

REPLICATION ORIGINS OF MAMMALIAN CHROMOSOMES: THE HAPPY FEW.

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1. ABSTRACT

Replication of eukaryotic cell genomes is a tightly controlled process occurring once and only once per cell cycle. Replication initiates at several thousand origins, whose cis-acting sequences and trans-acting proteins have been partially characterized in the yeast *S. cerevisiae* in the last few years. In contrast, identification of origins of DNA replication in mammalian cells have proven much more difficult. Currently, less than 20 bona fide mammalian origins have been identified, of which only few characterized in detail. Here we discuss the available methods for origin identification in mammalian DNA and the main results, sometimes controversial, so far generated by their application. In particular, we review the currently available information concerning the three best characterized origins, namely those in the lamin B2 and β -globin gene domains in human cells and the one located downstream of the dihydrofolate reductase gene in hamster cells.

2. INTRODUCTION

Eukaryotic DNA replication occurs in a series of tandemly repeated units along the chromosomes named replicons (1,2). Each replicon (10-300 kb in length) is characterized by an origin from which two opposite moving forks emanate (3). Clusters of adjacent replicons, most probably organized by attachment to the nuclear scaffold and in conjunction with replication proteins, are coordinately replicated during the S-phase at specific nuclear locations (for recent reviews, see references: 4,5). Efficient and accurate duplication of large eukaryotic genomes inside a relatively short S phase is therefore based on the spatially and temporally programmed firing of a multitude of origins. This process is tightly controlled by the cell-cycle regulating machinery and occurs only once-per S-phase.

In the last few years, important advances have been achieved in the understanding of the molecular events regulating origin firing in simpler eukaryotic cells. Work mainly conducted in *S. cerevisiae*, led to the identification of a number of proteins involved in the activation and regulation of yeast origins. The current model for this regulation involves the sequence-specific binding of a complex of six polypeptides - origin recognition complex, ORC (6) - to the yeast origins. In G1 phase, ORC proteins cooperate with the Cdc6 - in *S. cerevisiae* (7,8) - or Cdc18 - in *S. pombe* (9) - gene products to load a complex of six proteins - MCMs (10,11) - and form a pre-replicative complex. Once replication begins, the concordant removal of Cdc6/Cdc18 prevents the loading of MCM proteins onto the origin-bound ORC in G2, thereby preventing re-replication of DNA (for reviews, see references: 12-14). The pre-replication initiation complex thus formed in G1 is ready to initiate DNA replication upon the activation of S-phase promoting factors like cyclin-CDKs and Cdc7/Dbp4 kinases.

Not surprisingly, the Orc, Mcm, Cdc6, Cdc7, Dbp4, and Cdc45 proteins are quite conserved in evolution, and mammalian homologues for most of these proteins have been identified and cloned (15-25). However, very surprisingly indeed, no formal proof or even hint so far exists about the interactions of these proteins with any origin-specific sequences in mammalian DNA. On the other hand, computation of the ratio between replicon and genome sizes, as well as calculation of the rate of advancement of the replication fork, led to the conclusion that about 30,000 origins should exist in mammalian cells (3,26). However, less than 20 of these origins have been identified so far, while only very few, the happy few (27), have been characterized in detail. Essential questions such as how origins are spaced in the genome, which are their

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common features and how are they regulated are currently almost unexplored.

3. LESSONS FROM THE REPLICATION ORIGINS OF SIMPLER ORGANISMS

Historically, the first lessons about eukaryotic DNA replication were taken from studies of animal viruses that replicate their DNA in mammalian nuclei (in particular, papovaviruses). In most of these cases, viral DNA is organized into circular mini-chromosomes and is replicated by virtue of a variety of cellular proteins in conjunction with a trans-acting initiator protein encoded by the virus itself. The origins of these viruses are quite simple, consisting of a well defined core and regulatory region composed of several auxiliary elements which facilitate but are not essential for viral DNA replication (28). The core sequence is tripartite, containing the cis-acting replicator element which interacts with the initiator protein; a flanking easily unwound region which opens up, probably as a consequence of the structural modifications caused by the nearby bound origin-specific proteins, and eases helicase entry; and an A:T rich region made of T stretches on one side and A on the other side (28). Presence of complete viral origin core in plasmid DNA enables it to autonomously replicate, when the cognate initiator protein and other necessary replication factors are provided.

