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DNA Replication in *Xenopus*

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Cell-free extracts of eggs of the South African clawed toad *Xenopus laevis* support complete chromosome replication under normal cell cycle control (Blow and Laskey 1986). Apart from a similar system derived from embryos of the fruit fly *Drosophila melanogaster* (Crevel and Cotterill 1991), it is currently the only eukaryotic cell-free system that supports efficient chromosome replication in vitro. Since the *Xenopus* cell-free system progresses through the complete cell cycle in vitro (Hutchison et al. 1987; Murray and Kirschner 1989), it offers a unique opportunity to study the way that DNA replication is coordinated with other cell-cycle events. This subject has recently been reviewed in detail (Blow 1996); the major conclusions are summarized here.

When *Xenopus* eggs are crushed by low-speed centrifugation, the resultant low-speed supernatants, which contain abundant particulate material (including nuclear envelope precursors), can support all the major activities of the early embryonic cell cycle. During each in vitro cell cycle there is an ordered sequence of events as follows:

1. Activation of certain essential replication proteins and their assembly onto decondensing chromosomes
2. Assembly of chromosomal DNA into nuclei with an intact nuclear envelope
3. Import of proteins required for the initiation and progression of replication
4. Termination of replication forks and inactivation of certain replication proteins to prevent re-replication of DNA in the current cell cycle.

THE ROLE OF NUCLEAR ASSEMBLY IN DNA REPLICATION

The *Xenopus* system can replicate a wide range of different DNA templates, including demembranated *Xenopus* sperm nuclei (sperm chromatin: the natural substrate for DNA replication in the egg) and

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naked DNA. In each case the template DNA is assembled into interphase nuclei by the cell-free system, involving the assembly of nuclear pores and a double unit nuclear envelope around a chromatin mass (Lohka and Masui 1983, 1984; Vigers and Lohka 1992). Once nuclear assembly is complete, selective nuclear protein accumulation rapidly occurs, an early consequence of which is the assembly of a nuclear lamina (Newport 1987; Newport et al. 1990; Meier et al. 1991; Jenkins et al. 1993). Assembly of template DNA into a functional nucleus is crucial for the way that replication is controlled.

When low-speed supernatants are centrifuged hard to remove particulate material, the resultant high-speed supernatants neither assemble interphase nuclei nor initiate DNA replication (Lohka and Masui 1984; Newport 1987; Sheehan et al. 1988; Blow and Sleeman 1990). Both these activities can be restored by re-addition of pelleted membrane material to the supernatants. When naked DNA is incubated in low-speed supernatants, only a fraction is assembled into nuclei, and only this DNA is replicated (Blow and Sleeman 1990). These results strongly suggest that nuclear assembly is required before DNA replication can occur. Since high-speed supernatants support the elongation stage of DNA replication (Méchali and Harland 1982; Blow and Laskey 1986; Cox 1992; Shivji et al. 1994), it appears that nuclear assembly is specifically required for the initiation of DNA replication. A similar dependence of DNA replication on nuclear assembly is seen in extracts of *Drosophila* embryos (Crevel and Cotterill 1991).

One explanation for this nuclear envelope requirement is that it permits the selective nuclear accumulation of proteins involved in initiation. Consistent with this, when nuclear protein import is prevented in egg extract, the initiation of DNA replication does not occur (Cox 1992). Nuclear assembly may also provide structural components of the nucleus required for DNA replication. DNA synthesis in sperm nuclei replicating in *Xenopus* extract localizes to approximately 100–200 discrete foci in the nuclear interior, each containing about 1000 replication forks (Mills et al. 1989). Replication foci were also seen within nuclei assembled from naked DNA (Cox and Laskey 1991). The need to assemble replication forks into these foci may be part of the reason that initiation of replication is dependent on nuclear assembly. Sequential assembly of replication proteins into these foci is observed. RP-A associates with pre-replication foci prior to nuclear assembly (Adachi and Laemmli 1992, 1994). PCNA and DNA polymerase-α are observed in these foci once nuclear assembly has been completed, just prior to the initiation of replication (Hutchison and Kill 1989).

