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REVIEWS

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Regulation of DNA Replication Timing

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Abstract—Although replication of different chromatin types is long known to occur at different times in the S phase, it was not until recently that the inheritance of replication timing came to be considered from the epigenetic viewpoint. Significant changes in genome replication timing are now known to occur during differentiation and are intimately linked with changes in transcriptional activity and nuclear architecture. Replication domains delineate discrete units of chromosome structure and function. The functional significance of this tight control of replication timing is not fully understood, but it is clear that replication program is a basic feature of a given differentiation state. The review focuses on the molecular mechanisms of spatial and temporal control of replication timing at the levels of both individual replication origins and extended chromatin domains.

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INTRODUCTION

Genomic DNA replicates in every cell cycle to provide daughter cells with hereditary material. It is of immense importance that all nucleotide sequences are copied copied exactly once, and replication is consequently subject to a strict control.

Eukaryotic replication is initiated in every cycle at a number of the so-called replication origins. On the one hand, replication origin is defined as a genome site where replication is initiated. On the other hand, replication origins are often understood as DNA regions binding the proteins involved in replication initiation.

The replication initiation proteins are rather conserved. However, consensus DNA sequences characteristic of origins were only found in the yeast *Saccharomyces cerevisiae*. In multicellular organisms, replication initiation sites are presumably determined epigenetically, thus allowing a more flexible regulation to optimize genome replication for different cell types. The replicon sizes and the positions of origins are determined, to a great extent, by the chromatin context, are regulated during development, and change during cell differentiation. In addition, the distribution of active origins changes in response to genotoxic stress.

The time when an individual origin starts working in the S phase is another important parameter of replication initiation that is controlled epigenetically. Replication timing is regulated at the level of extended chromatin domains. Early origins usually occur in the genome regions that are marked with histone modifications characteristic of active genes, while origins in the regions with modifications characteristic of silent chromatin are usually activated in the late S phase. At the same time, there is growing evidence that the time of replication in the S phase acts as the factor that determines the maintenance of epigenetic status for chromatin domains of different types. Thus, the epigenetic state and replication timing of chromatin are tightly intertwined. As cells differentiate, the genome replication program undergoes substantial changes, which are linked with transcriptional activity of genes and the organization of the nucleus.

The review discusses the mechanisms of spatial and temporal regulation of replication. While the molecular mechanisms of action of individual proteins are omitted, emphasis is placed on the general principles of regulation at the levels of individual origins and extensive chromatin domains.

CONSEQUENCE OF EVENTS PRECEDING REPLICATION INITIATION

Replication initiation at an individual origin is an intricate process that proceeds through several steps and requires many proteins to bind to the origin. While conserved DNA sequences essential for DNA replication initiation were not found in higher eukaryotes, the steps of initiation and the proteins involved are conserved to a great extent [1].

The first step involves assembly of a six-subunit origin recognition complex (ORC). The ORC remains associated with the origin throughout most of the cell cycle and, therefore, may function to mark the replication initiation sites. ORC synthesis and binding to

chromosomes is regulated during the cell cycle in vertebrates and *Drosophila melanogaster.* In *S. cerevisiae*, ORC is permanently associated with chromatin [2].

At the second step, ORC triggers assembly of a multiprotein complex known as the pre-replication complex (pre-RC) on the origin. The pre-RC formation requires at least ORC, Cdc6, Cdt1, MCM9, and the heterohexameric complex MCM2-7 (Fig. 1). Cdc6 directly interacts with the ORC; these proteins and Cdt1 are essential for MCM landing [3]. The pre-RC is assembled in G1 [4].

The third step is pre-RC activation immediately followed by replication initiation at the given origin. Pre-RC consecutively recruits additional proteins to form a preinitiation complex (pre-IC). These proteins include Cdc45, RPA, and appropriate DNA polymerases. In contrast to the previous steps, the third step occurs at different times on different origins and is controlled by regulatory kinases. CDK-family kinases (CDK1 and CDK2, which are cyclin-dependent kinases and function in complex with cyclins) and Cdc7 in complex with the regulatory subunit Dbf4 are responsible for the positive control, while negative control is mediated by kinases involved in the cell response to DNA damage (intra-S-phase checkpoint) [3]. It is thought that the MCM2-7 heterohexamer together with Cdc45 and the GINS complex act as a main helicase during replication [5]. The binding of Cdc45 and GINS results in helicase activation. Cdc45 is necessary for both replication initiation and subsequent progression of the replication fork. Cdc45 presumably recruits CDK2, which leads to phosphorylation of histone H1 and chromatin decondensation. This makes the initiation possible and facilitates the progression of the replication fork [6].

Licensing of replication origins. Cdc6, Cdt1, MCM9, and MCM2-7 form a group of the so-called licensing replication factors, and the mechanism that allows origins to be activated only once per cell cycle is known as licensing. This mechanism is based on the fact that the pre-RC assembly is only possible at a certain cell cycle step, when cyclin-dependent kinases are lacking or present at low concentrations; the step corresponds to G1 and, thus, immediately precedes replication. Cyclin-dependent kinases (CDKs) are activated prior to the S phase to allow the replication initiation. After replication is initiated, the origin is converted into an unlicensed state, and CDK-dependent phosphorylation of licensing factors prevents a new licensing. This phosphorylation inhibits chromatin-binding activity of the licensing factors and leads to their proteolysis or export from the nucleus into the cytoplasm. Metazoan cells additionally have the Geminin protein, which is present in the cell from the early S phase until the end of mitosis and binds Cdt1 to prevent licensing. When DNA replication and chromosome segregation are complete, CDKs are inactivated and Geminin is degraded [7].

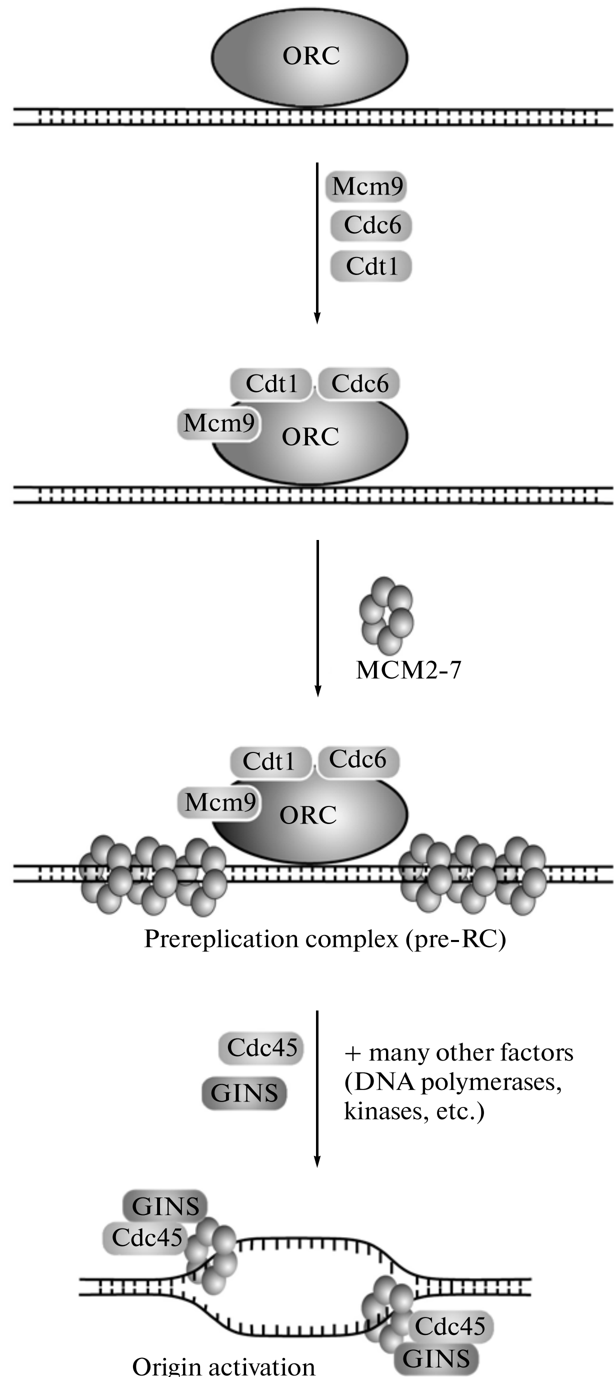

Fig. 1. Order of events in the formation of the pre-replication complex and activation of a replication origin in mammals (modified from [1]).

PLASTICITY OF REPLICATION INITIATION

The proportion of cell cycles when a given origin is active is known as the efficiency of the origin. Each origin has a certain efficiency and activation time. The two parameters are programmed in every cell cycle; at the same time, they are stable characteristics of the origin and are inherited epigenetically. The activation

time and efficiency of an origin are not directly associated with each other, i.e., some late origins are efficient, while some others are not. Some origins are inefficient because they neighbor earlier ones, while some are inefficient by themselves. The efficiency of yeast origins varies within a broad range and may reach 90% [8, 9]. Metazoan origins are far less efficient; the best characterized ones have efficiencies ranging from 5 to 20% [10, 11]. This low efficiency means that replication initiation events occur stochastically in each particular region and that their distribution may vary among cells of one type or among cell cycles of one cell.

Replication origins are usually clustered to form the so-called replication initiation zones [1, 12]. A zone includes many origins, and the localization of individual initiation events varies among cells because the origins have extremely low efficiencies. When the first origin is activated within a cluster, its neighbor origins are inactivated via interference [10].

