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The Organisation of Replisomes

Anna Ligasová and Karel Koberna

*Institute of Experimental Medicine, Academy of Sciences of the Czech Republic
Czech Republic*

1. Introduction

The eukaryotic chromosomal DNA is divided into hundreds to thousands of independent replication segments called replicons. Each replicon is replicated from one replication origin. In the S phase of the cell cycle, individual replicons are gradually activated and subsequently replicated (Edenberg & Huberman, 1975; Hand, 1978). The size of particular replicons varies and is usually within the range of 30–450 kbp. On the other hand, much smaller (shorter than 10 kbp) or much longer (longer than 1 Mbp) replicons have also been observed (Berezney et al., 2000; Edenberg & Huberman, 1975; Hand, 1978; Hyrien & Mechali, 1993; Jackson & Pombo, 1998; Yurov & Liapunova, 1977). It is supposed that several adjacent replicons are synchronously activated in the S phase (Edenberg & Huberman, 1975; Hand, 1978), whereas the number of replicons in one such group is lower than ten (Jackson & Pombo, 1998; Ma et al., 1998). The evidence of such replicon organisation comes mainly from studies mapping the newly-synthesised DNA on stretched DNA fibres (Edenberg & Huberman, 1975; Hand, 1978; Jackson & Pombo, 1998).

The replication of replicons proceeds bi-directionally by means of two replication forks and is terminated when the replication forks of two adjacent replicons meet (Blow & Dutta, 2005; Heintz, 1996). The so-called “licensing” of replication origins is performed before the actual initiation of DNA synthesis. First, many different proteins such as the ORC complex, Cdc6 protein, Cdt1 protein, MCM 2-7 protein complex bind in that exact order at the sites of replication origins (Bell & Dutta, 2002; Blow & Dutta, 2005; DePamphilis, 2003; Diffley, 2004; Chesnokov, 2007; Lei & Tye, 2001; Sasaki & Gilbert, 2007; Stillman, 2005; Takahashi et al., 2005). Later, due to the regulation mechanisms, some of the proteins are removed and other new proteins are bound to DNA instead of them. Examples include the Cdc45 protein, MCM10 protein or GINS protein complex (Bauerschmidt et al., 2007; Diffley & Labib, 2002). Cyclin-dependent kinases and Dbf4-dependent kinase are important for the changes in the protein-DNA interactions (Bauerschmidt et al., 2007; Diffley & Labib, 2002). All of these processes result in the formation of two replication complexes, also referred to as replisomes, at the site of the active replication origin that ensure the synthesis of DNA in mutually opposite directions (Baker & Bell, 1998; Johnson & O'Donnell, 2005; Waga & Stillman, 1998). The main components of replisomes are a helicase complex enabling the unwinding of the parental DNA strands, DNA polymerases responsible for the duplication of DNA, and a complex of polymerase and primase (Langston et al., 2009). It is supposed that the MCM2-7 protein complex, which is necessary for the “licensing” of replication origins, plays also the role of a helicase in the common complex with Cdc45 protein and GINS complex (Aparicio et al., 2006).

On the cellular level, the individual active replicons or groups of simultaneously replicated replicons were localised to the discrete domains (Dimitrova & Gilbert, 1999; Fox et al., 1991; Hozak et al., 1993; Leonhardt et al., 2000; Ma et al., 1998; Nakamura et al., 1986; Nakayasu & Berezney, 1989; O'Keefe et al., 1992). At the light microscopy level (LM), these domains are referred to as replication foci. In the case of electron microscopy (EM) localisation, these domains are commonly called replication factories. Presently, the term replication factory is used also for the description of the complex of replication proteins and is frequently substituted by the term replisome. Alternatively, replication factory can designate a complex where besides replication proteins other proteins such as proteins for DNA recombination and DNA repair are present (Migocki et al., 2004). It is evident that the number, size and localisation of the replication foci are changed during the S phase whereas several different replication patterns have been described by various groups. Some of them distinguish between three basic replication patterns (Jackson, 1995; Manders et al., 1992; Nakayasu & Berezney, 1989), others describe five replication patterns (Dimitrova & Gilbert, 1999; O'Keefe et al., 1992; van Dierendonck et al., 1989). Basically, at the onset of the S phase, small replication foci scattered throughout the nucleoplasm except the nucleoli are observed. In the middle part of the S phase, the foci are less numerous; on the other hand, they are larger and localised mainly in the perinucleolar and perinuclear parts of the cell nucleus. At the end of the S phase, heterochromatin is replicated. In this part of the S phase, replication typically proceeds via large and not very numerous foci. The number and size of replication foci was measured by means of several techniques of light microscopy (Ma et al., 1998; Nakayasu & Berezney, 1989; Tomilin et al., 1995). The use of various techniques contributed to the high variability in the obtained numbers and sizes of replication foci in the early replicated cells (0.1–0.5 μm ; 120–1500; Jackson, 1995; Ma et al., 1998; Mills et al., 1989; Nakamura et al., 1986; Nakayasu & Berezney, 1989; Tomilin et al., 1995).

