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Roles of Nuclear Structure in DNA Replication

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The cell nucleus is the defining feature of eukaryotes. It is bounded by a nuclear envelope consisting of two concentric layers of membrane perforated by nuclear pores that serve as channels of communication between the nucleus and the cytoplasm. DNA is not randomly packed into the nucleus but packaged precisely in such a way that all regions are accessible for replication each cell cycle. Partial access and therefore partial replication would result in chromosome breakage or nondisjunction at mitosis, with disastrous consequences. The packing hierarchy involves radial loop organization from an axial scaffold, as well as the compaction resulting from coiling DNA twice around the nucleosome subunits of chromatin (Schedl and Grosveld 1995; Van Holde et al. 1995).

A crucial feature of eukaryotic chromosomal DNA replication is that it always occurs within a nucleus. In lower eukaryotes such as fungi or *Physarum*, the nuclear membrane remains intact throughout the cell cycle, whereas it breaks down during mitosis of higher eukaryotes. Nevertheless, replication is constrained to interphase when the nuclear membrane is intact. We argue that this constraint has important regulatory consequences.

Further key features of eukaryotic DNA replication are that multiple initiations occur within a single chromosome and that these initiations are coordinated so that each region of the chromosome replicates, but replicates only once in any cell cycle. We argue that nuclear structure has essential roles to play in coordinating multiple initiations to replicate the chromosome exactly once.

DNA Replication in Eukaryotic Cells

NUCLEAR STRUCTURE IS REQUIRED FOR CELLULAR DNA REPLICATION

Studies of eukaryotic DNA replication have been held back by a shortage of cell-free systems that initiate chromosomal DNA replication efficiently in vitro. Efficient replication systems have been developed for several viruses (see other chapters in this volume), but the only systems that clearly initiate efficiently on cellular DNA are derived from animal eggs. Initiating cell-free replication systems have been developed from unfertilized eggs of *Xenopus* and *Drosophila* (Blow and Laskey 1986; Newport 1987; Crevel and Cotterill 1991). In both cases, the egg contains a prefabricated stockpile of materials for exceptionally rapid DNA replication and nuclear assembly. *Xenopus* and *Drosophila* embryos reach 10,000 cells faster than a proliferating mammalian cell divides once.

These features make animal eggs attractive systems to study cell proliferation (see Blow and Chong, this volume), but they have some compensating disadvantages. For example, they lack G_0, G_1, and G_2 phases of the cell cycle, making them unsuitable for studies of transition between normal cell-cycle phases. A cell-free system that initiates cellular chromosomal replication efficiently in extracts of mammalian cells or yeast would have important applications.

A conspicuous feature of both the replication systems from *Xenopus* eggs is their dependence on nuclear structure to initiate DNA replication. Initiation is only observed when the template DNA is enclosed within a complete nuclear membrane (Newport 1987; Sheehan et al. 1988; Blow and Sleeman 1990). Nuclei whose membranes have been permeabilized by nonionic detergents or lysolecithin must be repaired by membrane vesicles before initiation is observed (Leno et al. 1992; Coverley et al. 1993).

Two further lines of evidence emphasize the importance of nuclear structure in initiation of DNA replication. First, purified DNA is replicated reasonably efficiently when added to *Xenopus* egg extracts (Blow and Laskey 1986; Newport 1987; Blow and Sleeman 1990; Newport et al. 1990; Cox and Laskey 1991), but replication is observed only when the DNA is assembled into pseudonuclei. Furthermore, the efficiency of replication depends on the efficiency of nuclear assembly, and only the DNA that is enclosed within the pseudonuclei replicates (Blow and Sleeman 1990).

The second line of evidence that nuclear structure is crucial for DNA replication comes from studies of nuclear membrane assembly in vitro. These indicate that the nuclear membrane defines the DNA it encloses as

an integral unit of replication. Thus, Blow and Watson (1987) found that individual sperm nuclei in a common egg-extract environment act as integrated and independent units of replication. All the DNA in a nucleus replicates roughly synchronously, but not synchronously with its neighbors. This conclusion was strengthened by Leno and Laskey (1991), who exploited an unusual behavior of chicken erythrocyte nuclei in egg extract. These nuclei tend to clump to form aggregates. The response of the extract is to assemble a single nuclear membrane around the entire clump, not around the individual nuclei it contains. In this circumstance, all the nuclei enclosed within a single nuclear membrane replicated in precise synchrony, even though different clumps replicated at different times, and even though individual nuclei outside the clumps replicated asynchronously. Therefore, the nuclear membrane defines the DNA it encloses as a unit for initiation of replication.

