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PROSPECT

DNA Replication and Nuclear Architecture

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Abstract The model of in situ DNA replication provided by immunofluorescence and confocal imaging is compared with observations obtained by electron microscopic studies. Discrepancies between both types of observations call into question the replication focus as a persistent nuclear structure and as a replication entity where DNA replication takes place. Most electron microscopic analyses reveal that replication sites are confined to dispersed chromatin areas at the periphery of condensed chromatin, and the distribution of replication factors exhibits the same localization pattern. Moreover, rapid migration of newly synthesized DNA from the replication sites towards the interior of condensed chromatin regions obviously takes place during S-phase. It implies modifications of replication domains, hardly detectable by fluorescence microscopy. The confrontation of different observations carried out at light microscopic or electron microscopic levels of resolution lead to a conclusion that a combination of in vivo fluorescence analysis with a subsequent ultrastructural investigation performed on the same cells will represent an optimal approach in future studies of nuclear functions in situ. J. Cell. Biochem. 85: 1–9, 2002. © 2002 Wiley-Liss, Inc.

Key words: DNA replication; replication foci; replication sites; fluorescence microscopy; electron microscopy; nuclear domains

DNA replication is an essential process that must be achieved within a limited time period before mitosis, so that each daughter cell can receive only one full identical copy of the genomic material. DNA replication begins at multiple specific sites (origins) or specific sequences on eucaryotic chromosomes [Ritzi and Knippers, 2000]. After recognition of the origins, two replication forks are established, which move in opposite directions untill adjacent replication units or replicons meet. A number of proteins (prereplicative complex), which have been characterized in yeast and Xenopus are involved in the initiation of DNA replication. The origin recognition complex (ORC) proteins associate with the DNA at origins during G1 and then recruit CDC6p, which facilitates the association of Mcm factors acting probably as a helicase. This step allows the cell to enter the S-phase and CDC6p is directly released and replaced by Cd45p contributing to attract the protein RPA, whereas the Mcm proteins are gradually dissociated [Ritzi and Knippers, 2000]. These initiation steps are regulated by cyclin dependent kinases (CDK2, cyclin A). The elongation phase involves a multiprotein replication complex containing the DNA polymerases (Polδ, Polϵ), with the combined action of PCNA (proliferating cell nuclear antigen), RF-C (replication factor-C) and DNA ligase I depending on the strand [Burgers, 1998]. It is thought that the choice of replication origins, and therefore, temporal order of replication is partly controlled by modifications in chromatin structure [Wintersberger, 2000]. Moreover, specific replication initiation was shown to be activated by some components of the nuclear structure [Gilbert et al., 1995], and the elongation phase of replication was dependent on nuclear lamin organization [Moir et al., 2000], hence the importance of analysing replication at the subnuclear and ultrastructural levels. Extensive investigation of the subnuclear localization of the replication sites has been carried out in the past using incorporation of [^{3}H]-thymidine into newly synthesized DNA followed by autoradiography and light microscopy; this technique requires relatively long periods of exposure and the use of very thin specimens (< 1 μm) in order

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to reach the best resolution. Now the indirect immunofluorescence technique is widely applied, and the DNA precursors often used are either halogenated deoxyuridines, which do not need cell permeabilization for their incorporation or, for instance, biotin or Cy5-labeled dUTP, which must be introduced into cells by different techniques. In addition, it requires a primary antibody that specifically recognizes the incorporated DNA precursor in conjunction with a secondary antibody recognizing the primary antibody and labeled with a fluorochrome. This technique allows one to visualize newly replicated DNA in the whole nucleus using fluorescence microscopy and confocal laser scanning microscopy (CLSM). However, the discrete and limited fluorescent domains observed must be carefully interpreted since degradation of cell structure occurs during cell permeabilization, denaturation of DNA or further processing for immunofluorescence microscopy, as visualized by electron microscopic analysis [Visser et al., 2000]. Moreover, false negative results can be obtained due to insufficient penetration of antibodies into the nuclear compartments [Wansink et al., 1993]. These problems can be partly avoided by introducing directly, by microinjection or similar methods, fluorescent analogues such as Cy5-dUTP or FITC-dUTP. Yet the best x/y resolution of a confocal image remains in the order of 220 nm [Stelzer, 1995]. At the ultrastructural level, replication sites were observed by means of high-resolution autoradiography using [^{3}H]-thymidine labeled DNA [Fakan and Hancock, 1974], whose resolving power is limited to about 140 nm. Better results were obtained after incorporation by cells of halogenated deoxyuridines and indirect immunolabeling with specific antibodies and gold-labeled secondary markers [Jaunin et al., 1998]. The resolution power of the electron microscope being about 0.2 nm, the distance between the epitope and the center of the gold particle can be around 19–22 nm when using secondary colloidal gold markers of 6 or 12 nm diameter considering the size of both antibodies employed in the indirect technique.

