

Review

Eukaryotic origins of DNA replication: could you please be more specific?

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Available online 25 February 2005

Abstract

Initiation of eukaryotic DNA replication commences when the origin recognition complex (ORC) binds to DNA, recruiting helicases, polymerases, and necessary cofactors. While the biochemical mechanism and factors involved in replication initiation appear to be highly conserved, the DNA sequences at which these events take place in different organisms are not. Thus, while ORC appears to bind to specific DNA sequences in budding yeast, there is increasing new evidence that metazoan ORC complexes do not rely on sequence to be directed to origins of replication. Here, we review examples of specific and non-specific initiation, and we consider what, if not DNA sequence, accounts for DNA binding of ORC to defined regions in eukaryotic genomes.

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Keywords: ORC; Origins; DNA replication; Cell cycle; Initiation

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1. Introduction

Precise duplication of the eukaryotic genome during each cell cycle is critically important for cellular survival. As such, mechanisms that control how replication initiates are highly regulated by the cell. Initial models of DNA duplication suggested that replication would begin when a sequence-specific DNA binding protein known as an initiator bound to a defined region of the genome called the replicator [1]. The initiator protein would then recruit the factors that unwind DNA and assemble a replisome. Examples from bacterial and viral systems supported this notion, as did studies in *Saccharomyces cerevisiae*, where the eukaryotic initiator protein was first discovered. This six-subunit protein complex, called the origin recognition complex, or ORC, is required for replication in budding yeast, and it carries out its function by binding to defined regions of the genome known as autonomously replicating sequences, or ARSs [2].

Further investigation demonstrated that ORC has homologs in all eukaryotic organisms examined, and indeed most biochemical factors required for initiation of DNA replication are highly conserved [3]. ORC acts to recruit two more proteins, *cdc6* and *cdt1*, to the DNA. These proteins, in turn, are required for the chromatin loading of the MCM2–7 complex, which is believed to be the replicative DNA helicase [4–6]. Together these proteins form a structure known as the pre-replication complex, or pre-RC, so termed because it generates a characteristic DNA footprint prior to replication initiation (pre-replicative footprint) [7]. As the cell progresses from G1 into S phase, the pre-RC is activated by two protein kinases, *cdc7/dbf4* and a cyclin dependent kinase (Cdk), whose activities rise in S phase. These kinases initiate a cascade of events that ultimately leads to origin unwinding, polymerase recruitment, and duplication of the cellular genome [3]. Initiation of DNA replication leads to disassembly of the pre-RCs, causing a smaller footprinting pattern, known as the post-replicative state of the origin [7].

Although many proteins involved in the initiation of DNA replication have been identified, much of the mechanistic detail of this process remains unclear. In addition to providing a model for where replication begins, bacterial systems were also initially used as a model for ORC function. In bacteria, replication begins when the DnaA protein binds to the bacterial origin of replication, *oriC* [8]. This binding induces local melting of the DNA. DnaA, assisted by DnaC, then loads DnaB, the replicative helicase, onto the single-stranded DNA. The helicase then further unwinds the DNA from the melted region and allows DNA synthesis to begin. Originally, it was proposed that ORC might work through a similar mechanism as DnaA, inducing origin melting. However, single-stranded DNA has not been detected as a result of ORC binding, and as such it appears that the eukaryotic initiator might play a different role than its prokaryotic counterpart. One hypothesis is that ORC acts as a clamp loader for the MCM2–7 complex, allowing the MCM2–7 complex to

encircle double-stranded DNA [9]. This hypothesis is attractive given that three out of six ORC subunits (Orc1, 4, and 5) contain AAA⁺ ATPase domains, as does the *cdc6* protein [10]. Precedence for this type of loading mechanism comes from the AAA⁺ ATPase RFC, which loads the processivity factor PCNA onto double-stranded DNA. Additionally, recent evidence showing an archaeal MCM complex as a ring structure with an inner circle large enough to accommodate double-stranded DNA is in keeping with this hypothesis [11,12].