An invaluable contribution to the in depth characterization of eukaryotic DNA replication origins and of the cognate proteins was given by the studies on the budding yeast *Saccharomyces cerevisiae*. Similar to eukaryotic viruses, also in *S. cerevisiae* the identification of origins was essentially facilitated by the availability of a functional assay for origin function, based on the ability of certain genomic DNA fragments to drive autonomous replication of plasmids containing them (29). Several, but not all of such autonomously replicating sequences (ARSs) were later shown to function as replication origins also when embedded in the chromosome (reviewed in refs. 12, 13, 30-32). A complete ARS element (<150 bp) contains a highly conserved, 11bp long A:T rich sequence (core element), and two or three associated motifs (B domains) that are divergent in sequence but functionally conserved, one of those essential functions being a DNA-unwinding-element (DUE).

4. MAPPING THE LOCALIZATION OF MAMMALIAN ORIGINS

Despite these early successes in the identification of microbial eukaryotic origins, during the past years it appeared clearly that the identification of metazoan DNA replication origins was not as simple. First of all, addressing the existence of defined origin sequences in metazoan chromosomes is a complex matter that has to take into account the much greater complexity of metazoan genomes as well as their very composite development. The main reason for the elusiveness of origin sequences in animal cells is probably related to the lack of a successful functional assay, possibly related to the structural complexity of mammalian origins. In mammalian cells,

several groups have tried to obtain autonomous replication of reporter plasmids driven by specific mammalian DNA sequences, in analogy to the approach that led to the identification of yeast ARSs. Despite sporadic reports that mammalian sequences can specifically support episomal replication of recombinant plasmids (33-38), the overall outcome of the majority of these attempts has been disappointing.

The lack of simple genetic assays largely limited the identification of mammalian origins to methods for mapping replication initiation sites in the chromosomes. All these methods are based on some conserved features of replication forks. Initiation of DNA synthesis generates specific structures (replication bubbles) and proceeds bidirectionally in a semidiscontinuous manner. Thus, an origin of DNA replication can be operationally defined as the site where: i) transition between continuous DNA synthesis of the leading strand and discontinuous DNA synthesis of the lagging strand occurs; ii) nascent DNA synthesis and iii) replication bubbles are created. These three features have all been utilized in the last years for origin mapping in eukaryotic chromosomes (for extensive reviews, see: (39-41)).

The definition of the transition point between leading and lagging strand was achieved by the isolation of either Okazaki fragments to be used as probes against single stranded DNA or RNA segments of the genomic region of interest, or by purification of leading strand DNA and hybridization to labeled single strand probes. Both procedures are somewhat cumbersome and artifact prone. The former one relies on labeling of Okazaki fragments at high specificity in permeabilized nuclei (42), while the latter technique requires inhibition of lagging strand synthesis by the use of emetin, a protein synthesis inhibitor also exerting a number of pleiotropic effects on cell metabolism and affecting cell viability (43). Among the origins mapped by Okazaki fragment hybridization are the hamster DHFR ori-beta (42,44), and the origins located in the murine ADA (45), hamster RPS14 (46), and hamster CAD (47) genetic loci. Mapping resolution has been different with the two techniques: when applied to DHFR ori-beta, Okazaki fragment hybridization allowed mapping of leading-to-lagging strand transition point within a DNA segment of ~450 bp. On the contrary, leading strand analysis allowed mapping only at several kb resolution (43,47-49).