Replisome complexes are of course assembled not only in eukaryotic cells but also in prokaryotic cells. In both of the groups of organisms, there are two basic views of the organisation of sister replisomes during replication. According to the first one, the sister replisomes move independently in opposite directions along the DNA (Bates & Kleckner, 2005; Berkmen & Grossman, 2006; Hiraga et al., 2000; Kongsuwan et al., 2002; Reyes-Lamothe et al., 2008; Yardimci et al., 2010). On the contrary, the second view supposes that the sister replisomes are tightly associated during replication (Dingman, 1974; Falaschi, 2000; Jensen et al., 2001; Kitamura et al., 2006; Lau et al., 2003; Lemon & Grossman, 2000; Ligasová et al., 2009; Migocki et al., 2004; Pardoll et al., 1980; Wessel et al., 1992).

In the chapter, a procedure enabling the distinction between the above-mentioned models of replisome organisation in human HeLa cells is described. This procedure can be used universally for other eukaryotic systems. The method is based on the pulse-chase labelling of the short segments of DNA and their localisation by means of the pre-embedding approach followed by electron tomography. Presently, the pre-embedding approach is the only method that allows the localisation of labelled DNA in the sections and provides 3D information at sufficient resolution by means of the EM tomography. The EM tomography approach is based on the stepwise tilting of the section in the electron beam followed by the mathematical analysis of the obtained data. This method provides high resolution of structures (5–10 nm) in three dimensions as the plastic sections are cut enough (200–1,000 nm) to contain the sufficient amount of information. In the case of serial sections, the resolution of the third dimension (the depth of the section) cannot be more than twice the thickness of the section (McEwen & Marko, 2001). The thickness of the common EM sections is about 70 nm, although it is possible

to prepare sections with approximately 10-nm thickness (McEwen & Marko, 2001). However, the resolution is still 20 nm as opposed to 5-10 nm for EM tomography. Moreover, an obligatory problem is the ordering of the serial section by processing the data.

The whole experiment is illustrated in Figure 1. From the scheme, it is apparent that the most significant difference between both models is a change in the number of the labelled domains after the different lengths of incubation: in the case of independent replisomes, the number of the labelled domains in mitosis is at most doubled; in the tightly associated replisomes, this number is almost quadrupled (Figure 1).

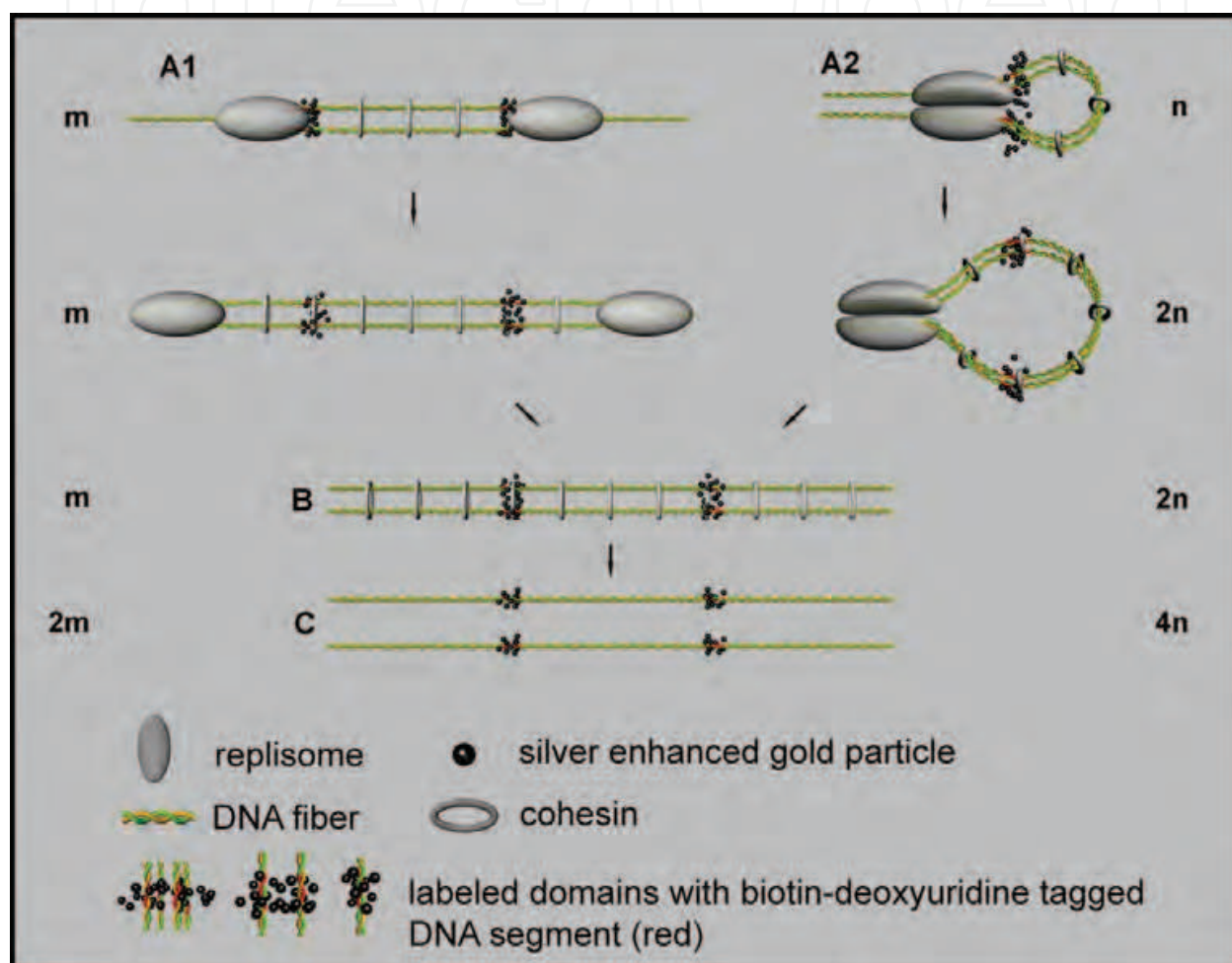


Fig. 1. The explanatory scheme depicting two models of the arrangement of “sister” replisomes in HeLa cells and the effect of different organisations of the biotin-16-2'-deoxy-uridine-tagged segments on the number of labelled domains during various pulse-chase experiments.