The cell nucleus is compartmentalized into domains, whose roles in nuclear functions are still only partially understood. When seen at the ultrastructural level, the nucleus consists of condensed chromatin areas surrounded by perichromatin regions including RNP containing-perichromatin fibrils and perichromatin granules and separated by a domain largely devoid of chromatin, the interchromatin space. In this region, interchromatin granules as well as different nuclear bodies (e.g., coiled (Cajal) bodies, PML bodies, or other nuclear bodies) can be observed. However, most of these domains, when visualized by immunofluorescence microscopy, appear as foci, dots or speckles, the limits of light microscopy precluding a further definition of such domains. Finally, numerous nuclear components and functions were reported to be associated with the nuclear matrix obtained by subsequent extractions of nuclei with detergents, nucleases, and high salt solutions. However, its correspondence with an in situ nuclear architecture was not so far demonstrated [Hancock, 2000]. This article focuses on the intranuclear distribution of replication sites and of the newly synthesized DNA. We attempt to critically compare light microscopic and electron microscopic data on DNA replication with regards to in situ nuclear architecture.

REPLICATION FOCI AND REPLICATION SITES

From immunofluorescence and confocal imaging, a model of in situ replication based on discrete and separate domains has been proposed. At the level of fluorescence microscopy resolution, the term of replication foci is defined as solid bodies or dots resulting from the detection of the labeled nucleotides incorporated into newly synthesized DNA [Cossmann et al., 2000]. Such foci are often considered as replication sites resulting from the clustering of small replicons [Berezney et al., 2000]. However, this definition must be refined, since some findings have shown horseshoe and ropeshaped domains [Nakamura et al., 1986]; whether these differences are due to image acquisition conditions, to the progression into S-phase, or to the length of the labeling period still remains to be elucidated. A great variability of size (0.2–0.4 μm) and number (500–1,500) of foci was described for early S-phase [Tomilin et al., 1995]. As an example, foci observed in embryonic quail cells were larger than in embryonic chick cells and also less confluent [Cossmann et al., 2000]. Moreover, foci in primary fibroblasts are limited in number at the onset of S-phase compared to immortalized cells [Kennedy et al., 2000]. Patterns and size of foci are certainly influenced by the method of detection of incorporated labeled nucleotides since, for

example, HCl, widely used for DNA denaturation, led to a redistribution of BrdU sites [Kennedy et al., 2000] and to some collapse or alteration of the nuclear structures [Rizzoli et al., 1992]. However, foci observed in fixed cells can also be revealed in vivo using fluorescent nucleotide analogues (Cy5-dUTP), which do not need any further specimen treatment for their visualization [Manders et al., 1999]. Light microscopists seem to have reached a consensus that foci represent functionally and structurally stable units. Five different reproducible intranuclear distribution patterns of newly synthesized DNA were described for exponentially growing and synchronized CHO or HeLa cells labeled for 10 min with BrdU [O'Keefe et al., 1992]. The distribution of replication foci was quite different during early S-phase compared to late S-phase. Generally, the replication pattern of early S-phase consisted of many small domains scattered throughout the nucleoplasm, whereas during late S-phase replication domains were larger and confined especially to perinuclear and intranuclear dense chromatin and in particular to chromatin surrounding the nucleolus. A punctuate pattern revealed at a precise time of S-phase was reported to persist throughout the cell cycle and independently of DNA synthesis [Jackson and Pombo, 1998] suggesting that there are synchronized waves of synthesis, with different groups of foci activated in turn. Moreover, it was suggested that DNA in one "focus" is replicated in less than 1 h [Jackson and Pombo, 1998], which has led to the idea that foci could be considered as stable entities like beads filled with DNA and that replicons are regularly distributed throughout the constant volume of such foci independently of labeling time duration [Berezney et al., 2000]. However, this does not seem compatible with the findings of Manders et al. [1996] showing that when cells are labeled with two different DNA precursors during various periods of S-phase, domains containing nascent DNA and domains containing previously replicated DNA (termed newborn DNA) were observed not to overlap, when the time between each pulse was longer than 25 min. These two fractions of DNA separated from each other at a rate of about 0.5 μm/h. It is also not clear to what type of chromatin (active or inactive, dispersed or condensed chromatin) different replication foci correspond. Previous work making use of two different thymidine analogues incorporated during distinct periods of S-phase showed that early and late replicating DNA formed separate chromatin foci in the interphase nuclei corresponding to R/G banding patterns on metaphase chromosomes, R bands corresponding to early replication and G bands to late replication patterns [Ferreira et al., 1997; Sadoni et al., 1999]. A model for chromatin organization consisting of euchromatin containing transcriptionnaly active genes (R bands) and heterochromatin containing non coding DNA and silent genes has been proposed [Wintersberger, 2000], even though some findings do not seem to be in agreement with it [e.g., Gartler et al., 1999]. When replication patterns were confronted with chromatin structure at the light microscopic level, it was shown that replication foci in early S-phase consisted of both lightly and intensely stained DNA regions while late replicated chromatin was visualized as strongly stained compact areas [Nakayasu and Berezney, 1989].

