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INITIATION OF DNA REPLICATION IN *XENOPUS* EGG EXTRACTS

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1. ABSTRACT

In the last decade, extraordinary advances in our understanding of the initiation step of eukaryotic DNA replication have been achieved. Many factors required for replication initiation have been identified, and an elegant model to explain how DNA replication is restricted to a single round per cell cycle has emerged. Of the many experimental approaches used to study DNA replication, egg extracts from *Xenopus laevis* are among the most powerful, since they recapitulate a complete round of cell-cycle regulated chromosomal DNA replication *in vitro*. In this review, we discuss current models for how DNA replication is initiated and regulated in *Xenopus* eggs, and we highlight similarities and differences seen between this and the other most common experimental organisms, yeast and humans.

2. INTRODUCTION

During each S phase, eukaryotic cells initiate DNA replication from a large number of sites on each chromosome called origins. To maintain ploidy, not a single origin should be allowed to initiate DNA replication ("fire") more than once per cell cycle. In recent years, work in *S. cerevisiae*, *S. pombe*, *X. laevis*, and *D. melanogaster* has led to a "two-step" model for replication initiation which explains why origins fire only once per cell cycle (1, 2). The first step involves the assembly at origins of a pre-replication complex (pre-RC). The pre-RC is assembled through the sequential recruitment of at least four factors: the origin recognition complex (ORC), Cdc6, Cdt1, and the 6 minichromosome maintenance proteins that make up the MCM2-7 complex. The second initiation step occurs in S phase, when the pre-RC is converted into an active DNA replication fork through the action of Cyclin dependent kinase (Cdk) and Cdc7/Dbf4 protein kinase, both of which are required for binding of the initiation factor Cdc45 to the pre-RC. After Cdc45 is loaded, the origin is unwound by a DNA helicase, DNA polymerase α/primase loads, and DNA synthesis commences. When DNA replication initiates, the MCM2-7 complex is lost from the origin, and it is prevented from re-binding until the following G1 phase by the high level of Cdk activity that is present in S, G2, and M. Therefore, pre-RCs that are established at origins in G1 phase are allowed to fire exactly once during the ensuing S phase, and they are not allowed to re-bind until cells pass through mitosis.

Our current knowledge of the initiation of DNA replication comes from work in numerous experimental systems. Genetic studies in the yeasts *S. cerevisiae* and *S. pombe* resulted in identification of the large majority of the replication initiation factors that are known, and the application of chromatin immunoprecipitation (ChIP) techniques, as well as studies with purified proteins have yielded substantial biochemical information about the initiation process. Experiments in human tissue culture cells have been powerful in elucidating the subcellular localization of replication complexes. Genetic and cell biological studies in *Drosophila* have been powerful tools to understand the developmental regulation of DNA replication. In this review, we focus on extracts derived from unfertilized eggs of the African clawed toad, *Xenopus laevis* (3, 4). These extracts currently represent the most powerful cell-free system to study eukaryotic DNA replication *in vitro*. We first discuss the main features of the early embryonic cell cycles in *Xenopus*, as these form the biological context for the cell-free egg extracts. We then summarize the most common methods for preparing replication-competent extracts from eggs. Third, we review current models for how DNA replication is initiated in egg

extracts, including how replication initiation sites are selected on the chromosome, how ORC leads to the assembly of pre-RCs, how pre-RCs are activated at the onset of S phase, and how a single, complete round of DNA replication is achieved during each embryonic cell cycle. Throughout, we compare the findings in *Xenopus* with the data from other systems, particularly yeast and humans, as there are fundamental similarities and differences between these systems.

3. DNA REPLICATION DURING THE EARLY EMBRYONIC CELL CYCLES

Before fertilization, mature oocytes are arrested in metaphase of meiosis II by cytostatic factor (CSF), which maintains high intra-cellular levels of Cdk1/Cyclin B (traditionally known as maturation promoting factor, MPF) (reviewed in (5)). The mature oocytes pass down the oviduct and emerge from the frog as unfertilized eggs. Upon fertilization by sperm, a transient increase in cytoplasmic calcium concentration leads to destruction of CSF and Cyclin B, and concomitant transit of the egg from metaphase, through anaphase, to interphase. The first cell cycle after fertilization involves fusion of the male and female pronuclei, DNA replication of the zygotic genome, and mitosis. This cell cycle lasts about 75 minutes. The next 11 cell cycles are more rapid, each lasting about 30 minutes. During these early embryonic cell cycles, a ~20 minute S phase alternates with a ~10 minute M phase, and there are no G1 or G2 phases. These initial cell divisions are synchronous, do not involve cell growth, and effectively increase both cell number and nuclear content (DNA) of the embryo in a short period of time. After 12 cell cycles, the embryo progresses through the midblastula transition (MBT), a key developmental milestone characterized by elongation of the cell cycle and onset of zygotic transcription (6, 7). Entry into the MBT is triggered once a specific ratio of nuclear content to cytoplasm is reached. As an illustration of how the nucleocytoplasmic ratio changes, a fertilized *Xenopus* egg initially contains the equivalent of 1 diploid nucleus in a volume of ~1uL. After 12 cell cycles and no cell growth, the concentration of diploid nuclei approaches 2^{11} or ~2000/uL. With respect to how the nucleocytoplasmic ratio controls the MBT, it has been postulated that a critical regulator of the cell cycle that is present in excess in the unfertilized egg becomes limiting as nuclear content increases.

The reason why DNA can be replicated so quickly during the embryonic S phase is that the distance between initiation events (the replicon size) is only about 10 kb (8). This contrasts with somatic cells of *Xenopus* and other organisms where the replicon size is 100-200 kb and S phase lasts several hours (9, 10). The transition between a rapid embryonic S phase with short replicons and a slower somatic S phase with large replicons is presumed to occur at the MBT. Consistent with this idea, the replicon size at the rDNA locus was seen to increase after the MBT (11). In addition, the transition from small to large replicon size can be recapitulated by titrating DNA into replication-competent egg extracts (see below), and the transition occurs at a nucleocytoplasmic ratio similar to that observed at the MBT *in vivo* (12). The initiation of DNA replication is sequence-independent before the MBT (see Section 6). However, after the MBT, it becomes restricted to certain regions of the chromosome. Thus, at the rDNA locus, the initiation of DNA replication after the MBT is localized to the intergenic region and is excluded from the transcription units (11). Interestingly, it has been proposed that the increase in the length of S phase is the underlying trigger for the MBT because the longer interphase which results allows expression of genes whose transcripts would normally be aborted in mitosis (13, 14). As such, the change in replicon size may represent a key regulator of development.

4. *IN VITRO* DNA REPLICATION: INTERPHASE, CYCLING, AND MITOTIC *XENOPUS* EGG EXTRACTS

Extracts derived from unfertilized eggs recapitulate DNA replication with characteristics similar to what is seen in the pre-MBT cell-cycles. There are three ways to prepare replication-competent extracts from unfertilized eggs (for detailed procedures, see (15-17)). The simplest approach is to crush the unfertilized eggs at 16,000 x g in the presence of cycloheximide (Figure 1A, I; (3, 4)). Crushing leads to release of Ca^{2+} and degradation of Cyclin B. A low speed cytoplasmic fraction referred to as "Low Speed S Phase extract" or LSS is harvested. The presence of cycloheximide prevents re-synthesis of Cyclin B, and the extracts are permanently arrested in interphase. To carry out DNA replication, demembranated sperm chromatin is added as a DNA template. Upon addition to LSS, sperm chromatin undergoes a series of distinctive transformations. In the first five minutes, nucleoplasmin leads to removal of protamines from the sperm and deposition of histones H2A and H2B (18). Within ~15 minutes, pre-RCs form on the chromatin. Finally, nuclear membrane vesicles bind to the surface of the sperm chromatin, fuse, and insert nuclear pore complexes to generate a transport-competent nuclear envelope (19), an apparent pre-requisite for DNA replication (20, 21). The two major steps in the assembly of a replication competent nucleus, pre-RC and nuclear envelope formation, can be separated by further fractionation of the LSS at 260,000 x *g* into "High Speed Supernatant" (HSS) and a fraction of nuclear membrane vesicles (4). HSS assembles pre-RCs on added sperm chromatin (22), but no nucleus formation or DNA replication occurs unless membrane vesicles are also supplied (4).