Eukaryotes additionally have the so-called dormant origins, which never initiate replication in normal cell cycles and are only activated in certain conditions, in particular, in response to replication stress [1].

The localization, efficiencies, and order of activation of replication origins are subject to epigenetic regulation and to a great extent determine the spatial and temporal pattern of genome replication. At the earliest steps of embryo development, replication origins are distributed relatively stochastically and are activated relatively synchronously. Transition to site-specific replication initiation coincides with the onset of zygotic transcription [13].

CLUSTERS OF CO-REGULATED REPLICONS

Replication foci. Groups of replicons are replicated within “factories”, which can be seen as spots (known as foci) via pulse incorporation of nucleotide precursors (e.g., BrdU) in DNA or by immunostaining for replication proteins. The nuclear positions and sizes of replication foci produce specific patterns at different stages of the S phase. The number of active foci corresponding to euchromatic regions in the nucleoplasm gradually decreases to zero during the S phase, while an increase is observed for the number of active foci associated with heterochromatic regions in the nucleoplasm, around the nucleoli, and close to the nuclear membrane. Foci detectable in the mid- or late S phase are far less numerous than in the early S phase, and their average size is greater. These discrete replication domains probably correspond to the so-called replication bands, which are observed in mammalian metaphase chromosomes and coincide with Giemsa-positive (G, late-replicating) and Giemsa-negative (R, early replicating) bands of metaphase chromosomes [14, 15].

Replication foci are thought to be a universal feature of eukaryotic DNA replication and a characteristic of the nuclear architecture [16, 17]. Replication foci reflect coordinate activation of DNA replication at neighboring origins. Replication foci are considered to be discrete sites of the interphase nucleus where DNA replication enzymes are assembled to allow simultaneous progression of replication forks in neighbor replicons. The foci greatly vary in size. It is believed that an average replication focus corresponds to approximately 1000 kb [16], but recent analysis of replicating nuclei by new-generation super-resolution 3D-structured illumination microscopy (3D-SIM) showed that structures earlier believed to represent individual foci have a more intricate spatial organization and probably consist of several smaller foci [18]. Thus, it is still an open question whether the replication foci that are cytologically detectable in intact nuclei correspond to clusters of co-activated origins observed on spread DNA fibers and to replication domains of the genome.

Replication foci are stable structures whose chromosomal localization is preserved throughout the cell cycle and through cell generations. Replication foci are presumably basic units of the chromosomal organization [17, 19].

The spatial organization of origins into replication factories makes it possible to coordinate not only the initiation events at adjacent origins, but also the entire process of replication in the locus. Coordinate regulation was demonstrated for the progression rates of replication forks initiated from one origin in different directions and the rates of replication forks initiated from origins belonging to one cluster [20]. Arrest or slowing down of replication fork progression leads to activation of additional origins within the cluster. Thus, neighboring replicons within one replication factory seem to possess information about the distances to adjacent origins and their replication rates.

Sequential replication initiation in neighboring foci. To initiate replication, replication machinery is assembled de novo in each new focus. At the same time, it is of interest that completion of replication in one focus tends to stimulate initiation in the adjacent focus [17, 21]. For instance, after DNA synthesis has started (the earliest replication origins have been activated) in HeLa cells, only 10% of all de novo replication initiation events occur at the sites that do not neighbor a site where replication has just completed. This regulatory mechanism acts at the level of replication factories [22].

It is thought that movement of a replication fork into the vicinity of a cluster of spatially close origins triggers their activation. For instance, studies of replication of the immunoglobulin heavy chain (IgH) gene locus in mouse lymphoblasts showed that one replication fork is responsible for replication of approximately 400 kb [23]. The fork starts in a cluster of early origins and reaches a cluster of late origins. It is pre-

sumably the replication fork entering the cluster of late origins that activates their replication. Origins are not clustered in *S. cerevisiae*. However, studies of the order of replication initiation at all of the nine origins of *S. cerevisiae* chromosome VI showed that, at each origin (apart from the first one), replication is not initiated until the replication fork initiated at an earlier origin moves into its vicinity [24].

Thus, replication is regulated at several levels in higher eukaryotes. First, there is control at the level of individual origins. Second, a regulation occurs at the level of replication foci, which are organized as groups of several looped domains (replicons) anchored on the nuclear matrix. Finally, a temporal regulation of genome replication occurs at the level of extensive chromatin domains and nuclear compartments.

WHAT DETERMINES THE LOCALIZATION OF ORIGINS?

Origin mapping is rather difficult in higher eukaryotes because of the absence of consensus sequences for replication initiation. Consequently, until recently studies of replication initiation were mostly restricted until recently to individual model origins associated with certain loci [25, 26]. An increasing number of publications focus now on the mapping of replication origins at the genomic level, in particular, in individual chromosomes of mouse and human cell lines [12, 27–29]. Genome-wide mapping of replication origins was carried out in various *Drosophila* cultured cells and tissues [30, 31] and mouse cells [32]. Whole-genome mapping of replication origins was reported for *Arabidopsis thaliana* [33]. Comparison of the localization of replication origins in different cells from one organism showed that origin distribution is tissue specific in both *Drosophila* and mammals [12, 34].

To the definition of origins. We have defined an origin as a genome region where replication initiation (synthesis of the leading DNA strand) occurs. However, what is meant by an "origin" when the origin mapping or number of origins are considered? The answer depends on the method used to identify the origins.

Details of various mapping techniques are out of the scope of this review (for a review, see [35]). It is only important to note that virtually all techniques of genomic-scale mapping are based on averaging the results obtained from many cells [1, 35, 36].

Mapping of short newly synthesized leading strands makes it possible to identify the genome regions where replication was initiated in at least some cells. Since the majority of origins are low-efficient in higher eukaryotes, the origin number estimated by this analysis substantially differs from the number of origins activated in each particular cell cycle.

When origins are identified by a characteristic set of origin-associated proteins, potential origins are found, while it remains unclear whether they actually initiate replication. First, only a minor fraction of all pre-RCs assembled is activated in each cell cycle. Second, certain pre-RC components (e.g., MCM2-7) occur in a great excess in chromatin, and other coponents (e.g., ORC) may have additional functions unrelated to replication initiation.

Cell synchronization (e.g., with hydroxyurea (HU) treatment) is involved in many techniques. However, any manipulation with the cell cycle and activation of the ATR/CHK1 pathway (see below) may substantially affect the pattern of active origins. For instance, HU triggers dormant origins.

Thus, it is necessary to clearly specify the criterion used to define origins when speaking about them. For instance, an origin may be defined as a genome region that binds with the ORC.

The ORC binds to nucleosome-free DNA regions. Generally, the metazoan ORC does not display a nucleotide sequence specificity in vitro, but has higher affinity for supercoiled DNA [37, 38]. Based on this, it was assumed that changes in DNA topology and, in particular, those resulting from nucleosome removal affect the position of a potential origin. Pilot experiments in *S. cerevisiae* showed that a nucleosome occurring at an origin position inhibits replication initiation on the given origin [39]. *S. cerevisiae* histone deacetylase Sir2 inhibits origin activity by stabilizing the positions of nucleosomes, which are less tightly associated with DNA in its absence [40]. Genome-wide ORC mapping in *S. cerevisiae* and *D. melanogaster* cells showed that the ORC interacts with DNA regions that are free from nucleosomes or contain nucleosomes that are easy to displace [34, 41–45]. ATP-dependent chromatin-remodeling complexes were found to play an important role in the distribution of replication origins [34, 46]. The complexes are involved in changing the nucleosome positions relative to DNA, replacing nucleosomal histones with their variants, and removing nucleosomes from DNA. Taken together, these findings indicate that the DNA nucleosome positioning may determine origins in all eukaryotes.

ORC is also capable of affecting the nucleosome positioning [47, 48]. The pre-RC formation requires landing of a number of proteins, and some of them have to bind to DNA. The ORC seems to recruit new chromatin-remodeling complexes to its landing site, thus leading to the formation of a more open nucleosome arrangement and allowing the other pre-RC components to bind to DNA.

Replication origins often occur in the promoter regions of genes. Eukaryotic genes typically have the following nucleosomal organization: their promoters usually have a nucleosome-free region at a fixed distance from the transcription start site (TSS). Consequently, the first nucleosome covering the gene body is also at a fixed distance from the TSS. The following nucleosomes are arranged in an ordered manner, each at a certain distance from the other [49]. Because

active promoter always has a nucleosome-free region, promoters often act as replication origins in eukaryotes.

In *S. cerevisiae*, origins harbor binding sites for the Abf1 transcription factor, which may play a role in activating the origin [50]. In *Schizosaccharomyces pombe* and higher eukaryotes, replication origins often map to gene promoters [27, 28, 30, 34, 51]. There are many examples of transcription factors affecting the localization or activation of origins [46]. The effect may be achieved via recruitment of chromatin-remodeling machinery or complexes responsible for covalent histone modifications or via direct interactions of transcription factors with pre-RC components. However, the most important is that histones H3 and H4 are hyperacetylated in transcriptionally active promoters to maintain the open chromatin structure, which is thought to be preferable for replication initiation.