The role of ATP binding and hydrolysis in ORC function has been characterized to different degrees in different organisms. In budding yeast, ATP binding by the ScOrc1 subunit is essential for viability *in vivo* and DNA binding *in vitro* [13]. Recently, ATP hydrolysis by ScOrc1 has also been shown to be essential for viability. Mutation of a conserved arginine residue in ScOrc4 leads to abrogation of ScOrc1's ATPase activity, which in turn leads to a reduction of MCM loading and cell death [118]. Mutation of the AAA⁺ ATPase domains in ScOrc4 and 5 elicits no phenotype, leading to the conclusion that in budding yeast, ATP binding by ScOrc1 is primarily responsible for ATP-dependent ORC function [13]. The interaction of *Drosophila* ORC with ATP is similar to that of *S. cerevisiae* ORC. Mutations in the Walker A motif of DmOrc4 or 5 have no effect on DmORC's ability to support DNA replication, whereas mutation of the Walker A motif in DmOrc1 completely abolishes DNA replication in an *in vitro* system [14]. Despite this apparent requirement for ATP binding to the DmOrc1 subunit, a recent study suggests that WT DmORC can bind DNA in the absence of ATP, and this binding is stimulated approximately three-fold by the addition of ATP [15]. This result is similar to that seen with HsORC, which can also bind DNA in the absence of ATP, but undergoes a three- to five-fold increase in DNA binding in the presence of ATP [16]. While it seems that most ORC complexes studied require ATP to some degree for their activity, further study will be required to validate or disprove the idea of ORC as an ATP-dependent MCM2–7 clamp loader.

Despite conservation of the proteins involved in eukaryotic DNA replication, the DNA sequences on which initiation takes place are highly divergent, and in many organisms, poorly characterized [17]. On the one hand, the yeast ORC complex binds to specific DNA sequences, which direct initiation events *in vivo*. On the other hand, metazoan ORC complexes exhibit virtually no sequence-specificity [15,16]. However, replication initiation events in these organisms are not random, and can in some cases be quite localized. Additionally, while in yeast the genetically defined replicator element is coincident with the biochemical origin, or initiation site, in other organisms the relationship between the replicator and the origin is not as well defined. Here, we examine what defines a replicator element in various eukaryotic species. We then discuss models for what directs initiation events to defined locations in the absence of a sequence specific initiator protein.

2. *Saccharomyces cerevisiae*

The prototype for sequence-specific DNA binding by a eukaryotic initiator protein comes from *S. cerevisiae*. Yeast replicators were initially described as genomic sequences that confer on a plasmid the ability to be maintained extrachromosomally. These DNA elements were termed autonomously replicating sequences, or ARSs [18]. In addition to allowing extrachromosomal replication of plasmid DNA, many of these elements also function as origins in their native chromosomal context [19]. Purification of proteins that caused a specific DnaseI footprint on ARS1 led to the discovery of the eukaryotic initiator protein, ORC [2]. Molecular dissection of ARS1 revealed that it contains four motifs that are required for replication, and thus plasmid propagation [20]. The most important element of ARS1 is the A element, which contains the ARS consensus sequence, or ACS. This 11 bp sequence, 5'(A/T)TTTA(T/C)(A/G)TTT(A/T)3', is an essential feature of all known ARSs [21]. The ACS constitutes half of the bipartite ORC binding site. ARS1 contains three additional elements, B1, B2, and B3 that together are also essential for function [20]. Combinations of these B elements are also found in other ARS sequences [21]. The first, B1, comprises the second half of the ORC binding site [22,23]. Careful mapping of initiation sites revealed a precise transition from leading to lagging strand synthesis at ARS1 between the B1 and B2 elements [24]. B2 was originally proposed to be a DNA unwinding element based on studies that showed functional substitution of the element with other easily unwound sequences and also studies demonstrating a correlation between free energy of unwinding of this region and origin function [21]. Another recent study, however, demonstrated a lack of correlation between helical stability and origin function based on a large number of mutant B2 elements [25]. Instead, the authors find that a specific sequence element, consisting of an imperfect match to the ACS, is important for B2 function. This finding suggests that B2 might in fact contain a sequence element important for binding of a pre-RC component, and not just an easily unwound sequence. Finally, B3 is a binding site for the transcription factor Abf1p which is present in ARS1 but not in every ARS. The Abf1p binding site can be functionally replaced at ARS1 with binding sites for several other transcription factors, suggesting a link between transcriptional activation and DNA replication [20].

