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Nuclear Matrix Support of DNA Replication

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Abstract In higher eukaryotic cells, DNA is tandemly arranged into 10^4 replicons that are replicated once per cell cycle during the S phase. To achieve this, DNA is organized into loops attached to the nuclear matrix. Each loop represents one individual replicon with the origin of replication localized within the loop and the ends of the replicon attached to the nuclear matrix at the bases of the loop. During late G_1 phase, the replication origins are associated with the nuclear matrix and dissociated after initiation of replication in S phase. Clusters of several replicons are operated together by replication factories, assembled at the nuclear matrix. During replication, DNA of each replicon is spooled through these factories, and after completion of DNA synthesis of any cluster of replicons, the respective replication factories are dismantled and assembled at the next cluster to be replicated. Upon completion of replication of any replicon cluster, the resulting entangled loops of the newly synthesized DNA are resolved by topoisomerases present in the nuclear matrix at the sites of attachment of the loops. Thus, the nuclear matrix plays a dual role in the process of DNA replication: on one hand, it represents structural support for the replication machinery and on the other, provides key protein factors for initiation, elongation, and termination of the replication of eukaryotic DNA. J. Cell. Biochem. 96: 951–961, 2005.

Key words: nuclear matrix; DNA replication; chromatin organization

The most important discovery in life sciences during the twentieth century was that DNA is composed of a double helix of two complementary polynucleotide chains wound around each other in opposite directions [Watson and Crick, 1953]. This opened an exciting opportunity to understand how the genetic information is stored, copied, and passed to the progeny. The term replication refers to all molecular events leading to the separation of the two DNA strands and their use as templates for the synthesis of new strands. Thus, two identical DNA molecules are produced, each containing a parental and a new strand (semiconservative replication). Many of the individual steps in this process have been discovered, as have the enzymes and other proteins taking part in them. However, the spatial aspects of DNA replication are still poorly understood. In prokaryotic cells, which contain about 10^6 bp DNA, replication begins from a single origin and the two replication forks moving in opposite direction at a rate of 10^4 bp/min are able to complete the replication of the prokaryotic genome for less than an hour. Eukaryotic genomes are 2 to 3 orders of magnitude larger and DNA is complexed with histones to form nucleosomes. During replication of eukaryotic DNA, it has to be unwrapped from around the histone octamers, which have to be moved behind the replication fork to the daughter DNA molecules and this slows down the process of replication considerably. In order to be able to replicate the eukaryotic genome during the short few hours of the S phase at this reduced rate, in eukaryotic cells DNA is arranged into individual replicating units called replicons and is tightly packed in a hierarchical fashion in a special organelle—the nucleus. This makes replication in eukaryotic cells very complex. It was obvious that such a complex and strictly regulated process is not possible to be successfully performed time and again outside certain structural frame which to coordinate it. For this

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reason, the discovery of the nuclear matrix and in particular, the fact that the newly synthesized DNA was attached to it [Berezney and Coffey, 1975] was met with enthusiasm since it provided the missing stage for the choreography of the replicating eukaryotic DNA. In the years that followed a rapid progress was made in our understanding of all aspects of DNA metabolism, which were invariably found connected in some way with the nuclear matrix. Thus the nuclear matrix was loaded with the conceptual task to organize and coordinate the common DNA processing activities in the otherwise compartmentalized nuclear environment.

On the other hand, contrary to the cases with the cytoskeleton and the extracellular matrices, which were found to play similar organizing roles, the material carrier of this organizing function was not studied in depth in the case of the nuclear matrix. For example soon after the cytoskeleton was discovered, the cytoskeleton major proteins were identified and isolated and their properties were studied and found consistent with their function in vivo. The major constituents of the internal nuclear matrix have not been identified so far and that gave rise to opinions that although a useful concept, the nuclear matrix does not exist in reality. Thus it has been hypothesized, that the information for the complex pattern of chromatin replication may reside entirely in its structure [reviewed in Pederson, 2000], and even that all nuclear structures were self organized by concentration dependent "crowding forces" through creation of phase boundaries [Hancock, 2004]. Although the "self assembly" could account for the formation of some of the structures such as assemblies of pre-RNA nucleoproteins to be spliced and exported from the nucleus, the concentration driven self assembly of the DNA replication machinery with its precise and dynamic stoichiometry and regulation seems much less probable.

In this review, we will try to present and discuss the experimental evidence in support of the role of the nuclear matrix as an organizing frame for the replication of DNA in higher eukaryotic cells.