One conspicuous way in which the nuclear membrane could regulate initiation of DNA replication is by concentrating nuclear proteins in the nucleus from the cytoplasm. The importance of this process for initiation of replication has been demonstrated by use of the inhibitor wheat germ agglutinin (WGA). This inhibits protein import and also prevents initiation of replication (Cox 1992).

A second way in which replication depends on an aspect of nuclear structure concerns the nuclear lamina. When lamins are removed from egg extract by immunodepletion, nuclear assembly and protein import are not inhibited, but the nuclei formed are unable to initiate replication (Newport et al. 1990; Meier et al. 1991; Jenkins et al. 1993).

The conclusion we offer from this section is that nuclear structure is essential for initiation of DNA replication in eukaryotes. This in turn suggests that attempts to obtain cell-free initiation of DNA replication from mammalian cells should focus on using nuclei as the template.

REPLICATION OCCURS AT DISCRETE SITES WITHIN THE NUCLEUS

Replication forks are not distributed diffusely throughout the nucleus. Instead, they are clustered in replication foci or factories (Fig. 1) (Nakamura et al. 1986; Mills et al. 1989; Nakayasu and Berezney 1989; Cox and Laskey 1991; Fox et al. 1991; O'Keefe et al. 1992; Hozak et al. 1993). These may contain tens to several hundreds of replication forks, depending on the type of cell. They are revealed by pulse-labeling sites of DNA replication with precursors that can be detected fluorescently.

The significance of replication fork clustering is not clear. It might facilitate coordination between polymerases and accessory proteins on the

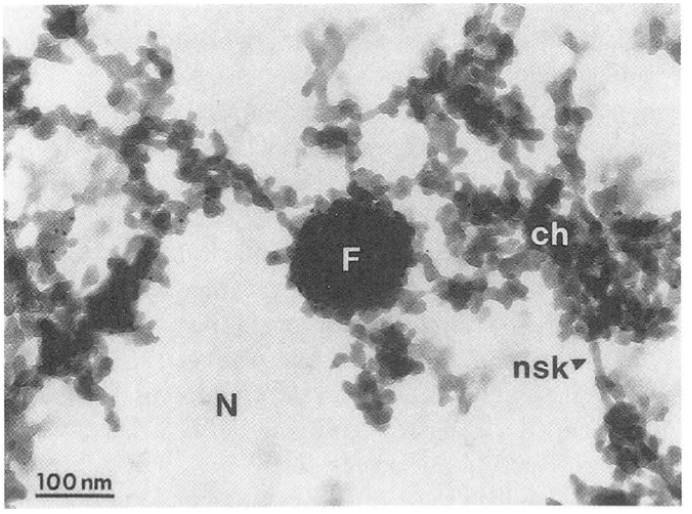

Figure 1 A replication factory. Resinless electron microscopy of HeLa nuclei following chromatin digestion reveals residual chromatin (ch) attached to an underlying nucleoskeleton (nsk). In S-phase cells, dense "factories" (F) are sites of DNA synthesis. Bar, 100 nm. (By courtesy of P. Hozak and P. Cook.)

two sides of each fork and on the two forks resulting from each initiation. More interestingly, it might provide a structural framework for the task of ensuring that all DNA is completely replicated.

The clustering of replication forks has added fuel to the debate of whether mobile DNA polymerases move along a fixed DNA track, or alternatively, whether mobile DNA is spooled through immobilized replication machinery (Hozak et al. 1993; Jackson 1995). Opinions remain divided on this issue, although it appears to us that evidence for spooling through fixed replication sites is slowly growing stronger. Electron microscopy of the initial DNA unwinding reaction for SV40 DNA replication clearly illustrates two T-antigen complexes at the replication forks which remain together while loops of single-stranded DNA are extruded (Wessel et al. 1992). If spooling is the correct interpretation, then one particular problem arises from the need to replicate the last regions of DNA between two replication foci or factories. Are they pulled in two directions? Are they completed by mobile polymerases detaching from the cluster? Alternatively, does DNA move in the same direction through consecutive clusters? Answers to these questions and the underlying issue of spooling would help us to understand the structural basics of replication.