Although most *S. cerevisiae* origins conform to the paradigm discussed above, there are several examples of so-called compound origins. At the HMR-E locus, three separate fragments located within a small region each have ARS activity [26,27]. Several compound origins (including ARS101 and ARS310) contain multiple ACS elements, which must all be mutated in order to abrogate replication [28]. These compound origins are similar to initiation zones found in higher eukaryotes (see below), in that replication can begin from multiple sites within a defined region. However, despite their relative complexity, compound origins in yeast contain

sequence specific DNA binding elements, and this would appear to set them apart from initiation zones in mammalian cells.

3. *Schizosaccharomyces pombe*

Like *S. cerevisiae* ARS elements, *S. pombe* replicator sequences were identified through plasmid transformation studies ([29] and references therein). Additionally, many *S. pombe* ARSs function as replicators at their endogenous chromosomal loci. However, *S. pombe* ARS elements are much larger than their *S. cerevisiae* counterparts (0.5–1 kb versus 100–200 bp), and no well-defined consensus sequence analogous to the ACS has been identified that is essential for ARS function in *S. pombe*. Instead, *S. pombe* replicator elements are composed of asymmetric stretches of adenine and thymine bases. Although in each ARS tested, deletion of small elements of ~50 bp completely abrogates ARS function, these elements are not the same among different ARS elements, and they have no homology with one another aside from an unusually high A–T content [30]. One study showed that essential regions of ars2004 can be replaced with poly(dA/dT) tracts [31]. Indeed, a computational genome wide analysis based on locating regions of DNA with higher A–T content than average identified 384 “A+T-rich islands” [32]. Twenty of these islands chosen at random were tested for ARS activity, and 18 were active origins, showing that A–T content is an excellent predictor of functional ARS elements. This method estimates 345 ARSs in the *S. pombe* genome, which is similar to the estimates reached by two genome wide approaches in *S. cerevisiae* of 332–429 ARSs [33,34]. Since these organisms have similar genome sizes and lengths of S phase, it is logical that they contain similar numbers of replication origins.

A unique feature of the *S. pombe* ORC protein accounts for its high preference for A–T rich DNA. Unlike ScORC, where multiple ORC subunits contact the DNA, DNA binding by SpORC is mediated through a single subunit, SpOrc4. This subunit is unique among Orc4 proteins in that it contains an N-terminal DNA binding domain containing nine copies of the HMG-I (Y)-related AT-hook motif [35]. These AT-hook domains bind to the minor groove of A–T rich DNA stretches sequence non-specifically [36]. As stated above, *S. pombe* ARS elements have multiple functional domains that contribute to origin activity, and likewise, SpORC can bind to various elements within an ARS with similar affinity [37–39]. A recent study at ars2004 shows that ORC binds synergistically to two elements of this origin, and this double-binding event is important for MCM loading and origin firing in *S. pombe* [40]. This mode of action is reminiscent of ORC binding at the *Drosophila* chorion gene locus on chromosome three, where DmORC binds to multiple sites, but initiation takes place at just one of these locations (see below).

4. Embryonic systems

The most extreme examples of origin usage come from *Xenopus* and *Drosophila* embryonic systems where any sequence can be used as an origin of DNA replication. Two-dimensional gel analysis reveals that in these systems, replication begins at random throughout various genomic and plasmid sequences tested [41–43]. As development continues, however, replication begins to be localized to more specific sites in both *Drosophila* and *Xenopus* cells. Hyrien et al. demonstrated that although early *Xenopus* embryos replicate the rDNA locus from random sites, the start of zygotic transcription at the mid blastula transition marks a concomitant shift in origin usage in this region [44,45]. After this time, replication is seen to initiate only from an intergenic spacer region found between rDNA transcription units. Likewise, in *Drosophila*, origin usage at the 65-kb *DNApolα-dE2F* region changes dramatically when compared between 1 and 5 h after fertilization. Yet another pattern was detected at this locus in cultured cells. The authors conclude that the change in origin usage can be correlated with transcriptional activity in this region [46].

Even before the midblastula transition, *Xenopus* ORC can be made to recognize some sequences over others. In diluted *Xenopus* egg extracts, addition of SpOrc4, the DNA binding subunit of SpORC, reduces XIORC binding, causing partial inhibition of DNA replication [47]. These studies suggest that like SpORC, XIORC has some preference for AT-rich DNA, but this specificity is masked in undiluted extracts due to the high concentration of XIORC. In somatic cells, the concentration of XIORC is less than in embryos suggesting that in these cells, there may be a preference of ORC for AT-rich DNA.