A simple variation of the above procedure is to omit cycloheximide (Figure 1A, II; (23-25)). In this case, extracts enter interphase upon crushing, and they immediately begin to re-synthesize Cyclin B. As a result, they re-enter mitosis. Sperm chromatin added to these extracts can undergo up to 5 rounds of DNA replication and mitosis, making them ideal to study the cell cycle regulation of DNA replication. A third variation involves crushing the unfertilized eggs in the presence of EGTA, which chelates Ca^{2+} (Figure 1A, III; (26)). The resulting egg extract is arrested in metaphase of meiosis II with high Cdk1/Cyclin B activity. Sperm chromatin added to this

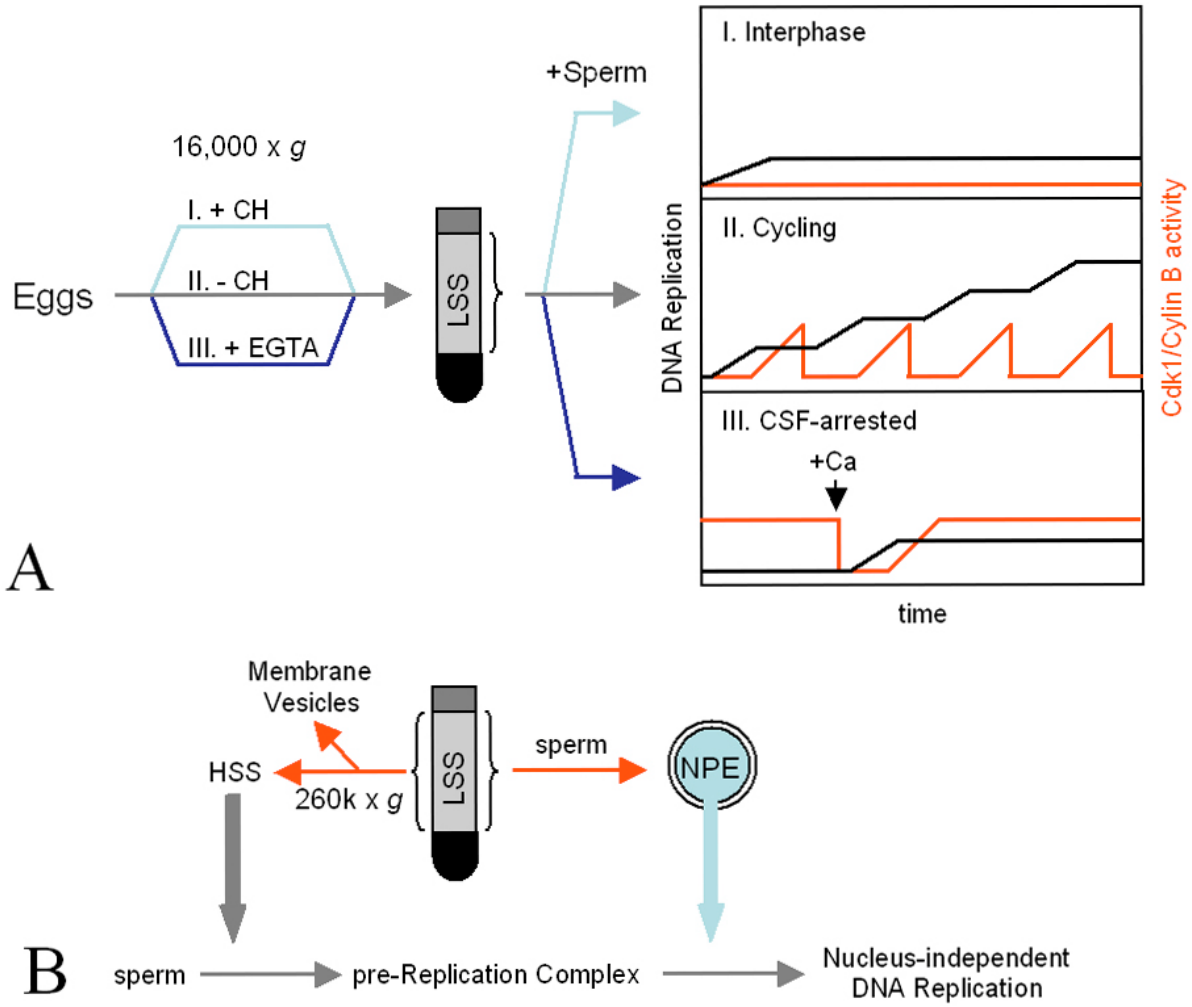

Figure 1. Preparation of *Xenopus* egg extracts. (A) Nuclear assembly extracts. Eggs are crushed in the presence of cycloheximide to prevent Cyclin B synthesis (I, Interphase extract), no additives (II, Cycling Extracts), or EGTA to chelate calcium (III, CSF arrested extract). The crude cytoplasm (Low speed supernatant, "LSS") is recovered, and sperm chromatin is added. The graph indicates DNA replication (Black) and Cdk1/Cyclin B activity (Red). (B) Nucleus-free extracts. Left red arrow: LSS is fractionated at 260,000 x g to yield high speed supernatant ("HSS") and purified membrane vesicle ("M"). Right red arrow: sperm chromatin is added to LSS to form nuclei, the nuclei are harvested, and centrifuged at 260,000 x g to separate the chromatin and nuclear envelopes from the nucleoplasmic extract (NPE). To carry out DNA replication in the absence of nuclei, sperm chromatin is first mixed with HSS to form pre-RCs. Subsequently, 2 volumes of NPE is added to stimulate DNA replication.

"CSF-arrested" extract assumes a highly condensed state characteristic of mitosis. Subsequent addition of calcium leads to Cyclin B degradation and entry into interphase, at which time a nucleus forms around the sperm and DNA replication takes place. These extracts will reenter mitosis, where they usually arrest (16). An important feature of all three approaches described above is that DNA replication requires nuclear envelope formation, and we refer to these as "nuclear assembly extracts."

The cell-free systems described above recapitulate the properties of the pre-MBT cell cycle, in that they replicate their DNA rapidly (20-30 minutes) using small replicons. However, egg extracts can be made to exhibit certain properties of somatic cells. When aphidicolin is used to arrest DNA replication in the early *Xenopus* embryo, or in a cycling egg extract containing low concentrations of sperm, there is no cell cycle arrest (27-29). This contrasts with the situation in somatic cells where blocks to DNA replication trigger a cell-cycle checkpoint that causes a G2-arrest. However, when enough sperm chromatin is added to a cycling egg extract in the presence of aphidicolin (about 500-1000/µl), a cell-cycle checkpoint is triggered that downregulates Cdk1/Cyclin B and delays mitosis (27). Therefore, while *Xenopus* embryos contain the checkpoint machinery, a minimum number of stalled replication forks is needed to subdue the cell cycle engine. In addition, at high sperm concentrations, DNA replication

takes longer due to an increase in replicon size, as seen in somatic cells (12, 27). Therefore, at high nucleocytoplasmic ratios, egg extracts can take on properties of the post-MBT cell cycle.

5. INDIRECT REQUIREMENT FOR THE NUCLEUS IN DNA REPLICATION

The requirement for a nuclear envelope in DNA replication could be explained in two ways. First, nuclear transport of a select group of proteins into the nucleus via the nuclear pore complex might create a biochemical environment that is permissive for DNA replication. In support of this model, manipulations that block nuclear transport block DNA replication (20, 21). In addition, Cdk2/Cyclin E, a protein kinase that is critical for DNA replication initiation (see below), is concentrated 200-fold in synthetic nuclei assembled in *Xenopus* egg extracts (22), and the high nuclear concentration of this kinase is critical to achieve DNA replication (30). Unlike in somatic cells, Cyclin E in embryos is not degraded, and it appears to be regulated primarily via nuclear transport. The second explanation is that DNA replication may require higher order nuclear structures. In apparent agreement with this idea, disruption of lamins, a structural component of the nuclear matrix, disrupted DNA replication in *Xenopus* nuclear assembly egg extracts (31-33). However, the lamin-deficient nuclei are small and fragile, so the defect in DNA replication might be traceable to problems in nuclear transport.