Extracts immunodepleted of lamin B_3 do not assemble a lamina, nor do they support DNA replication, although functional nuclear envelopes are assembled (Newport et al. 1990; Meier et al. 1991; Jenkins et al. 1993). The involvement of the lamina in the initiation of DNA replication is unexpected, since its position underneath the nuclear envelope places it far from the replication foci in the center of the nucleus (Mills et al. 1989). In somatic cells, B-type lamins may colocalize to replication foci (Moir et al. 1994), which could give them a direct role in DNA replication (Hutchison et al. 1994).

Nuclear Structure and Replication Origins

Xenopus eggs and egg extracts replicate a wide variety of DNA templates introduced into them (Harland and Laskey 1980; Méchali and Kearsey 1984; Blow and Laskey 1986; Newport 1987). When normalized for size, the DNA sequence of the template DNA has little effect on the efficiency with which it is replicated (Méchali and Kearsey 1984). Neutral/neutral two-dimensional gel analysis (Hyrien and Méchali 1992; Mahbubani et al. 1992) and electron microscopy (McTiernan and Stambrook 1984) showed that different copies of replicating plasmid molecules contained single initiation bubbles at many different locations. Similar results were obtained by two-dimensional gel analysis of replicating sperm chromatin, showing initiation bubbles scattered throughout the rDNA of sperm chromatin (Hyrien and Méchali 1993). These results suggest that chromosomal DNA is replicated by a series of semi-discontinuous forks (Blow and Laskey 1986) initiated at sites that are not primarily dictated by DNA sequence (Fig. 1A).

Some mechanism must exist to regulate replicon size, because if initiation events occurred at random sites on the genome, there would be some excessively large replicons (Mahbubani et al. 1992). Instead of being dictated by DNA sequence, replicon size may be directly controlled by chromosome structure (Fig. 1B). Chromosomal loop size correlates well with the average replicon size as this increases during *Xenopus* development (Buongiorno Nardelli et al. 1982). In particular, each copy of rDNA appears to comprise one supercoiled loop (Marilley and Gassend Bonnet 1989), and each supports only a single initiation event (Mahbubani et al. 1992; Hyrien and Méchali 1993). Consistent with a role for nuclear structure in determining origin usage, intact hamster nuclei incubated in *Xenopus* extract continued to use the dihydrofolate reductase origin of replication, although naked DNA containing this region showed no preferential initiation (Gilbert et al. 1993, 1995).

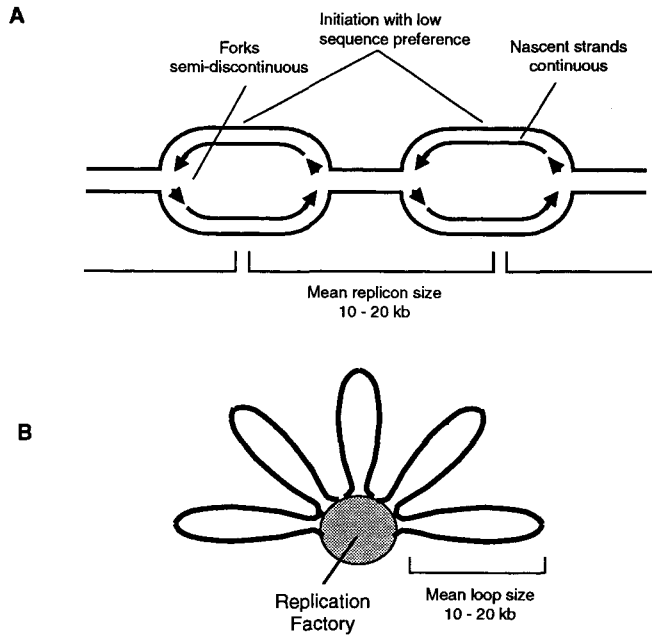

Figure 1 Replication origin usage in the *Xenopus* system. (*A*) Cartoon summarizing experiments analyzing replication origin usage and the structure of replicative intermediates in the *Xenopus* system. (*B*) Cartoon showing a possible role for chromosome looping in generating a relatively constant replicon size in the *Xenopus* system. See text for more details. (Redrawn from Blow 1996.)