5. *Drosophila*

A classic example of sequence-specific replication initiation comes from a unique replication scheme utilized by *Drosophila melanogaster*. In order to ensure that sufficient levels of the eggshell protein are made during *Drosophila* oogenesis, follicle cells surrounding the oocyte undergo a process known as chorion gene amplification (reviewed in [48]). Amplification of four loci containing genes expressed in follicle cells depends on the same replication proteins that are necessary for genomic replication [48,49]. The best characterized of these regions is located on the third chromosome, where two DNA elements are necessary and sufficient for amplification. These elements, ACE3 and oriβ, together can direct amplification when inserted into exogenous genomic locations [50]. Additionally, two-dimensional gel analysis indicates that replication begins from multiple origins within this region [51,52]. Further dissection of the locus revealed that while DmORC binds to both ACE3 and oriβ, the majority of replication initiates from oriβ, leading to the idea that ACE3 is a replicator element that activates initiation from

a downstream origin, oriβ [50,53]. Initial sequence analysis seemed to indicate that like ScORC, DmORC was a sequence specific DNA binding protein, as DmORC was shown to bind to ACE3 and AER-d (a larger element encompassing oriβ) but not to flanking genomic sequences [53]. Additionally, mutational analysis has further narrowed the critical element within ACE3 to an evolutionarily conserved 142 bp sequence and the oriβ element to a 140 bp sequence and a 226 bp A/T rich element [54]. The oriβ sequence cannot be replaced by ARS1 from *S. cerevisiae*, suggesting that DmORC is not simply recognizing an A/T rich sequence [54].

The above data suggested sequence specific DNA binding of DmORC to the third chorion amplification region. Recent biochemical data, however, argues that something other than sequence directs DmORC to this origin. A quantitative study of DmORC DNA binding found that DmORC bound to origin and several non-origin sequences with similar affinities [15]. Fragments tested included ACE3 and oriβ, which would be expected to bind to ORC, as well as *S. pombe* and *S. cerevisiae* WT and mutant ARS fragments, and most strikingly, a P element sequence, which was bound by ORC at the same level as ACE3. The largest difference in binding affinities measured was six-fold, which is clearly not sufficient to allow DmORC to distinguish between origin and non-origin DNA in cells. These data suggest that DmORC is not a sequence specific DNA binding protein. Consistent with this idea, any plasmid transfected into *Drosophila* Schneider cells undergoes autonomous replication, regardless of the sequence contained on the plasmid [55]. Furthermore, two-dimensional gel analysis of these plasmids reveals that replication begins from many dispersed sites. Although it is clear that in vivo DmORC is recruited to ACE3 and oriβ to direct chorion gene amplification, it appears as though a mechanism other than sequence is responsible for delivering ORC to its required place.

6. Mammalian cells

Studies of replication origins in mammalian cells have revealed similar findings to the studies in *Drosophila*; although examples of genetic elements that seem to direct replication from defined sites exist, biochemical and plasmid maintenance studies suggest that the HsORC protein does not exhibit sequence specificity. After the description of the ARS in *S. cerevisiae*, similar plasmid transformation screens were performed to attempt to identify autonomously replicating sequences in human cells. Instead of finding particular sequences that allow plasmid propagation, investigators discovered that any piece of human DNA of sufficient length was able to confer upon the plasmid the ability to be replicated [56]. Furthermore, replication was shown by two-dimensional gel analysis to begin from random sequences within the plasmid. Interestingly, replication initiation occurred as frequently in the inserted human sequences as it did in the bacterial plasmid backbone [57]. These studies

seem to suggest that a consensus sequence is not required for replication initiation in human cells.

Despite the inability of investigators to isolate an autonomously replicating sequence in mammalian systems, approximately 20 mammalian origins (sites of replication initiation) have been identified [58]. In some cases, DNA elements derived from these loci can direct replication at ectopic sites, and as such they are termed replicators. Mammalian origins fall into two general classes [17]. The first class contains regions referred to as zones of initiation, where replication begins from one or several of many potential sites within a large region of DNA. The second class includes origins where replication initiates from a localized site in each cell cycle.

