

Replication origins in yeast versus metazoa: separation of the haves and the have nots

David M Gilbert

The recent flood of information concerning *Saccharomyces cerevisiae* replication origins and the proteins that interact with them contrasts alarmingly to the trickle of progress in our understanding of metazoan origins. In mammalian cells, origins are complex and heterogeneous, and appear to be selected by features of nuclear architecture that are re-established after each mitosis. Studies in *Xenopus* egg extracts have shown that once per cell cycle replication does not require specific origin sequences, despite the identification of functional homologues to yeast origin-binding proteins. These observations suggest that initiation of DNA replication in higher eukaryotes is focused to specific genomic regions by features of chromosome structure.

Addresses

Department of Biochemistry and Molecular Biology, SUNY Health Science Center, 750 East Adams Street, Syracuse, New York 13210, USA; e-mail: gilbertd@vax.cs.hscsyr.edu

Current Opinion in Genetics & Development 1998, 8:194–199

<http://biomednet.com/eleceref/0959437X00800194>

© Current Biology Ltd ISSN 0959-437X

Abbreviations

2D	two-dimensional
ACS	ARS consensus sequence
ARS	autonomously replicating sequence
DHFR	dihydrofolate reductase
DUE	DNA-unwinding element
LCR	locus control region
ODP	origin decision point
ORC	origin recognition complex
pre-RC	replication complex

Introduction

Every field of research has its golden age. For example, the 'eukaryotic-gene-expression' world experienced an explosion in the identification of transactivators and basal transcription factors from the late '80s through the early '90s. Functional assays such as transient transfection of reporter gene constructs and techniques to identify protein–DNA interactions allowed studies in mammalian cells to keep pace with powerful yeast genetic systems, revealing a transcriptional initiation mechanism largely conserved from yeast to man. In the replication field, the golden age has now arrived. However, a glance at the past year's literature reveals an explosion of information in yeast systems that is not shared by metazoan systems. The lack of functional assays for replication origin activity and the inability to conclusively identify specific DNA sequences that function as replication origins continue to plague efforts to define metazoan origins. In this review, I briefly outline our understanding of replication origin

assembly in *Saccharomyces cerevisiae*. I then describe some recent reports that illustrate the difficulties experienced with the identification of mammalian replication origins and describe studies in *Xenopus* that demonstrate how replication can be studied in an origin-less world. Finally, I address some of the reasons for these differences. I will focus the discussion on chromosomal origins that initiate once-per-cell-cycle.

Yeast: the 'haves'

In *S. cerevisiae*, specific ARS (autonomously replicating sequence) elements were identified on the basis of their ability to direct the autonomous replication of cloned plasmid DNA (recently reviewed in [1–3]). Each ARS element (<150 bp) contains an essential 11 bp ARS consensus sequence (ACS) associated with two or three short stimulatory motifs that are divergent in sequence but functionally conserved; one of those essential functions being a region of low thermodynamic stability known as a DNA-unwinding element (DUE) [4•]. Using two-dimensional (2D) gel electrophoresis techniques under conditions that can identify replication bubble and fork structures, ARS elements were shown to be coincident with the sites where bi-directional replication begins both in ARS containing plasmids and within yeast chromosomes. Thus, in *S. cerevisiae*, origins consist of specific sequences that are both required for replication and serve as the start sites for DNA synthesis.

The availability of clearly defined origin sequences allowed for the identification of proteins that interact with yeast origins and the dissection of their functions during the cell-cycle (reviewed in [1–3]). Perhaps most important of these is a complex of six proteins—the origin recognition complex (ORC)—that is bound to the ACS and adjacent B1 motif throughout the cell-cycle [5•,6•]. Origin recognition requires ATP as a co-factor and origin binding stimulates the ATPase activity of ORC [7•]. Recently, the 5' ends of nascent strands at ARS1 were shown to originate from an 18 bp initiation zone adjacent to the ORC-binding site [8•]. Thus, ORC-binding may promote a local DNA distortion that leads to unwinding in a manner analogous to the initiator proteins of several bacterial, plasmid and viral systems [9].