The Nucleus as a Fundamental Unit of DNA Replication

Initiation occurs virtually as soon as nuclear assembly has been completed. Since nuclei are not all assembled at the same rate, this means that different nuclei may start to replicate at different times. Analysis of replication kinetics showed that nuclei act as individual units, each receiving a signal to replicate from the cytoplasm, which causes them to undergo once and only once a burst of near-synchronous initiation events (Blow and Watson 1987). The feature that defines this unit of replication is likely to be the nuclear envelope, since all the DNA surrounded by an intact nuclear envelope starts to replicate at the same time, even if this DNA was originally derived from more than one nucleus (Leno and Laskey 1991).

As outlined in Figure 2, these results provide a model for the way in which DNA replication is controlled in the *Xenopus* cell cycle (Blow and Watson 1987; Blow and Laskey 1988; Blow 1996). Replication control

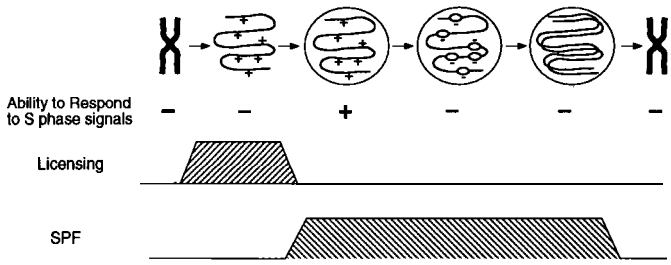

Figure 2 Model to explain replication control in the *Xenopus* system. A single nucleus is shown as it passes through a complete cell cycle. During late mitosis, prior to nuclear envelope assembly, the DNA becomes "licensed" (+) to undergo DNA replication. Once assembled into a nucleus, the licensed DNA is capable of initiating DNA replication in response to the presence of the S-phase inducer SPF. However, the license is destroyed (–) as the DNA is replicated. Only following passage through mitosis does the nucleus once again become competent to undergo further DNA replication. (Redrawn from Blow 1996.)

is divided into two distinct components: the DNA template in the nucleus, and activities present in the cytoplasm that act on this nuclear substrate. The nucleus can be in one of two states: either capable or incapable of responding to S-phase inducers by undergoing DNA replication. The ability of nuclei to respond to the S-phase inducers requires DNA to have been "licensed" for DNA replication during the previous mitosis. The cytoplasm also provides an S-phase-promoting factor that acts on intact licensed nuclei to induce them to initiate DNA replication. The license is then inactivated or destroyed in the process of DNA replication.

ROLE OF LICENSING FACTOR IN REPLICATION CONTROL

The inability of replicated nuclei to respond to S-phase inducers present in *Xenopus* extracts can be demonstrated directly. Replicated G_2 nuclei that were transferred to fresh extract did not undergo further replication (Blow and Laskey 1988). However, if these nuclei were allowed to pass into mitosis, the DNA efficiently re-replicated on transfer to interphase extract. The effect of passage through mitosis could be mimicked by agents that caused nuclear envelope permeabilization, such as lysolecithin or phospholipase (Blow and Laskey 1988). Similar results were obtained in *Drosophila* extracts (Crevel and Cotterill 1991) and on addition of nuclei from mammalian tissue-culture cells into *Xenopus* eggs (De Roeper et al. 1977) or egg extracts (Leno et al. 1992; Coverley et al. 1993).