Contrary to the above techniques, analysis of nascent DNA is more amenable to procedures that avoid the use of chemical treatment of cell cultures or cell synchronization. Nascent DNA issuing from a replication origin consists of a population of single-stranded DNA molecules centered over the origin itself. This DNA can be isolated according to its length (50-54) or by its property of being RNA primed (55). When obtained from asynchronously growing cells, it presents as a complete collection of all fired (early as well as late S phase) origin sequences, in which each specific origin sequence is contributed only by the small proportion of cells which are actually duplicating its replicon at the moment of

harvesting and is diluted among all the other origins and the Okazaki fragment ligation intermediates of similar sizes. To overcome the limitation in sensitivity imposed by this starting material, early mapping studies have been performed mostly on highly amplified domains (DHFR region in CHO400 cells, mouse ADA gene amplicon, mouse and human rDNA repeats region) and usually exploiting techniques such as cell synchronization, cell permeabilization, and psoralen cross-linking (56,57,45,53,58). The introduction of the polymerase chain reaction (PCR) – first in a qualitative (50,51,37) and later in a strictly quantitative manner (52,59) – for the amplification of DNA segments in nascent DNA preparations has enabled origin-mapping also in single copy regions of asynchronously growing cells.

Origin identification by quantification of nascent DNA using competitive PCR relies on the functional definition of an origin of DNA replication as the genomic site where newly replicated DNA of increasing length emanates bidirectionally and progressively covers adjacent sequences. As a consequence, the representation of defined markers within a certain genomic area in samples of short nascent DNA fragments is inversely correlated with their distance from the origin: the more abundant the marker, the closer it is to the origin. By measuring the abundance of selected DNA fragments along a genetic region, it is possible to precisely map the movement of the replicative fork along the region and hence locate the origin. This technique relies on the possibility of obtaining a very precise quantification of low abundance DNA molecules using a competitive PCR procedure, by which PCR amplification is carried out on the DNA template to be quantified together with competitor DNA fragments sharing the primer recognition sites (59). Using this mapping technique, several origins have been mapped at high resolution, including those located close to the lamin B2 (52,60) and the DNA-methyltransferase (61) genes in human cells; ori-beta and ori-beta' downstream of the DHFR gene in hamster cells (54,55); and, again in hamster cells, the origins located downstream of the GNAI3 (62) and upstream of APRT, GADD and TK genes (63). The last three hamster origins as well as the human Trk A origin (63) were all shown to co-localize with CpG islands, a property which is also shared by the lamin B2 origin (64). This finding raised the interesting possibility that CpG islands – G+C-rich regions approximately 1 kb long that are free of methylation and contain the promoters of many mammalian genes – could represent essential genetic determinants not only for transcription of housekeeping genes but also for initiation of DNA replication (63).

Specific structures of replicating DNA (such as replication bubbles and forks) can be detected by means of 2D (two-dimensional) gel electrophoretic methods. Two complementary 2D-gel methods (neutral/neutral) (65) and neutral/alkaline (66) are providing different views of replication intermediates. In the neutral/neutral 2D-gel method, replication intermediates are separated in the first dimension on the basis of molecular mass and in the second dimension on the basis of both mass and shape. Replication intermediates are transferred to a membrane and visualized

by hybridization with probes from the genomic region of interest. Different hybridization arcs are characteristic for fragments containing a single fork, a replication bubble (origin) or a termination structure. The neutral/alkaline 2D-gel method determines the direction of fork movement through a given genomic region (for details, see ref. 67). In the first dimension at neutral pH, replication intermediates are separated by their size. The nascent strands are released from the parental DNA under denaturing conditions and then separated by size in the second dimension. Hence, for each investigated DNA fragment, the probes that are closer to the origin can hybridize to the short nascent strands while those further away from the origin hybridize only to the long ones. Origin and fork movement mapping by 2D-gel analysis turned out to be very successful in yeast, and has been extensively used for the identification of genomic origins both in *S. cerevisiae* (68,69); reviewed in ref. (30) and *S. pombe* (70,71), for the definition of replication timing (72), and for the analysis of origin function in several DNA replication and checkpoint mutants (73). However, application of this technique to mammalian cells is more technically demanding, since it usually requires cell synchronization and previous purification of DNA replication intermediates. A striking result was obtained by the analysis of the replication intermediates in the region downstream of the hamster DHFR gene. Both in CHO400 cells (containing ~1,000 copies of the region) (74,75) and in CHO K1 cells with single copies (76), replication bubbles were detected throughout a large “initiation zone” up to 55 kb that included the previously recognized ori-beta region. These results suggested that DNA replication can initiate in a broader area rather than at fixed points, and have been the subject of an intense intellectual debate over the last years (67,77,78); see also below. Using 2D-gel analysis, similar broad initiation zones have been also suggested for the region containing the rDNA repeat in human cells (79).