ORC appears to be the landing pad for the assembly of pre-replication complexes (pre-RCs) during late mitosis. Mitosis is followed by a period of low B-cyclin-dependent kinase (B-cdk) activity, during which the highly unstable Cdc6 protein is synthesized [10•] and rapidly associates with ORC-bound origins [5•,6•]. This interaction is followed by loading of the Cdc45 protein [6•,11•,12•] and

another six-member family of proteins, the Mcm complex [5••,6••], onto origins as part of the pre-RC. Once loaded, the Mcm complex interacts with something in addition to ORC and Cdc6, as both can be selectively washed off chromatin after Mcms are bound [13•]. Subsequently, a rise in B-cdk activity is followed by activation of the Cdc7 kinase; both of these kinase activities are required for entry into S-phase and may interact directly with pre-RCs [5••,14,15,16•,17•]. A novel gene product, Mcm10, may also be involved in converting the pre-RC to an active RC [18••]. After initiation, ORC remains bound to the origin and the Mcm complex appears to follow the replication fork [6••], possibly even providing the still elusive helicase activity [19•]. Importantly, if Cdc6 expression is experimentally delayed until after the rise in B-cdk activity, the ability of Cdc6 to load Mcm complexes onto origins is prevented [5••,20]. These high levels of B-cdk activity persist until the end of mitosis. Thus, pre-RCs form at origins during a period of low kinase activity, whereas initiation requires a period of high kinase activity that simultaneously converts pre-RCs to RCs and prevents their re-formation, ensuring once-per-cell-cycle replication. This series of events is largely conserved in the fission yeast *Schizosaccharomyces pombe*, with some notable discrepancies (reviewed in [21,22]).

The first cell-free system for initiating ORC- and origin-dependent *S. cerevisiae* DNA replication was reported this year [23••]. This system employs a soluble nuclear extract from yeast synchronized in S-phase to initiate replication specifically at chromosomal origins and requires whole G₁-phase yeast nuclei as a substrate. Replication is dependent upon a functional ORC within the substrate nuclei but does not require functional ORC in the extract. This system will be useful for separating gene products required for the assembly and maintenance of pre-RCs within G₁-phase nuclei from those that trigger the initiation of replication. The inability to define a soluble system for regulated replication of purified DNA templates could be explained by a requirement for the ordered sequence of events described above, the first step of which is bypassed by using G₁-phase nuclei as substrate. If conditions for the assembly of pre-RCs on purified DNA templates can be defined, the subsequent addition of S-phase-promoting factors may finally allow the development of a soluble eukaryotic replication system.

Metazoa: the have nots

In metazoa, a much more complex picture of origin structure is sluggishly emerging. The most extensively mapped initiation zone in metazoa resides downstream of the dihydrofolate reductase (DHFR) gene in Chinese hamster ovary (CHO) fibroblasts. A variety of methods, which first purify nascent DNA strands and then either localize their 5' ends or determine their polarity, suggest that replication forks travel bidirectionally away from a site ~17 kb downstream of the DHFR gene (ori-β), and possibly a second specific site (ori-γ) 20 kb further downstream

(reviewed in [24]). By contrast, 2D gel methods reveal the presence of replication bubble structures throughout a 55 kb 'initiation zone' and replication forks moving in both directions throughout this same region [25]. This year, the positions of small (<0.8 kb) 5'-RNA-primed nascent strands were mapped at 21 sites across 13 kb surrounding ori-β, revealing the presence of a second (albeit less frequently utilized) initiation site, 5 kb downstream of ori-β [26••]. Although the 2D gel results are still not compatible with two discrete origins [27], these results at least bring the various mapping techniques in closer agreement. In any case, these studies have introduced the concept of a metazoan 'origin locus'—a chromosomal region permissive for initiation, within which some sites are utilized more frequently than others.

Origin-mapping studies at other loci suggest that metazoan origins are highly heterogeneous both in size and behavior. Whereas the human rDNA [28] and *c-myc* [29] loci, as well as the *Drosophila* polymerase α locus [30], appear to have 10–30 kb initiation loci, the human β-globin locus [31] and the lamin B2 locus [32] contain origins that may be localized to within a few kilobases or less. Origin usage can also vary during development. The entire mouse immunoglobulin heavy chain (Igh) gene locus is replicated from a single origin located downstream of the gene cluster in non-B-cell types. However, in B-cells expressing the Igh genes, replication initiates from multiple origins scattered throughout the gene cluster [33•]. At the β-globin locus, the same origin is utilized whether the genes are transcriptionally silent or expressed but its timing of initiation during S-phase is developmentally regulated [31].