Many examples of initiation zones exist, including the human rRNA locus, the Chinese hamster rhodopsin and DHFR loci, the *Drosophila oriDα* origin, and the *S. pombe ura4* origin region [59–62]. Even in budding yeast where replication is defined by a specific sequence element, the compound origins at ARS101 and 310 described above fit into the category of zones of initiation [28]. The best-characterized example of an initiation zone is the Chinese hamster ovary DHFR locus. This locus was first described as an early replicating sequence within a highly amplified region of the Chinese hamster ovary genome [63]. Early studies indicated that the DHFR locus incorporates radioactively labeled nucleotides into at least three distinct regions of the DNA early in S phase [64]. Further analysis of this region led to some controversy. High resolution labeling studies in an amplified cell line identified two loci called *oriβ* and *oriγ* as preferred initiation sites [65,66]. Various methods confirmed that *oriβ* indeed appeared to be an origin of bidirectional replication, with one study demonstrating that 80% of all initiations within DHFR arose from the 500 bp region surrounding *oriβ* [67–69]. Indeed, a demonstration that *oriβ* represents a bona fide replicator element resulted from later studies where *oriβ* was placed at random ectopic locations in hamster and human cells and shown to direct replication initiation [70,71]. However, in apparent contrast to *oriβ* acting as a unique replicator, two-dimensional gel mapping of a single copy DHFR locus in CHO cells indicated that replication begins from a multitude of sites spanning the 55 kb intergenic region between the DHFR and 2BE2121 loci. Preference for initiation was seen inside the central 35–40 kb region, known to contain *oriβ* and *oriγ* [72]. An extension of the competitive PCR technique previously utilized to show discrete initiation at *oriβ* further confounded the issue by describing a second initiation site 5 kb downstream from *oriβ* termed *oriβ'*. This origin appeared to be used at lower frequency than *oriβ* [73]. This study suggested that mammalian initiation zones were composed of a primary initiation site coupled with multiple lower frequency sites which can be detected by two-dimensional gel analysis but not necessarily nascent strand analysis. Deletion analysis delivered a further blow to the idea of a single initiation start site by demonstrating that removal of *oriβ* has no effect on the overall efficiency of initiation of the DHFR locus, indicating that other origins within the region can be activated

in the absence of *oriβ* [74]. Further analysis indicates that even deletion of the 40 kb core of the DHFR intergenic region encompassing 90% of known initiation sites does not abrogate initiation in the remaining sequence, and does not change S phase timing [75]. One confusing finding of the former deletion study was the demonstration that deletion of the 3' end of the DHFR gene completely abrogated early S phase replication initiation from any region of this locus. These results are explained in a later study showing that deletions of the DHFR promoter which abrogate transcription of the gene lead to reduced initiation within the intergenic region, with the initiation that does take place occurring throughout the body of the DHFR gene, as well as the intergenic region. This information indicates a role for transcription in determining the efficiency and location of origin firing from the DHFR locus [76]. Resolution of the disparate data regarding this locus has been reached through a comprehensive study that demonstrated that 30 of 31 restriction fragments tested by two-dimensional gel analysis contained bubble arcs, and 14 out of 15 fragments tested by a PCR-based nascent strand abundance assay tested positive for initiation. The fragment that did not show any indication of initiation was the same in both assays [77]. These experiments seem to demonstrate with finality that the DHFR intergenic region is composed of many potential initiation sites that are used with varying degrees of efficiency.

One key piece of data that is lacking in the many DHFR studies is where ORC binds within the origin region. If conventional replicators (here thought of as the DNA where ORC binds) direct initiation at DHFR, there must be a large number of these elements spaced closely together. Alternatively, if ORC can direct replication initiation at remote sites, for example by depositing MCM complexes at a considerable distance from its own binding site (see below), the replicator element might lie outside of the intergenic region. Identification of ORC binding sites within and outside the DHFR locus might help to determine the location of the replicator sequences that direct initiation from this locus.

A markedly less complicated example of mammalian replicators exists near the human lamin B2 gene. The lamin B2 origin was mapped to a ~500 bp region 3' of the lamin B2 gene by competitive PCR quantitation of nascent DNA stands [78]. Like yeast ARS1, the lamin B2 origin displays a cell cycle dependent footprint that is present at G1 and which shrinks as cells progress into S phase [79]. Contained within this footprint is a finely mapped origin of bidirectional replication, where the transition between leading and lagging strand synthesis has been mapped to a single nucleotide [80]. Recently, in vivo crosslinking followed by chromatin immunoprecipitation demonstrated that the cell cycle dependent footprint of the lamin B2 origin is likely to be mediated by pre-RC components [81]. The G1 chromatin surrounding the lamin B2 origin contains Orc1 and 2, cdc6, and MCM3. S phase chromatin is only bound by Orc2, and M phase chromatin contains none of these pre-RC components. These data indicate that a mammalian origin is bound by the same repli-

cation machinery as is used in many other eukaryotic systems, and this machinery follows the same cell cycle patterns as in yeast. Finally, it has recently been shown that the lamin B2 origin can direct replication at ectopic loci in both human and hamster cells, demonstrating that lamin B2 is a true mammalian replicator [71,82].