The scheme shows the expected results of the consecutive mapping (indicated by arrows) of the segments tagged during a short pulse of biotin-16-2'-deoxy-uridine-5'-triphosphate (biotin-dUTP) in the early S phase followed by the chase period of a different length from the time immediately after the pulse (the upper part of the scheme) to the complete mitotic segregation of the sister chromatids (the lower part of the scheme). Note that some clusters of silver-enhanced gold particles belonging to the mutually close segments can “fuse”. Therefore, the domain labelled by silver-enhanced gold particles, used as markers in the present study, can contain between one and four segments, depending on the model and the

length of the chase. This “fusion” is a result of the “large” size of the antibody complex with the silver-enhanced gold particle as against the distance between the segments. The expected number of domains for the individual stages of replicon organisation is shown as a multiple of the initial number of domains. The initial number is designated by **m** for the model of replisome singles and by **n** for the model of replisome couples. Note that the number of labelled domains is doubled in the model of replisome singles (A1) and quadrupled in the model of replisome couples (A2) in mitosis. In fact, the increase in the model of replisome couples is lower as the labelled segments of replicons early after initiation cannot contribute to this increase (see below). Several simplifications have been used in the model such as chromatin being shown as a DNA double helix in all the models, although the DNA in chromatin is more condensed. In addition, the partial segregation of chromatids is not taken into account in the model before mitosis.

(A1) A model of replisome singles. “Sister” replisomes move in opposite directions during replication. The two tagged segments of the sister chromatids are close to each other both during and after replication because of the cohesion of the sister chromatids mediated by a cohesin complex. Each labelled domain contains one pair of “sister” segments. The number of the labelled domains remains unaltered during this process.

(A2) A model of replisome couples. “Sister” replisomes form a closely associated complex, resulting in the formation of a DNA loop. The four tagged segments are in close proximity at the time of their replication and are visualised as one labelled domain. Later, the loop inflates, as a consequence of which the distance between both “sister” pairs of the tagged segments of chromatids is gradually prolonged and the number of labelled domains increases. Each labelled domain contains only one pair of segments at this point.

(B) Two sister chromatids bound together by cohesin complexes after the termination of replicon synthesis and dissociation of replisomes are shown. No difference in the organisation of the tagged segments is visible in the case of the model of replisome singles. The number of the labelled domains is also the same when compared with the ongoing replicon replication shown in A1. On the other hand, the relaxation of the loops shown in the model of replisome couples (A2) resulted in an increase in the distances between the pairs of tagged chromatin segments, which facilitates the recognition of previously less distant “sister” pairs. Consequently, the number of the labelled domains is nearly doubled with respect to the number of domains found immediately after the biotin-dUTP labelling pulse. The increase is lower as labelled segments of replicons which began DNA synthesis during the pulse are not separated by non-labelled DNA strand.

(C) In mitosis, sister chromatid cohesion is broken and the pairs of the tagged segments separate. Mitotic segregation results in the twofold increase of labelled domains with respect to (B). Each individual domain contains only one biotin-16-2'-deoxy-uridine-tagged (biotin-dU) chromatin segment. (From Ligasová, et al., 2009).

2. EM tomography analysis of the organisation of replisomes in human HeLa cells

2.1 Material and methods

2.1.1 Cell culture and synchronisation

Human HeLa cells were incubated in cell culture flasks or on coverslips in Dulbecco's modified Eagle's medium with L-glutamine supplemented with 10% fetal calf serum 1% gentamicin and 0.85 g/l NaHCO₃ at 37 °C in a humidified atmosphere containing 5% CO₂.

In the most of experiments, the cells were synchronised at the G1/S border by means of the double block with 2'-deoxythymidine (dT; Koberna et al., 2005). After the release from the block, the cells were labelled with biotin-dUTP (Koberna et al., 1999; Ligasová et al., 2009). In short, the cells were rinsed with the hypotonic buffer (30 mM KCl, 10 mM Hepes, pH 7.4) and subsequently incubated in the hypotonic buffer supplemented with 0.2 mM biotin-dUTP for 10 minutes. Next, the cells were incubated in culture medium for 10 minutes unless otherwise stated.

For the analysis of mitotic chromosomes, the cells were first synchronised by means of a double block with dT. Then, the cells were incubated for 100 minutes in fresh medium. After the 100-minute incubation, biotin-dUTP was introduced into the cells by means of hypotonic delivery. The cells were subsequently incubated for 9 hours in fresh medium and then the medium was changed for a medium supplemented with nocodazol (0.04 µg/ml, 5 hours; Zieve et al., 1980).