Overall, the methods described above were used for origin-mapping with different success in terms of resolution and sensitivity. Obviously, if an origin of replication is operationally defined as the site or zone at which replication initiates, the highest possible resolution is the determination of the actual nucleotides at which leading strand synthesis starts. Very recently, a highly sensitive method (RIP-replication initiation point mapping) has been developed for mapping DNA replication initiation sites at the nucleotide level. By this method, it was shown that the replication start sites of *S. cerevisiae* ARS1 in an episomal context span a 18 bp region, adjacent to the ORC-binding site (80). Using the same method, the same authors also demonstrated that leading strand initiation in chromosomal ARS1 occurs at a single distinct site (81). Application of this technique to mammalian cells is much more demanding, due to genome size considerations. Recent work performed on the lamin B2 origin of DNA replication using ligation-mediated PCR in the authors' laboratories led to the identification of the precise start site of leading strands at this origin in the same region that is involved in cell cycle-regulated protein-DNA interactions (82), with analogy to the *S. cerevisiae* findings (M. Deganuto *et al.*, manuscript in preparation).

5. MAMMALIAN ORIGINS OF DNA REPLICATION

As outlined above, the identification of mammalian origins is currently restricted to the application of chromosomal mapping techniques. In virtually all cases, these techniques are technically demanding. As a result, only few origins of replication have been identified so far and very few characterized in detail. Most of the mapped origins are presented in table 1. They are quite heterogeneous in size, chromosome-localization, activation-time inside the S phase and cell-type specificity. Most of these origins are mapped within a promoter region of different genes (lamin B2, beta-globin, c-myc, hsp70), whereas some of them are mapped in the coding region (hamster CAD and rhodopsin genes). In one case, origin usage was shown to vary during development. While in cell lines of B lineage expressing the IgH genes replication initiated from a multitude of origins scattered throughout the gene cluster, in non-B cells the entire locus was shown to be replicated by a single replication fork and formed a transition region between early and late replication domains (83,84). On the contrary, at the beta-globin locus DNA synthesis initiates both in expressing cells, where the globin domain replicates early, and in non-expressing cells, which are characterized by late replication of the same region (85,86). In general, the available information on most of these origins is fragmentary and specific, and does not allow to draw any generalized conclusion about the presence of common features. In this respect, it should also be emphasized that for only one of these origins (the lamin B2 origin) the existence of specific, cell cycle regulated protein-DNA interactions has been determined (82,87).

5.1. Origin(s) of DNA replication in the hamster DHFR locus

The most extensively mapped replication initiation region in mammalian cells resides downstream of the dihydrofolate reductase (DHFR) gene in Chinese hamster ovary (CHO) fibroblasts. The presence of a DNA replication origin in that zone was first suggested by the discovery that two fragments within a 28 kb region incorporate radioactive DNA precursors early in the S phase (88). Later on, a variety of methods have shown that replication forks travel bidirectionally away from a site positioned 17 kb downstream of the DHFR gene (ori-beta), and from another specific site (ori-gamma) 23 kb further downstream (56,57). Specific initiation of DNA replication at ori-beta was also suggested by the results of experiments employing most of the above mentioned mapping techniques, including Okazaki fragment analysis (42), leading strand analysis (43, 89), competitive PCR quantitation of nascent DNA (54,55) and an assay based on hybridization of labeled nascent DNA fragments (44,90-92). However, as mentioned above, 2D-gel studies of a 55 kb long spacer region between the DHFR and 2BE2121 genes, suggested that initiation events are spread in the entire region (74,75). Although some attempts have been performed in the last years to try to reconcile these apparently opposite results (fixed versus dispersed initiation; refs 41,67,77), we believe that none of the proposed models is entirely satisfactory. In this respect, it should be considered that the outcome of all the techniques