NUCLEAR MATRIX

On the ground of electron microscopic observations in non-extracted eukaryotic cells, the nuclear matrix was first defined as the non-chromatin structures of the nucleus [Fawcett, 1966]. The nuclear matrix is also referred to as nucleoskeleton or scaffold. The last definition emphasizes the similarity between the DNA organization in interphase and in mitosis, when chromatin loops are anchored to a structure termed the chromosomal scaffold. Electron microscopy experiments showed that the nuclear matrix consists of ribonucleoprotein (RNP) particles and protein network. The removal of the nuclear envelope did not release the RNP particles from the nuclear interior, which showed that they were firmly bound to some internal structure. The digestion of RNA with RNase removed the granular component revealing the existence of extensive branched fibrilar network. This network was not degraded by DNase treatment, which showed that it represented a protein structure [reviewed in Nickerson, 2001]. In a pioneering work, Berezney and Coffey [1974] described a procedure for isolation of this structure by digesting nuclei with DNase I, followed by extraction with high salt to remove the histones and the fragmented DNA. Electron microscopic images showed that the remaining nuclear ghosts, which preserved the overall nuclear form and size, represented proteinacious network filling the nuclear interior and attached to the nuclear lamina [reviewed in Berezney et al., 1995]. Later many modifications were made in the original method for matrix isolation, using different DNA degrading enzymes and different ions and/or ionic concentrations to dissociate chromatin proteins. Methods avoiding hypertonic salt concentrations have also been developed [Jackson and Cook, 1988] and they revealed ultrastructural features similar to those seen in the traditional matrix preparations. Studies on the higher order chromatin organization confirmed the existence of the nuclear matrix. DNA is complexed with histones to form the 10 nm and the 30 nm chromatin fibers condensing the DNA length about 50-fold. There is strong evidence that the next level of higher order chromatin structure is the packaging of the 30 nm chromatin fibers into loops ranging in size between 50 and 200 kb in length [Vogelstein et al., 1980; Jackson et al., 1990]. Similar loop structure is evident at mitosis, when attachments are made to a proteinaceous chromosomal scaffold [Mirkovitch et al., 1988]. The loops were microscopically visualized after stripping DNA of the histones and it was shown that the loops remained anchored to the nuclear matrix. Autoradiography of in vivo pulse-labeled and chased

DNA showed that the newly synthesized DNA moved away from the loop bases to the periphery of the loops [Pardoll et al., 1980]. This suggested that DNA is replicated at the bases of the loops by DNA polymerase complexes attached to the nuclear matrix. Since the size of the loops is consistent with the estimated replicon size, it was suggested that each loop represents one replicon. Another strong evidence for the existence of the nuclear matrix is the discovery of replication foci that represent stable sub-chromosomal regions and occupy fixed positions in the cell nucleus. These foci stay in their original places even after removing most of the chromatin and soluble proteins from the nucleus (discussed below). Finally, chromosomes are arranged in defined and non-overlapping chromosomal territories in the nuclear volume during interphase and these territories are well preserved in the small fraction of DNA retained at the loop bases in nuclear matrix preparations [Ma et al., 1999]. The nuclear matrix is therefore involved both in the topological organization of DNA and in its functioning, which are mutually dependent.

The analysis of about 3,000 studies from the literature dealing with different aspects of the nuclear matrix showed that approximately 400 individual proteins could be identified as matrix proteins including enzymes, structural proteins, different transcription, RNA processing, repair and replication factors [reviewed in Mika and Rost, 2005]. About 300 of the proteins are sequenced partially or fully. Only 13 proteins were classified as part of the nuclear lamina and 42 as internal nuclear matrix proteins. The majority of the proteins (198) were classified as associated with the internal nuclear matrix and 130 as proteins, whose affinity to the internal nuclear matrix changes depending on the cell type or the stage of the cell cycle. Many proteins involved in DNA replication have been classified as matrix-attached: DNA polymerases, primases, PCNA, RPA, topoisomerases, etc. It has to be pointed out that individual cell lines could be distinguished based on their matrix protein composition and this has opened a way to use specific matrix proteins as markers for different types of cancer. So far, specific matrix protein patterns characteristic for prostate, bladder, renal, and column cancers have been identified that could be used as diagnostic tools. Another important trend in the nuclear matrix studies is the search for protein sequences responsible for nuclear matrix binding [Stein et al., 2003]. For 53 of the proteins in the database so far there is information for nuclear matrix targeting signals.

