close to where XORC is bound (Fig. 7D). Consistent with this mode of binding, XMcm3 (and other MCM/P1 proteins, data not shown) remain associated with chromatin when XORC has been removed by exposure to either high salt or metaphase extract (this paper), or cyclin A kinase (Hua and Newport, 1998). A similar effect of salt-washing chromatin has been demonstrated in *S. cerevisiae* (Donovan et al., 1997). In *Xenopus*, the MCM/P1 proteins remain fully functional under these treatments (Blow, 1993; Hua and Newport, 1998; this paper).

Whilst this work was underway, Hua and Newport (1998) showed that when XOrc2 is removed from chromatin by exposure to high cyclin A kinase levels, the resultant chromatin, which still contains XMcm3, is competent to replicate in XOrc2-depleted extract. They suggested that the essential function of XORC in DNA replication is to load XMcm3 and related proteins onto chromatin prior to entry into S phase. However, cyclin A kinase can provide ‘SPF activity’ and promote the initiation of DNA replication (Strausfeld et al., 1994, 1996; Jackson et al., 1995). Therefore a limitation to this interpretation is that by exposing chromatin to high cyclin A kinase levels, other proteins that normally only associate with chromatin very close to the initiation of replication under the action of SPF will be loaded onto chromatin. For example, in *S. cerevisiae* the Cdc45 protein is assembled onto replication origins following activation of the Clb-Cdc28 kinase, the major initiating Cdk activity in these cells (Zou and Stillman, 1998). A similar Cdk-dependent loading of the *Xenopus* Cdc45 homologue (XCdc45) onto chromatin has also been described (Mimura and Takisawa, 1998).

We have used two different techniques to remove XORC and XCdc6 from chromatin that do not involve exposing the chromatin to S-phase inducing Cdks. In the first technique licensed chromatin was exposed to high salt, which efficiently removed XOrc1, XOrc2 and XCdc6 without affecting the ability of the chromatin to replicate in either XOrc1- or XCdc6-depleted extract. In the second technique licensed chromatin was exposed to metaphase extract, containing active Cdc2-cyclin B which cannot supply SPF activity (Strausfeld et al., 1994, 1996); again this chromatin remained fully competent to replicate in XOrc1-depleted extract. These results are consistent with the idea that the only essential function of XORC and XCdc6 in DNA replication is to support licensing (Fig. 7E), and strengthen the conclusion of Hua and Newport (1998). However, it is still possible that XORC and XCdc6 are also required for the assembly of further, so far unidentified, replication proteins onto chromatin and that these unidentified proteins are also not removed by the high salt treatment. However, two considerations suggest that few, if any, other proteins apart from RLF-M need to be assembled onto chromatin before XORC and XCdc6 can be removed. First, the time required for chromatin assembly in

these experiments was restricted to the minimum required for efficient licensing to occur (Blow, 1993; Thömmes et al., 1997). Second, replication of salt-washed chromatin in XOrc1-depleted or XCdc6-depleted extract was blocked by $p21^{Cip1/Waf1}$, and so the chromatin has still to complete the Cdk-dependent SPF function.

The licensing of replication origins is regulated to ensure that no re-licensing of origins occurs after replication is complete, thus ensuring that re-replication of DNA does not occur (Blow and Laskey, 1988). In yeast, the activation of Cdks at the end of G_1 plays an key role in preventing re-licensing and hence re-replication of DNA (Broek et al., 1991; Piatti et al., 1996; reviewed by Diffley, 1996). In *Xenopus*, Cdks driven by cyclins A, B and E have all been shown to block the licensing of DNA (Blow, 1993; Mahbubani et al., 1997; Hua et al., 1997). However, the precise mechanism by which Cdks block licensing remains unclear. The licensing-dependent origin inactivation that we demonstrate here and which allows Cdks to remove XOrc1 chromatin only once it has been licensed, provides one potential mechanism by which Cdks could prevent re-licensing of replication origins.

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