The most comprehensive data on the role of the chromatin landscape for ORC localization were obtained in the Model Organism Encyclopedia of DNA Elements (modENCODE) project [34, 42] for *D. melanogaster.* The project was aimed at identifying various functional DNA regions in the genomes of two model organisms, *D. melanogaster* and *Caenorhabditis elegans*. In *D. melanogaster*, as well as in all of the eukaryotes examined, many origins coincide with TSSs or occur in their vicinity. To understand which of the origin-surrounding elements are essential for ORC binding and which are determined by the origin location in TSS, the differences were studied between the ORC-binding sites that are within 1 kb of a TSS and the sites that are far away from TSSs [34].

It was found that, regardless of the localization of the nearest TSS, all of the ORC-binding sites are enriched in chromatin-remodeling proteins, for instance, components of the NURF complex (NURF301 or ISWI). In line with the concept of ORC binding to dynamic active chromatin, rapid turnover of nucleosomes and histone variant H3.3 was demonstrated for all of the ORC-binding sites. Many histone modifications proved to be equally characteristic of the ORC-binding sites located in promoter regions and those distant from promoters. However, certain differences were observed. The ORC-binding sites close to TSSs were enriched in histone modifications typical of promoters, in particular, H3K9ac, H3K27ac, H3K4me2, and H3K4me3. Distant ORC-binding sites had less H3K4me3 and more H3K418ac and H3K4me1. In addition, the origin regions were not enriched in chromatin marks characteristic of gene bodies (for instance, H3K79me1, H3K36me1, and H3K36me3), while the ORC-binding sites distant from TSSs were specifically enriched in H3K36me1.

To identify the features that are of special importance for a promoter to function as an origin, it is necessary to understand the difference between the promoters that do or do not bind ORC. Studies showed that promoters acting as origins are associated, on average, with a greater amount of chromatin-remodeling proteins and have a higher nucleosome turnover rate. The finding testifies again that an easy removal of nucleosomes from chromatin is the most important factor for ORC binding [34].

ORIGIN PROGRAMMING IN THE CELL CYCLE

The origin licensing model suggests that, to avoid re-replication of the genome, pre-RC assembly and activation must occur at different steps of the cell cycle and that assembly should not occur in the S phase. Hence, both early and late origins should be charged before the S phase starts. ORC binding to DNA commences as early as late mitosis and continues in early G1. The pre-RC is gradually assembled in G1. The number of pre-RCs whose assembly is completed by the early S phase is far greater than the number of replication initiation events in one cell cycle [52]. It seems that the cell already “knows” by the start of the S phase which origins will be activated (unless replication stress occurs) and at which of the S-phase steps activation of a particular origin will take place (Fig. 2).

Elegant experiments with isolated mammalian nuclei replicating in a *Xenopus* egg extract showed that within G1 there is so-called origin decision point which is the time that as a time interval that determines the choice of the origins to be activated in the nearest S phase among all origins with the pre-RC assembled [53]. The time interval coincides with the completion of a postmitotic reorganization of the nucleus and fixation of the nuclear architecture until the next mitosis. Mitosis is another important step that is necessary for establishing the pattern of origins to be active in the nearest S phase. Mitosis is associated with a global nuclear reorganization, which ends in early G1.

The importance of passing through mitosis for the organization of replication is evident from the results of reprogramming differentiated mammalian nuclei into embryonic stem cells upon their incubation in *Xenopus* egg extracts made to arrest the cell cycle at mitosis (a mitotically competent extract) [54]. To be completely reprogrammed, the cell should start rapid embryonic-type divisions, which utilize far more active origins. Additional treatment with the cell extracts corresponding to mitosis apparently facilitates the reprogramming. Moreover, the reprogramming efficiency is higher when the transfer of mouse somatic nuclei into zygotes is carried out during mitosis [55–57].

Timing decision point is programmed relatively independently from origin selection. This programming occurs earlier in the cell cycle and at the level of larger domains, rather than at the level of individual origins [4, 53, 58–60] (Fig. 2). Interestingly, the programmed state is only preserved until replication is

complete. Replication timing markers are absent from chromosome regions in G2 and are restored again in early G1, after the necessary transition through mitosis [61].

From the standpoint of replication foci model origin selection defines which origins will be activated within a given focus in a given cell cycle. In its turn, replication timing sets the specific order and stage of cell cycle when clusters of co-firing origins will become activated.

FACTORS DETERMINING THE ORIGIN EFFICIENCY

What differences at the molecular level may lead to different efficiencies of origins in activating replication? Why some origins activate replication extremely rare, others are tissue specific, and still other ones work only in response to replication stress? The molecular mechanisms responsible for these differences are considered below.

The origin efficiency depends on the ORC binding time. The efficiency of origins depends on the affinity of the corresponding chromosome segments for the ORC in some cases. ORC binding depends on the local chromatin conditions and transcriptional activity; hence, it is not surprising that ORC binding is distinct in different tissues. In *S. pombe*, the origin efficiency depends on the cell-cycle stage when the ORC binds to chromatin. ORCs assembled in chromatin early, during M, tend to be more efficient than ORCs assembled later, in G1 [62]. ORC binding to chromatin is periodical in *S. pombe*, increasing during mitosis and reaching its maximum during the M/G1 transition. The pre-RC formation is also periodical in the cell cycle, starting and reaching its maximum in G1. A cell arrest in mitosis leads to a partial loss of ORC1 binding specificity, and origins consequently become more homogeneous in properties. An excess of pre-IC components enhances replication on both previously efficient and inefficient origins [62].

A dependence on the MCM2-7 loading. The amount of MCM2-7 complexes loaded onto a given origin is another factor that may affect the efficiency of origin activation. It was demonstrated that, by the S phase, the MCM2-7 complexes are found in chromatin in a large excess to the number of replication initiation events, to the number of ORCs, and to the MCM2-7 number minimally necessary for genome replication in normal conditions [1]. The excess plays a dual role.

First, excess of MCM2-7 on a particular origin may improve its efficiency. Once helicase is activated in one of the MCM2-7 complexes bound to a given ORC and DNA melting starts, the mechanism of origin interference is triggered, and replication initiation on the neighboring MCM2-7 complexes is inhibited [63].

Second, studies with mammalian cells showed that binding sites of excess MCM, which is not associated

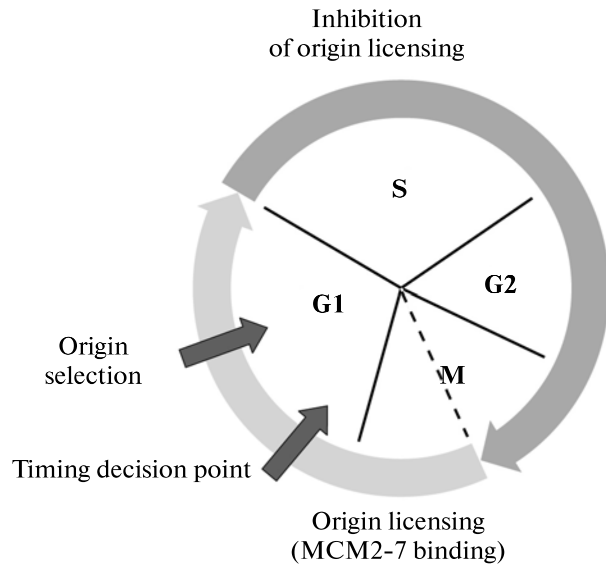

Fig. 2. Establishment of the replication program in the cell cycle. The inner circle marks the cell-cycle phases. The outer circle shows the cell-cycle periods when replication licensing is possible (light arrow) or inhibited (dark arrow). Indicated are the cell-cycle stages when the replication timing program is established and the origins are selected that are to fire in the nearest S phase.

with ORC, correspond to dormant origins, which are only activated upon replication stress [63, 64]. It is thought that, once loaded on chromatin in ORC localization sites, a portion of MCM2-7 complexes moves along the chromatin fiber travel far away along and that these excessive MCM2-7 complexes are capable of activating replication initiation in stress [13]. Full-genome analysis of the origin distribution in cultured *D. melanogaster* cells showed that only 82% of the origins detectable in HU-treated cells correspond to ORC binding sites [34].

Limiting factors that determine the number of replicons active simultaneously. The role played by the amounts and stoichiometric proportion of components necessary for replication initiation can be seen from replication initiation in somatic and embryonic mammalian cells.

The high density and high efficiency of origins in embryonic chromatin is thought to be due, to a great extent, to an excess of all of the pre-RC components and Cdc45 at the embryonic stage of development. Somatic mammalian cells contain, on average, one ORC hexamer, two Cdc6 molecules, and four or five MCM hexamers per 100 kb; i.e., their contents are approximately tenfold lower than in embryonic cells. The additional and probably most important factor is that pre-RCs have to compete for Cdc45 as a limiting factor in somatic cells [13]. Cdc45 available in the cell only ensures activation of a certain proportion of origins, and new replicons are not activated until Cdc45 is released. As a component of the helicase complex,

Cdc45 is involved not only in initiation, but also in elongation of replication, and it takes some time for Cdc45 to be released. Consequently, the Cdc45 concentration substantially affects the temporal characteristics of the S phase. An excess of purified Cdc45 microinjected in cell nuclei in the S phase activates additional origins, demonstrating again that Cdc45 acts as a factor that determines the number of replicons simultaneously utilized in mammalian cells [13].