Another mammalian origin where origin firing is restricted to a discrete site is the human β -globin locus, which was discovered through similar radioactive labeling studies as were used to discover the DHFR origin region [83]. In contrast to the DHFR locus, however, replication from the β -globin locus was initially thought to emanate from a single bidirectional origin of replication. This replication is independent of transcription of the β -globin genes, although gene expression does determine timing of the origin [84,85]. Replication from this origin is sensitive to deletions 50 kb upstream of the origin as well as internal deletions [83,86–88]. Like the lamin B2 and DHFR ori β loci, the β -globin origin can direct replication at ectopic locations [87]. Surprisingly, more detailed studies of the β -globin locus at ectopic chromosomal locations revealed that the locus is actually composed of two non-overlapping replicators that can direct replication independent of one another at non-native locations [89]. This finding suggests that even seemingly well-defined single origins of replication may be more complex than once thought.

Despite careful study of these three mammalian origins as well as others, evidence for initiator binding to these regions is lacking in all but a few cases. While human ORC has been localized to the lamin B2 locus, the MCM4/PRKDC intergenic origin, and the TOP1 gene, ORC binding has not been observed at other mammalian origins [81,90,91]. Although it is likely that ORC does bind to and initiate replication from these regions, it is unlikely that ORC is directed to these regions by binding to a specific DNA sequence. First, among the approximately 20 known mammalian replication origins, no consensus sequence emerges to tie them together. Further, two recent studies, one biochemical and one based on plasmid maintenance in human cells demonstrate that HsORC is likely to act without regard to DNA sequence. In the first study, baculovirus expressed HsORC containing all six subunits was shown to bind to DNA in a manner that was stimulated by, but not dependent on ATP [16]. By filter binding assays, HsORC was shown to have a preference for A–T rich DNA. Beyond this preference, HsORC was unable to distinguish between human origin and control sequences, in both filter binding assays and replication assays carried out in *Xenopus* egg extracts. These data indicate that HsORC has no intrinsic DNA binding specificity, that it does not require a specific sequence to function, and that something other than sequence is likely to direct HsORC to origins of DNA replication in human cells. A second study shows that HsORC does not require a specific sequence to direct replication in vivo. This study utilized an extrachromosomal plasmid maintained in cells through many generations via a scaffold attachment region that allows the plasmid to associate with mitotic chro-

mosomes [92]. This plasmid was replicated in a cell cycle dependent manner, was bound by components of the ORC and MCM complexes, and showed characteristic dissociation of Orc1 and MCM that occurs after origin firing in mammalian cells. Importantly, ORC bound at many regions throughout the plasmid, and consistent with this finding, nascent strand analysis revealed that replication also began from many sites within the plasmid. Taken together, these two studies demonstrate in vivo and in vitro that the human ORC complex can initiate DNA synthesis without a requirement for a specific sequence. How these findings can be reconciled with very specific initiation events like those at the lamin B2 and β -globin locus is the subject of further study and will be discussed below.

7. Mechanisms of origin specification

Even if the metazoan ORC complex binds to DNA with low sequence preference, as indicated by the studies discussed above, there must still be mechanisms to insure that ORC is distributed on chromosomes in such a way as to allow timely replication of all chromosomes. Indeed, if origin selection were completely random, there would be a small but significant probability that in each cell cycle, large regions of chromosomes would enter S phase without pre-RCs. This situation is potentially fatal since experiments in yeast have shown that limiting pre-RC formation causes cells to enter mitosis with unreplicated DNA, presumably because they are unable to detect unreplicated DNA in an otherwise unperturbed cell cycle [93–95]. Even if the S phase checkpoint in metazoans were sensitive enough to detect small amounts of ongoing DNA replication, assembling pre-RCs in a completely random fashion would be expected to lead to a high degree of variability in the length of S phase, which in turn would probably have adverse effects on development. Indeed, current evidence suggests that even in the absence of a sequence-specific ORC complex, metazoans have developed strategies to direct pre-RC formation to particular sites on the chromosome, and these are discussed below.