The nuclear matrix preparations also contain some DNA firmly attached to the protein network. Progressive digestion with DNase I decreases the size of the matrix attached DNA, but not below 200 bp, which was accepted as the approximate length of the DNA-matrix contact. These DNA fragments did not share extensive homology but are rather AT rich and have the ability to bind specifically to isolated matrix preparations. For this reason, they were designated as matrix attachment regions (MARs) or scaffold attachment regions (SARs) [Izaurralde et al., 1988]. It was shown that in chromosomal DNA there are more S/MARs, and that in the matrix, there were more S/MAR attachment sites than involved in the actual matrix-DNA attachment at any given moment [Cockerill and Garrard, 1986; Hakes and Berezney, 1991]. This ensures the dynamics of association and dissociation of different DNA regions to different matrix attachment sites during the cell cycle.

DNA REPLICATION

Replication Foci

A characteristic feature of DNA replication in higher eukaryotic cells is that it occurs at a few hundred discrete foci. The replication foci were first observed by Nakamura et al. [1986] when replicating DNA was pulse-labeled with bromodeoxyuridine and then stained with fluorescently labeled antibodies against the precursor. The results revealed that the label was not diffusely distributed in the nucleus, but formed well-defined fluorescent foci. A typical replication focus contains a cluster of several adjacent replicons that fire simultaneously in S phase and comprise on the average about 1 MB of DNA. Double pulse-labeling experiments have shown that DNA sequences that were replicated at given foci are replicated at the same foci and during the same period of S phase in the following generations. This indicates that chromosomes possess specific organization that is reproduced in the cell cycle. They are organized into stable units of replicon clusters, which are manifested as replication foci in S phase and as sub-chromosomal bands during mitosis [Jackson and Pombo, 1998; Ma et al., 1998; Sadoni et al., 2004]. In living cells replication foci do not

change their position in the nucleus and after replication of the respective replicons is completed, they fade and disappear while new foci appear at the next sites [Sadoni et al., 2004]. The stable localization of the replication foci in the nucleus rises the question what holds them at their respective positions. There are many lines of evidence that point to the possibility that replication foci are attached to the nuclear matrix. Thus, replication foci exhibit specific patterns during the progression of S phase and it was shown that after digestion of most of DNA, the residual structures preserved their S phase specific pattern observed in vivo [Nakayasu and Berezney, 1989; Hozak et al., 1994; Ma et al., 1998]. On the ground of that observation, it was suggested that the individual foci are formed by the aggregation of several 50–200 kb DNA loops attached to the nuclear matrix [Berezney et al., 2000]. Co-localization of DNA polymerase (and the replication protein PCNA with replication foci after exhaustive DNase digestion, demonstrated that the replication machinery is also associated with the nuclear matrix [Hozak et al., 1993]. When DNA is digested and extracted under mild conditions, the residual structures retained their functional activity. They were found capable of synthesizing DNA without addition of protein extracts and the replication foci visualized by labeling in vitro at the matrix were indistinguishable from those visualized in intact cells by in vivo labeling [Nakayasu and Berezney, 1989; Hozak et al., 1993; Hozak et al., 1994; Djeliova et al., 2001a; Radichev et al., 2005]. These data suggest that the replication machinery that would handle several replication forks simultaneously is assembled at the nuclear matrix. The replication foci were directly visualized by high-resolution electron microscopy. Human cells at different phases of the cell cycle were permeabilized and incubated with precursors of DNA synthesis and the distribution of the labeled DNA was followed in thick resinless sections from which approximately 90% of the chromatin had been removed. The results revealed morphologically discrete dense structures attached to a nucleoskeleton. Their number and pattern during S phase were similar to the replication foci observed by light microscopy [Hozak et al., 1993, 1994].

Initiation

The replication of DNA has three stages—initiation, elongation, and termination. The initiation step begins at the so-called origins of replication, which are genetically, or epigenetically determined and the timing of their firing is precisely controlled. A typical eukaryotic cell has about 10^4 origins more or less evenly distributed along the chromosomal DNA. DNA segments replicated from a single origin are called replicons. The average length of the eukaryotic replicons is 50–200 kbp and taking into account that the replication forks move at a rate of about 1–2 kb/min, an average replicon is replicated for about 2 h. The two replication forks formed at the site of initiation travel in opposite directions until they reach the ends of the replicon where they are dismantled and replication terminated.