We recently showed that the requirement for nuclei during DNA replication in *Xenopus* egg extracts can be bypassed if sperm chromatin is exposed sequentially to HSS and then to a highly concentrated nucleoplasmic extract or NPE (34). To prepare NPE (Figure 1B), sperm chromatin is added to interphase-arrested LSS to assemble nuclei. The nuclei are harvested and the nucleoplasm is separated from chromatin and nuclear envelopes, yielding a nucleoplasmic extract (NPE). When added to pre-replication complexes formed in HSS, the NPE supports a complete round of DNA replication in the absence of a nuclear envelope or chromatin associated lamins (Figure 1B). Unlike nuclear assembly extracts, this nucleus-free DNA replication also supports 100% efficient DNA replication of small circular plasmids. NPE is highly enriched in many DNA replication factors including Cdk2/Cyclin E and Cdc7/Dbf4, and the high concentrations of these protein kinases in NPE are essential for efficient DNA replication (35-37). Therefore, the key property of nuclei that allows DNA replication in *Xenopus* egg extracts is that they generate a soluble biochemical environment that is permissive for DNA replication.

6. SEQUENCE-INDEPENDENT REPLICATION INITIATION IN *XENOPUS* EMBRYOS

Jacob proposed the replicon model for DNA replication in which an initiator protein recognizes a specific DNA sequence called a replicator (38). Binding of the initiator to the replicator sets in motion a cascade of events that leads to initiation of DNA replication. The replicator model has been confirmed in yeast, where well-defined DNA sequences called autonomously replicating sequences (ARSs) interact specifically with ORC, the eukaryotic initiator protein (39).

As ARS elements were being discovered, the nature of replicators in *Xenopus* was being determined by injection of different DNA sequences into unfertilized *Xenopus* eggs. Strikingly, any DNA injected into these eggs (including from prokaryotic organisms) underwent efficient DNA replication that was limited to a single round per cell cycle (40, 41). These results were corroborated by the finding that any DNA could serve as a DNA substrate for ORC-dependent and cell-cycle regulated DNA replication in *Xenopus* egg extracts (3, 4, 34), and that initiation on these templates was truly random with respect to DNA sequence (42, 43). Importantly, replication initiation on the chromosomes of early embryos is also sequence-independent (8), arguing that the experiments performed with prokaryotic DNA templates were physiologically significant. Because DNA replication in *Xenopus* egg extracts requires ORC (44), it can be inferred from these studies that *Xenopus* ORC is able to interact functionally with any DNA sequence, although a recent study suggests that *Xenopus* ORC may interact preferentially with AT rich DNA (45).

The question arises to what extent the low sequence-dependence of replication initiation seen in early *Xenopus* embryos applies to somatic cells. Clearly, replication initiation in somatic cells is non-random. Loci where high frequencies of initiation events are observed are called origins (46). Experiments in mammalian cells have lead to the description of two classes of origins, those where replication initiation is highly localized (exemplified by human lamin B2), and those where replication initiates in a broad zone (exemplified by DHFR). More recently, several of these origins were shown to contain a replicator element that is sufficient to stimulate replication initiation at ectopic locations. Finally, ChIP assays have shown that ORC binds to several of these origins (47, 48). Together, the data suggest that the replicon model can be extended to somatic cells. However, other data suggest that the highly sequence-specific interaction between initiator and replicator envisioned in the replicon model may not apply in somatic cells. We recently reported that a human ORC (HsORC) complex purified from insect cells binds preferentially to AT-rich DNA, but otherwise exhibits no observable sequence-specificity, being unable to discriminate between origin-containing and control DNA fragments (49). Furthermore, when added to *Xenopus* egg extracts, HsORC is able to support replication initiation from any DNA sequence. In addition, an earlier study by Calos and colleagues demonstrated that any DNA sequence greater than 10 kb in length was able to serve as a replicator in human somatic cells (50). Collectively, these data suggest that vertebrate ORC complexes generally exhibit little sequence specificity and they raise the question of how initiation sites are selected in somatic cells. A possible model is that chromatin structure plays a major role in determining where ORC binds. Thus, embryonic cells, which are largely devoid of higher order chromatin

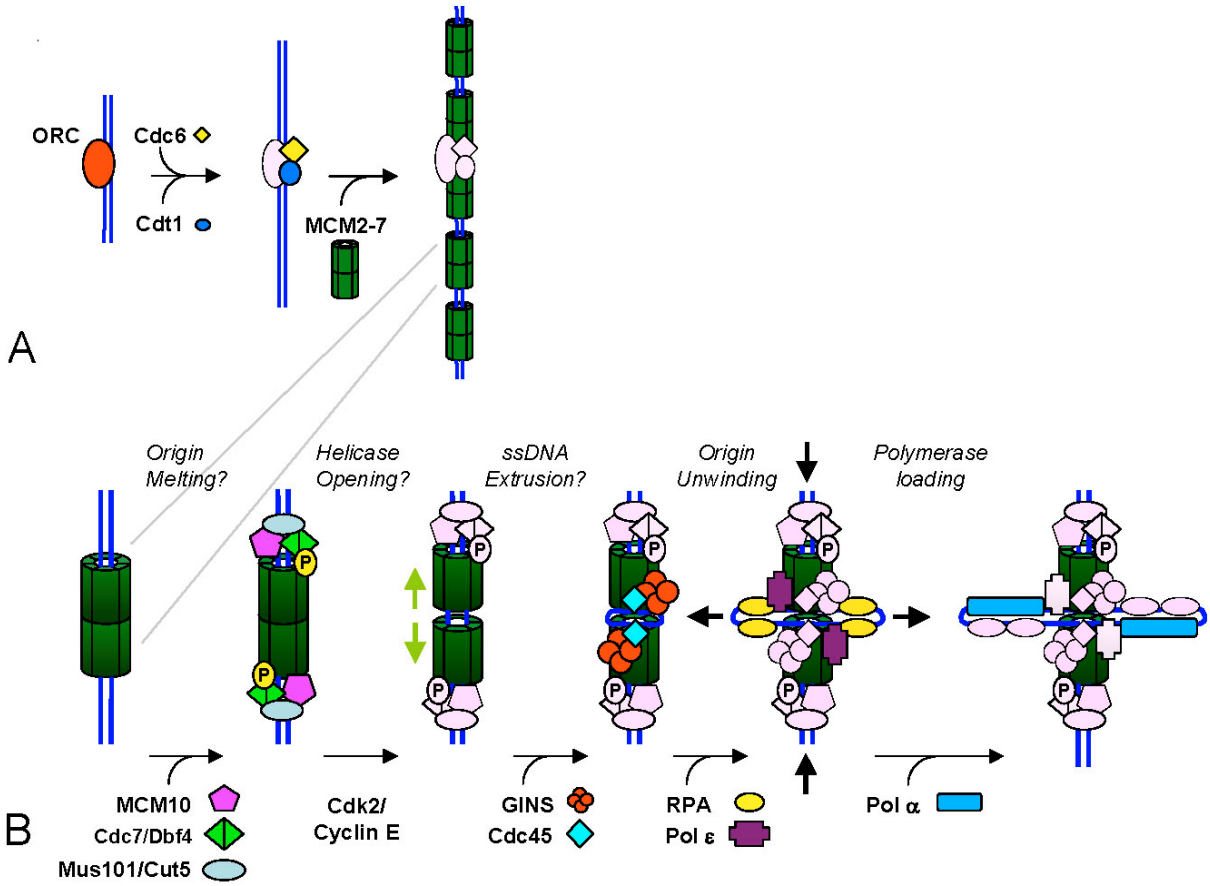

Figure 2. Model for replication initiation in *Xenopus* egg extracts. (A) Assembly of the pre-RC in egg cytosol. (B) Mechanism of pre-RC activation at the G1/S transition. One of the many MCM2-7 double hexamers shown in Figure 2A is shown to undergo activation. Each initiation factor is a different color at the time when it loads onto the origin, after which it is colored pink. The movement of DNA during unwinding is indicated by black arrows. The model is based on the assumption that MCM2-7 encircles duplex DNA both in G1 and in S phase, when it is active as a DNA helicase.

structure, are able to initiate DNA replication anywhere. By contrast, in somatic cells, DNA replication would initiate at sites where chromatin structure is conducive for pre-RC formation. In this view, a key function of origins of DNA replication would be to establish such a chromatin structure. Within a region of open chromatin structure, the preference of ORC for AT-rich DNA may help to determine where it binds, and where DNA replication initiates.