Many factors play a role in origin activation. Apart from Cdc45, whose role as a limiting factor was demonstrated for mammalian and *S. pombe* cells [62, 65], candidate limiting factors include Cdc7 in complex with Dbf4 (DDK) (*S. pombe*), CDK1, CDK2 (vertebrates) [66, 67], and Cdc28/Clb5 (*S. cerevisiae*) [68].

DNA attachment to the nuclear matrix plays an important role in origin activation. High-salt treatment of nuclei removes a substantial portion of chromatin proteins. The remaining nuclear structures consist of loop domains, which are anchored in the nuclear matrix and are detectable microscopically, allowing their sizes to be estimated. There is a tight association among the chromatin loops, replicons, and origins of DNA replication. For instance, the sizes of chromatin loops were found to coincide with the sizes of replicons in many animals and plants [46, 69]. Incubation of differentiated nuclei in a *Xenopus* egg extract corresponding to mitosis leads to global changes in chromosome architecture. In particular, when erythrocyte nuclei were treated with the extract, the average size of DNA loops changed from 97 to 15 kb, correlating with the replicon size [46].

A similar correlation between the replicon and loop sizes was observed in mammalian cells [70]. The distribution of active origins proved to depend not only on the replication fork progression rate, but also on the organization of chromatin loops in Chinese hamster cells. When the replication fork progression rate was modulated with various chemical agents, a substantial correlation was observed between the replication rate in the given S phase and the chromatin loop size in the next G1 phase. Origins corresponding to the sites of anchorage of chromatin loops in G1 were activated preferentially in the subsequent S phase. These data suggest a mechanism of origin programming in which replication rate determines the distribution of chromatin loops, and this distribution, in turn, controls the choice of origins in the next cycle [70].

When the amplified dihydrofolate reductase (DHFR) locus is replicated in cultured CHOC 400 Chinese hamster cells, only 15% of origins are activated in each cell cycle, while the other repeat units are replicated passively by forks traveling from the active origins of flanking repeats [71]. During the G1/S transition, changes in micrococcal nuclease sensitivity, which reflect the changes in chromatin structure, are only observed in the repeat units associated with the nuclear matrix [72].

The concentrations of the ORC2–5 subunits remain constant throughout the cell cycle in mammalian cells, while the ORC1 level oscillates. The phenomenon is known as the ORC cycle [2]. The ORC1 level starts to increase in mid-G1, reaches its maximum during the G1/S transition when OCR1 becomes detectable in the nuclear matrix, and decreases to the basal level in the S phase. An ORC2–5 fraction associated with the nuclear matrix appears in the nucleus concomitantly with the increase in ORC1 concentration [73]. Thus, ORC1 regulates the ORC status in mammalian cells by recruiting it to the nuclear matrix [74].

How does attachment to the matrix regulate the initiation of replication? In *Xenopus*, the distribution of replication origins and loop size remodeling depend on DNA topoisomerase II [57], which is associated with the matrix [75]. A reprogramming of replication origin distribution correlates with ORC recruitment to chromatin, which also depends on topoisomerase II [57]. The cause-and-effect relationship is not completely clear in this case, but the loop organization of chromatin may be assumed to play an important role in both ORC localization and the regulation of origin efficiency during activation. Vice versa, ORC binding may determine the formation of loops by anchoring origins in the matrix. Attachment to the matrix helps to combine several replication origins to organize a replication factory, thus facilitating their coordinate regulation [76].

Chromatin features affecting the efficiency of origins. Origin activation depends on acetylation of histone tails. All of the origins within rDNA repeats region are activated in almost every cell cycle in *S. cerevisiae* mutant for *Sir2* histone deacetylase gene, while only 20% of the origins are involved in replication initiation normally [77]. Differences in local histone acetylation in the mammalian β-globin gene locus are responsible for the fact that the corresponding replication origin is active in erythroid cells and inactive in nonerythroid cells [78]. Pre-RC proteins are associated with acetyltransferases in both mammals and *D. melanogaster* [79].

Targeted binding of histone acetyltransferase Chameau to the *Drosophila* chorionic origin inserted in an artificial construct locally stimulated origin activity, while histone deacetylase Rpd3 or Pc binding inhibited it (Pc is a subunit of multiprotein complexes involved in maintaining chromatin domains of a certain type with repressed transcription). Therefore, targeted recruitment of acetylases and deacetylases to the origin may program its developmental and cell cycle-dependent activity patterns. The Rb protein is a candidate targeting agent. Mammalian histone deacetylases, such as Rpd3, are known to bind with Rb to ensure transcriptional repression. In *D. melanogaster*, dE2F1 and Rbf, which is a *D. melanogaster* homolog of Rb, act in complex to repress transcription and are capable of interacting with DmORC. In mammalian

cells, E2F and Rb bind to replication foci and origins [79].

It is of interest that mammalian histone acetyltransferase HBO1, which is homologous to Chameau, also binds to replication origins and is thought to act as an important regulator of their licensing. Recent studies show that HBO1 interacts with Cdt1 and regulates the landing of the MCM2-7 complex. The HBO1 concentration in human fibroblasts is virtually equimolar to the number of active origins, suggesting an important role in regulating replication initiation for the enzyme [80, 81].

At the same time, acetylation is not a universal property of replication origins. Acetylation is probably used to select certain origins, but its involvement in the regulation of initiation timing is far tighter than in the setting of efficiency of origins [52].

When initiation occurs in closed chromatin domains with a low histone acetylase level, alternative mechanisms are possibly used to recruit the pre-RC components to chromatin. In *S. pombe*, activation of replication origins in heterochromatic regions involves SWI6, which is homologous to HP1 and recruits the DDK/Cdc7 complex to the regions in a targeted manner [82]. An interaction between the ORC and HP1 was observed in *D. melanogaster* [83]. The ORC function in the establishment of heterochromatin domains is presumably independent of replication, but the ORC–HP1 interaction may be thought to facilitate the pre-RC formation in heterochromatin regions.

REPLICATION INITIATION AND CELL-CYCLE REGULATORS. EARLY AND LATE ORIGINS

Early and late origins differently respond to the intra-S-phase checkpoint. New origins are continuously activated throughout the S phase. However, the terms early and late origins are commonly accepted. Origins are divided into two classes depending on how the so-called intra-S-phase checkpoint, which a system that controls DNA damage in the S phase, affects the replication initiation on a given origin [84, 85].

To artificially initiate a cell cycle arrest in the S phase cells are treated with HU. HU is thought to block ribonucleotide reductase; this leads to exhaustion of the nucleotide pool in the cell and, consequently, slows down or totally stops the progression of DNA replication forks [86]. Early replication origins remain capable of initiating replication after HU treatment, while replication initiation on late origins is blocked.

Interestingly, this is accompanied by activation of many dormant origins, which remain inactive in normal conditions. Activation of the intra-S-phase checkpoint stabilizes the stalled replication forks, which preserve the polymerase complex as a result. In the absence of this stabilization, a fork most likely collapses to induce DNA double-stranded breaks [87]. When damage is substantial, the cell enters apoptosis.

The system of response to DNA damage is conserved among eukaryotes. A block of replication initiation on late origins in the presence of HU was observed in *S. cerevisiae* [84], mammals [88], a *Xenopus* egg extract [89], and *D. melanogaster* [90]. For instance, a genome-wide study with *S. cerevisiae* showed that, of 260 replication origins, 143 (early) fire in the presence of HU, while 104 origins do not [91]. In *D. melanogaster*, 30% of origins are capable of replication initiation in the presence of HU [30, 90].

In mammals, stably early and stably late origins account for a substantial fraction of the genome, while almost half of the origins are activated in the early or late S phase at equal probabilities [92]. In *S. pombe*, HU exerts only a minor effect on origin activation [9, 93]. It is of interest that stably late origins are nearly absent from *S. pombe*, and its origins are activated stochastically with respect to the time in the S phase [51, 94].

The ATR/CHK1 pathway regulates the activation of origins. The order of activation of replication origins during the normal S phase is regulated by the same regulatory pathway that plays a role in the cell response to replication stress [1, 95]. Replication stress is defined as the conditions that lead to a replication fork arrest, in particular, when DNA lesions are too many or replication-associated enzymes, such as ribonucleotide reductase (HU treatment) or DNA polymerases (aphidicolin), are blocked.

The key components of the pathway are as follows. ATM kinase (ataxia telangiectasia mutated) acts as a main sensor of DNA damage. Stalled replication forks activate ATR (ATM- and Rad3-related) kinase. ATM and ATR kinases affect several targets whose phosphorylation is necessary for the regulation of the normal cell cycle, the response to DNA damage, and apoptosis. The role of these kinases in the regulation of replication origins is mostly related to activating phosphorylation of checkpoint kinase 1 (CHK1) [96]. CHK1 activation leads to phosphorylation of Cdc25A phosphatase, which triggers its own degradation. In turn, Cdc25A regulates activity of cyclin-dependent kinases. CHK1-dependent degradation of Cdc25A phosphatase prevents dephosphorylation of CDK1 and CDK2. This prevents Cdc45 from binding to origins, which is necessary for their activation [85, 97] (Fig. 3).

In the normal cell cycle, the ATR-dependent pathway inhibits a premature activation of late origins and limits the use of origins in early origin clusters (lateral inhibition). A block of ATM and ATR activities with caffeine or specific antibodies substantially increases the number of origins in normal cells [98, 99].