Genetic elements that specify the initiation of replication in metazoa have remained completely elusive. ARS assays are extremely inefficient and/or indiscriminate, necessitating the molecular dissection of origins in their native chromosomal environment. At the human β-globin locus, a naturally occurring deletion that includes the origin results in the loss of initiation activity in this region of the chromosome [31]. However, deletion of the locus control region (LCR)—a DNA sequence element required to organize replication timing, chromatin structure, and transcription of the globin gene cluster and located 50 kb upstream of the origin—also eliminates origin activity [31], suggesting that genetic elements controlling initiation of replication in metazoa may be separated by large distances. A simple relationship between the LCR and origin activity cannot be ascribed, since an insertion into the globin LCR eliminated transcriptional activity but did not eliminate origin activity [31]. Furthermore, as reported this past year [33•], deletion of a putative LCR at the murine Igh locus had no effect on timing of replication or replication fork polarity.

At the CHO DHFR origin, the discovery of a 500 bp densely methylated island in which every dC was

methylated in proliferating cells (but not in stationary cells) gave hope that epigenetic modifications of DNA might be the missing element required for recognition of mammalian origins [34]. Recently, more careful analysis of the bases encompassing the putative DMI revealed that its detection was most likely an artifact, although an adjacent segment of DNA revealed a cluster of mCpG residues [35•,36•]. Taken together, these studies illustrate some of the frustrations experienced by investigators trying to identify mammalian origins.

***Xenopus*: 'proud to be originless'**

In the rapid early cleavage stages of *Xenopus* development, replication does not initiate within specific DNA sequences, nor does it require specific DNA sequences. Any purified DNA sequence microinjected into *Xenopus* eggs or introduced into *Xenopus* egg extracts is replicated efficiently and once per cell cycle [37]. With de-membranated *Xenopus* sperm nuclei (sperm chromatin) as a substrate, nearly 100% of template DNA is replicated exactly once and many investigators have exploited this efficient replication system without regard for the nature of origin sequences. The *Xenopus* homologues of yeast ORC, Cdc6 and Mcm proteins have been isolated and shown to be essential for replication in *Xenopus* egg extracts and to assemble onto sperm chromatin in the same order as in yeast (reviewed in [3]). *Xenopus* pre-RCs are activated by the A- and E-cdks [38], and require a period of low cdk activity for re-assembly [39•], as in yeast. Loading of the *Xenopus* Mcm complex onto chromatin requires an additional partially purified component (replication licensing factor B; RLFB) that has not yet been assigned a yeast homologue [39•].

The nuclear envelope

The nuclear envelope is proposed to play two roles in regulating initiation, both of which were challenged recently. In *Xenopus* egg extracts, initiation requires a completely assembled nuclear envelope, with functional nuclear pores and a nuclear lamina. It did not appear that nuclear assembly was required simply to concentrate initiation factors because certain disruptions in nuclear envelope function allowed nuclear import but inhibited initiation [40–42,43•]. Moreover, disruptions of the nuclear lamina alter the distribution of imported replication factors, suggesting that a disruption in the internal organization of the nucleus inhibits replication [41,43•]. Once assembled, the envelope is presumed to prevent re-initiation by serving as a barrier to a positive replication licensing factor(s) (RLF), required for the assembly of pre-RCs [44]. This hypothesis explained the fact that re-replication of G₂ nuclei in *Xenopus* egg extract requires permeabilization of the nuclear membrane, either by experimental reagents or during mitosis [44,45]. The possibility that a replication inhibitor is removed upon permeabilization seemed unlikely, as permeabilized G₂ nuclei would not initiate replication if they were re-sealed with purified membranes prior to introduction into *Xenopus*

egg extract [46]. In this past year, Newport and co-workers [47••] demonstrated that high concentrations of active A- or E-cdk complexes added to *Xenopus* egg extracts prevent association of Mcm3 with sperm chromatin and inhibit subsequent DNA replication. As these same cdks also initiate replication [38], these investigators propose that the nuclear envelope is required to concentrate cdk activity (through active import) to levels that promote initiation and prevent pre-RC assembly. Breakdown of the nuclear envelope during mitosis would cause a dilution of the inhibitory cdk activity to levels that promote pre-RC assembly but do not allow initiation. This 'inhibitor model' predicts that high concentrations of cdks added to chromatin after pre-RC formation will both trigger the initiation of replication and prevent re-initiation, in the complete absence of a nuclear envelope. Such a finding would warrant a thorough re-investigation of the experiments described above.