The events at the replication origins preceding the beginning of DNA synthesis are collectively called initiation. The process of initiation is best understood in Saccharomyces cerivisiae, but the initiation factors are conserved from yeasts to humans and the sequence of events at the initiation complexes are similar among eukaryotic cells. Initiation begins with binding of the six-subunit origin recognition complex (ORC) to the origins of replication. In G_1, the chromatin bound ORC recruits the initiation factors Cdc6 and Cdtl that are required to load the hexameric MCM 2-7 complex to form the pre-replication complex (pre-RC). MCM 2-7 is considered the helicase, responsible for unwinding of the parental DNA strands. As cells progress from G_1 to S phase, the pre-RC is activated by the S phase promoting kinases Cdk2 and Cdc7 leading to association of Cdc45 with MCM. Upon the formation of the MCM-Cdc45 complex, the duplex DNA is unwound and various replication proteins, including DNA polymerases, are recruited onto the unwound DNA [reviewed in Bell and Dutta, 2002]. In S. cerevisiae the replication origins are genetically determined, that is, they represent definite DNA sequences (replicators) able to ensure autonomous replication of the plasmids that contain them. Actually, this property of the yeast origins was first used to isolate them by cloning randomly fragmented yeast DNA into plasmids without replication origin and screening for plasmids that have acquired the ability for autonomous replication in yeast cells. Several such sequences have been isolated from S. cerevisiae and designated ARS (autonomous replication sequence) elements. The minimal replicator in S. cerevisiae is a 100–150 bp region

that includes three to four AT-rich 10–12 bp sequence elements that are required for origin function, one of which is conserved between different origins and referred to as ARS consensus sequence [reviewed in Bell and Dutta, 2002]. This approach has been applied to isolate origins from mammalian cells as well, but despite some reports of successful cloning of mammalian ARS, it was generally not very successful [Krysan et al., 1993]. Despite tremendous efforts only about 20 origins of bidirectional replication have been localized at specific sites in mammalian genomes by different methods mapping the sites of initiation of nascent DNA chains and only for four of them genetic evidence that they serve as replicators when placed at ectopic chromosomal locations have been provided. No extensive homology has been reported between the mammalian origins of replication, but they share some common functional elements [Aladjem and Fanning, 2004]. They are AT-rich sequences, contain transcription factors binding sites and the so called DNA unwinding elements (DUE), which due to the specific primary structure or/and torsional strain melt more easily than the surrounding DNA. Other characteristic elements that are found in the vicinity of origins are S/MARs. The importance of the S/MARs for initiation is underlined in the following experiment. In an attempt to define the minimal requirements for a plasmid vector to replicate extrachromosonally in mammalian cells, it has been demonstrated that S/MAR modules linked to an upstream active transcription unit are sufficient for episomal replication and mitotic stability of the vector, which associates with the nuclear matrix in vivo by means of specific interaction with the nuclear matrix protein SAF-A [Jenke et al., 2004].

Mammalian cells contain 10^4–10^5 molecules of ORC per cell, suggesting that they would be bound to DNA once per 60–600 kb, which is consistent with the average size of replicons in mammalian cells [reviewed in DePamphilis, 2003]. Thus, it could be expected that most of the ORCs would be bound to replication origins. For three human origins of replication it has been shown that they contain in vivo binding sites for ORC proteins, but there are data that imply that specific DNA sequences are not always required for ORC binding. Thus, when the in vivo established ORC-binding region in the upstream promoter region of the *MCM4* gene was inserted in the extrachromosomally replicating plasmid pEPI-1, it was shown that ORC and MCM proteins were bound over the entire plasmid circle, without a preference for the inserted origin sequence [Schaarschmidt et al., 2004]. Furthermore, in experiments for in vitro binding of purified human ORC it was shown that except for preferential binding to AT-rich polydeoxynucleotides, ORC did not discriminate between natural origin containing, ORC-binding in vivo DNA sequences [the human lamin B2 origin, Abdurashidova et al., 2003] and control sequences [Vashee et al., 2003]. ORC binds specifically to a well defined *Drosophila* origin of replication in vivo, but in vitro binding experiments have shown that the highest DNA-binding affinity of *Drosophila* ORC is towards negatively supercoiled DNA and not to specific DNA sequences [Remus et al., 2004]. Thus, it seems that the intrinsic DNA-binding activity of ORC may not be sufficient to target ORC to specific origins of replication.

On the ground of these data, the emerging concept about metazoan origins of replication is that they are not exclusively genetically determined by their primary DNA sequence, and that their function depends on epigenetic factors, such as chromatin structure and nuclear localization. There are three lines of evidence that nuclear matrix attachment step is necessary for the initiation events. First, origins of replication were found attached to the nuclear matrix prior to their firing, second, crucial initiation factors were found attached to the nuclear matrix and third, it was possible to initiate replication on isolated matrix preparations in which most of the chromatin has been removed.