8. Transcriptional activity

Possibly the most well characterized mechanism directing ORC to particular genomic locations is transcriptional activity. Ties between replication, transcription, and chromatin structure exist in nearly every experimental system and this has been reviewed extensively elsewhere [17,96,97]. Strong evidence for a role of transcriptional activity in origin specification comes from *Xenopus* and *Drosophila* where replication initiates at random prior to the mid-blastula transition and becomes more specific concomitantly with the start of transcription after the MBT [44,46]. Likewise, in budding yeast, nearly every origin of replication is found in an intergenic region, suggesting a mutual exclusivity between

replication and transcription [33,34]. These studies suggest a role for transcription in negatively regulating ORC binding. However, examples of a positive interplay also exist. For example, transcription factor binding plays an important role in *S. cerevisiae* origin usage at ARS1, where binding of Abf1 facilitates plasmid maintenance of an ARS1 containing plasmid [20]. Abf1 was found to regulate replication from this origin by limiting nucleosome binding within the origin [98]. Additionally, as discussed above, recent evidence suggests a role for transcription in regulating replication initiation from the many initiation sites of the DHFR locus [76].

9. Methylation and acetylation

Another mechanism that appears to negatively regulate ORC binding, is DNA methylation. Methylation of plasmid DNA at CpG sites inhibits replication in *Xenopus* egg extracts, due to inhibition of ORC DNA binding [99]. Consistent with this observation, it has previously been shown that in mammalian cells, undermethylated regions of the genome often coincide with origins of replication [100]. In this study, nascent strands were analyzed for the presence of CpG islands, which are regions of the genome with high CpG content that, paradoxically, are undermethylated compared with the rest of the genome. Nascent strand analysis revealed that these undermethylated regions are often associated with origins of replication, consistent with the notion that methylation is inhibitory for ORC binding, as found in *Xenopus*. Additionally, since CpG islands are often associated with mammalian promoters, the correlation between these islands and replication may be indicative of a link between transcription and replication, as discussed above [101].

Finally, strong evidence for a tie between histone acetylation and origin activity has recently been shown in two model systems. In *Drosophila*, acetylated histones are localized to active origins at amplification foci, coincident with ORC [102]. In this system, hyperacetylation of histone H4 leads to redistribution of ORC from amplification foci to a genome-wide staining pattern. Conversely, tethering of deacetylases to the DNA leads to a decrease in origin activity. In *Xenopus* eggs, injection of a plasmid containing a TATA-box and five GAL4-VP16 binding sites leads to localization of replication forks, with concurrent acetylation of histones in this transcriptional domain [103]. In both of these systems, it is not clear whether the correlation between origin activity and acetylation is mediated solely at the level of ORC binding or whether it involves other replication proteins, but what is clear is that epigenetic events have a definite role in origin localization.

10. Tethering of ORC to another protein

Another possibility, especially for origins that are highly localized, is that another, more sequence-specific DNA bind-

ing protein directs ORC to specific sites in the genome. This mechanism is utilized by the Epstein–Barr virus, which uses cellular replication proteins and appears to require recruitment of ORC by a viral protein, EBNA-1, to direct replication from the viral origin, oriP [104–106]. Additionally, the mechanism by which SpORC binds to DNA might support the notion of a separate sequence specific protein directing ORC to origins of replication. In this case, an early version of SpORC would have been separate from an A–T hook motif containing protein that bound to SpORC and rendered it sequence-specific. At some point these proteins would have fused to give us the modern day SpORC. Some evidence for ORC recruitment by another protein also exists in *Drosophila*. DmORC does not localize to amplification foci in follicle cells that are mutant for the *chiffon* gene, suggesting a role for *chiffon* protein in DmORC binding [54]. This finding is somewhat surprising, given that *chiffon* is the *Drosophila* homolog of Dbf4, a protein normally thought of as executing its activity after pre-RC formation. The authors suggest that initial ORC binding might take place without assistance from the *chiffon* protein, but that binding of increased levels of ORC necessary for amplification might be facilitated by the *chiffon* protein. Additionally, dE2F also appears to have a role in DmORC targeting, albeit indirect [107,108]. It should be noted, however, that if ORC is directed to origins by additional proteins, different sequence specific proteins must be responsible at different loci because if the same protein were used in all cases, a unifying sequence might have been expected to emerge among known human origins.