that were applied for origin mapping in this (as well as other) genomic regions are possibly prone to technical difficulties that could at least partially account for the results obtained. Among these potential problems, in addition to cell synchronization and cell treatment with drugs having pleiotropic effects, 2D-gel analysis is potentially biased by the enrichment for replication intermediates, which can select for events occurring only in a subset of cells. On the contrary, competitive PCR mapping, which can be performed in asynchronously growing and untreated cell populations, still relies on the analysis of short stretches of nascent DNA which can also contain DNA segments of the same size but deriving from DNA transactions other than DNA replication. Clearly, only the identification of several other origins and their validation by different techniques will permit to draw definite conclusions in this respect.

A possible clarification of the molecular events that are occurring for DNA replication of the DHFR locus derived from the detailed quantification of RNA-primed nascent DNA for 21 different genomic sequences scattered in a 13 kb long region encompassing ori-beta. This study revealed the presence of an additional initiation site (named ori-beta') lying 5 kb further downstream from ori-beta and used at lower frequency (55). This finding led to the suggestion that an apparently broad initiation zone could be composed of several closely spaced individual origins, some of which could be preferentially activated (78). Similarly, also the origin of replication located near the *ura4* gene on chromosome III of *S. pombe* was shown to contain multiple initiation sites (71).

Interesting insights into the genetic elements determining origin activity in the DHFR locus were recently obtained by a study analyzing the effects on origin function of different deletions in this region (93). The deletion of a region encompassing the 3' end of the DHFR gene and the 5' portion of the intergenic spacer, but not ori-beta itself, abolished early firing at the ori-beta initiation zone. On the contrary, the timing and efficiency of initiation in this region was retained when ori-beta itself was deleted. These results suggest that ori-beta does not fulfill the criteria of a genetic replicator (defined as the cis-acting genetic element which is necessary and sufficient for origin activity). Possible molecular explanations for these findings could be that the deletion of the 3' end of the DHFR gene allows transcription to travel into the intergenic spacer and clash with the replication machinery, or alternatively, that it interferes with origin timing by switching its activity from early to late S. Finally, it is possible that the deleted segments contain some still unidentified cis-acting sequences important for ori-beta function, similarly to the still speculative relationship between the LCR (Locus Control Region) and the origin of replication in the human beta-globin locus (see below).

5.2. The human beta-globin origin

In an extensive search for origins of bidirectional replication in more than 200 kb in the human beta-globin locus, a single origin was located within a 2 kb region lying upstream of the beta-globin gene itself (48).

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Table 1. Origin mapping studies in animal cells