The reports that the newly synthesized DNA is attached to the nuclear matrix, which presumes that the synthesis is taking place there, led to the conclusion that at the time of initiation of DNA synthesis, replication origins should be attached to the matrix. This raised the question whether origins are permanently attached to the nuclear matrix or the attachment is dynamic. This point was checked out by isolation of matrix-attached and loop DNA and their probing for the abundance for origin sequences. By using a collective origin fraction consisting of short DNA fragments originating from the regions of initiation of DNA synthesis throughout the genome and one specific origin of replication (ori-β from the dihydrofolate reductase domain in Chinese hamster cells), it

was found that replication origins in exponentially growing cells were randomly distributed between matrix-attached and loop DNA [Djeliova et al., 2001b]. These results overthrow the possibility for permanent attachment of the origins of replication to the nuclear matrix and indicate that the attachment is dynamic. To follow the dynamics of association of the origins to the nuclear matrix during the cell cycle, the abundance of sequences from ori-β and the human β-globin replicator was determined in the matrix-attached DNA isolated from cells synchronized at different stages of G_1 and S phase. The matrix-attached DNA isolated from cells in late G_1 phase was enriched in origin sequences in comparison with the matrix-attached DNA from early G_1 phase cells. The concentration of sequences from the early firing ori-β in DNA attached to the matrix decreased in early S phase, while the late firing β-globin origin remained attached to the matrix until late S phase. A conclusion was drawn that replication origins associate with the nuclear matrix in late G_1 phase and dissociate after initiation of DNA replication has been completed in S phase [Djeliova et al., 2001a]. These results suggest that association of the origin sequences to the nuclear matrix in late G_1 phase may play a role in the establishment of initiation competent state of the origins. A question arises how the changes in the association with the nuclear matrix occur. A number of reports have shown that chromatin moves locally as well as on a large scale during G_1. The large-scale movements in early G_1 are connected with the repositioning of chromosomal territories [Zink and Cremer, 1998]. It can be speculated that because of the small-scale refolding of subchromosomal regions mediated by protein-DNA and/or protein–protein interactions, the origins of replication can associate and dissociate with the nuclear matrix.

The second line of evidence that the nuclear matrix plays a role in the process of DNA initiation comes from the finding that crucial proteins of the pre-replication complex were found attached to the nuclear matrix. In mammals, both the cellular concentration of the ORC 2–5 proteins and the amount of each protein bound to chromatin appear constant throughout the cell cycle. On the other hand, the level of the largest subunit ORC 1 of the human ORC oscillates during the cell cycle, a phenomenon termed "the ORC cycle" [reviewed in DePamphilis, 2003]. ORC 1 starts to accumulate in middle G_1 phase, reaches a peak at the G_1/S boundary when it is found associated with nuclease-insoluble, non-chromatin nuclear structures, and decreases to a basal level in S phase. The appearance of nuclease-insoluble ORC 2–5 parallels the increase in the level of ORC 1. Thus, it appears that ORC 2–5 are temporally recruited and tethered to the nuclear matrix by formation of the ORC 1–5 complex. An artificial reduction in the level of ORC 1 in human cells by RNA interference resulted in a shift of ORC 2 to the nuclease-soluble fraction, and the association of MCM proteins with chromatin fractions was blocked by this treatment [Ohta et al., 2003; Tatsumi et al., 2003]. These results indicate that ORC 1 regulates the status of the ORC complex in human nuclei by tethering ORCs to the nuclear matrix. Due to the coincidence of the association of the origins of replication and the binding of the ORC subunits with the nuclear matrix in late G_1, it was suggested that ORC 1 might determine the binding of the potential origin sequences associated with ORC 2–5 to the nuclear matrix (Fig. 1). As the origins of replication and ORC 1 dissociate from the matrix after initiation, this mechanism may be involved in the restriction of re-initiation at the same origin during a given cell cycle [Ohta et al., 2003].

To address further the nuclear matrix support of initiation of DNA replication a cell-free replication system was developed, in which the nuclear matrix along with the residual matrix-attached chromatin was used as a substrate for DNA replication [Radichev et al., 2005]. Biochemical and immunofluorescent microscopy analyses showed that the chromatin fraction attached to the nuclear matrix was capable of initiation of DNA replication under cell-cycle control and in a sequence-specific manner. To prove that the observed initiation takes place at legitimate DNA replication origins, the in vitro synthesized nascent DNA strands were isolated and analyzed. It was shown that they were enriched in sequences from the core origin region of the early firing, dihydrofolate reductase origin of replication ori-β and not in distal to the origin sequences. The replication foci exhibit specific patterns during the different stages of S phase. Early S phase patterns are characterized by foci dispersed throughout the nucleus, middle S phase patterns are characterized by foci predominantly located at the

periphery of the nucleus and internal areas, and late S phase patterns are characterized by a few large foci [Nakayasu and Berezney, 1989]. According to these criteria DNA synthesis on the chromatin fraction isolated from exponentially growing cells where S phase cells were continuing elongation of DNA in vitro, displayed patterns of replication foci typical for all stages of S phase. In contrast, the replication foci visualized after in vitro initiation at the residual chromatin structures derived from late G_1 cells, displayed predominantly a pattern typical for early S phase. These results indicated that initiation of DNA replication in the in vitro system might follow the temporal pattern of origin usage in vivo, and second, that once established and anchored to the nuclear matrix the pre-replication foci exhibit structural and functional independence from the rest of chromatin [Radichev et al., 2005].