How does the pathway, which is activated in response to replication stress, function in the normal S phase in the absence of stalled forks and DNA lesions?

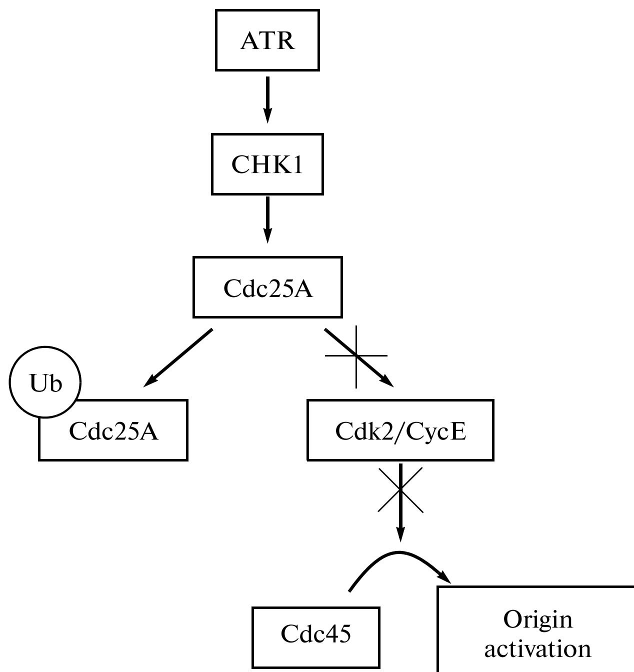

Fig. 3. Main ATR-cascade components involved in regulating activation of replication origins.

It was found that a complex of ssDNA with the ssDNA-binding RPA proteins, which is a replication intermediate, acts as an ATR activator [100]. For instance, RPA depletion from a *Xenopus* egg extract prevents the number of active origins from increasing in response to caffeine.

There are several interesting sequels to this mechanism of ATR activation. First, the slower the DNA synthesis, the higher is the ssDNA amount associated with the replication fork and the higher is the level of ATR induction. Second, if RPA activates ATR, then ATR is induced locally immediately after the start of DNA synthesis. This circumstance may explain the interference between closely spaced origins [97].

In addition, the progression of replication forks presumably has physiological genomic limitations. First, certain nucleotide sequences are more difficult to replicate than others. Second, chromatin-associated factors, insulator elements, and specific nuclear structures may slow down or stop a replication fork in the absence of replication stress, also activating the ATR pathway.

CHK1 mediates the effect of ATR on origin interference and suppression of late origins during normal replication and on suppression of late origin activation in response to replication stress [99, 101, 102]. As with ATR, inhibition or removal of CHK1 increases the number of active origins, slows down the progression of replication forks, and distorts the temporal pattern of genome replication. It is important to note that the origins that normally act late to initiate replication are activated in the early S phase in the absence of CHK1 [103–105]. In addition, a decrease in CHK1 suppresses origin interference, leading to shorter intervals between origins [98].

In *S. cerevisiae* and metazoans, RAD53 and CHK1, respectively, prevent activation of late origins until approximately half of the genome is replicated [99, 106]. Replication of the other half of the genome requires a restoration from the checkpoint, that is, CHK1 neutralization. It was shown indeed that CHK1 undergoes ubiquitination and proteasomal degradation in the mid- and late S phase [107].

The role of CHK1 in the S phase is not limited to affecting the initiation of origins by regulating cyclin-dependent kinase activity. CHK1 additionally plays an important role in regulating transcription of the genes necessary for the normal progress of the S phase [108]. For instance, CHK1 activation leads to repression of the gene for acetyltransferase GCN5, which may be involved in arresting the cell cycle in response to replication stress.

It is of special interest that CHK1 plays a role in repression of *RNR2*, which codes for a ribonucleotide reductase [109]. The *RNR2* expression level determines the levels of DNA precursors and, consequently, the rate of DNA synthesis. The replication rate directly depends on the number of active origins [70]. CHK1 probably coordinates the origin density and replication fork progression rate in both normal S phase and replication stress [109–111].

Activation of origins in replication stress. A response to DNA damage and stalled replication forks involves several events, which seem contradictory at first glance.

On the one hand, when a replication fork is stalled, dormant origins are initiated [112] in its immediate vicinity [64, 113]. Likewise, DNA double-strand breaks increase the use of origins located close to a break [114]. Thus, not only do the checkpoints prevent the progress of the S phase in conditions interfering with the cell cycle, but they also make activation of inefficient origins more likely and activate dormant origins. This is probably essential for preventing underreplication. DNA repair takes rather a long while, and this mode of continuing replication provides for a minimal possible delay in replication of the total genome in response to the events that occur occasionally during the progression of individual replication forks.

On the other hand, replication stress must activate ATR and its direct target CHK1, which must suppress activation of replication origins. How it is that some origins are suppressed, while some others are simultaneously activated?

One of the models is based on the dual effect of ATM/ATR and suggests that, in replication stress, another ATM/ATR-dependent regulatory pathway is triggered and overcomes the inhibitory effect on dormant origins.

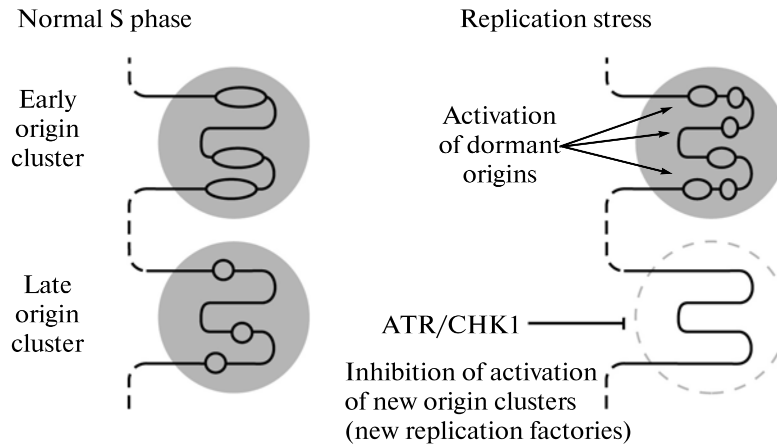

Fig. 4. Model demonstrating how replication factories respond to moderate replication stress (Cited from [115]; first published in JCB, doi: 10.1083/jcb.201007074).

In addition, different effects of the ATR pathway are related to the regulation of replication initiation at the levels of origin clusters organized in replication factories [115]. In low-level replication stress, ATR/CHK1 predominantly acts to inhibit the activation of new replication factories, thus reducing the total number of active origin clusters and replication factories. The replication rate in the replicons that are already active decreases at this time, which was shown to result in activation of dormant origin within active replication foci [70]. Thus, inhibition of new factories by ATR/CHK1 leads to a redistribution of resources to the factories that are already active (Fig. 4), minimizing the harmful effect of stalled forks and preventing the problem of the regions that are between stalled forks and fail to complete replication by the end of the S phase.

These results demonstrate that all origins located in the replication factories that have still not been activated in the given S phase are sensitive to CHK1-mediated inhibition in replication stress. The question arises as to whether early and late origins exist in fact or experimental treatment with HU synchronizes the cells at the stage when activation only involves the first replication factories that correspond to the start of the S phase.

It was found that early and late origins not only differ in sensitivity to the ATR/CHK1-mediated regulation, but they are also activated by different cyclin-dependent kinases.

Early and late origins are activated by different cyclin-dependent kinases. The assumption that early and late origins are activated by different complexes of cyclins and cyclin-dependent kinases is based on studies of *S. cerevisiae* replication. In *S. cerevisiae*, Cdk1–Clb6 plays a major role in the activation of early origins. Cyclin Clb6 is degraded during the S phase, while Clb5 is necessary for the activation of late origins [116, 117]. When the Clb5p cyclin gene is affected by a mutation, the all of the genome is replicated using early origins. Early and late origins are similarly activated by different Cyc/CDK complexes in mammalian cells.

Cyclins A and E in complex with CDK2 regulate the replication initiation on early origins in somatic mammalian cells. The regulation additionally involves Cdc7 kinase in complex with the Cbf4 regulatory subunit. These two kinases phosphorylate MCM2-7 subunits, allowing the origin to bind Cdc45. Another complex, cyclin A2–CDK1, plays an important role in the activation of late origins in mammalian cells [66].

The cyclin A2–CDK2 complex is active during the early S phase in mouse embryo fibroblasts. The formation of CDK2 complexes reaches a plateau by the mid-S phase, and then activity of the cyclin A2–CDK1 complex becomes detectable and increases gradually [66]. It is of interest that this differential activity is associated with CHK1 to a great extent. Activity of the cyclin A2–CDK1 complex become detectable earlier and is higher, while activity of the cyclin A2–CDK2 complex remains unchanged in cells with a mutant CHK1. CDK2 binds only to early origins, while CDK1 is capable of binding to both early and late origins in these cells [66].

Thus, a qualitative transition from CDK2-mediated activation, which involves only early origins, to CDK1-mediated activation, which is less specific with respect to origins, occurs in the mid-S phase. The transition requires that CHK1 be neutralized.

The role of CHK1 in regulating activity of the cyclin A2–CDK1 complex, but not the cyclin A2–CDK2 complex, is associated with the regulation of the Cdc25A level. A CHK1 removal substantially increases the Cdc25A level and leads to hyperactivation of the cyclin A2–CDK1 complex. The replication origin density becomes almost thrice higher in this case [66]. Overexpression of the cyclin A2–CDK1

complex exerts a similar effect. Hence, the CDK1 level most likely modulates the efficiency of origins and the timing of their activation [99, 102].