Patterns of replication fork clusters remain the same throughout S

phase of *Xenopus* sperm nuclei replicating in *Xenopus* egg extracts (Mills et al. 1989), but in somatic mammalian cells they change as S phase progresses (Nakayasu and Berezney 1989; Fox et al. 1991; O'Keefe et al. 1992). Some foci appear late, and further clustering together of entire foci occurs late in replication. Perhaps this aggregation of foci contributes to solving the problem referred to above of how the last stretch of DNA between adjacent foci is replicated.

What specifies the pattern of foci? This question has been addressed by two extreme examples seen in Figure 2. At one extreme, purified DNA from bacteriophage has been used as a template for reassembly into pseudonuclei and replication in egg extracts. Even phage DNA is replicated under strict cell-cycle control in *Xenopus* eggs (Harland and Laskey 1980; Méchali et al. 1983; Newport 1987). As shown in Figure 2, phage DNA is also replicated under the egg's spatial control. Even though it lacks eukaryotic sequences, the pattern of clustered replication forks strikingly resembles that of *Xenopus* sperm nuclei, indicating that specific eukaryotic DNA sequences are not required for this level of spatial regulation.

At the opposite extreme, polytene nuclei from salivary glands of *Drosophila* larvae also have a similar pattern of clustered replication forks superimposed on them by *Xenopus* egg extract (Fig. 2). The polytene chromatin is remodeled and decondensed by the extract during this process, losing its banded appearance. From these examples, we conclude that the pattern of clustered replication forks can arise de novo even on prokaryotic DNA that has never been subjected to such patterns, or on highly organized polytene chromosomes. The pattern is specified by the egg extract, not by the incoming nuclei, and it must be imposed by a structural measuring mechanism that is independent of DNA sequence.

One series of experiments demonstrates that *Xenopus* eggs can recognize and use preexisting nuclear structure, at least transitionally, for initiation of replication. Gilbert et al. (1995) showed that nuclei from Chinese hamster ovary cells with highly amplified genes for dihydrofolate reductase initiate replication nonrandomly at preferred sites. These sites are the same as those used by cells in vivo. However, when the nuclear membrane was disrupted or naked DNA was used as the substrate, specificity of initiation was lost.

REPLICATION LICENSING AND THE NUCLEAR MEMBRANE

Above, we argued that the nuclear membrane plays a regulatory role by coordinating the replication of all the DNA it contains. Here we argue

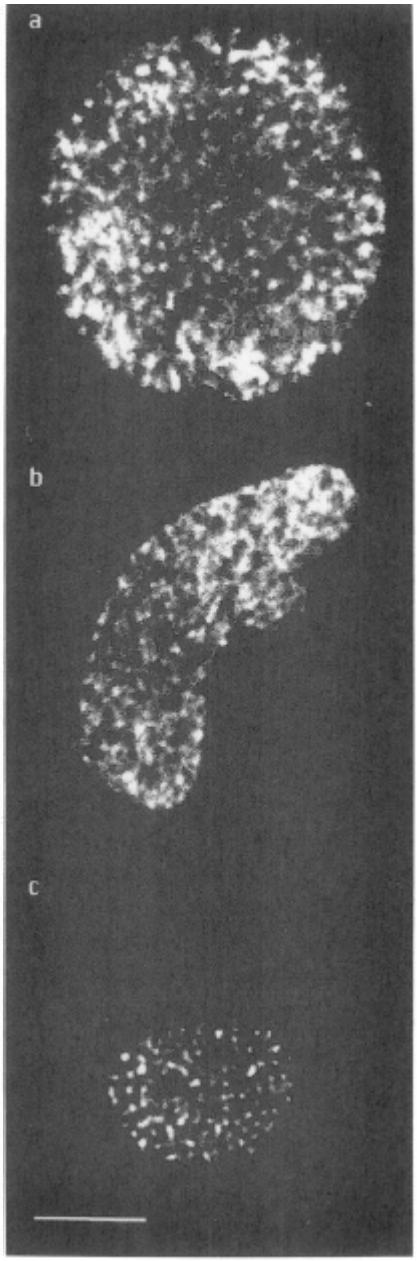

Figure 2 Discrete sites of replication seen as fluorescent foci in three types of nuclei replicating in *Xenopus* egg extracts: (*a*) *Drosophila* polytene nuclei, (*b*) *Xenopus* sperm nuclei, and (*c*) bacteriophage λ DNA pseudonuclei. Bar, 5 μm. (Original photographs provided by A.D. Mills and L.S. Cox.)