The further elaboration of cell-free systems for initiation of DNA replication will open new possibilities for studies of the assembly of mammalian pre-replication complexes at the nuclear matrix. They can be manipulated by introducing chromatin preparations from which specific pre-replication proteins have been dissociated to allow evaluation of the role of individual pre-replication proteins and their attachment to the nuclear matrix in the process of initiation of DNA replication.

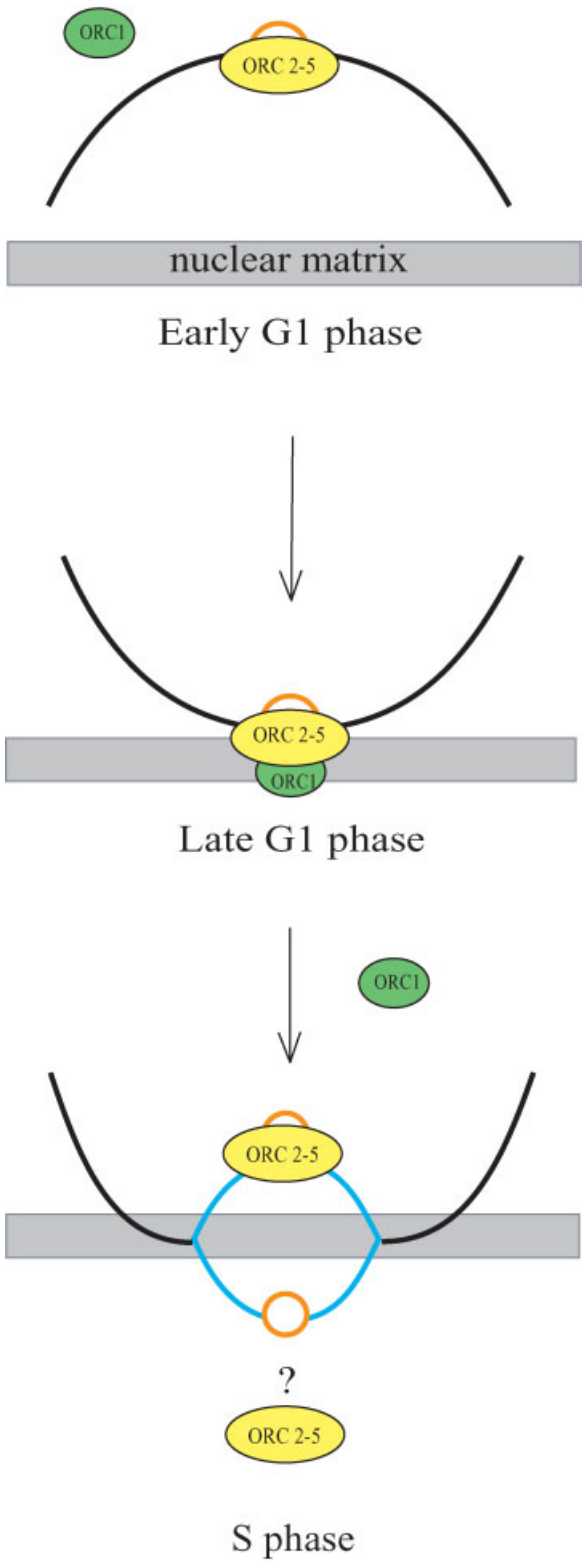

Fig. 1. Cell cycle-dependent assembly of the human ORC 1–5 and its association with the nuclear matrix. ORC 2–5 (yellow) is stably bound to DNA origin sequence (orange circle) throughout the cell cycle. At late G_1 phase ORC 1–5 is formed by association of ORC 1 (green) to ORC 2–5 and the complex is tethered to the nuclear matrix (grey). The dissociation of ORC 1 after the initiation of DNA replication in S phase returns the ORC 2–5 complex to its early G_1 state and prevents reinitiating at the same origin. It is not clear neither to which one of the two replication origin sequences formed after the replication ORC 2–5 remains attached, nor when another ORC 2–5 complex is attached to the second replication origin.