Why CDK1 exerts this specific effect on late origins? CDK1 binds preferentially to late origins, although both CDK1 and CDK2 can interact with early origins. It seems that cyclin-dependent kinases are somehow capable of differential recognition of the *cis*-acting factors that determine origin activation. Since the timing of origin activation is programmed at the level of extended chromatin domains, the chromatin state must play an important role in this recognition.

Gradual sequential activation of origins is necessary for maintaining a balance between the number of active replication forks and the DNA synthesis rate. The density of active origins (the frequency of initiation events) and the progression of replication forks (the elongation rate) should be regulated in a concerted manner to ensure efficient and complete duplication of every chromosome domain. This hypothesis is supported by the finding that the ATR/CHK1 pathway plays an important role in the coregulation of the initiation frequency and elongation rate [110].

MECHANISMS OF PROGRAMMING THE TIME OF ORIGIN ACTIVATION

If early and late origins differently respond to cell-cycle signals, then they should somehow be marked as early and late by the start of the S phase. Below we consider how and at what step of the cell cycle this programming occurs.

In almost all cases, origins cloned as autonomously replicating plasmids initiate replication early regardless of the time of their replication in the native position [3]. Therefore, the replication origin timing should be determined by the surrounding nucleotide sequences or the state of adjacent chromatin. There are specific sequences that bind the factors responsible for late replication initiation. Such conserved sequences were identified in *S. pombe*. They are found in the vicinity of all late origins known in *S. pombe* and affect the time of their replication [118]. In higher eukaryotes, several *cis*-regulatory elements are known to determine the activation time for neighboring origins, but these elements are specific to the corresponding origins [119–121].

Position in the nucleus plays an important role in the programming of origins. Replication timing is programmed in early G1 in mammalian cells. Postmitotic chromosome movements are complete by this time, indirectly suggesting an association between the programming of the replication time for chromosome regions and their positions in the nucleus [122, 123].

Developmental changes in replication timing profile were recently mapped at the whole-genome level in mammals. The changes proved to correlate with the maps of spatial interactions between regions [124]. A model was put forward that the spatial reorganization of the nucleus, which occurs simultaneously with the programming of replication domains, leads to the formation of nuclear compartments that provide different conditions for replication initiation. Extensive domains of early and late replication correspond to the genome regions that reside in different compartments. The domains remain programmable to initiate their replication at a certain time during the S phase until they pass through replication [61, 125].

In yeast cells, late-firing origins tend to occur at the periphery of the nucleus at a certain time of G1, while early-firing origins are localized more stochastically.

For instance, the late-firing origin ARS501 normally occurs at the periphery of the nucleus in *S. cerevisiae*. If its excision from the chromosome is induced, the resulting episome moves away from the periphery of the nucleus, but the late time of origin firing is still preserved in the nearest S phase [122, 126]. Human chromosome 18 replicates late to a considerable extent. The chromosome was shown to occur at the periphery of the nucleus in actively dividing cells; however, its nuclear localization changes when the cell leaves the cell cycle. If the cell is stimulated to reenter the S phase, chromosome 18 preserves its capability of late replication without resuming its peripheral localization until the next mitosis [127]. The spatial organization of the nucleus may undergo substantial changes when the cell leaves the cell cycle in G0. However, when the cell returns to the cell cycle, replication follows the same scenario; i.e., the programmed state of domains is preserved in spite of the visible changes in the distribution of late and early replicating domains through the nucleus. Therefore, the peripheral position in the nucleus is necessary for establishing, rather than maintaining, the late replication program.

How can the position within the nucleus play a role in programming replication? In early G1, a postmitotic redistribution of chromosomes coincides with the binding of structural chromatin proteins, which were removed from chromatin during mitosis. A reassociation of the proteins that determine the chromatin architecture at the periphery of the nucleus may increase the local concentrations of these proteins, thus providing microconditions for certain chromosome architecture to form. The architecture may be rather stable during the rest of the cell cycle regardless of the nuclear position and may determine the timing of origin firing.

The histone acetylation status of origins determines the time of replication initiation. As mentioned above, origins are somehow marked at the periphery of the nucleus in early G1, and then the marks are recognized by cell-cycle kinases in the S phase. What are the marks?

Whole-genome studies showed that local chromatin properties determine the origins that are to fire predominantly in the early S phase [27, 28, 36, 128–130].

The regions that are marked with histone modifications characteristic of active genes usually contain early origins, while origins located in regions with repressive modifications corresponding to heterochromatin usually fire in the late S phase [131]. The finding agrees with the data that early replication correlates with transcriptional activity to a substantial extent [59, 129]. Early origins occur predominantly in the vicinity of actively expressed genes, the origins that fire in the mid-S phase occur near genes expressed to a moderate extent, and late origins are far away from transcribed genes [36]. Early-replicating genome regions are characterized by a high DNase I sensitivity [132] and a packing with acetylated histones [42]. Late-replicating regions have a closed chromatin conformation and predominantly contain nontranscribed sequences [90, 92, 128, 133, 134].

Because replication origins occur in the vicinity of actively transcribed genes and often coincide with promoters or other transcriptional regulatory elements, a great variety of chromatin marks can be found in origin regions. Consequently, it is rather difficult to identify the role that individual chromatin components play in replication initiation. Even neighboring origins firing synchronously may have different sets of modifications [135].

The character of histone acetylation is thought to act as a main marker of replication time. For instance, when histone deacetylase Rpd3 or its interaction partner Sin3 is lacking in *S. cerevisiae*, late origins in intrachromosomal regions bind replication factor Cdc45p earlier and, consequently, initiate replication earlier [136]. Targeted recruitment of HAT/HDAC to the replication origin of the human β-globin gene region changes the replication time approximately by 20% of the total S-phase duration [78]. Histone deacetylase inhibitors change the replication timing pattern in mammalian cells [137]. Taken together, these findings indicate that the regulation of replication initiation via histone deacetylation is conserved among eukaryotes.

Whole-genome studies showed that early-replicating regions of *D. melanogaster* chromosomes are marked with histones acetylated at H4K16 and that the enrichment in H4K16ac is observed even in the absence of transcription and is especially high in replication initiation regions. It is of interest that late replication is absent from the H4K16ac-rich dosage-compensated X chromosome in *D. melanogaster* males [59].

Other chromatin components may mediate fine-tuning regulation of the replication timing program. It is thought that other components and modifications of chromatin are also capable of a direct effect on the replication timing program, although the effect of each individual factor is relatively small [138]. For instance, only moderate changes in the replication time of individual pericentric heterochromatin regions were observed in mouse embryo cells with mutant chromatin proteins, such as DNA methyltransferase Dnmt1, histone methyltransferase G9a, the Polycomb group protein Eed, or histone methyltransferase Suv39h1/h2. However, the replication time of 20 genes examined was not changed [139]. A certain effect of methyltransferase Suv39h1/h2 on the replication time of heterochromatic sequences was observed in human embryo fibroblasts [140]. Replication of heterochromatic regions was slower in *S. pombe* with mutations of the genes for HP1 and SU(VAR)3–9 orthologs (Swi6 and Clr4, respectively) [82, 141]. *Drosophila* HP1 was shown to perform two functions in regulating the temporal characteristics of replication. The protein controls very late replication of centromere DNA and is necessary for earlier replication of euchromatic regions with a high content of repetitive sequences [142]. These findings demonstrate that the effects of many chromatin factors supplement the global mechanisms that control the replication timing program, providing for its fine tuning [140, 142].

It is important to note than none of the epigenetic marks correlates with replication timing stronger than transcription [82].

Cascade activation of replication domains. A domino model. As we noted when considering the replication foci, neighbor clusters of replicons display a tendency to consecutive activation during replication of mammalian genomes. It is possible to assume that replication of a particular genome region is facilitated by the consecutive binding of replication factors to the neighboring initiation sites. The replication act probably induces local changes in chromatin condensation, and, in turn, these changes ensure the access/recruitment of replication factors and new initiation events. The model suggests that replication starts in sites with the open chromatin conformation. Initiation events lead to chromatin decondensation in neighboring regions so that their chromatin becomes accessible to replication initiation factors. The model, which implies a cascade mechanism for preparing chromatin to replication initiation, is known as the domino model [18].

There is indirect evidence that this gradual conversion of chromatin to a replication initiation-competent state may involve helicase complex components, in particular, MCM2-7 or Cdc45. The latter is involved in specific phosphorylation of histone H1 and phosphorylation-associated chromatin decondensation, which is necessary for replication initiation. In addition, the distribution of the two proteins on cytological preparations of replicating cells reflects the general progress of the S phase, but the proteins become detectable in replication foci before replication is initiated [6].

EXTENSIVE REPLICATION DOMAINS

Studies of the genomic replication profile in various higher eukaryotic cells (other than embryo cells at the stage of rapid cleavage divisions) showed that early- and late-replicating domains are distinct in each

cell type [58, 131]. The domains generally reflect well the genome organization in chromatin domains. It is possible to assume that relatively homogeneous chromatin properties within a domain determine a relatively synchronous replication initiation in the domain.