Nuclear architecture and origin specification

At the blastula stage of *Xenopus* development, the initiation of replication becomes focused to specific chromosomal sites [48]. One possible explanation for this developmental switch is that high concentrations of replication factors in the early embryo allow relaxed origin specification and these factors become titrated as DNA is replicated. In support of this hypothesis, high concentrations of sperm chromatin replicated at a reduced rate in *Xenopus* egg extracts, implying that origins were spaced at greater distances at low cytosol–DNA ratios [49•]; however, origin mapping was not performed in these experiments to determine whether initiation was focused to specific loci. An alternative explanation is that changes in nuclear structure that take place at the mid-blastula transition focus replication to specific sites. In support of this hypothesis, it has been shown that *Xenopus* egg extracts are capable of initiating replication specifically at the CHO *DHFR* origin locus, providing the substrate for replication is introduced in the form of an intact fibroblast nucleus [37,50••,51,52••,53]. With damaged nuclei or naked DNA, replication initiated at apparently random sites. Most strikingly, when metaphase chromatin was introduced into these same extracts, replication initiated at a novel specific site within the *DHFR* gene itself [50••]. Recognition of this specific site required topoisomerase-II-mediated chromosome condensation. These observations suggest that initiation of DNA replication in higher eukaryotes is focused to specific genomic regions by higher-order features of chromosome architecture.

Mammalian replication origins are specified at a distinct point during G₁-phase

Recognition of the CHO *DHFR* origin by *Xenopus* egg extract requires nuclei from cells synchronized after a specific point during G₁-phase, designated the origin decision point (ODP) [51,52••]. With nuclei from cells synchronized prior to this point, replication initiates efficiently but without any detectable preference for the

DHFR origin. Since intact G₂ nuclei do not replicate at all in *Xenopus* egg extract, pre-ODP nuclei have already acquired the potential to replicate (replication licensing) but have not yet specified where replication should initiate. It was shown recently that passage through the ODP takes place prior to restriction point control and is independent of the presence of serum mitogens [52**]. Treatment of CHO cells with protein kinase inhibitors prevented the specification of origins, suggesting that a mitogen-independent protein kinase pathway is required for origin specification. Thus, the ODP represents a distinct early G₁-phase replication regulatory point that specifies which of many potential chromosomal sites will function as an origin in the upcoming S-phase. Interestingly, *Xenopus* embryos lack a G₁-phase until after the mid-blastula transition, coincident with the specification of origins. Perhaps origin selection in higher eukaryotes requires a proper G₁-phase, during which the functional re-organization of the nucleus focuses pre-RC formation to specific origin loci.

What gene products might specify origins at the ODP? Perhaps the most pressing question for the coming year is whether metazoan ORC binds to specific DNA sequences. If we make the assumption that ORC functions as an initiator protein to promote adjacent DNA unwinding, then some modification of the *S. cerevisiae* paradigm must be invoked. In *Xenopus* embryos, ORC cannot bind specific sequences. In mammalian cells, ORC must bind to many sequences. Considering all the data, the most inclusive working hypothesis is that metazoan ORC binds DNA non-specifically but is focused to specific sequences by features of chromosome structure. The ODP—and the blastula stage of *Xenopus* development—could represent the establishment of a nuclear structure that favors ORC–DNA interactions at particular chromosomal sites. If this model is correct, specific ORC-binding sites will be found only in post-ODP chromatin and not on naked DNA.

Conclusions

Why have metazoan replication origins been so elusive? Studies with *Xenopus* egg extracts provide a clear demonstration that 'once-per-cell-cycle' replication of DNA does not require specific DNA sequences. All that is needed is a signal to assemble pre-RCs ('licensing') separated from a signal to convert those pre-RCs to RCs ('initiation'). As specific origin sequences are not necessary for replication, what then might be their function and why are they so site-specific in yeast? Recent experiments suggest that origin specification requires features of chromosome architecture that are re-assembled after each mitosis. The heterogeneity of origins suggests that different loci require different degrees of specificity. The position of replication origins in relation to the functional activities of a chromosome may be very important. In organisms with streamlined genomes such as yeast and viruses, it may be critical that replication begin at a very defined

site, requiring a specific initiator–replicator interaction. In higher eukaryotes, distances between functional elements are generally much greater and it may be sufficient to focus replication to less well-defined chromosomal loci. In *Xenopus* embryos, there are few functional considerations other than rapid replication. In this context, a chromosome architecture that limits inter-origin distance is more important than one that regulates sequence specificity.