Elongation

After the initiation step, during which the replication forks are established, DNA is replicated bidirectionally outward from the respective replication origins. Most of the biochemistry of this process is well known and here we will dwell on some topological aspects in relation with the nuclear matrix. The fact that the individual replicons do not replicate individually, but in synchronously regulated clusters of adjacent replicons presents extremely complex spatial problem and it is difficult to envisage the choreography of the replicating DNA even from a single replication origin let

alone the coordinated and simultaneous replication of clusters of replicons. The findings that in the course of DNA replication replicating DNA is always associated with the nuclear matrix, while the replicated DNA is moved away, led to the revolutionary new concept about the mechanics of DNA replication. Until then it was accepted that DNA remains static in the course of replication, while the two replication forks travel in opposite directions, gradually moving apart from each other. Now it becomes clear that the replication forks remain stationary during the process, while the replicating DNA is reeled through them [Cook, 1999]. Support for this concept comes from high resolution electron microscopy studies following both the movement of DNA during replication and the localization of replication proteins during this movement. They showed that the replication machinery is fixed to the nuclear matrix and that during replication DNA is spooled through the replication machinery [Hozak et al., 1993, 1994]. Recently the process of replication was directly observed in living cells. Visualization of the replicating DNA and the replication machinery simultaneously have shown that the newly synthesized DNA performs some local rearrangements but is not redistributed to other nuclear sites, which is consistent with spooling of DNA of replicon size through fixed replication factories [Sadoni et al., 2004].

Several authors have suggested hypothetical mechanisms by which the DNA segments with fixed ends can be replicated, but not all the details are fully worked out. There are data in the literature showing that replication origins may be permanently attached to the nuclear matrix [Lagarkova et al., 1998]. Accordingly, models have been proposed to explain how a DNA loop anchored at a nuclear structure could replicate by reeling through the anchoring site and the region where replication had begun to remain attached to the same site [Dingman, 1974; Cook, 1991]. However, in imaging DNA replication on the nuclear matrix, one has to keep in mind that there are more sequences in chromosomal DNA that can be attached to the matrix and more matrix sites than the actually engaged in matrix-DNA association at any given time. Furthermore, S/MAR sequences have been identified in the vicinity of all replication origins from higher eukaryotes analyzed so far. This, and the results showing that origins are attached to the nuclear matrix during late G_1 phase and are released after firing in S phase, are in favor of a model in which the association of the origins with the matrix is temporary and dynamic [Cook, 1999]. According to this model, the origins are localized in the periphery of the DNA loops and before initiation of replication they become attached to the nuclear matrix. Upon this attachment, the original loop would be transformed into two new loops, each containing the DNA from one end of the replicon to the origin. With the progress of replication, two more loops of daughter DNA will grow like "rabbit ears" from the site of replication, giving rise to a configuration of four loops emanating from a single point (Fig. 2).

There are also data that the newly synthesized DNA is not leaving the nuclear matrix immediately after its synthesis. It has been shown repeatedly that the newly synthesized DNA is hypersensitive to digestion with micrococcal nuclease. This hypersensitivity is transient and after between 10 and 20 min during which time the newly synthesized DNA has moved some 10–20 kb away from the site of synthesis the hypersensitivity is lost. At the same time, it has been reported that although more susceptible to nuclease digestion, the newly synthesized chromatin was nevertheless resistant to extractions and release from the nucleus [Pospelov et al., 1982]. Taken together, these two groups of data show that the newly synthesized DNA remains attached to the nuclear matrix for some time after its synthesis during which it is complexed with histones and nonhistone proteins to obtain the structure of the mature chromatin. During this period of reestablishing of the chromatin structure the epigenetic labeling of the sites of initiation of replication could occur. Since in mammals the amount of ORC 2–5 proteins bound to chromatin appears constant throughout the cell cycle and they are supposed not to dissociate from the parental strand during replication, it can be suggested that they are good candidates to tag the origins in the daughter strands. Some support for this speculation comes from the observation of nuclease sensitive sites in origins of replication found in the fraction of chromatin bound to the nuclear matrix [Djeliova et al., 2002].

Termination

The termination of DNA replication also poses several problems, some of them connected with