2.1.2 Antibodies

The rabbit anti-biotin primary antibody (Enzo Biochem Inc.) and secondary antibody conjugated with 1nm gold particles (Aurion) were used for the detection of incorporated biotin-dU.

2.1.3 Electron tomography and the evaluation of the tomograms

The ultrastructural localisation of the biotin-tagged DNA was performed using the synchronised cells by means of the pre-embedding approach (Koberna et al., 2005). Briefly, the cells were fixed by 2% formaldehyde and subsequently permeabilised by 0.2% Triton X-100. After the incubation with primary and secondary antibodies and silver intensification of the ultra-small gold following Danscher (Danscher, 1981), the samples were dehydrated and embedded in Epon resin. After the polymerisation, ultra-thin sections (of 70 and 200 nm) were cut on a Leica UltraCut S microtome with a diamond knife and then contrasted in 3% uranyl acetate. The 70-nm-thick sections were cut as a ribbon of three and more adjacent sections. The sections were analysed by means of a Morgagni 268 transmission electron microscope equipped with a Megaview II camera (a resolution: 1280 × 1024 pixels, a magnification: 14,000×). The mutual position of the neighbouring sections was adjusted by means of the Adobe Photoshop software. The electron tomography analysis of the 200-nm sections was performed by means of a Tecnai G2 Sphera electron tomography microscope equipped with a Gatan Ultrascan camera 894 US1000 (resolution: 2048 × 2048 pixels, magnification: 5000×) at 200 kV. The picture series were scanned within the range of angles -64° to +64° with the increment of 2°. The scanned picture series were reconstructed in IMOD software (Kremer et al., 1996). The final 3D models were created in Amira software (Figure 2).

To minimise the possible inaccuracies at the edges of the tomograms, every side of the original tomogram was reduced by 10–20 nm. The 300–500 labelled domains in the 3D model were measured in the case of the evaluation of the size of labelled domains. In this evaluation, we have excluded those domains found at the borders of the tomogram. The length of the labelled domains was measured as the longest distance between the outer margins of silver-enhanced gold particles. In the case of the analysis of the number of labelled domains, we did not evaluate the domains crossing the left, bottom and front sides of the model. In both analyses, we have analysed 100 sections from more than fifty different cells.

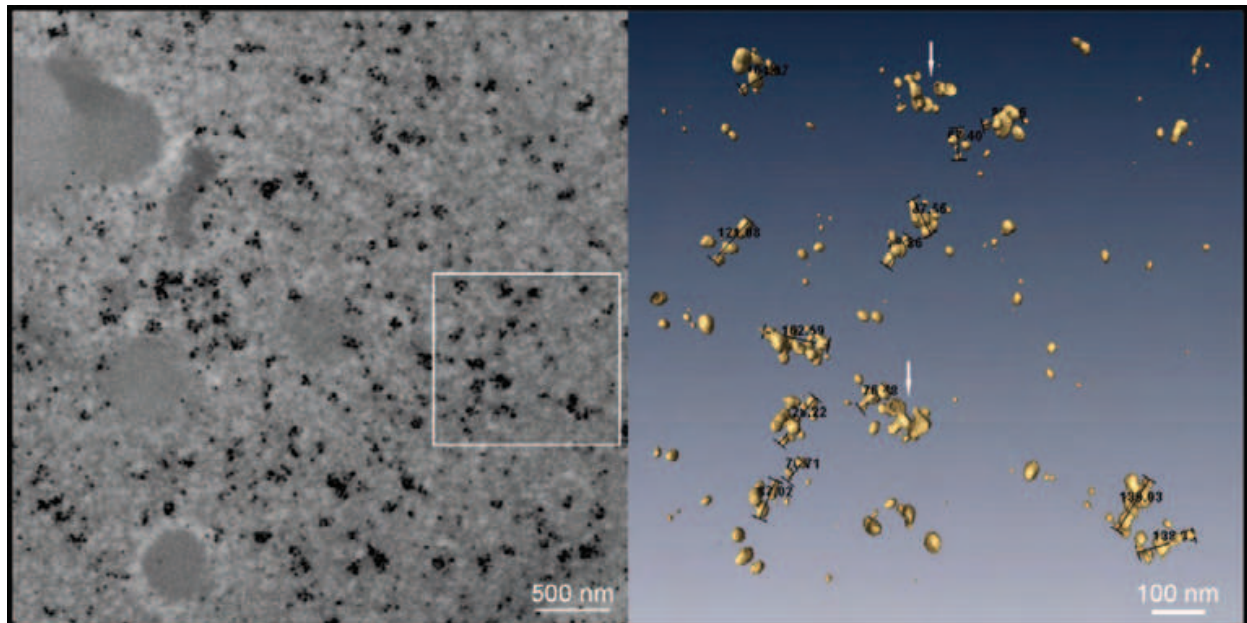


Fig. 2. A 3D reconstruction of the labelled domains.

The original image of a 200-nm-thick section of the cell nucleus from the 2-h experiment is shown on the left (scale bar: 500 nm), whereas a 3D reconstruction of the labelled domains reconstructed from the insert is shown on the right (scale bar: 100 nm). Only clusters of silver-enhanced gold particles in the outlined area of the electron microscopy image were reconstructed using Amira software. The length measurement is demonstrated. The arrows indicate the labelled domains traversing the section faces. (From Ligasová, et al., 2009).