Acknowledgements

Special thanks to J Huberman, C Newlon, and D Dimitrova for critically reading this article.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Diffley JFX: **Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells.** *Genes Dev* 1996, **10**:2819-2830.
 2. Stillman B: **Cell cycle control of DNA replication.** *Science* 1996, **274**:1659-1664.
 3. Rowles A, Blow J: **Chromatin proteins involved in the initiation of DNA replication.** *Curr Opin Gen Dev* 1997, **7**:152-157.
 4. Lin S, Kowalski D: **Functional equivalency and diversity of cis-acting elements among yeast replication origins.** *Mol Cell Biol* 1997, **17**:5473-5484.
- Domain-swapping experiments between ARS305 and ARS1 demonstrate the diverse nature of the essential B-domain motifs between different *S. cerevisiae* origins.
5. Tanaka T, Knapp D, Nasmyth K: **Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs.** *Cell* 1997, **90**:649-660.
- See annotation [6**].
6. Aparicio O, Weinstein D, Bell S: **Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S-phase.** *Cell* 1997, **91**:1-20.
- This paper and [5**] employ formaldehyde crosslinking to examine directly the association between replication proteins and replication origins. [5**] shows that Cdc6p and Mcm7p associate with origins during G₁, but not G₂. Origin-association of Mcm7p in G₁ was dependent on the presence of Cdc6p and its dissociation from origin DNA during S was dependent upon active Dbf4/Cdc7 kinase. In the presence of B-cdk activity, Cdc6p could associate with origins but could not load Mcm7p. [6**] shows that ORC is associated with origins throughout the cell-cycle, whereas Cdc45p and Mcm4p, 7p are associated with origins only during G₁-phase. After S-phase begins, Mcm and Cdc45 dissociate from origins and appear to move with the replication fork in concert with DNA polymerase ϵ .
7. Klemm R, Austin R, Bell S: **Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex.** *Cell* 1997, **88**:493-502.
- Origin recognition by ORC requires ATP. Here it is shown that ATP binding and hydrolysis by ORC are regulated by origin DNA. ATP binding, but not hydrolysis, was required for an ORC–origin interaction. This suggests that ATP functions as a cofactor to lock ORC onto origin DNA whereas its hydrolysis may drive transitions between functional states of ORC.
8. Belinsky A-K, Gerbi SA: **Discrete start sites for DNA synthesis in the yeast ARS1 origin.** *Science* 1998, **279**:95-98.
- Mapping the 5' ends of RNA primed nascent DNA in the vicinity of ARS1 localized the site of initiation of replication to an 18 bp site adjacent to the ORC-binding site. These results suggest that ORC functions like other well characterized initiator proteins to dictate the sites of initiation of replication through local helix distortion.
9. Kornberg A, Baker T (Editors): *DNA Replication – Second edition.* New York: Freeman, WH and Company; 1992.
 10. Detweiler C, Li J: **Cdc6p establishes and maintains a state of replication competence during G₁ phase.** *J Cell Sci* 1997, **110**:753-763.

Cell-cycle analysis of Cdc6 expression and function. Cdc6 is expressed primarily between mitosis and Start, apparently by post-transcriptional controls.