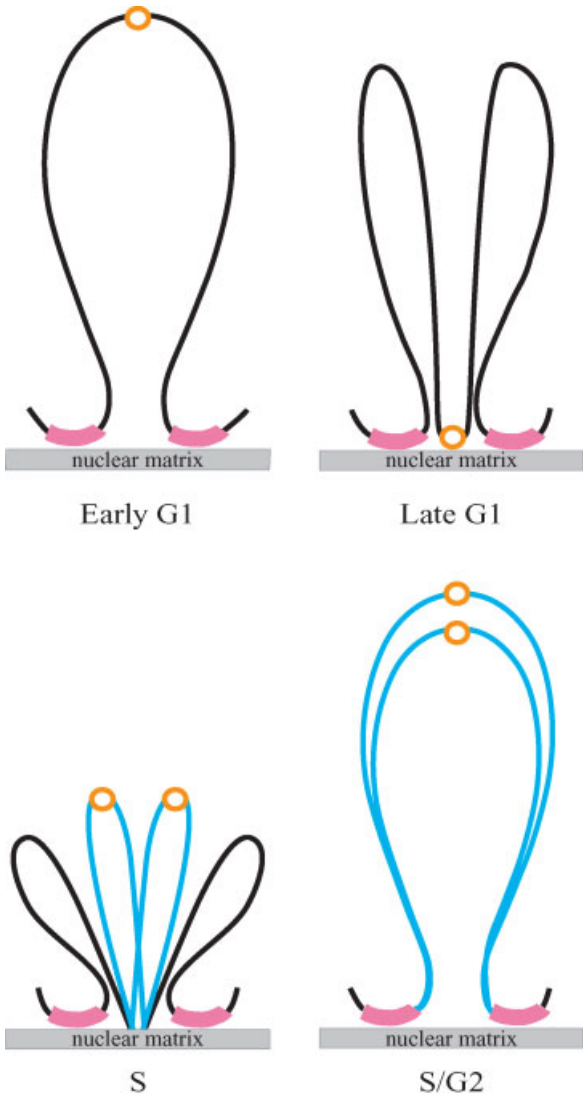

Fig. 2. A model for DNA replication. Parental DNA of replicon size (black line) forms a loop attached to the nuclear matrix (grey) by two MARs (magenta). The origin of DNA replication (orange circle) is localized at the top of the loop in early G_1 phase. At late G_1 phase, the origin is brought to the nuclear matrix where the replication machinery is assembled. As DNA is reeled through the matrix attached replication machinery, two loops of newly synthesized DNA (blue) are formed and grow outward from the matrix attachment site. When replication is completed at the end of the S phase, the replication machinery is dismantled and the pre-replication loop organization of DNA restored.

the attachment of DNA to the nuclear matrix. Stop codons for the replication (termination sequences) have been identified in viruses and yeast [Fields-Berry and DePamphilis, 1989]. They promote the formation of catenated intertwines when two converging replication forks enter to complete replication. It seems that there are no such termination sequences in the higher eukaryotes. This leaves the cells with two possibilities to terminate successfully replication: either the ends of replicons are epigenetically marked, or the replication stops when two replication forks from neighboring replicons moving in opposite directions collide with each other. The second possibility seems less plausible since the last few nucleotides between the colliding replication forks will remain unreplicated and the DNA loops unresolved. It is more probable that the ends of the replicons are properly earmarked and that provisions are made to replicate the last DNA sequences at the border of any two replicons and to resolve the newly generated DNA loops. There are indirect data that this task is performed by the nuclear matrix where the ends of the replicons are anchored to the nuclear matrix by S/MARs. The benefits of this organization are twofold. First, S/MARs like origins of replication are AT rich and have low melting energy, which would permit their easy opening to complete replication. Second, topoisomerase II, which is essential for the resolving the replicated DNA is intrinsic component of the nuclear matrix and is found at the loop bases [Iarovaia et al., 2004]. Thus, it seems that S/MAR sequences when in proper spatial and functional context, that is, at the ends of the replicons and attached to the matrix, could play the role of termination sequences in higher eukaryotic cells.

CONCLUSIONS

The nuclear matrix represents a proteinacious network attached to the nuclear lamina, to which in addition to the nascent RNP particles that are being spliced and transported from the nucleus, the DNA sequences that have been replicated, transcribed, repaired, or recombined at the moment of isolation, are attached [reviewed in Berezney et al., 1995]. There is ample experimental evidence that the nuclear matrix plays an important role in the process of DNA replication both by providing a structural support and key protein factors. Nevertheless, periodically its role and its very existence are challenged. A serious reason for concern connected with the proposed nuclear matrix support of DNA replication mechanisms is that they are based mainly on circumstantial and correlative evidence. Indeed, there are still no direct experiments demonstrating either the attachment of origin sequences to specific matrix

components, or the association of the replication factories to specific matrix proteins, nor the path of the replicating DNA through the replication factories. Our belief is that these gaps in our knowledge are result of inadequate experimental techniques rather than of erroneous hypothesis. The development of new, more powerful, biochemical techniques combined with different genetic approaches to study the matrix proteins and their properties would reveal their functions and significance. On the other hand the development of more sophisticated cell-free systems combined with modern "single-molecule techniques" able to work in the range between the light and electron microscopy, opens the possibility to observe the replication of single DNA molecules. There is no doubt that these developments will soon solve the remaining uncertainties about the DNA replication on the nuclear matrix.

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