that it plays a further regulatory role by preventing reinitiation of DNA replication within a single cell cycle. The evidence for this assertion comes from experiments in which replicated, G_2 nuclei are transferred to fresh egg extract. They do not reinitiate replication if their nuclear membranes are intact. If, however, their nuclear membranes are permeabilized by nonionic detergents or lysolecithin before they are transferred, they reinitiate replication efficiently (Fig. 3). These experiments have been performed using *Xenopus* sperm nuclei as the templates or using synchronized HeLa nuclei (Blow and Laskey 1988; Leno et al. 1992; Coverley et al. 1993; Madine et al. 1995). These observations can be explained by a licensing model (Fig. 4A) (Blow and Laskey 1988). This model postulates an essential initiation factor (originally called "licensing factor," but see below) that is necessary, but not sufficient, for initiation of replication and that is unable to cross the nuclear envelope because it lacks a nuclear localization signal. Therefore, it could only bind to chromatin when the nuclear membrane breaks down in mitosis.

The model postulates that the factor would be inactivated by the act of replication so, in this way, each round of replication would need to be individually licensed by nuclear envelope breakdown at mitosis. Permeabilizing the nuclear membrane artificially would simply mimic this effect of mitosis, allowing the factor to act on chromatin to generate

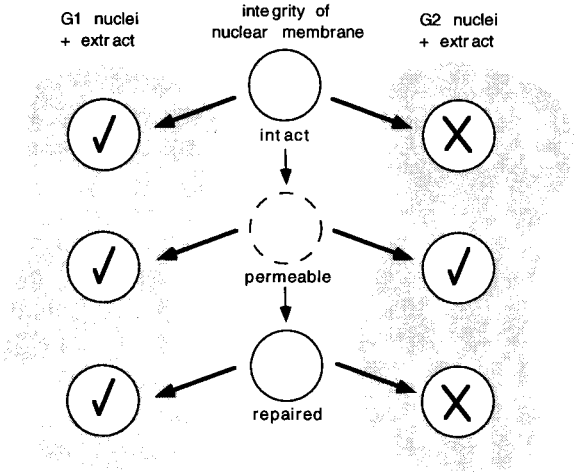

Figure 3 Summary of replication capacity of synchronized HeLa nuclei in *Xenopus* egg extract. G_1 nuclei replicate whether or not their nuclear membranes are intact, whereas G_2 nuclei are only able to replicate when their nuclear membrane is permeable (for references, see text).

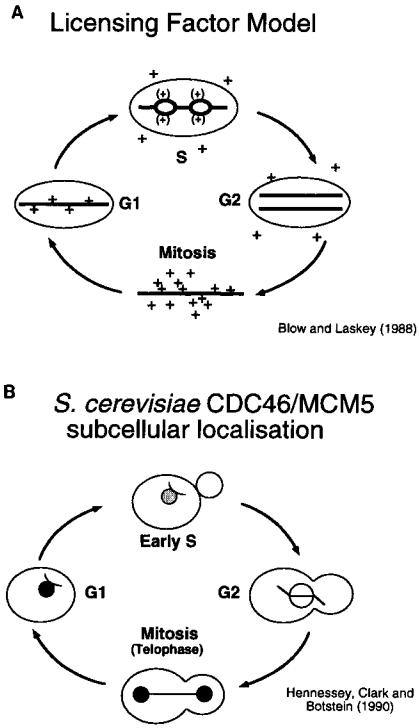

Figure 4 (*A*) "Licensing factor" model for mechanism limiting DNA replication to once per cell cycle. Licensing factor binds to DNA during mitosis but is unable to enter the nuclear envelope during interphase. It is inactivated by initiation or passage of a replication fork so that replication cannot reinitiate until the nuclear envelope breaks down again in the next mitosis. (Modified from Blow and Laskey 1988.) (*B*) Cartoon depicting nuclear localization of CDC46/MCM5 during the cell cycle of *S. cerevisiae* (Hennessey et al. 1990). Dark shading represents nuclear localization.

a new license. Evidence supporting a positive factor of this type has come from three laboratories (Blow 1993; Coverley et al. 1993; Kubota and Takisawa 1993) and has been extended recently (Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995). These studies suggest, however, that the activity can be resolved into at least two components.