11. Zou L, Mitchell J, Stillman B: **CDC45, a novel yeast gene that functions with the origins recognition complex and Mcm proteins in initiation of DNA replication.** *Mol Cell Biol* 1997, 17:553-563.
See annotation [12*].
12. Dalton S, Hopwood B: **Characterization of Cdc47p-minichromosome maintenance complexes in *Saccharomyces cerevisiae*: identification of Cdc45p as a subunit.** *Mol Cell Biol* 1997, 17:5869-5875.
Cdc45 interacts genetically with the Mcm genes and is further characterized in this report and [11*]. Cdc45 is synthetically lethal with ORC, Mcm2 and Mcm3 [12*]. It is a component of high molecular-weight MCM complexes in cell lysates, and remains nuclear throughout the cell cycle [12*].
13. Donovan S, Harwood J, Drury L, Diffley J: **Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast.** *Proc Natl Acad Sci USA* 1997, 94:5611-5616.
Mcm complexes are found associated with *S. cerevisiae* chromatin in a Cdc6-dependent fashion. Once bound, conditions were found to selectively wash ORC and Cdc6 from chromatin, without removing Mcm complexes. Thus, Cdc6 loads Mcm onto chromatin, after which Mcms interact with another tightly associated component of chromatin.
14. Dowell SJ, Romanowski P, Diffley JF: **Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins *in vivo*.** *Science* 1994, 265:1243-1246.
15. Elasser S, Lou F, Wang B, Campbell J, Jong A: **Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases.** *Mol Biol Cell* 1996, 7:1723-1735.
16. Brown G, Jallepalli P, Huneycutt B, Kelly T: **Interaction of the S phase regulator cd18 with cyclin dependent kinase in fission yeast.** *Proc Natl Acad Sci USA* 1997, 94:6142-6147.
A fusion protein of Cdc18, the *S. pombe* homologue of Cdc6, copurifies with an active B-cdk complex. This interaction suggests that the B-cdk complex may interact directly with the pre-RC.
17. Hardy C, Dryga O, Seematter S, Pahl P, Solafani R: **mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p.** *Proc Natl Acad Sci USA* 1997, 94:3151-3155.
Mutations in one of the members of the Mcm complex suppress mutations in Cdc7. This genetic interaction suggests that Cdc7 may interact directly with the pre-RC.
18. Merchant A, Dawasaki Y, Chen Y, Lei M, Tye B: **A lesion in the DNA replication initiation factor Mcm10 induces pausing of elongation forks through chromosomal replication origins in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1997, 17:3261-3271.
A novel gene product is shown to be essential for DNA replication in *S. cerevisiae*. Mcm10 mutants show defects in ARS plasmid maintenance and Mcm10 interacts physically with the Mcm complex members. Mcm10 mutants appear to cause replication forks to pause at origins, as if Mcm10 is required to disassemble pre-RCs.
19. Ishimi Y: **A DNA helicase activity is associated with an Mcm4, -6, and -7 protein complex.** *J Biol Chem* 1997, 272:24508-24513.
The replicative helicase has yet to be identified. This paper reveals that both an ATPase activity and DNA helicase activity co-purifies with a complex of three Mcm proteins. Taken together with [6**], this makes the Mcm complex an attractive candidate for a helicase at the replication fork.
20. Piatti S, Bohm T, Cocker J, Diffley J, Nasmyth K: **Activation of S-phase-promoting CDKs in late G₁ defines a 'point of no return' after which Cdc6 synthesis cannot promote DNA replication in yeast.** *Genes Dev* 1996, 10:1516-1531.
21. Huberman J: **Cell-cycle control of S phase: a comparison of two yeasts.** *Chromosoma* 1996, 105:197-203.
22. Jallepalli P, Kelly T: **Cyclin-dependent kinase and initiation at eukaryotic origin: a replication switch?** *Curr Opin Cell Biol* 1997, 9:358-363.
23. Pasero P, Braguglia D, Gasser S: **ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei.** *Genes Dev* 1997, 11:1504-1518.
The first *in vitro* system for the initiation of replication specifically at yeast origins is described, employing intact G₁-phase nuclei as a substrate. Replication is blocked at the restrictive temperature in nuclei from an *orc2-1* mutant and cannot be complemented in trans by an extract containing wild-type ORC. The results demonstrate that—once a pre-RC has been assembled within early G₁-phase nuclei—initiation of replication can be elicited with soluble S-phase promoting factors.