7. PRE-REPLICATION COMPLEX ASSEMBLY: THE HELICASE DELIVERY MECHANISM

Once ORC has loaded onto chromatin, the next step in the initiation of DNA replication involves the loading of Cdc6, Cdt1, and MCM2-7 to form the pre-RC (Figure 2). Using immunodepletion of specific proteins from egg cytoplasm, it was shown that Cdc6 loading requires the presence of ORC but not MCM2-7, whereas the loading of MCM2-7 requires ORC and Cdc6 (51-53). Subsequently, Cdt1 was identified, and shown to bind chromatin after ORC and to be required for MCM2-7 loading (54). The loading of Cdc6 and Cdt1 appear to be independent of one another (54, 55). Recently, pre-RC formation has been reconstituted using purified ORC, Cdc6, Cdt1, and MCM2-7 (55). Because the ORC and MCM2-7 preparations used were endogenous, it cannot be ruled out that they contained additional proteins required for pre-RC formation. For example, a new pre-RC component called Noc3 was recently identified in budding yeast (56). Noc3 is highly conserved in all eukaryotes, and it will be interesting to determine whether it plays a role in pre-RC formation in *Xenopus* egg extracts.

Once ORC, Cdc6, and Cdt1 have delivered the MCM2-7 complex to chromatin, they appear to be dispensable for subsequent initiation. Thus, after the MCM2-7 complex has been loaded onto chromatin in HSS, ORC can be removed by salt extraction or addition of Cyclin A (57, 58). Upon transfer of the chromatin to ORC depleted extract containing membranes, DNA replication still occurs. Cdt1 and Cdc6 are also removed during salt extraction and are unlikely to rebind in the absence of ORC. Similar findings have now been reported in yeast (59). There is a growing consensus that MCM2-7 is the eukaryotic replicative DNA helicase (60). Experiments in *Xenopus* extracts are consistent with this idea. For example, when MCM2-7 is depleted from egg extracts, there is no

Table 1. Essential Xenopus DNA replication initiation factors

Factor	# subunits	Biochemical Activity	Proposed Role in Initiation
ORC	6	ATPase, DNA binding protein,	Initiator, MCM2-7 recruitment
Cdc6	1	ATPase, clamp loader	MCM2-7 recruitment
Cdt1	1	?	MCM2-7 recruitment
MCM2-7	6	ATPase, Helicase	Replicative DNA Helicase?
MCM10	1	?	Cdc7/Dbf4 co-factor?
Cdc7/Dbf4	2	Protein kinase	MCM2-7 phosphorylation
Mus101/Cut5	1	?	Cdc45 recruitment
Cdk2/Cyclin E	2	Protein kinase	Cdc45 recruitment, substrate unknown
GINS (Sld5, Psf1, 2, 3)	4	?	Cdc45 recruitment
Cdc45	1	?	MCM2-7 cofactor?
RPA	3	single-stranded DNA binding	ssDNA binding, polymerase recruitment

origin unwinding, as seen in a plasmid supercoiling assay, and by a lack of RPA binding to chromatin (61, 62). Moreover, immunoprecipitates of MCM2-7 from solubilized chromatin exhibit modest helicase activity (63). Therefore, pre-RC formation represents an elaborate mechanism to recruit the replicative DNA helicase to chromatin.

Measuring the number of ORC and MCM complexes on chromatin has yielded unexpected results. When sperm chromatin is incubated in HSS to form pre-RCs, one ORC complex is bound on average every 10 kb (12, 52), or about once per replicon. Strikingly, the number of chromatin-bound MCM complexes was found to be in a 20-40 fold excess over ORC (12, 64). These MCM2-7 complexes are distributed over several kb of DNA surrounding ORC, even before they have become activated, and each MCM2-7 complex can be used as a replication initiation site (Figure 2A; (65, 142)). In this view, the pre-RC in *Xenopus* embryos is a highly redundant structure that consists of many replication-competent MCM2-7 complexes. In yeast, ChIP experiments suggest that the MCM2-7 complex binds in a more localized fashion (66, 67). In vertebrate somatic cells, there are several lines of evidence which suggest that MCM complexes may bind in a distributed fashion to chromatin: chromatin-bound MCM2-7 complexes are present in excess over the number of origins (68), the large majority of MCM complexes do not co-localize with sites of DNA replication (69-71), and cross-linking experiments show that MCM2-7 and ORC do not co-localize on DNA fragments smaller than 500-1000 bp (72). In section 11, we discuss how distributed MCM2-7 complexes may contribute to faithful DNA replication.

Despite the progress in understanding the assembly pathway of pre-RCs, little is known about the molecular mechanisms by which ORC, Cdc6, and Cdt1 recruit MCM2-7 onto chromatin. While it is clear that ORC serves as a landing pad for other pre-RC components, it is not clear what other function, if any, it plays during replication initiation. The analogy with prokaryotic and viral initiators such as dnaA and SV40 large T antigen suggested that ORC might denature, or "melt," the origin to form an open complex, which then serves as a binding site for the helicase. However, no melting activity of purified yeast ORC has been detected (73), and no ORC-dependent changes in DNA topology of circular plasmids were observed in HSS (61). Similarly, there is currently no model for the mechanism of action of Cdt1, a coiled-coil domain protein. Cdc6 is an ATPase that shows limited homology to the "clamp-loader" RFC, a protein required to deposit the PCNA processivity factor onto double stranded DNA (74). The ATPase activity of Cdc6 is required for MCM2-7 loading in *Xenopus*, yeast, and human cells ((75) and references therein). The similarity of Cdc6 to RFC is intriguing because it suggests that Cdc6 might contribute to the topological engagement of MCM2-7 with duplex DNA. If this model is correct, a major question is whether MCM2-7 is engaged with single-stranded or double stranded DNA in the pre-RC. If MCM2-7 were bound to ssDNA within the pre-RC, significant amounts of ssDNA would be present for extended periods of time, given that in somatic cells MCM2-7 loads in G1 and only becomes activated in S phase hours later. Moreover, there are up to 40 MCM complexes per origin in *Xenopus* egg extracts, so the amount of ssDNA present in a pre-RC in this system would have to be extensive. Assuming that exposure of ssDNA can cause DNA damage, we suggest that MCMs may encircle double stranded DNA (Figure 2A). Consistent with this model, MCM complexes loaded onto chromatin in egg cytosol are extremely stable, being resistant to extraction by ~1 M salt (65, 76).

8. PRE-INITIATION COMPLEX ASSEMBLY: THE HELICASE ACTIVATION STEP

At the onset of S phase, the pre-RC is activated by two protein kinases, Cdk2/Cyclin E and Cdc7/Dbf4, which collaborate with a number of other factors to recruit the initiation factor Cdc45 to the origin (Figure 2B). The complex of Cdc45 bound to the origin has been called the pre-Initiation Complex (77). The formation of the pre-IC appears to be a key event because it immediately precedes initiation and is required for origin unwinding (61, 62). In this section, we will review the factors that are known to contribute to pre-IC formation (see Table 1), the order in which they assemble onto the pre-RC, and the mechanisms by which they are thought to act (Figure 2B).