The whole volume of the cell nuclei in the S-phase cells and the volume of mitotic cells was calculated by means of Cavalieri's method (Gundersen et al., 1988). An analysis was performed on fifteen cells from three different experiments.

In order to evaluate the distance between the pairs of labelled domains, we analysed only such pairs that had similar size (the difference in their length was smaller than 20%), similar labelling intensity (the difference in the labelling intensity was below 25%), similar shape and whose mutual distance was less than 400 nm. The number of domains in pairs was calculated as a percentage of the domains in pairs to the overall number of labelled domains.

2.2 Results

2.2.1 Around 5400 domains in one cell nucleus are labelled in the 10-minute experiment

Data from our experiments showed that around twenty-one labelled domains are in $1 \mu\text{m}^3$ of the cell nucleus in the early S phase after the 10-minute labelling pulse. Every such domain represents several tagged segments of DNA (Koberna et al., 2005; Figure 1). As the total volume of the cell nucleus in the early S phase was $260 \pm 44 \mu\text{m}^3$, the number of labelled domains in one cell nucleus was 5460 ± 923 . In fact, the number of the labelled domains of concurrently active replicons is lower, because some of the labelled domains contain also the tagged segments of the replicons which began synthesis during the labelling pulse. To determine the number of domains labelled during the pulse, we supposed that this number is inversely proportional to the length of the replication of one average replicon

and directly proportional to the length of labelling (for a more detailed description of the calculation see Ligasová et al., 2009). On the basis of the performed calculations, it is clear that the average number of the labelled domains after the above-mentioned correction was 4890 ± 827 and the number of the domains labelled during the pulse was 570.

An analysis of the size of the labelled domains after the ten-minute labelling pulse showed that the average size of these domains was 113 ± 40 nm. This value was corrected with respect to the size of the antibody complex used for the detection of biotin-dU-tagged DNA segments and also with respect to the different degree of the intensification of the ultra-small gold particles (for the more detailed explanation see Ligasová et al., 2009). The maximum diameter of the tagged segments of DNA in domains after this correction was $\geq 74 \pm 45$ nm. This data surprisingly corresponds to the thickness of one or two pairs of 30 nm-chromatin fibres associated for example with the help of cohesin molecules and sister replisomes (cf. Fig. 1A1 and A2).

2.2.2 The number of the labelled domains is doubled after the two-hour labelling pulse and quadruples after the sister chromatid separation in mitosis.

To assess which of the two models of the mutual organisation of replisomes is correct, it was crucial to determine the number of domains after the various lengths of the incubation of cells in medium after a biotin pulse (see Fig. 1). In this case, the cells were incubated in the culture medium alternatively for 30 minutes, 1 hour, 2 hours or until mitosis (approximately fourteen hours, Figure 3). The number of domains after the 30-minute incubation was similar to that found in the ten-minute experiment. This finding is in absolute agreement with our conclusion that the pool of biotin-dUTP introduced into the cells during the hypotonic shift is depleted in less than 10 minutes (for a more detailed description see Ligasová et al., 2009).

In the case of the one-hour and two-hour experiments, we observed a gradual increase of the number of labelled domains (around 7040 ± 1191 and $11,000 \pm 1875$ domains were labelled in one cell nucleus in one-hour and two-hour experiments, respectively). Moreover, during the analysis of the tomograms, we found the presence of pairs of labelled domains with a similar shape and intensity of labelling. These pairs of domains were observed mainly in the two-hour experiment. In the analysis of the distances between the paired domains, we evaluated only pairs with a similar size, similar intensity of labelling, shape and with a mutual distance of less than 400 nm. The average measured distance was approximately 227 ± 96 nm (Fig. 3). When we take into account that the replication of an average replicon is around one hour (for example Jackson & Pombo, 1998; Manders et al., 1992; Nakamura et al., 1986) and the speed of replication fork in the S phase is 0.6 kbp/minute (Malinsky et al., 2001), then the average size of the replicon in the early S phase does not exceed 72 kbp. The length of a 2.6 kbp-long fragment of stretched DNA is around 1 μ m (Jackson & Pombo, 1998). As the compaction of a 30-nm chromatin fibre is around 40 (Wagner et al., 2005), the length of a 72-kbp-long replicon in the form of a 30-nm fibre is around 700 nm. The determined distance between domains in pairs would then correspond to one-third of such a replicon. It is a very realistic estimation that indicates the possibility that the maximum condensation of a replicon during its replication is not higher than the condensation of a 30-nm chromatin fibre, at least at the replicon level.