24. Hamlin JL, Mosca PJ, Levenson VV: **Defining origins of replication in mammalian cells.** *Biochim Biophys Acta* 1994, 1198:85-111.
25. Dijkwel PA, Hamlin JL: **The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites.** *Mol Cell Biol* 1995, 15:3023-3031.
26. Kobayashi T, Rein T, DePamphilis ML: **Identification of primary sites for DNA replication in the hamster DHFR gene initiation zone.** *Mol Cell Biol* 1998, in press.
Extensive mapping of the 5' ends of nascent DNA strands at the DHFR origin locus reveals a novel preferred initiation site (ori-β') 5 kb downstream of ori-β. Strict interpretation of 2D gel analyses [27] is still not compatible with two distinct initiation sites in this region but this represents the first time that a non-2D gel technique has corroborated the presence of additional initiation sites near ori-β and suggests that this technique could reveal other preferred initiation sites throughout the broad initiation zone defined by 2D gels. 2D gel analyses of DNA segments between ori-β and ori-β' should help to clarify whether data from these two techniques can finally be reconciled.
27. Kalejta R, Lin H-B, Dijkwel P, Hamlin J: **Characterizing replication intermediates in the amplified CHO dihydrofolate reductase domain by two novel gel electrophoretic techniques.** *Mol Cell Biol* 1996, 16:4923-4931.
28. Yoon Y, Sanchez A, Brun C, Huberman JA: **Mapping of replication initiation sites in human ribosomal DNA by nascent-strand abundance analysis.** *Mol Cell Biol* 1995, 15:2482-2489.
29. Waltz S, Trivedi A, Lefkowitz M: **DNA replication initiates non-randomly at multiple sites near the c-myc gene in HeLa cells.** *Nucleic Acids Res* 1996, 24:1887-1894.
30. Shinomiya T, Ina S: **Mapping an initiation region of DNA replication at a single-copy chromosomal locus in *Drosophila melanogaster* cells by two-dimensional gel methods and PCR-mediated nascent-strand analysis: multiple replication origins in a broad zone.** *Mol Cell Biol* 1994, 14: 7394-7403.
31. Aladjem M, Groudine M, Brody L, Dieken E, Fournier R, Wahl G, Epner E: **Participation of the human β-globin locus control region in initiation of DNA replication.** *Science* 1995, 270:815-819.
32. Kumar S, Giacca M, Norio P, Biamonti G, Riva S, Falaschi A: **Utilization of the same DNA replication origin by human cells of different derivation.** *Nucleic Acids Res* 1996, 24:3289-3294.
33. Michaelson J, Ermakova O, Birshtein B, Ashouian N, Chevillard C, Riblet R, Schildkraut C: **Regulation of the murine immunoglobulin heavy chain gene locus: evaluation of the role of the 3' regulatory region.** *Mol Cell Biol* 1997, 17:6167-6174.
Previous studies showed that a translocation dissociating the *Igh* genes from a putative LCR located 40 kb downstream of *Cα* alters the replication pattern at this locus. In this report, a B-cell line with a 34 kb deletion encompassing the LCR is examined. Although there is a marked reduction in expression of the *Igh* genes, no alteration in the *Igh* replication pattern was detected.
34. Tasheva ES, Roufa DJ: **Densely methylated DNA islands in mammalian chromosomal replication origins.** *Mol Cell Biol* 1994, 14:5636-5644.
35. Rein T, Natale D, Gartner U, Niggemann M, DePamphilis M, Zorbas H: **Absence of an unusual "densely methylated island" at the hamster dhfr ori-β.** *J Biol Chem* 1997, 272:10021-10029.
See annotation [36*].
36. Rein T, Zorbas H, DePamphilis ML: **Active mammalian replication origins are associated with a high-density cluster of mCpG dinucleotides.** *Mol Cell Biol* 1997, 17:416-426.
This paper and [35*] thoroughly re-analyze the methylation status of sequences near the CHO DHFR ori-β. In [35*], the 'densely methylated island' reported in [34] was not identified and appears to be an artifact of the technique used in that study. In [36*], the authors identify a separate but adjacent DNA segment where mCpGs were found at an unusually high frequency.
37. Gilbert DM, Miyazawa H, DePamphilis ML: **Site-specific initiation of DNA replication in *Xenopus* egg extract requires nuclear structure.** *Mol Cell Biol* 1995, 15:2942-2954.
38. Strausfeld U, Howell M, Descombes P, Chevalier S, Rempel R, Adamczewski J, Maller J, Hunt T, Blow J: **Both cyclin A and cyclin E have S-phase promoting SPF activity in *Xenopus* egg extracts.** *J Cell Sci* 1996, 109:1555-1563.
39. Mahbubani H, Chong J, Chevalier S, Thommes P, Blow J: **Cell cycle regulation of the replication licensing system: involvement of a Cdk-dependent inhibitor.** *J Cell Biol* 1997, 136:125-135.

Fractionation of *Xenopus* egg extracts separates replication licensing factor activity into two components, RLFM, which corresponds to the Mcm complex, and RLFB, a partially purified activity. Here, it is shown that there are at least two ways that RLF activity is regulated during the *Xenopus* embryonic cell-cycle. First, an RLF inhibitor, which appears to be Cdc2/CyclinB, prevents licensing activity during metaphase. Second, RLFB activity, which rises rapidly after metaphase, declines during S-phase in the absence of the RLF inhibitor.