11. Other origin specification mechanisms

When considering what defines metazoan origins of replication, it may be necessary to look beyond ORC. It is important to consider parameters that alter origin usage after pre-RC formation when contemplating origins of replication, especially those that have not been shown to bind ORC. In these cases, sites that appear to be origins of replication, and thus would be expected to bind ORC, might actually represent sites where MCM complexes have been deposited distally from ORC. This hypothesis might help to explain the myriad sites from which replication takes place in the DHFR locus. Usage of MCM complexes that are located at a distance from ORC binding sites is discussed below.

12. Many pre-RCs in embryonic systems

Nowhere does the need to faithfully duplicate the genome in a short time appear as crucial as in early embryonic systems. *Xenopus* embryos must duplicate their entire genome in less than 20 min using a completely random initiator protein and without the luxury of an S phase checkpoint. This challenge is known as the “random completion problem” [109,110]. Taking into account the rate of fork progression

(~500 bp/min) and the length of S phase, replication origins can be situated no more than 20 kb apart in order to ensure complete genome duplication. However, random placement of origins would result in some inter-origin distances that exceed this value. These calculations indicate that some mechanism must exist to limit inter-origin distance, so that no portion of the DNA remains unreplicated at the end of S phase. Indeed, measurements of inter-origin distances give values of 8–15 kb. Since there is a vast excess of all replication factors in *Xenopus* embryos, these results suggest that mechanisms exist to prevent initiation events from being too close, or too distant. This semi-regular distribution could be established by a defined chromatin structure, which promotes or inhibits ORC binding along certain regions of the genome. However, it has been shown that although ORC binding occurs at approximately the same frequency and distance as origin firing events, MCM complexes bind in excess to the ORC complex [111,112]. These MCM complexes are present in a widely distributed fashion, with one MCM complex bound every ~250 bp. This finding is consistent with a model where each MCM complex is a potential replication start site, and where origin spacing in the *Xenopus* system is regulated by interference between adjacent MCM complexes. Recently, a mathematical study proposed a DNA looping model that takes into account both of the above hypotheses [113]. In this study, measurements of the stiffness of the chromatin predict an optimal loop size of 11 kb, which would be in strong agreement with previously measured inter-origin distances. The prediction from this model is that looped regions fall into “replication factories”, where initiation occurs preferentially, but since MCMs could in theory be coating the entire DNA, all sites are potential initiation sites. This situation would create permissive and non-permissive regions that set the upper and lower limitations of origin spacing. Additionally, since MCM complexes are bound to a great number of sites, if a portion of the genome remains unreplicated towards the end of S phase, these MCMs could be activated later in S phase to ensure complete genome duplication [114].

13. Many pre-RCs in non-embryonic systems

The notion of creating many potential initiation sites by hyperloading pre-RCs may be a mechanism whose usage is not limited to just early embryonic systems. Experiments in which Chinese hamster ovary nuclei are incubated in *Xenopus* egg extracts support this idea. In these studies, origin usage within the DHFR locus is highly dependent on the time at which the nuclei are placed into the extracts during G1 phase [115]. One interpretation of these data is that many pre-RCs are deposited on the DNA during the end of mitosis, and that chromatin changes in G1 ultimately dictate which of these potential initiation sites will be utilized. Additionally, reiterative MCM loading has recently been shown to be required for DNA replication in budding yeast [118]. Other factors influencing origin usage presumably after formation of the

pre-RC are nucleotide pool availability and acetylation state of the chromatin [116,117].

14. Conclusions

Since the first description of the eukaryotic replicator protein over a decade ago, much progress has been made in identifying the molecular players involved in the initiation of eukaryotic DNA replication. However, study of the DNA sequences on which these proteins act has led to a plethora of confusing and controversial information which has yet to be resolved in higher eukaryotes. One theme that is emerging is that sequence-specific DNA binding by ORC may not be important for origin specification in higher eukaryotes. Although many possibilities exist to explain what directs ORC to its required positions in metazoan cells, no satisfying universal answer has been agreed upon. Most likely, ORC binding is regulated by different mechanisms at different origins. We await further studies to conclusively answer this intriguing question in a comprehensive manner.

Acknowledgement

We thank Tatsuro Takahashi for helpful comments on the manuscript.

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