Region studied	Organism	Method	Main conclusions	References
β -globin gene	Human	Imbalanced DNA synthesis PCR of nascent DNA strands	Origin mapped in a 2 kb fragment	(48,49)
Lamin B2 gene	Human	Quantitation of nascent DNA strands	Origin mapped in a ~500 bp region at the end of the gene, at a site with cell-cycle regulated protein-DNA interactions	(52,82)
HSP70 gene	Human	PCR of nascent DNA strands	Origin region mapped in a ~400 bp fragment in the promoter region	(37)
rRNA genes	Human Mouse Rat	Analysis of nascent strand abundance 2-D gel electrophoresis	Multiple initiation sites in the 31 kb non transcribed spacer, with a preferred region upstream of the rDNA transcription start site	(53,58,79,108, 109)
DNA-methyltransferase gene	Human	Quantitation of nascent DNA strands	Origin mapped within the gene	(61)
c-myc gene	Human	PCR of nascent DNA strands	Origin mapped within a 2.5 kb region upstream of c-myc gene	(38,50)
DHFR gene	Hamster	Analysis of Okazaki fragments Earliest replicated DNA Imbalanced DNA synthesis 2-D gel electrophoresis PCR of nascent DNA strands	Initiation zone in 55 kb (2-D gel studies) Three fixed origins (ori- β , β' and γ) localized at 0.5-8 kb resolution (all the other studies)	(42-44, 51,54, 55,75,76,89)
Ribosomal protein S14 (RSP14) gene	Hamster	PCR amplification of nascent DNA strands Analysis of Okazaki fragments	Origin mapped in ~2.5 kb region overlapping the gene	(110)
Rhodopsin gene	Hamster	Earliest replicated DNA	Origin contained in a 10 kb region overlapping the gene	(111)
APRT locus	Hamster	Imbalanced DNA synthesis	Origin mapped in a ~6 kb region	(89)
CAD gene	Hamster	Analysis of Okazaki fragments	Origin localized in a 5 kb region in the CAD transcriptional unit	(47)
APRT gene	Hamster	PCR quantitation of nascent DNA strands	Origin localized the promoter region encompassing a CpG island	(63)
GADD gene	Hamster	PCR amplification of nascent DNA strands	Origin localized the promoter region encompassing a CpG island	(63)
TK gene	Hamster	PCR amplification of nascent DNA strands	Origin localized the promoter region encompassing a CpG island	(63)
GNAI3 and GNAT2 genes locus	Hamster	2D-gel electrophoresis Quantitation of nascent DNA strands	~1.7 kb origin in the intergenic region between GNAI3 and GNAT2	(62)
Aldolase B locus	Rat	Hybridization of nascent DNA	Origin mapped in a 1 kb region containing the AldB promoter	(112)
ADA gene (early S)	Mouse	Analysis of Okazaki fragments	11 kb origin region at 28.5 kb 5' of ADA gene	(45)
ADA gene (late S)	Mouse	PCR of nascent DNA strands	1-2 kb origin region at 150 kb 3' of ADA gene	(36)
Ig heavy chain locus	Mouse	Semiquantitative PCR	Origin mapped in a ~600 bp region within the enhancer	(35)
Ig heavy chain locus	Mouse (non B cells)	2-D gel electrophoresis	One/multiple origins within 90kb downstream of the 3' regulatory region	(83)

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This origin is active in a variety of cells of different derivation, regardless of expression of the gene, and independently of replication timing. Deletion of 8 kb in this region, as occurs in the haemoglobin Lepore syndrome, cancels bidirectional DNA synthesis at this site and leads to passive replication of the region from an unidentified origin lying upstream (48). Another naturally occurring 35 kb deletion, present in Hispanic thalassemia patients in a region located approximately 50 kb upstream of the beta-globin gene and including the majority of the LCR, revealed the regulatory importance of this element on origin function in the native chromosomal context (49). Absence of this region not only prevents beta-globin expression in erythroid cells, but also shuts off origin function and shifts replication timing from early to late S phase. Contrary to the Lepore defect, in cells harboring this deletion replication fork movement through the beta-globin gene is in the 3' to 5' direction, starting from an origin located downstream of the gene.

Recently, a novel approach was developed for the dissection of the genetic elements required for beta-globin origin function. When an 8 kb DNA fragment containing the origin was targeted to a new chromosomal location using the Cre/Lox recombination system, it retained origin function and replication timing, despite the absence of the LCR element (94). Analysis of deletion mutants derived from the transferred 8 kb DNA fragment suggested the existence, within this region, of a core replicator sequence, which is necessary for origin function, and two flanking auxiliary sequences.