These results strongly support the model of associated replisome pairs (Model A2 in the Fig. 1). If we take into account that the sister replisomes operate as independent units, the

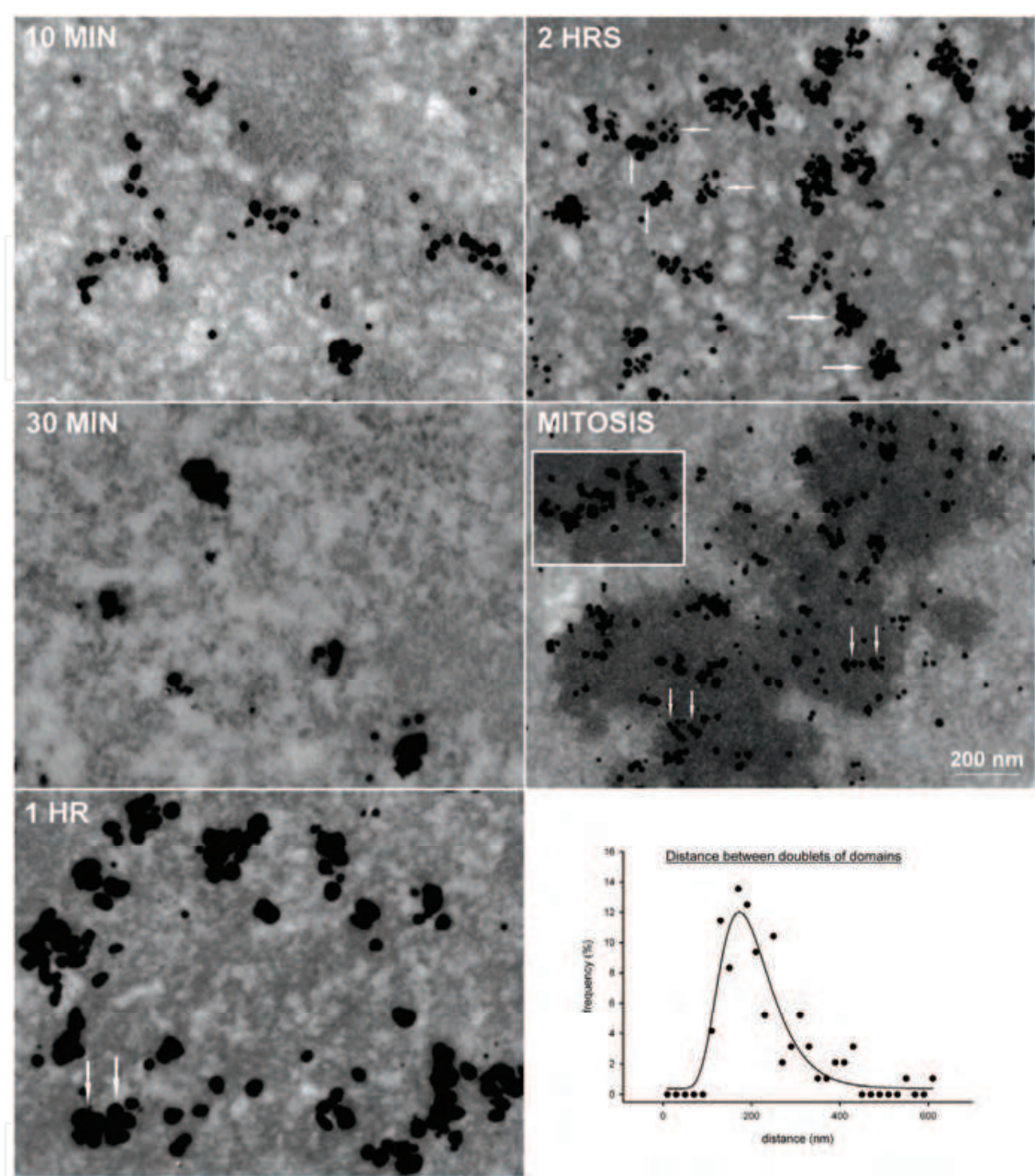


Fig. 3. EM images of thin sections of HeLa cell nuclei with labelled domains and a graph of the distances between the doublets of labelled domains. Images of the 70-nm-thick sections of the nuclei from the 10-min, 30-min, 1-h, 2-h and mitotic experiments are shown. The clusters of the silver-enhanced gold particles correspond to the labelled domains. The number of the labelled domains increases substantially between the 1-h and mitotic experiments. The arrows in the images from the 1- and 2-h and mitotic experiments indicate doublets of the labelled domains. The insert in the image of the mitotic-cell nucleus shows an example of a cluster of several labelled domains from a different cell. Seventy-nanometre sections were chosen instead of 200-nm sections as they have higher contrast and accommodate a much lower number of labelled domains. In this respect, they are much more suitable for the demonstration of individual doublets although they cannot reflect their overall organisation. Scale bar: 200 nm. The graph shows the frequency of the distances between the doublets of “sister” domains from the 2-h experiment. (From Ligasová, et al., 2009).

double increase of the number of labelled domains would correspond to the complete separation of sister chromatids. However, regarding the fact that the analysed cells were in the S phase, it is unlikely. This conclusion is also in complete agreement with the results obtained from the analysis of the mitotic cells. To achieve the complete segregation of sister chromatids, we analysed the number of labelled domains in metaphase cells (Rieder & Cole, 1999). We found that a $1\text{-}\mu\text{m}^3$ mitotic cell contains eleven labelled domains. The volume of mitotic cells was around $1919 \pm 310 \mu\text{m}^3$, thus the total number of labelled domains in mitotic cells was around $21,109 \pm 3420$. Our theoretic calculation of the number of labelled domains after sister-chromatid segregation was 20,700 ($4 \times 4890 + 2 \times 570$) for the model of replisome pairs, which is in agreement with the measured value.