A family of proteins that were discovered in yeast have become excellent candidates for one of the components required for replication licensing. These proteins are becoming a growth industry. They are called MCM or *m*ini *c*hromosome *m*aintenance proteins. They have been discovered independently in *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, mouse, *Pleurodeles*, and *Notophthalmus*, and have

also been cloned from several other species, including human and *Xenopus*. In yeast, they are required for DNA replication, and their localization is strikingly similar to that predicted for a licensing factor. Not only are they required for replication, but they are cytoplasmic until mitosis, when they enter the nucleus at anaphase and remain there until S phase, when they are removed (Fig. 4B).

This coincidence was investigated by cloning, expressing, and raising antibodies against *Xenopus* MCM3 (Madine et al. 1995). Affinity-purified antibodies precipitated a complex containing several MCM proteins. Removal of this complex by immunodepletion inhibits replication of *Xenopus* sperm nuclei (Fig. 5). Replication is restored by re-addition of the MCM complex. When synchronized HeLa cells were used as templates, permeable G_2 nuclei replicated in mock-depleted extract as expected, but not in extract depleted of the MCM complex. In contrast, G_1 HeLa nuclei replicated whether or not the *Xenopus* MCM complex was present, implicating the MCM complex in the mechanism that distinguishes G_1 from G_2 nuclei and preventing further replication of G_2 nuclei (Fig. 6). Similar conclusions were reached independently by Kubota et al. (1995) and Chong et al. (1995) using different approaches but also implicating the MCM complexes.

An obvious question is, Can the MCM proteins cross the nuclear membrane? Experiments with an MCM3 fusion protein indicated that it cannot cross an interphase nuclear membrane (Kubota et al. 1995). How-

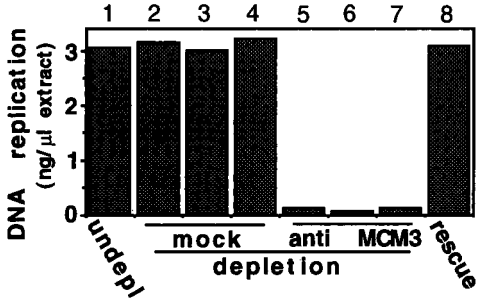

Figure 5 Immunodepletion by anti-XMCM3 antibodies inhibits DNA replication of *Xenopus* sperm nuclei in *Xenopus* egg extracts. Sperm nuclei replicate efficiently in undepleted (track *1*) extract or mock immunodepleted extracts (tracks *2–4*). In contrast, immunodepletion of the MCM protein complex with any one of three anti-XMCM3 antibodies abolishes replication (tracks *5–7*). Readdition of MCM complex purified from a fourth antibody restores replication (for details, see Madine et al. 1995). (Reprinted, with permission, from Madine et al. 1995 [copyright Macmillan].)

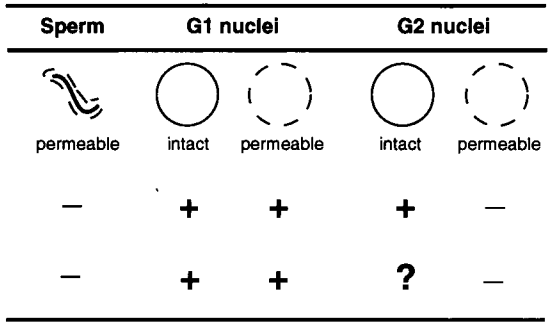

Figure 6 Summary of the MCM status of nuclei from *Xenopus* sperm or synchronized G_1 and G_2 HeLa cells. "MCM stain" refers to immunofluorescence with antibodies against MCM3. "MCMs active" refers to the ability of nuclei to replicate in an egg extract from which the MCM complex has been depleted (for details, see Madine et al. 1995).

ever, when transport of the native MCM complex is examined, MCM3 does cross an intact nuclear membrane (Madine et al. 1995). One possible explanation of this difference is a folding problem of the recombinant fusion protein, but a more interesting possibility is that another member of the MCM family is responsible for targeting the whole MCM complex to the nucleus.

If the MCM complex can cross an intact nuclear membrane, then how does it relate to the requirement for nuclear membrane breakdown or permeability to reinitiate DNA replication? Clues may come from two directions. First, M.A. Madine et al. (in prep.) have shown that nuclear envelope permeability is needed to allow binding of MCM3 to chromatin, rather than for entry into the nucleus. Second, Chong et al. (1995) have shown by fractionation that a second activity is required for replication licensing. Thus, it appears that two separate activities are required for replication licensing. It is possible to argue semantically about which of these should be called "licensing factor." We suggest that this argument is unhelpful and that the term licensing factor should be replaced by referring instead to "replication licensing," since at least two classes of factors are involved.

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