MCM10: MCM10 is an initiation factor with a highly conserved Zinc binding motif that was initially identified in yeast. In budding yeast, MCM10 binds to chromatin throughout the cell cycle, and is required for binding of MCM2-7 to origins (78). As such, it was

classified as a pre-RC component. In contrast, immunodepletion of *Xenopus* MCM10 from the nucleus-free DNA replication system showed that MCM10 is not required for MCM2-7 binding, but rather for Cdc45 loading to form pre-ICs (35). Consistent with its post-pre-RC role in replication initiation, MCM10 does not bind to chromatin during pre-RC formation in HSS, but only after addition of NPE. Similarly, in humans MCM10 binds chromatin at the G1/S transition (79). Binding of MCM10 to chromatin in NPE is dependent on the previous association of MCM2-7 with chromatin, and is independent of Cdc7 and Cdk2 activities, indicating that it loads at an early step during the G1/S transition. Interestingly, a recent report shows that like *Xenopus* MCM10, *S. pombe* MCM10 is not required for MCM2-7 chromatin loading in G1, but rather for Cdc45 loading at G1/S (80). The molecular function of MCM10 in initiation remains unknown. A hint comes from a recent report showing that SpMCM10 stimulates phosphorylation of MCM2 and MCM4 by Cdc7/Dbf4 *in vitro* (81), suggesting that MCM10 may function as a Cdc7 co-factor *in vivo*.

Cdc7/Dbf4: Like MCM10, the protein kinase Cdc7 is required for Cdc45 loading in *Xenopus* egg extracts (37, 82). Cdc7 binds to chromatin dependent on the prior presence of the MCM2-7 complex, and its binding is independent of Cdk2 activity. A *Xenopus* Dbf4 homolog has been isolated which is recruited to chromatin, and which also binds to and activates the kinase activity of Cdc7 (83, 84). In budding yeast, a point mutation in MCM5 (the *bob-1* allele) bypasses the requirement for Cdc7 in DNA replication, suggesting that MCM2-7 is a substrate of Cdc7 (85). Consistent with this, various members of the MCM2-7 complex serve as good substrates for Cdc7/Dbf4 in a wide range of organisms (reviewed in (86)). For example, in *Xenopus* egg extracts, MCM2 and MCM4 phosphorylation depends on Cdc7 ((82) and our unpublished results). Cdc7 acts independently of Cdk2, and Cdc7 must exert its function in initiation before Cdk2 (37, 82). Interestingly, reciprocal shift experiments using temperature sensitive alleles of Cdc7 and Cdc28 in yeast concluded that Cdk must act before Cdc7 (87). It remains to be determined whether the different outcomes of these experiments represent artifacts or real differences in the order of action of these protein kinases. The functional consequences of Cdc7 action on the pre-RC remain uncertain. However, the *bob-1* allele suggests that the major consequence of Cdc7 action may be a conformational change in the MCM2-7 complex. Interestingly, *bob-1* yeast cells arrested in the G1 phase of the cell cycle experience changes in origin structure that could reflect denaturation of origin strands (88). Therefore, an attractive model is that the action of Cdc7 on the MCM2-7 complex leads to origin melting (Figure 2B).

Mus101: The *Xenopus* Mus101 protein (also referred to as Cut5) is homologous to the *Drosophila* Mus101 protein, the fission yeast Cut5 protein, the budding yeast Dpb11 protein, and the human TopBp1 proteins (89, 90). It contains eight copies of the BRCA1 C-terminus (BRCT) domain, which are thought to be involved in protein-protein interactions. In *Xenopus* egg extracts, Mus101/Cut5 is required for the loading of Cdc45 (but not MCM10 or Cdc7) onto chromatin, and therefore is a bona fide replication initiation factor. It is also required for the elongation phase of DNA replication. Mus101/Cut5 loading onto chromatin is Cdk2 and MCM2-7 independent while requiring ORC. The specific role of Mus101/Cut5 in DNA replication is not understood, but the data suggest its action is a pre-requisite for the subsequent modification of the replication complex by Cdk2/Cyclin E (89).

The protein most similar to Mus101 in yeast is Dpb11. At the non-permissive temperature, yeast cells harboring a temperature sensitive allele of *dpb11* exhibit no defect in RPA binding, indicating that origin unwinding occurs normally, whereas loading of DNA pol ε and α is deficient (91). These results contrast with the findings in *Xenopus*, which indicate that Mus101 is required before origin unwinding. It is presently unclear whether these two proteins are true homologues, whether their functions have diverged, or whether a null allele of *dpb11* would give an earlier arrest in yeast.

Cdk2/Cyclin E: Immunodepletion of Cdk2 from interphase egg extracts abolishes DNA replication (92), as does depletion of Cyclin E (93). When Cdk2/Cyclin E is removed, other Cdks can substitute. Initially, it was reported that Cdk2/Cyclin A and Cdk1/Cyclin A, but not Cdk1/Cyclin B, could support DNA replication in egg extracts (93-95). A likely explanation for the inability of Cdk1/Cyclin B to support DNA replication was that it causes nuclear envelope breakdown (NEB). Indeed, when Cdk1/Cyclin B activity is adjusted to intermediate levels that do not cause NEB, it supports DNA replication (96). Moreover, in the nucleus-free system, concentrations of Cdk1/Cyclin B that would normally cause NEB are fully active for DNA replication (our unpublished results). Therefore, Cdk2/Cyclin E can be replaced by all the other major Cdks that are expressed in S, G2, and, M.

When Cdk2 is inhibited by $p21^{Cip}$ or $p27^{Kip}$, DNA replication is abolished (61, 97), but the Cdc7-dependent modification of replication complexes takes place, as does the loading of MCM10 and Mus101/Cut5. In contrast, Cdc45 loading is blocked. Hashimoto and colleagues reported that the Cdk2-dependent initiation step can be carried out in the absence of Cdc45 (89), suggesting that the action of Cdk2/Cyclin E generates a stable, chromatin-bound intermediate which then forms a binding site for Cdc45. The nature of this intermediate is unknown, but we speculate it may involve reconfiguration of the MCM2-7 complex such that ssDNA can be extruded (Figure 2B and see below). After Cdc45 has loaded, Cdk2 activity is not required for subsequent initiation events (61, 97). These observations indicate that Cdk2 is required during a narrow window in replication initiation. At present the substrate(s) of Cdk2/Cyclin E which must be phosphorylated for replication initiation to occur in *Xenopus* egg extracts are unknown. Experiments performed in budding yeast show that DNA replication initiation requires the Cdk-dependent phosphorylation of the Sld2 protein (98, 99). Phosphorylation of Sld2 controls its association with the

Mus101/Cut5 relative, Dpb11, which is thought to act after origin unwinding (see above). Presently, a vertebrate Sld2 protein has not been identified.

GINS: Recently characterized in *Xenopus* and in *S. cerevisiae* as an essential replication factor, GINS is a tetrameric complex composed of four subunits, Sld5, and Psf1-3 (76, 100). In *Xenopus*, chromatin loading of GINS requires pre-RC assembly, Mus101, and Cdk2/Cyclin E activity. Additionally, chromatin loading of *Xenopus* GINS and Cdc45 appear to be interdependent. *Xenopus* GINS interacts with Cdc45 and MCM2-7 on replicating chromatin, and ChIP experiments in *S. cerevisiae* indicate that GINS travels with MCM2-7 and Cdc45, initially associating with replication origins and later with origin-distal sequences. These results suggest that GINS is present initially as part of the pre-IC at origins and later as a component of the replication fork machinery. Electron microscopy of recombinant *Xenopus* GINS reveals that the tetramer forms a ring-like structure. Based on work in both *Xenopus* and yeast, it has been proposed that GINS could cooperate with Cdc45 to activate the MCM2-7 complex. In addition, it may function as a loading factor for DNA pol ε.

Sld3: In budding and fission yeasts, Cdc45 forms a complex with Sld3, which is essential for Cdc45 loading onto chromatin (101, 102). To date, a metazoan Sld3 protein has not been identified.