What events take place at the boundaries of replication domains? What factors determine the replication time at the boundaries of discrete replication domains? If there are physical barriers between domains, the replication fork can be expected to stop, and the replication profile should be discontinuous between neighboring domains. Otherwise the replication profile should be smoother.

Indeed, a discontinuous replication profile is possible for the boundaries of replication domains in higher eukaryotes. A substantial difference in replication time between relatively closely spaced regions (less than 100 kb apart) was demonstrated for several boundary regions between mammalian R and G bands [143, 144]. For instance, a difference of 1 h was observed for replication of sequences separated only by 16 kb in the human major histocompatibility complex (MHC) locus [144], suggesting a barrier at which the replication fork is either stopped or substantially slowed down. The nucleotide sequences of such regions share certain properties, in particular, a high density of the Alu repeats and LINEs and the presence of polypurine/polypyrimidine tracts; di-, tri-, and tetranucleotide repeats; and SARs [143, 144].

At the same time, a gradual change in replication time is also possible for the boundaries between G and R bands. This pattern was observed between early-replicating R band 13q14.3 and late-replicating G band 13q21.1 of human chromosome 13 [145]. It is of interest that the change is stepwise, and groups of replicons of approximately 1 Mb in size (which corresponds to the average size of replication foci) are replicated in a coordinated manner.

Examples of a replication time gradient are also known. For instance, in mouse embrionic stem cells, the 400-kb *IgH* gene region is flanked, on one side, by a region that is always replicated extremely early in the S phase and, on the other side, by a region that is always replicated late. The *IgH* region is replicated by one fork, which arrives from the early-replicating region and moves toward the late-replicating region. Hence, the proximal early origin directs a replication fork through the *IgH* region, and the fork progresses until it meets the fork moving from the origin that fires later [23, 146]. Thus, the distance between clusters of synchronously firing origins may be substantially greater than the distance between origins with a cluster, and the boundaries of late-replicating domains may be determined by the progression rate of replication forks starting from early origins.

Genome-wide replication profiles obtained for various mammalian cells showed that regions of up to 1.5 Mb in length that lack replication initiation events occur at the boundaries of many replication domains [7, 36, 58, 124, 128, 147]. The regions are known as the temporal transition regions (TTRs). It is important to note that TTRs not merely lack replication origins, but they are probably characterized by active inhibition of origins. When inserted in the TTR of the mouse *IgH* locus, replication origins (DNA fragments capable of efficient replication initiation in other genome regions) did not initiate replication [147].

Results were published recently to contradict the concept that extended replicons are widespread in mammalian TTRs [148]. As the results indicate, such replicons are only characteristic of embryonic stem cells, while replicons exceeding 100 kb are not found in differentiated and cancer cells. A replication gradient observed in the TTR is thought to be due to a consecutive cascade activation of origins in the corresponding regions. In particular, the *IgH* locus is replicated using many origins that fire consecutively in HeLa cells.

Differentiation-related changes in replication occur at the level of extended domains. Replication domains where replication proceeds in a coordinated manner are discrete units of chromatin structure and function. The properties of the domains may change during cell differentiation [138]. It is safe to think that a substantial portion of the genome is subject to a developmental regulation of replication timing and that a specific replication program is established in each cell type [58, 59, 131, 149]. For instance, replication timing is changed for approximately 20% of the genome during differentiation of mouse embryonic stem cells into neural precursor cells. In total, almost half of the mammalian genome undergoes changes in replication timing at one or another developmental stage.

An interesting observation was made when studying the time course of genome replication profiles during mammalian cell differentiation. While replication domains may greatly vary in size (reaching several megabases) in each particular type of mouse or human cells, the genome fragments whose replication timing changes during differentiation are almost the same size, approximately 400–800 kb. The finding may be thought to indicate that a certain minimal domain size is necessary for the coordinate regulation of replication timing [58, 124, 125, 149, 150].

Similar studies were carried out with *D. melanogaster* cells [30, 59]. The replication profile was compared for two cell types, Kc cells, which are of an embryonic origin, and Cl8 cells, which originate from a wing imaginal disk, and the comparison revealed a 20% difference again. It is important to note that differentially replicating regions are also rather large in *D. melanogaster*, averaging 100 kb. At the same time, the replication time may change within small (several kilobases in size) genome regions in *D. melanogaster* [151].

The general replication timing characteristic of a particular cell type is conserved to a substantial extent.

For instance, similar replication profiles were observed for extensive syntenic regions of mouse and human cells of one type [152]. Tissue-specific replication profiles are more conserved than other properties of the same genome regions, including the GC content. This finding indicates that the maintenance of an exact temporal replication program is an important characteristic of the epigenetic state of the cell, although the exact significance of the program remains unclear.

Replication domains are a highly stable and informative epigenetic feature of the cell type and differentiation stage. The common problems of stem cell biology include checking quality of induced pluripotent cells, assessing their differentiation potential, and determining the cell identity because cells rapidly change their properties and epigenetic status in early embryo development. Recent experiments showed that temporal features of replication are suitable for solving these problems [153]. The replication profile proved to be a less dynamic characteristic than features of transcription or chromatin content. In addition, the characteristic changes simultaneously within extensive replication domains, whose organization is specific to each cell type. Differentiation-related changes in replication profile follow a strict order and occur at the level of extended genome domains.

Gilbert and colleagues [130] examined several dozens of cell types corresponding to different stages of embryo development and constructed a dendrogram that reflected the order of cell differentiation and cell relationships.

It is noteworthy that replication profiles of induced pluripotent cells reprogrammed from human or mouse fibroblasts are almost the same as those in embryonic stem cells. Moreover, the replication profile significantly changes with loss of pluripotency [138]. Thus, the replication profile provides a good marker of pluripotency. Individual marker regions were isolated whose local replication profile allows a classification of mouse or human cells. The regions were termed replication fingerprints [153]. Replication fingerprints provide great opportunities for characterizing various cell types or the extent of cell differentiation [153].

Replication domains and regulation of gene transcription. Cell differentiation is accompanied by substantial changes in replication timing of extended chromatin domains. How does this correlate with the regulation of gene expression?

Studies of whole-genome replication and transcription profiles in *D. melanogaster* cells showed that the later the gene is replicated in the S phase of the cell cycle, the less likely is its transcriptional activity in cells of the given type. At the same time, there are active genes that reside in late-replicating regions and inactive genes that are replicated in the early S phase [59, 133]. However, a correlation between the replication time of a gene and the level of its transcription was not observed in *D. melanogaster*. The majority of active genes with various expression levels are replicated early, while fewer genes, including actively transcribed ones, are replicated late [154]. The level of gene transcription correlates with the level of euchromatin-specific histone modifications. It was observed that early- and late-replicating domains significantly differ in transcription density along the chromosomes in *D. melanogaster*. The density of RNA polymerase II-binding sites is also substantially higher in early-replicating regions compared with regions whose replication starts later. These findings indicate that the transcription status affects the replication profile at the level of large domains (>100 kb) rather than that of individual genes [90].

Similar conclusions were made in studies of replication in mammals [58, 124, 130]. A significant correlation between replication timing and transcription was observed for the genes whose replication takes place in the mid- and late S phase. At the same time, such a correlation was not detected for the genes that are replicated during the initial one-third of the S phase; i.e., these genes are equally likely to be expressed or inactive.

It is important to note that nucleotide sequences corresponding to genes tend to replicate in the early S phase regardless of the level of gene expression. Whole-genome analysis of the replication profiles in mammals showed that approximately 75% of genes are replicated in the first half of the S phase, while the major portion of nongenic DNA is replicated in the late S phase in all cell types examined [58].

Which genes occur in the domains whose replication timing changes during development? This most likely depends on the chromatin context, rather than function, of a gene and, consequently, on its molecular organization. An example is provided by the paralogous mammalian β- and α-globin genes, which are expressed exclusively in erythroid cells.

The β-globin gene locus resides in a chromatin domain of more than 1 Mb, which is sensitive to DNase I digestion (open chromatin conformation) in erythroid cells. The locus displays a higher level of histone H3 and H4 acetylation, is far away from pericentric heterochromatin, and is replicated in the early S phase. The globin genes are silenced in nonerythroid cells, and the locus is insensitive to endonuclease digestion (closed chromatin), is hypoacetylated, becomes closer to pericentric heterochromatin, and is replicated in the late S phase [155, 156]. The regulation of these tissue-specific changes is due to specific *cis*-acting sequences, within LCR (an element essential for the regulation of tissue-specific expression of the β-globin gene locus), and *trans*-acting factors, which switch the replication program in erythroid cells. Deletion analysis made it possible to separate the nucleotide sequences responsible for the temporal regulation of expression and replication of the locus and to demonstrate that replication timing of the locus

correlates with its nuclease sensitivity (i.e., the chromatin structure), but not with globin gene expression [157].

The α-globin gene locus belongs to a chromatin domain that displays an open conformation and early S-phase replication regardless of the transcription status of the globin genes. The locus harbors many active genes and CpG islands [155]. An origin or a region responsible for replication timing was not found in the region. Its replication pattern is probably related to the local chromatin structure that facilitates access to multiple replication origins. Likely, early replication initiation does not require a special programming. It is known that CpG islands (methylation-free G + C-rich regions of approximately 1 kb) are contained in the promoters of many mammalian genes. Such islands similarly constitute a substantial fraction of the mammalian replication origins that display a coordinate early replication initiation in the S phase [158].