5.3. The human lamin B2 origin

The origin of DNA replication for which more details are currently available concerning the cis-acting sequences involved in origin activity is the one located immediately downstream of the human lamin B2 gene. The genomic DNA segment encompassing the origin had been originally identified in a library of nascent DNA sequences obtained from aphidicolin-synchronized HL-60 immediately after the release in S-phase (95). Later on, origin activity was localized by competitive PCR mapping in an approximately 500 bp region corresponding to the 5' end of the lamin B2 and the 3' portion of the intergenic spacer separating the lamin B2 gene from another gene of unknown function located downstream (52). This origin is firing in the first two minutes of S-phase in HL-60 cells, and is active in all the cell types tested so far, including cells of myeloid, epithelial, neuronal, fibroblast origin and primary peripheral blood lymphocytes (60). When the origin region was analyzed *in vivo* by genomic footprinting experiments, a prominent footprint was detected in close correspondence to the replication initiation site, that disappeared in non proliferating cells (87). These protein-DNA interactions marking the origin undergo remarkable changes during the cell cycle. In G0 phase cells, no protection is present; as the cells progress into G1, an extended footprint covering over 100 bp appears, particularly marked at the G1/S border. When the cells enter S phase this protection shrinks to 70 bp and remains unchanged until G2/M. In mitosis, the protection totally disappears, only to reappear in its extended form as the

cells move into the next G1 (82). These variations are reminiscent of those corresponding to the formation of the pre- and post-replicative complexes described in yeast (96,8) and *Xenopus* cells (97). Which are the proteins responsible for these interactions is currently the subject of intensive investigation.

6. PERSPECTIVE

As already pointed out above, very limited information is available at the molecular level to understand which are the common features characterizing origins of replication of mammalian DNA and determining their function. Thus, one of the major goal of the current research is the identification of several more origins out of the postulated 30,000 ones driving replication of mammalian DNA. For this purpose, novel investigation procedures are most probably required, since all the available techniques can be easily applied only to 10-100 kb regions and are not amenable to the analysis of larger areas. Thus, questions such as what are the molecular determinants connecting origin localization to chromosomal loops (98), chromosomal domains (99), nuclear structure (100), and replication factories (101-103) will most probably await the localization of origins from continuous replicons in megabase regions of the genome.

Another very interesting topic of investigation is related to the understanding of the molecular events linking cell cycle control, replication regulatory proteins such as Orc, Cdc6 and Mcms, and origins of DNA replication. A large body of evidence suggest that, similar to budding yeast, also in mammalian cells the proteins involved in origin regulation are most likely the targets on which cell-cycle control mechanisms converge. This was indirectly suggested by the study of protein-DNA interactions at the lamin B2 origin, which showed a cell cycle regulated pattern (82), and by experiments in which *Xenopus* egg cytosol was used to stimulate hamster nuclei to enter S phase, which demonstrated that origin specification occurs at a distinct time in G1 prior to the restriction point (90,91). Direct interactions between cell cycle control proteins and replication proteins were also demonstrated in several cases. For example, in human cells the retinoblastoma Rb protein specifically targets and inhibits a member of the Mcm complex (MCM7) (104); again in human cells, the cyclin/cdk2 complex binds to Cdc6 (24); in *Drosophila* follicle cells, E2F proteins determine localization and function of Orc2 (105). Finally, the existence of a specific network of interactions between cell cycle regulation and origin activation can be also inferred from the elucidation of the mechanisms of S-phase checkpoint in yeast cells. In *S. cerevisiae*, the induction of DNA damage by genotoxic agents, or the stalling of replication forks, are sensed by the Rad 53 protein and block DNA replication by specifically preventing late origin firing (106,73). Similarly, in *S. pombe*, the *S. cerevisiae* Orc2 homologue Orp2 is required for the response to checkpoint signals preventing progression of the cell cycle after DNA damage (107). Most likely, these biochemical and functional interactions requiring replication initiation proteins will turn out to be conserved also in higher eukaryotic cells.

Altogether, these observations strongly indicate that regulation of origin activity is a crucial step and a convergent target in the control of cell cycle progression. In this respect, however, it should be emphasized again that formal proof is still lacking that any metazoan homologues of the origin-interacting proteins in *S. cerevisiae* actually bind to cis-acting origin sequences also in higher eukaryotes. Whether this is due to mere technical reasons, or hinders more evolved control mechanisms still needs to be elucidated.

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