Cdc45: The signature component of the pre-IC is Cdc45 (77), a protein lacking known functional motifs. When Cdc45 is depleted from egg extracts, there is no loading of the single-stranded DNA binding protein RPA, and no negative supercoiling of plasmids undergoing replication initiation in a nucleus-free system (61, 62). Conversely, when RPA is absent, Cdc45 loading is unaffected. These results argue strongly that Cdc45 is required for origin unwinding, perhaps as a helicase co-factor. This model is consistent with the observation that MCM2-7 and Cdc45 are found in the same complex on replicating chromatin (63). If Cdc45 is a helicase co-factor, it should be required for the elongation stage of DNA replication. Although this has not been shown in any vertebrate system, a "degron" mutant of Cdc45 in yeast showed that Cdc45 is required for replication fork progression (103). More work will be required to determine whether Cdc45 truly functions as a helicase co-factor, and if so, how it performs this function. As discussed above, pre-RCs formed in *Xenopus* egg extracts contain a large number of MCM2-7 complexes which are bound along the length of DNA at a density of about one per nucleosome (Figure 2A; (65)). Upon initiation of DNA replication, only a small subset of these chromatin-bound MCM2-7 complexes associates with Cdc45 (roughly 2 per 10kb; Figure 2B). Importantly, limiting chromatin loading of Cdc45 reduces the kinetics of S phase, indicating that Cdc45 binding is a rate limiting step for DNA replication (65).

The loading of Cdc45 to form the pre-IC is regulated by cell-cycle checkpoints. Thus, addition of double-stranded DNA breaks to nuclear assembly egg extracts leads to activation of the checkpoint protein kinase ATM, downregulation of Cdk2 activity, and impairment of Cdc45 loading (104). Similarly, in human tissue culture cells, γ-irradiation activates ATM kinase, leading to inhibition of Cdk2 activity and a block to Cdc45 loading (105). The other major checkpoint protein kinase, ATR, has also been shown to block Cdc45 loading in *Xenopus* egg extracts (83). Single-stranded DNA activates ATR, leading to inhibition of Cdc7 activity via dissociation of its regulatory subunit Dbf4. Thus, different types of DNA damage inhibit Cdc45 loading via different mechanisms that can be recapitulated in *Xenopus* egg extracts.

9. A SPECULATIVE MODEL FOR HELICASE ACTIVATION

The active conformation of the MCM2-7 complex is unknown. In conventional models, the MCM2-7 complex would form a ring around single-stranded DNA and use the energy from ATP hydrolysis to translocate along single-stranded DNA and thereby unwind the duplex. However, an alternative model is that the MCM2-7 complex remains engaged with double stranded DNA, even after activation. This mechanism is consistent with the finding that a fragment of the archeal MCM protein forms a stable dimer with a positively charged central channel that is wide enough (22 A°) to accommodate duplex DNA (106). Interestingly, the MCM complex is similar in its molecular organization to the mitochondrial F1-ATPase, suggesting it may rotate DNA through its central core (107). Thus, in one proposal, the MCM2-7 complex is a "rotary pump" (108). In this model, which seeks to account for the many distributed MCM2-7 complexes bound to chromatin in egg extracts, multiple MCM2-7 complexes bound at a distance from each other coordinately pump DNA into the space separating them. The pumping of DNA by the two flanking groups of MCM2-7 complexes causes rotation of DNA in opposite directions, and this in turn would lead to strand separation. However, in this model, it is unclear how topoisomerases could be prevented from relaxing the DNA prior to strand separation. Moreover, in *Xenopus* egg extracts, the vast majority of chromatin-bound MCM2-7 complexes are dispensable for efficient DNA replication, and only a small subset of these are normally activated by Cdc45 (64, 65). A variation of this model is that two MCM2-7 complexes that physically associate with each other on the DNA pump DNA towards their interface and then extrude ssDNA (Figure 2B). This mechanism has been observed during SV40 DNA replication, where two adjacent Large T antigen hexamers extrude DNA to form a "rabbit-ear" structure visible by electron microscopy (109, 110). If the MCM2-7 complex encircles duplex DNA in G1 and in S phase, then its activation at the G1/S transition may involve the following steps (figure 2B): (1) melting of a limited amount of origin DNA by a conformational change in the MCM2-7 complex that is catalyzed by Cdc7/Dbf4 and possibly assisted by MCM10 and Mus101/Cut5; (2) further reconfiguration of the MCM2-7 complex by Cdk2/Cyclin E such that ssDNA can be extruded; (3) Cdc45 and GINS assisted extrusion of

ssDNA to carry out DNA unwinding. To test these ideas, higher resolution analysis of origin DNA, as well the proteins bound to it, will be required.

10. ORIGIN- RECRUITMENT OF DNA POLYMERASES

The final step in replication initiation is the loading of the replicative DNA polymerases α, δ, and ε, and the commencement of DNA synthesis. These events have been studied most intensively in the SV40 cell-free DNA replication system (111). DNA pol α contains four subunits, a 180 kD DNA polymerase subunit, 55kD and 45 kD subunits that together comprise the RNA primase, and a 68 kD subunit of unknown function. In the SV40 system, origin-loading of the DNA pol α complex onto the origin requires the helicase activity of SV40 Large T Ag, as well as interactions between the polymerase, Large T Ag, and RPA. DNA pol α then synthesizes a short, ~7 nucleotide RNA which serves as a primer for DNA synthesis. The 180 kD subunit of DNA pol α then polymerizes ~30 deoxynucleotides. Finally, RFC recognizes the DNA primer and loads the processivity factor PCNA as well as DNA pol δ, a highly processive DNA polymerase.

Our knowledge of DNA polymerase recruitment and the commencement of DNA synthesis in *Xenopus* egg extracts is less complete than in the SV40 system. Loading of DNA pol α requires Cdc45 (112). Cdc45 may play a direct role in DNA pol α loading, since interactions between these proteins have been reported in *Xenopus* egg extracts and in human cells (112, 113). However, there is also evidence that DNA pol α loading depends on origin unwinding (61), which in turn requires Cdc45 (61, 62). Therefore, Cdc45 may play direct and indirect roles in recruiting DNA pol α. Interestingly, origin unwinding but not polymerase loading occurs when RPA is replaced by *E. coli* SSB, suggesting that specific interactions with RPA are important for polymerase loading (61). In fission yeast, Dpb11 and Sld2 are implicated in loading polymerases after the origin is unwound. While the Dpb11 homolog in *Xenopus*, Mus101/Cut5, acts upstream of origin unwinding (see above), a role further downstream cannot be ruled out. Initiation of DNA replication in *Xenopus* egg extracts is expected to involve synthesis of an RNA primer by DNA pol α. Consistent with this, DNA replication is blocked by actinomycin D, an inhibitor of DNA-dependent RNA synthesis (114). When DNA pol α is depleted from nuclear assembly extracts (62), or in the presence of 50 μg/ml aphidicolin (114), an inhibitor of DNA pol α, PCNA is not loaded, indicating that PCNA loading by RFC requires a DNA primer as it does in the SV40 system. Based on these results, it appears likely that the loading of DNA pol α and DNA pol δ in *Xenopus* egg extracts closely follows the paradigm established in the SV40 system.

The third DNA polymerase that is required for chromosomal DNA replication is DNA pol ε, but its precise role is uncertain. DNA pol ε is not required for SV40 DNA replication (115). In *S. pombe*, cells lacking the catalytic N-terminal domain of DNA pol ε are viable, whereas cells lacking the non-catalytic C-terminal region are not(116, 117). In DNA pol ε-depleted *Xenopus* egg extracts, overall DNA replication is reduced by 60-80%, but it is not clear whether this defect is due to the absence of the catalytic function of DNA pol ε (118). Chromatin loading of DNA pol ε requires Cdc45 but not RPA (62). These requirements are distinct from those of DNA pol α whose loading requires both Cdc45 and RPA. Therefore, although DNA pol ε is a processive DNA polymerase, it loads *before* DNA pol α. Despite its early loading, DNA pol ε is however not required for origin binding of RPA or DNA pol α (118). In summary, in *Xenopus* egg extracts, DNA pol ε loads onto origins at a relatively early step and it is required for efficient DNA replication, but its precise function remains to be determined.