A change in S-phase replication timing may provide a mechanism that reprograms the state of the region for further cell generations. Indeed, a switch to a late replication program is capable of determining a repressed (silent) state of the region. In contrast, the active chromatin state, which is associated with early replication, does not necessarily increases gene activity because transcriptional activation may require specific regulatory factors. Active chromatin state may facilitate the promoter binding with these factors. Transcriptional regulators can recruit the enzymes that are involved in chromatin remodeling and reorganization, and, in turn, this circumstance may act as one of the factors determining where and when replication starts in higher eukaryotic chromosomes.

SIGNIFICANCE OF REPLICATION TIMING CONTROL

As discussed above, the specifics and mechanisms of the replication timing regulation indicate that the replication program is a stable epigenetic characteristic of the cell and is subject to a strict control depending on the cell cycle and development. The most important questions are why the replication regulation is controlled in such a strict manner and what role it plays in the cell cycle, development of a multicellular organism, and genome evolution.

The role in maintaining the genome stability. It seems that the initial role of plastic replication initiation is related to maintaining the stability of the eukaryotic genome. By modulating the efficiencies of potential origins, it is possible to ensure a necessary duration of the S phase regardless of the progression rates of individual replication forks and a certain number of lesions present in DNA. This ensures a certain duration of the cell cycle, which is of immense importance for concerted development of a multicellular organism. A distorted regulation of replication timing in higher eukaryotes leads to genome instability, altered chromosome condensation in mitosis, sister chromatid cohesion, and cell malignant transformation [138].

The role in regulating gene expression and maintaining chromatin domains. A significant correlation between early replication and transcription was observed in all multicellular organisms examined to date, but there is no strict relationship between the replication time of a gene and the level of its transcription. It is thought that the temporal organization of replication is important for maintaining the epigenetic status of extended chromatin regions, which, in turn, determine transcriptional competence of individual genes in cells of each type.

It seems reasonable that different chromatin types are replicated and, consequently, assembled in separate places and at different times because this separation additionally protects the genome from a random redistribution of chromatin marks. For instance, it is important that a centromeric histone H3 variant be reassembled exclusively on the centromeric DNA. The presence of this histone variant in other chromosome regions may result in an ectopic centromere. Centromeric chromatin is thought to act as an independent replication domain in all eukaryotes, although the centromeric DNA replication time may substantially vary. For instance, centromeric chromatin is replicated in the immediate early S phase in yeasts *Candida albicans*, *S. cerevisiae*, and *S. pombe* [159, 160]. The centromere-associated replication origin is the first to fire in the chromosome in *C. albicans*. A transfer of centromeric determinants into an ectopic site similarly results in extremely early replication [160]. In higher eukaryotes, centromeric DNA is replicated at various stages of the S phase, but always asynchronously with adjacent pericentric heterochromatin [161].

In addition to its passive effect on chromatin packaging, replication timing provides an active mechanism of epigenetic inheritance. It was found that the set of proteins involved in chromatin packing and modification may greatly vary among replication forks working at different stages during the S phase.

The situation with histone acetylation provides the most brilliant illustration for chromatin assembly at different stages of the S phase [162]. It was found that acetylation of histones H3 and H4, which accompanies chromatin assembly in a replication fork, is determined by the replication time of the corresponding region. Newly synthesized H3/H4 dimers undergo the following modification prior to their incorporation into a nucleosome. Histone H3 is initially free from acetylation and is acetylated in the early S phase. Histone acetylases bind to the replication fork for this purpose. In the late S phase, the replication fork is associated with the deacetylases that deacetylate histone H4, which arrives in a preacetylated state into the nucleus.

Hence, DNA whose replication is programmed for the first half of the S phase is automatically repackaged using acetylated core histones, while late-replicating genome regions are packaged using deacetylated histones. If DNA initially packaged into nucleosomes with deacetylated histones is made to replicate in the early S phase, chromatin will be repackaged using acetylated histones. A change from early to late replication is accompanied by chromatin packaging with deacetylated histones [162].

Since DNA replication timing is regulated at the level of extended (approximately 1 Mb) domains in higher eukaryotes, the genome is divided during replication into domains of two discrete types, hypoacetylated and hyperacetylated. This agrees well with the organization of mammalian chromosomes, which include hyperacetylated early-replicating R bands and hypoacetylated late-replicating G bands.

DNA and H3K9 methyltransferases are found in replication forks in the late S phase, ensuring the packaging of all late-replicating sequences into heterochromatin.

Thus, there is a feedback loop here. The type of a chromatin domain determines the replication time, while the replication time determines the chromatin type for the domain. This mechanism probably maintains a rough separation of domains with open and closed chromatin structures, which functions directly in the replication fork. Replication is followed by gradual chromatin maturation, which may involve additional region-specific factors such as DNA-binding proteins that recognize certain target sites or noncoding RNAs that are recruited to genome regions with a certain nucleotide sequence.

Importantly, it is possible not only to reproduce the chromatin marks through cell generations, but also to reprogram the state of a region by regulating its replication timing. For instance, a change in replication timing is one of the first epigenetic events associated with inactivation of the mammalian X chromosome. The two alleles are expressed similarly and are replicated in a coordinate manner in the majority of higher eukaryotic genes. However, there is a small class of genes that are transcribed predominantly from one allele in every cell (a phenomenon called imprinting). The inactivated allele is replicated late in the majority of cases. Both late replication and inactivation are programmed during meiosis and maintained throughout development; the program is only erased in the next round of gametogenesis. It is thought that a temporal shift to late replication is used as a mechanism selecting the active allele [163, 164]. Direct evidence for the possibility of programming the transcriptional status of a gene by changing its replication time was obtained in experiments where cells were transformed with plasmids containing a reporter gene at various stages of the S phase. When introduced in the late S phase, the reporter gene was packaged into closed chromatin and was not transcribed in cultured mammalian cells. When the plasmid was introduced at earlier stages of the S phase, the reporter gene was stably transcribed through cell generations [162, 165].

The role of replication timing in genome evolution. The spatial and temporal organization of replication reflects the complexity of the eukaryotic genome. A distinct correlation was observed between the genome size and the extent of replication asynchrony, that is, the S-phase duration [166]. Replication asynchrony emerges simultaneously with the formation of heterochromatin domains during embryo development [167].

Genome division into early- and late-replicating domains corresponds to the separation of R and G bands in mammals [138]. In addition, mammalian replication domains clearly correlate with isochores, which are genome regions with a relatively homogeneous GC content [15]. As observed in one of the earliest studies on replication profiling of mammalian chromosomes, a shift from early to late replication corresponds to an appreciable change in GC content, early-replicating regions being GC rich in contrast to late-replicating ones [144]. Thus, specifics of the organization of the genome may predict many features of its replication timing.

Late-replicating domains have specific properties at the genome level and are predictable via nucleotide sequence analysis in *Drosophila* [168]. Late-replicating areas are usually flanked by regions that have a higher gene density and harbor short and often overlapping genes. Within the areas there are long genes, which have large introns and alternate with long intergenic regions. In addition, the areas contain extremely short genes, and many of them are genes expressed in the testis [168].

The replication timing profile reflects the nonuniform chromatin organization, which, in turn, is associated with the nonuniform organization of the genome. It is possible that asynchronous replication of different chromosome regions acts as an important factor of divergence in the organization of chromatin domains. The neutral evolution rate significantly differs between genome regions replicating at different stages during the S phase in both mammals and *Drosophila* [166, 169, 170]. Several independent mechanisms were described to underlie this difference. The main mechanism suggests that mutations (namely, $C \rightarrow T$ substitutions) accumulate in late-replicating CpG dinucleotides because high-level CpG methylation is characteristic of late-replicating genome regions (CpG methylation significantly increases the probability of $C \rightarrow T$ substitutions). This is true for the organisms in which DNA methylation plays an important role, for instance, for mammals. In addition, a positive correlation between the single nucleotide substitution rate and replication timing was observed outside CpG islands. A decrease in repair efficiency during the S phase is considered to be the main mechanism in this case. For instance, the CHK1-dependent

system, which controls DNA damage, should be neutralized to allow late origins to fire in the second half of the S phase (see above). Mismatch repair and, possibly, certain other repair systems seem to become less active in the late S phase. In addition, the probability for mutations to arise during DNA synthesis varies among different stages of the cell cycle [166, 169]. Thus, replication timing is an important factor that affects the genome evolution rate in mammals.

CONCLUSIONS

Replication of different chromatin types is long known to occur at different stages of the S phase, but it was not until recently that the regulation of replication came to be considered as an epigenetic phenomenon. As became clear, the genome replication program undergoes substantial changes during cell differentiation, and these changes are tightly associated with transcriptional activity and the organization of the nucleus. Domains of temporally coordinate replications delineate discrete units of chromosome structure and function. While the functional significance of this strict regulation of replication is still unclear, it is safe to say that the replication program is an epigenetic characteristic of a cell type.

Studies of replication timing are in rapid progress and will probably bring many unexpected results in the nearest future. New data are continuously reported for various organisms, cells, and tissues. On the one hand, the data include whole-genome replication profiles and their association with the organization of chromatin and the distribution of chromosomal proteins. On the other hand, there are data on the molecular mechanisms responsible for the epigenetic regulation of replication. We tried to consider all of the levels at which replication is regulated to orient the reader in this vast pool of information.

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