Besides the number of labelled domains, we also analysed the size of these domains for each experiment. The values after the correction of the effect of the various sizes of the gold-enhanced particles (Ligasová et al., 2009) in the individual experiments were: $92 \pm 45 \text{ nm}$ for the 10-minute experiment, $85 \pm 46 \text{ nm}$ for the 30-minute experiment, $83 \pm 58 \text{ nm}$ for the 1-hour experiment, $90 \pm 48 \text{ nm}$ for the 2-hour experiment and $48 \pm 26 \text{ nm}$ for mitotic cells. The similar size of the domains in the 10-minute to 2-hour experiments is in absolute agreement with the possibility that the maximum size of the labelled domains corresponds to the thickness one or two pairs of tightly associated 30-nm chromatin fibres. This hypothesis is also in agreement with our previous results showing that the size of the domains is independent of the time of the incorporation of biotin-dUTP in the 3- and 10-minute experiments (Koberna et al., 2005). According to the model of replisome couples (Fig. 1A2), two pairs of 30 nm fibres can be found in most labelled domains in the 10-min experiment. Later, as the segment pairs are moved away from the replisomes and the loop is finally relaxed, each labelled domain contains only one pair of the segments. In mitotic cells, only one labelled segment is accommodated in the labelled domain. The reduction in the number of segments in the individual domains between the 2-h and mitotic experiments is reflected in the steep decrease in the size of the domains labelled. Such a decrease was not observed between the 10-min and 2-h experiments, likely due to the similar thickness of the bundle of 4 or 2 parallel segments.

2.2.3 Model of replisome pairs

All of the above-mentioned data showed that in HeLa cells the sister replisomes are tightly associated during replication. Another important finding concerning the organisation of the DNA loops that are formed during replication was the same number of labelled domains in 10- and 30-minute experiments. On the basis of this result, we suppose that the arms of DNA loops are tightly associated during and even for a certain time after the replication of the replicon. According to these data, we have proposed the model of newly replicated DNA (Fig. 4).

3. Conclusions

Our results are in complete agreement with the model showing that sister replisomes are organised as tightly associated pairs. Similar findings have been published also for other organisms, both prokaryotic (Jensen et al., 2001; Lau et al., 2003; Lemon & Grossman, 2000; Migocki et al., 2004) and eukaryotic (Kitamura et al., 2006). On the other hand, there are several studies showing a high degree of independence of sister replisomes (Reyes-Lamothe et al., 2008; Yardimci et al., 2010). Moreover, some studies showing that the sister replisomes

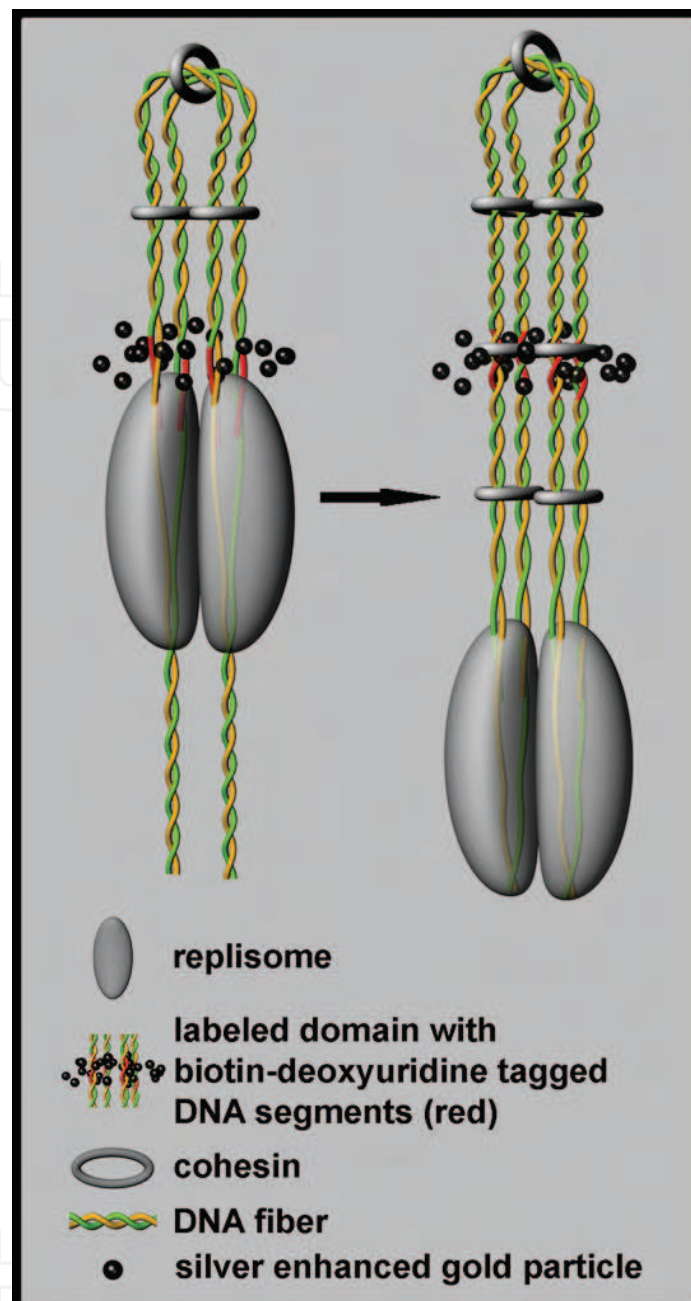


Fig. 4. The model of zipping loops.