40. Powers MA, Macaulay C, Masiarz FR, Forbes DJ: **Reconstituted nuclei depleted of a vertebrate GLFG nuclear pore protein, p97, import but are defective in nuclear growth and replication.** *J Cell Biol* 1995, **128**:721-736.
 41. Jenkins H, Holman T, Lyon C, Lane B, Stick R, Hutchison C: **Nuclei that lack a lamina accumulate karyophilic proteins and assemble a nuclear matrix.** *J Cell Sci* 1993, **106**:275-285.
 42. Goldberg M, Jenkins H, Allen T, Whitfield W, Hutchison C: ***Xenopus* lamin B3 has a direct role in the assembly of a replication competent nucleus: evidence from cell-free egg extracts.** *J Cell Sci* 1995, **108**:3451-3461.
 43. Spann T, Moir R, Goldman A, Stick R, Goldman R: **Disruption of nuclear lamin organization alters the distribution of replication factors and inhibits DNA synthesis.** *J Cell Biol* 1997, **136**:1201-1212.
- A truncated human lamin protein is used as a dominant negative mutant to perturb lamin organization. When added to *Xenopus* egg extracts, DNA replication was inhibited and the distributions of replication fork enzymes PCNA and RFC were dramatically altered within the nucleus. By contrast, the distribution of pre-RC proteins Mcm and ORC—as well as the initiation/replication fork enzyme DNA polymerase α —were not affected, suggesting an undefined specificity in the effects of the nuclear lamina on replication.
44. Blow JJ, Laskey RA: **A role for the nuclear envelope in controlling DNA replication within the cell cycle.** *Nature* 1988, **332**:546-548.
 45. Leno GH, Downes CS, Laskey RA: **The nuclear membrane prevents replication of human G₂ nuclei but not G₁ nuclei in *Xenopus* egg extract.** *Cell* 1992, **69**:151-158.
 46. Coverley D, Downes CS, Romanowski P, Laskey RA: **Reversible effects of nuclear membrane permeabilization on DNA replication: evidence for a positive licensing factor.** *J Cell Biol* 1993, **122**: 985-992.
 47. Hua XH, Yan H, Newport J: **A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell-cycle.** *J Cell Biol* 1997, **137**:183-192.

It is shown that assembly of a nuclear envelope in *Xenopus* egg extract is followed by a 200-fold concentration of cdk2 kinase in the nucleus. By experimentally increasing the concentration of A- or E-cdk2 in extracts prior to the introduction of sperm chromatin, both replication and the binding of

Mcm3 to chromatin were inhibited. These experiments suggest that the role of the nuclear envelope may be to concentrate cdk activity to levels that prevent re-initiation within one cell-cycle.

48. Hyrien O, Maric C, Mechali M: **Transition in specification of embryonic metazoan DNA replication origins.** *Science* 1995, **270**:994-997.
 49. Walter J, Newport J: **Regulation of replicon size in *Xenopus* egg extracts.** *Science* 1997, **275**:993-995.
- The concentration of sperm chromatin in *Xenopus* egg extracts was increased, resulting in a reduction in the rate of replication, presumably through an increase in replicon size. ORC was not the limiting factor, suggesting that some other factor controls how many ORC-DNA complexes initiate replication.
50. Lawlis SJ, Keezer SM, Wu J-R, Gilbert DM: **Chromosome architecture can dictate site-specific initiation of DNA replication in *Xenopus* egg extracts.** *J Cell Biol* 1996, **135**:1-12.
- Post-ODP CHO nuclei were cycled through an *in vitro* *Xenopus* egg mitosis, resulting in the assembly of a *Xenopus* embryonic nuclear envelope around CHO post-ODP chromatin. Replication within these chimeric nuclei initiated at a novel specific site in the 5' region of the DHFR structural gene. Inhibition of chromosome condensation during mitosis with either high concentrations of nuclei or a Topoisomerase II inhibitor prevented preferential initiation at this site. The same unusual site was recognized with CHO metaphase chromosomes as a substrate. Clearly, specification of origins in *Xenopus* egg extract is profoundly influenced by higher-order features of chromosome architecture.
51. Wu J-R, Gilbert DM: **A distinct G₁ step required to specify the Chinese hamster DHFR replication origin.** *Science* 1996, **271**:1270-1272.
 52. Wu J-R, Gilbert DM: **The replication origin decision point is a mitogen independent, 2-aminopurine sensitive, G₁-phase step that precedes restriction point control.** *Mol Cell Biol* 1997, **17**:4312-4321.
- Treatment of CHO cells with the protein kinase inhibitor 2-aminopurine inhibited passage of cells through the ODP. The protein kinase pathway required for the ODP did not appear to be related to Rb phosphorylation or the R-point, as the ODP took place several hours prior to these events and cells deprived of either serum mitogens or isoleucine throughout early G₁-phase passed through the ODP on schedule. After growth arrest at the R-point, nuclei experienced a gradual loss in the capacity to replicate, however, recognition of the DHFR origin remained intact until the point at which all nuclei had lost the capacity to replicate. Thus, the specification of origins at the ODP is a distinct, early G₁-phase, event that responds to internal cues and can be uncoupled from the signals that control licensing and cell proliferation.
53. Wu J-W, Yu G, Gilbert DM: **Origin-specific initiation of mammalian nuclear DNA replication in a *Xenopus* cell-free system.** *Methods, A Companion to Methods In Enzymology* 1997, **13**:313-324.