Figure 2 provides an explanation of these results (Blow and Laskey 1988; Blow 1993; Chong et al. 1996). An essential replication factor called replication licensing factor (RLF) binds DNA during late mitosis before nuclear assembly has occurred. RLF cannot cross the nuclear envelope, so once nuclear assembly is complete, RLF is only present in the nucleus where bound to DNA. On entry into S phase, RLF bound to DNA supports a single initiation event, after which it is inactivated or destroyed. Thus, in G_2, no active RLF remains in the nucleus and the nuclear envelope must be transiently permeabilized (as normally occurs during mitosis) to allow a further round of DNA replication to be licensed.

Identification of Licensing Factor Components

RLF has recently been subjected to biochemical fractionation by exploiting the ability of protein kinase inhibitors to block the activation of RLF that normally occurs during mitosis (Blow 1993; Kubota and Takisawa 1993; Vesely et al. 1994). RLF activity resolved into two components, RLF-M and RLF-B, both of which were required for licensing (Chong et al. 1995). RLF-M was purified to apparent homogeneity and consisted of a complex of at least three polypeptides, with molecular masses of 92 kD, 106 kD, and 115 kD. The 106-kD polypeptide is the product of the *Xenopus* MCM3 gene, and the other polypeptides seem likely to be other members of the MCM family (Chong et al. 1995). Both RLF-B and RLF-M were required for each successive round of DNA replication. *Xenopus* Mcm3 associated with chromatin in G_1 but was removed during replication, consistent with its involvement in the RLF system. Furthermore, the rebinding of Mcm3 to replicated chromatin was dependent on prior nuclear envelope permeabilization (Chong et al. 1995). Immunodepletion of *Xenopus* Mcm3 also resulted in replication defects consistent with these results (Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995). The role of nuclear envelope permeabilization is currently unclear. Although RLF activity does not cross the nuclear envelope in *Xenopus* (De Roeper et al. 1977; Blow and Laskey 1988; Leno et al. 1992; Coverley et al. 1993; Blow 1993; Chong et al. 1995) or *Drosophila* (Crevel and Cotterill 1991), Mcm proteins are nuclear throughout the mammalian cell cycle (Thömmes et al. 1992; Kimura et al. 1994; Todorov et al. 1994), and Mcm3 is imported into intact nuclei in *Xenopus* extracts (Madine et al. 1995). One possibility is that active RLF-B is required for RLF-M to associate with chromatin, and that RLF-B is incapable of crossing the nuclear envelope. Purification and characterization of the RLF-B fraction should elucidate this point.

SPF: THE SIGNAL TO INITIATE REPLICATION

Soon after nuclear assembly is complete, each nucleus undergoes a coordinated burst of initiation events. The intranuclear signal that generates this burst of initiation appears to be closely associated with cyclin-dependent kinases (cdks). Cdks are small protein kinase subunits, activated by complexing with a cyclin partner, that play an important role in cell-cycle regulation. In *Xenopus*, two classes of cdk (cdc2 and cdk2) and three classes of cyclin (A, B, and E) have been identified. The cdc2-cyclin B complex forms the mitotic inducer maturation promoting factor (MPF), which has a role in the activation of RLF during mitosis as described above. Other cdks generate the S-phase promoting factor (SPF) signal required for licensed nuclei to enter S phase.

Xenopus extracts affinity-depleted of cdks with the cell-cycle protein $p13^{suc1}$ were specifically unable to support the initiation of DNA replication (Blow and Nurse 1990). In extracts treated with protein synthesis inhibitors, SPF activity appears to be dependent on cdk2, as immunodepletion of cdk2 blocked DNA replication (Fang and Newport 1991), whereas the cdk inhibitor $p21^{Cip1}$ inhibited replication at concentrations comparable to that of endogenous cdk2 (Strausfeld et al. 1994; Chen et al. 1995; Jackson et al. 1995). No effect on replication fork movement or complementary strand synthesis was seen in cdk-inhibited extracts, implying that SPF function is specifically required for the initiation of replication (Blow and Nurse 1990; Fang and Newport 1991; Strausfeld et al. 1994).