11. REGULATION OF RE-REPLICATION

Eukaryotes take special care to insure that each origin of DNA replication fires only once per cell cycle, as re-duplication of even a small portion of the genome might lethal. The cell cycle regulation of replication is exerted on the chromatin-binding of the MCM2-7 complex. Thus, MCM2-7 binds to chromatin in G1, it leaves the origin when replication initiates, and multiple mechanisms insure that it is not able to re-bind to already fired origins in the S, G2, and M phases of the cell cycle.

Historically, *Xenopus* egg extracts have been an important system for the study of cell-cycle regulation of DNA replication because they support only a single round of replication (3). An early observation was that nuclei that had replicated once in LSS could be rendered competent to replicate again simply by permeablizing the nuclear envelope (119). Based on this observation, the existence of a replication "licensing factor" was postulated. The licensing factor was proposed to: (i) load onto chromatin and be required for initiation, (ii) be inactivated during origin firing, and (iii) be restricted from re-binding chromatin by nuclear exclusion until the cell has passed through mitosis. The MCM2-7 complex was considered a potential candidate for licensing factor because in *S. cerevisiae* it was found to be required for replication initiation, and it was excluded from nuclei once cells enter S phase (120). Subsequently, characterization of the *Xenopus* MCM2-7 complex showed that it was displaced from chromatin during the first round of DNA replication (121-123) and that it was not allowed to rebind to chromatin until cells passed through mitosis or the nuclear envelope was permeablized (124). However, surprisingly, MCM2-7 was found *not* to be excluded from the nucleus in Xenopus egg extracts (121, 125), leaving open the question as to how *de novo* MCM2-7 loading is prevented during the S phase.

Concurrent with the development of the licensing factor model, experiments in yeast showed that Cdk activity, which promotes origin firing, is also crucial to prevent re-replication before cell division. Thus, in budding and fission yeast, transient inactivation of Cdk activity during G2 is sufficient to induce MCM2-7 re-loading onto origins and to cause additional rounds of DNA replication

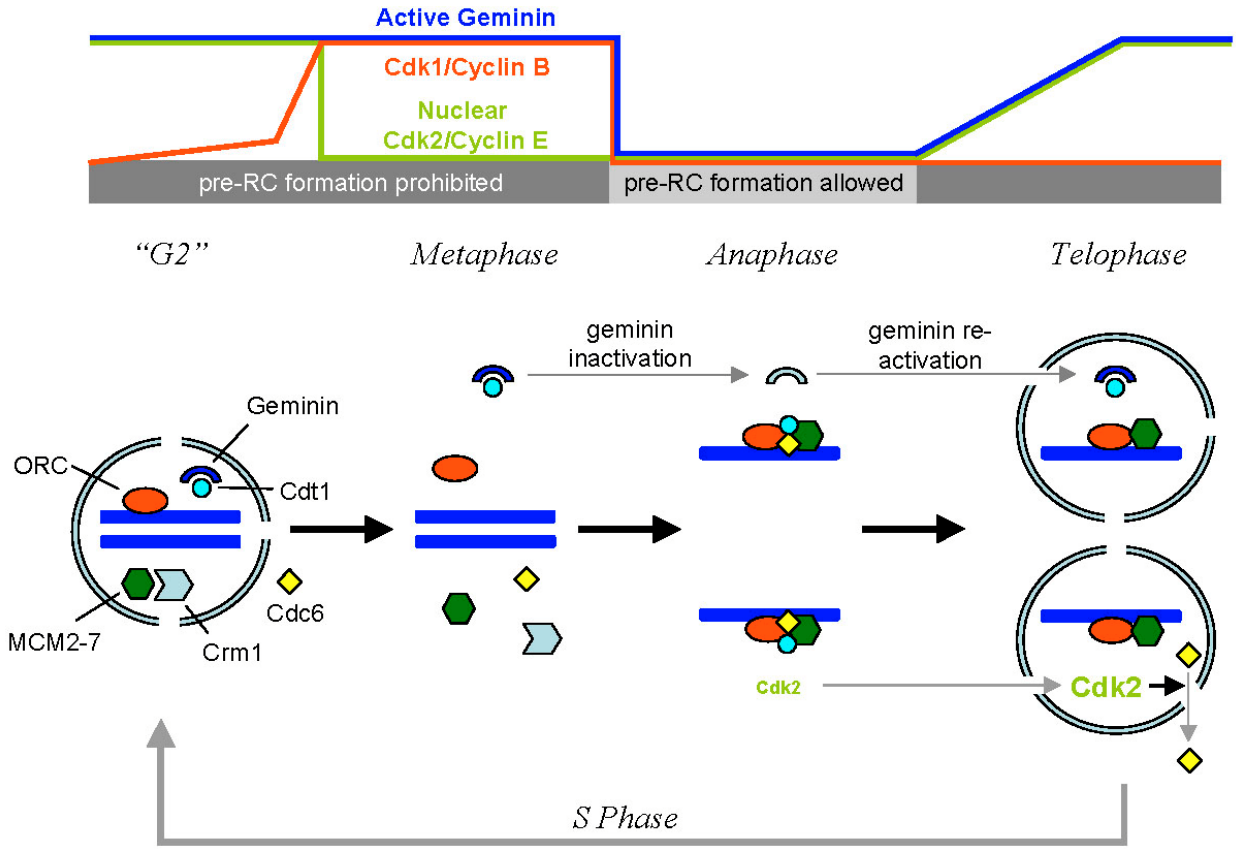

Figure 3. Cell-cycle regulation of DNA replication in Xenopus egg extracts. Pre-replication complexes assemble after anaphase, and before a nuclear envelope re-forms in telophase. At all other stages of the cell-cycle, various inhibitory mechanisms prevent MCM2-7 loading onto chromatin.

(67, 126, 127). Studies in *Xenopus* egg extracts showed that Cdk activity also inhibits MCM2-7 loading in vertebrates. As noted above, nuclear Cdk2/cyclin E is enriched ~200-fold relative to cytosol in nuclear assembly egg extracts. Importantly, when Cdk2/Cyclin E is added to HSS at concentrations approaching those seen in the nucleus, MCM2-7 loading is blocked (22). Based on these results, it appears that one likely consequence of nuclear envelope permeablization was the dilution of nuclear inhibitors of replication such as Cdk2/Cyclin E. Indeed, nucleoplasmic extract (NPE, Figure 1B) is a potent inhibitor of MCM2-7 loading (34).

How might Cdk2/Cyclin E prevent re-replication in *Xenopus* egg extracts? A report from Laskey and colleagues shows that Cdk2/Cyclin E phosphorylates Cdc6 on numerous sites in the N-terminus of the protein, leading to its export from the nucleus (Figure 3 (128)). Interestingly, a Cdc6 mutant lacking all phosphorylation sites that is constitutively nuclear does not induce re-replication, suggesting there might be other targets of Cdk2/Cyclin E. In yeast, Cdks negatively regulate at least three independent pre-RC components, ORC, Cdc6, and MCM2-7, and inactivation of any one of these proteins by Cdk is sufficient to prevent re-replication (129). However, in *Xenopus* egg extracts, addition of the Cdk2 inhibitor $p27^{Kip}$ to nuclei or NPE after the first round of DNA replication does not induce MCM2-7 re-loading, indicating there is a Cdk2-independent mechanism to prevent re-replication in egg extracts (our unpublished results).