The zipping of a DNA loop is shown. During replication, replisome couples produce a loop with the associated (zipped) arms probably in the form of four tightly associated 30-nm fibres. According to this model, “sister” pairs of biotin-dU-tagged segments of chromatids do not separate before the termination of the DNA synthesis of the replicon and the relaxation of the zipped arms. Immediately after labelling, the four tagged segments are present in one labelled domain (the left part of the image). Such an organisation of the tagged segments persists during the synthesis of the whole replicon (the right part of the image). Although the mutual changes of the replisome position between the left and right part of the figure can result in the impression of a movement of the replisome along DNA, this model does not reflect whether DNA or the replisome complex is moving during the replication. (From Ligasová, et al., 2009).

are tightly associated suppose the movement of DNA instead of DNA polymerase (Dingman, 1974; Lemon & Grossman, 1998, 2000; Pardoll et al., 1980). According to the above-mentioned studies, DNA is pulled through the static replisome and the newly-synthesised daughter DNA strains are released from the replication complex. The model of the coupled replisomes is also supported by the results of a time-lapse microscopic study focused on the microscopic analysis of the expressed GFP-PCNA in cell nuclei. This study (Leonhardt et al., 2000) showed that the replication foci form and again disintegrate during DNA replication, but no direct movement was observed.

Three basic studies dealing with the mutual position of sister replisomes were performed in eukaryotic cells. In the first one (Kitamura et al., 2006), the method that enables the tracking of specific chromosomal loci in an individual live cell was used to determine the organisation of replisomes in *Saccharomyces cerevisiae* yeast. The general disadvantage of such methods is the necessity of manipulation with the original genetic information of various proteins using GFP tags. Therefore, a high number of controls are required. Moreover, the number of cells inspected is usually relatively low and the resolution of light microscopy need not be sufficient, because chromatin is highly compacted in the cell nuclei. In the study described here, we have used the labelling of short DNA segments and analysed them during replication and mitosis. These tagged segments were analysed by means of electron tomography. Electron-microscopic studies generally provide high resolution. In addition, with respect to the possibility of using stereological approaches for evaluation, the obtained data are not burdened by high error owing to the high number of analysed cells. On the other hand, it is necessary to work with fixed and permeabilised cells, which results in volume changes and a corresponding error, whose value depends on the many factors. Apparently, the ratio values are less burdened by this error than the absolute values. From this point of view, the number of domains was selected as the crucial parameter for the testing of both models in the described approach. Independently of the methods used and their limitations, both studies brought data supporting the model of mutually associated replisomes.

In contrast to the above-mentioned studies, Yardimci et al. (Yardimci et al., 2010) tracked replication in an *in vitro* system. In this case, they used biotinylated DNA of λ phage, which was attached by one or both ends to the streptavidin-coated microfluid flow cell. The cell-free system based on the *Xenopus* egg extract was used to replicate these DNA molecules. From the results obtained, it was obvious that the studied DNA was replicated by two independent replication complexes. Despite the advantages of the mentioned arrangement consisting mainly in the high control of the described system, the main problem is the interpretation of the obtained data in terms of their application to the processes proceeding in the complex structure of the cell nucleus. In this respect, it seems that the nuclear structures are necessary for the replication of replicons by means of the pairs of tightly associated replisomes. These nuclear structures are, however, absent in the cell extracts (Yardimci et al., 2010).

In order to answer the question of the mutual organization of sister replisomes definitively, the development of a new approach enabling the acquisition of 3D data sets from well preserved cells at high resolution seems to be the next necessary step. In this regard, the recently developed procedure of labelling DNA via the incorporation of 5-ethynyl-2'-deoxyuridine represents a very promising base (Salic & Mitchison, 2008).

Independently of the question of the mutual organisation of replisomes, our results have shown that the method used has enabled distinction between individual replicons. The observed number of labelled domains in the 10-minute experiment was around 5000. This

number is several times higher than the number of the replication foci/factories published in the previous studies (120–1500; Jackson, 1995; Jackson & Pombo, 1998; Ma et al., 1998; Mills et al., 1989; Nakamura et al., 1986; Nakayasu & Berezney, 1989; Tomilin et al., 1995). It is supposed that at any time of the S phase approximately 10–15% of all the replicons are active (Jackson, 1995; Jackson & Pombo, 1998) and that the total number of replicons is around 40,000 (Singer et al., 1996). This is in absolute agreement with our results of the analysis of the number of labelled domains.

Our data also allowed us to speculate about the organisation of the replicon during its replication as well as to propose the model of the organisation of the replicon during replication. We suppose that the arms of DNA loop created during the duplication of replicon are tightly associated. The mechanism and the reason for this association remain unclear, and it is also not obvious whether the association of newly-replicated DNA is characteristic also for other organisms. Although our data have made it possible to test the model of the mutual organisation of replisomes in human cells, this method has not made it possible to decide whether the sister replisomes are moving during replication or not. In this respect, several studies have supposed that replisomes are attached e.g. to the nucleoskeleton in eukaryotic cells (Cook, 1999; Falaschi, 2000) or to cell structures such as the plasmatic membrane or cell wall in the gram-positive bacteria *Bacillus subtilis* (Lemon & Grossman, 2000) and that there are molecular motors mediating the movement of DNA. However, the direct evidence for such connection of replisomes does not exist.

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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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51000 Rijeka, Croatia
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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