Cyclins A and B are predominantly found complexed with cdc2 in the early embryo (Minshull et al. 1990; Howe et al. 1995; U.P. Strausfeld et al. in prep.), whereas cyclin E is found exclusively complexed with cdk2 (Rempel et al. 1995). Immunodepletion of cyclin E blocked DNA replication, further suggesting that a cdk2-cyclin E complex provides SPF activity (Jackson et al. 1995). However, DNA replication could be restored to cdk-defective extracts by both A- or E-type cyclins, but not B-type cyclins (Strausfeld et al. 1994 and in prep.; Jackson et al. 1995). Only low cyclin A concentrations could rescue DNA synthesis, however, as higher levels generated MPF activity and drove extracts into mitosis (U.P. Strausfeld et al., in prep.).

Figure 3 shows a model to integrate the roles of cyclins A, B, and E in controlling DNA replication in the *Xenopus* cell cycle. Both cyclin A and cyclin E can provide SPF activity (Strausfeld et al. 1994 and in prep.; Jackson et al. 1995). Since initiation occurs almost immediately once template DNA has been assembled into interphase nuclei, SPF does not appear to be rate-limiting for this process (Fig. 3, "nuclear assembly").

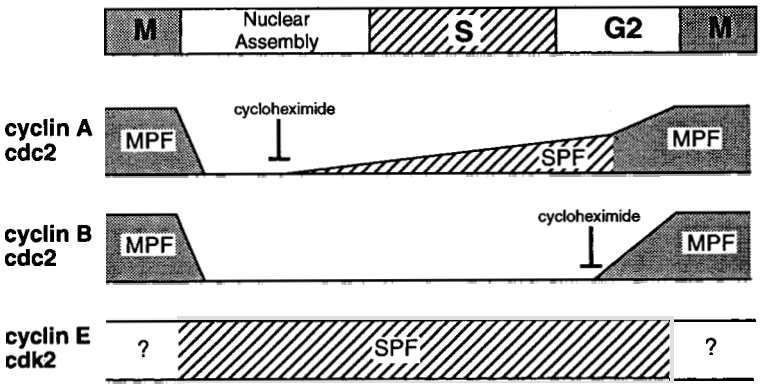

Figure 3 Cartoon showing the proposed roles of cyclins A, B, and E in *Xenopus* egg extracts. The top panel shows the cell-cycle events taking place in *Xenopus* extracts. Kinase activities of cyclin A-cdc2, cyclin B-cdc2, and cyclin E-cdk2 at the different times are shown by the boxed regions below. In the presence of cycloheximide, levels of cyclins A and B remain low, whereas cyclin E is largely unaffected. See text for more details. MPF (*stippled*) and SPF (*diagonal lines*) activities associated with these kinases are also indicated; SPF activity of cyclin E-cdk2 during mitosis is undetermined. (Redrawn from U.P. Strausfeld et al., in prep.)

On exit from mitosis in *Xenopus*, cyclin A but not cyclin E is degraded (Minshull et al. 1990; Gabrielli et al. 1992; Rempel et al. 1995), so that extracts prepared in the presence of protein synthesis inhibitors contain no cyclin A, and all SPF activity is provided by the cdk2-cyclin E complex (Fig. 3). However, cyclin A is abundantly translated and can form an active kinase with cdc2 (Minshull et al. 1990) to provide additional SPF activity (Strausfeld et al. 1994 and in prep.). Cyclin A is capable of inducing DNA synthesis even when added after nuclear assembly is complete (U.P. Strausfeld et al., in prep.), and its role may be to induce initiation at any replicons that have not already fired. Consistent with this, DNA replication in translationally active suc1-depleted extracts (which are likely to contain significantly more cyclin A than E) is more efficiently restored by addition of cdc2 mRNA than by cdk2 mRNA (Chevalier et al. 1995). As cyclin A and cyclin B kinase levels build up later in the cell cycle, this becomes sufficient to induce entry into mitosis. Correct passage through mitosis is necessary for DNA to become relicensed for DNA replication in the next cell cycle (Blow and Laskey 1988; Blow 1993). The coordination of the different cdks is therefore responsible for the regulated replication of DNA during the cell cycle.

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