A new inhibitor of re-replication, called geminin, was identified by McGarry and Kirschner (130). Geminin was first discovered in *Xenopus* egg extracts in a screen for proteins that are degraded at the metaphase to anaphase transition, and it was shown to block chromatin loading of MCM2-7 while not affecting ORC or Cdc6. Geminin is a nuclear protein, and in somatic cells, it is expressed at low levels during G1 when pre-RCs are assembled. Geminin accumulates during S, G2, and M phase, the times in the cell-cycle when new pre-RC formation is prohibited. As such, geminin exhibits the properties expected of a protein that prevents re-replication. Subsequently, it was shown that geminin binds to Cdt1 and that this interaction abrogates recruitment of MCM2-7 to chromatin (131, 132). Interestingly, in *Xenopus* egg extracts, endogenous geminin is only partially degraded upon mitotic exit, though it appears to be fully inactivated, and it is re-activated to bind Cdt1 upon import into the nucleus (Figure 3). This creates a window of opportunity for Cdt1 to recruit MCM2-7 to the origin. At present, the mechanisms of activation/inactivation of geminin are not known (133). Depletion of geminin from *Xenopus* extracts or embryos does not lead to re-replication, although geminin is highly active in mitotic

extracts and within interphase nuclei (131, 133). Therefore, the lack of re-replication in geminin-depleted extracts and embryos most likely points to the existence of other factors, such as Cdk2/Cyclin E and Cdk1/Cyclin B, that are sufficient to prevent re-replication in the absence of geminin. Direct evidence for an involvement of geminin in preventing re-replication comes from studies in *Drosophila* where depletion of geminin from embryos or tissue culture cells results in detectible re-replication (134, 135).

Recently, the small GTPase Ran has been linked to the cell-cycle regulation of DNA replication (125). In the nucleus, Ran binds to GTP, whereas in the cytoplasm it binds to GDP, and these two forms of Ran control the association of nuclear transport cargo with importins and exportins (136). Yamaguchi and Newport (125) showed that maintaining high concentrations of Ran:GTP in the nucleus is critical to prevent re-replication. Cdk2/Cyclin E stimulates the formation of a Ran:GTP/Crm1/MCM2-7 complex, which prevents re-replication by sequestering free MCM2-7.

In summary, during the early embryonic cell cycles in *Xenopus*, pre-RCs are only able to form during a short temporal window (Figure 3). The window begins to open upon nuclear envelope breakdown when the Ran gradient is dissipated, Cdk2/Cyclin E is diluted, and Cdc6 regains access to the chromatin. However, these events are not sufficient to allow MCM2-7 loading, since inhibitory levels of Cdk1/Cyclin B and geminin are still present. These two inhibitors are inactivated when cells go through anaphase. Therefore, MCM2-7 loads onto chromatin in late anaphase. The permissive window for pre-RC formation closes again when the nuclear envelope reforms in telophase, as this leads to nuclear import of Cdk2, import and activation of geminin. Importantly, the high nuclear concentration of Cdk2/Cyclin E also stimulates initiation. Therefore, pre-RC assembly and replication initiation occur at mutually exclusive times during the cell cycle, insuring that each origin fires only once.

A theme that emerges from these studies is that multiple mechanisms have evolved to prevent re-replication. An important challenge that remains is to understand the relative importance of each mechanism and to determine if additional mechanisms exist. In addition, it will be important to determine how the regulation of DNA replication differs in embryonic and somatic tissues.

12. THE RANDOM COMPLETION PROBLEM

Considering the characteristics of the early embryonic cell cycle, a dilemma arises with regard to how DNA replication is completed before mitosis (137, 138). The DNA replication fork moves at a rate of ~0.5 kb/min, and S phase lasts about 20 minutes (9, 42). The replication fork is therefore able to travel a maximum of ~10 kb during S phase, and the maximum replicon size that can be duplicated by two converging replication forks is 20 kb. Although the average replicon size in pre-MBT *Xenopus* embryos and in egg extracts is roughly 10 kb, replication initiation is sequence-independent. Therefore, assuming a completely random pattern of initiations, a Gaussian distribution of replicon sizes centered around 10 kb is expected, with a significant percentage of replicons being greater than 20 kb. Importantly, unreplicated DNA does not arrest the cell cycle during the early embryonic cell divisions (28, 29). As a result, any replicon greater than 20 kb is expected to cause cells to undergo anaphase with incompletely replicated DNA (mitotic catastrophe). The question of how DNA replication is completed before mitosis under these circumstances (sequence-independent replication initiation, short S phase, no S phase checkpoint), has been called the "random completion problem" (137).

There are several possible solutions. One is to maximize the amount of time that is available for DNA replication. A recent report shows that pre-RCs and nuclear envelopes form on individual chromosomes ("karyomeres"), allowing DNA replication to initiate on individual chromosomes in telophase before a complete nucleus has been assembled (139). This mechanism lengthens the time available for DNA replication relative to the total length of the cell cycle. Another solution is to insure that initiation events, while being random with respect to DNA sequence, are regularly spaced to maintain replicon size below 20 kb. Although the evidence is somewhat conflicting, it appears that the distribution of replicon sizes is not completely random, and this non-randomness could be encoded by chromatin structure (8, 137). However, with ~300,000 initiation events per S phase, some replicons are likely to exceed 20 kb, and entering mitosis with *any* unreplicated DNA is a lethal event. A third possibility is that replicon size during S phase is flexible. Work from Hyrien and colleagues suggests that replicon sizes vary widely at the onset of S phase, but that the frequency of replication initiation increases later in S phase in regions containing unreplicated DNA (140, 141). This model was considered to be problematic because it cannot be anticipated where on the chromosome a high frequency of initiation events will be required and because of the strict injunction against *de novo* pre-RC formation once cells enter S phase (see last section). However, the recent finding that MCM complexes are widely distributed on chromatin in egg extracts renders the "flexible initiation" model possible ((65, 142). In the most extreme form of this model, the entire chromosome is coated with MCM2-7 complexes. If all of these complexes are initiation competent as the data suggests, then any significant portions of the chromosome that remain unreplicated late in S phase could sustain many closely spaced replication initiation events without violating any rules of the cell cycle. One can envision several ways in which initiation frequency is increased late in S phase. One possibility is that continuous import of Cdk2/Cyclin E makes initiation increasingly frequent. Another is that changes in chromosome structure that result from DNA replication make further initiation events more likely. In Section 7 above, we discussed the evidence that a large excess of replication competent MCM complexes are bound to chromatin in somatic cells. If this is the case, the purpose

may be to insure that each origin will undergo at least one replication initiation event.

13. CONCLUSIONS AND PERSPECTIVES

The rapid embryonic cell cycles in *Xenopus* have evolved to generate a critical cell mass in a short time. However, we are still struggling to understand how mitotic catastrophes are prevented in these cell cycles because the time allotted for DNA replication is so brief and because they do not arrest in response to unreplicated DNA. Clearly, the solution lies in a fail-proof and extremely efficient mechanism of DNA replication. First, the activity of DNA replication factors such as Cdk2/Cyclin E is regulated through nuclear transport, which is more efficient and presumably more rapid than repeated cycles of protein synthesis and degradation, as seen in somatic cells. A second strategy involves the use of closely spaced DNA replication initiation events, as well as an apparently radical mechanism in which potential replication start sites, represented by MCM2-7 complexes, are spaced a few hundred base pairs apart, to be used as necessary. Third, *Xenopus* eggs contain a vast stockpile of DNA replication factors, which presumably results in optimal reaction kinetics. It is the latter property of unfertilized eggs that makes them such powerful tools for the biochemist. Together with data from other systems, experiments performed in egg extracts are helping to elucidate a highly-conserved biochemical machinery that underlies DNA replication initiation. The results show that to initiate DNA synthesis, at least 13 factors (including DNA polymerase α) bind to chromatin in a highly ordered cascade where each new binding step generally depends on the preceding step. It is now clear that a major function of this intricate machinery is the recruitment and activation of the MCM2-7 complex. However, the specific roles that the various initiation factors play in this reaction remain obscure. In addition, it is unclear how many replication initiation factors still remain to be identified. Experiments in *Xenopus* egg extracts will undoubtedly continue to contribute to the elucidation of this fascinating problem.

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Abbreviations: Cdc, cell division cycle, Cdk, Cyclin dependent kinase, MPF, maturation promoting factor, MCM, minichromosome maintenance, ORC, origin recognition complex, Pre-IC, pre-initiation complex, Pre-RC, pre-replication complex

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