How can the above microscopic data be compared with observations obtained by electron microscopic in situ analyses carried out on ultrathin sections of immunolabeled cells? Before making such a comparison, one has to keep in mind the advantages and weaknesses of the two approaches. While confocal microscopy allows one to investigate the three-dimensional (3-D) arrangment of intracellular features, its resolution is rather low and the intranuclear architecture can be affected by treatments often required for label visualization (see Introduction). Electron microscopic in situ observations are performed on ultrathin sections of structurally well preserved cells, and offer an excellent definition of structural and labeling details. Although immunogold labeling takes place only on epitopes occurring on the surface of the section, its sensitivity is very good and the signal can easily be quantified. Nevertheless, the image remains two dimensional and for 3-D studies, a reconstruction of successive serial sections is required. It is therefore important to employ, as much as possible, the complementarity of the two approaches [e.g., Visser et al., 2000]. Let us analyse, how we can visualize replication sites and what is a replication focus at the ultrastructural level. The rapid rate of replication (1–2 μm/min) revealed in vitro for chinese hamster DNA [Huberman and Riggs, 1968] like the above mentioned experiments on separation of nascent and newborn DNA

[Manders et al., 1996] clearly show that analyses of nascent DNA, and therefore, replication sites require the use of short pulses with precursors. Thus, it was shown [Jaunin et al., 2000] that a 2 min BrdU pulse (Fig. 1A) allows one to catch labeled DNA and consequently replication sites at the periphery of dense chromatin areas, associated with dispersed chromatin domains. On the other hand, labeling pulses of 5 min (Fig. 1B) or longer (Fig. 1C) do not show only replication sites, since a part of DNA which has already been replicated is internalized into the condensed chromatin areas. Moreover, a 25 min chase is enough for most of the DNA which has been replicated during 5 min to be internalized into dense chromatin domains. Separation between nacent DNA belonging to dispersed chromatin areas and newborn DNA integrating into condensed chromatin is confirmed using incorporation of two halogenated precursors (Fig. 2). This is in agreement with assays performed on mice after BrdU injection [Liu et al., 1995]. Therefore, most foci described at the light microscopic level do not reflect replication sites only, since the duration of precursor pulses reported in the literature was never shorter than 5 min, and often even longer. Moreover, the idea of a persistence and stability of foci throughout the cell cycle is not evident when considering electron microscopic observations. Indeed, the latter suggest motion of nascent DNA from replication sites towards the interior of dense chromatin aggregates. Although motion of chromatin was not always detectable in interphase nuclei using fluorescence microscopy or fluorescence recovery after photobleaching, it was demonstrated in S-phase CHO nuclei for some late replicating domains together with changes in the degree of chromatin condensation [Li et al., 1998]. In this regard, it is difficult to admit that foci visualized in S-phase directly after pulse labeling could be the same as those followed in G2 nuclei. However, at the ultrastructural level the term of foci for labeled newly replicated DNA does not seem to be appropriate. In many cell types, chromatin appears rather dispersed in early S-phase and becomes more condensed as cells progress through S-phase [Leblond and El-Alfi, 1996]. Consequently, it was shown that replicated DNA was confined to dispersed chromatin during early S-phase, labeling by gold particles occurring separately or in small groups throughout the nucleus as irregular and dispersed labeling [O'Keefe et al., 1992; Liu et al., 1995; Tamatani et al., 1995]. This reveals that the "replication focus" does not have an equivalent at the ultrastructural level with regard to early S-phase. It also strongly suggests that a replication focus does not represent a recognizable structure, but could rather represent the smallest particulate signal detectable by fluorescence and CLSM microscopy. Some authors reported newly synthesized DNA organized into ring like units of about 0.15 μm [Mazzotti et al., 1998], but these structures were not convincingly demonstrated and were not revealed during earlier observations on the same cells [Rizzoli et al., 1992]. Using relatively long pulses of DNA precursors, labeled DNA at the EM level was observed on small patches of chromatin giving rise to a punctuate pattern in early S-phase following the degree of chromatin condensation [Jaunin et al., 1998]. In mid and late S-phase, after pulses longer than 5 min, labeled DNA consisting of irregular shaped domains was also observed at the EM level on large aggregates of condensed chromatin [O'Keefe et al., 1992; Jaunin et al., 1998; Mazzotti et al., 1998]. Progressive condensation of chromatin occurring during S-phase can explain the increased size of foci in mid and late S-phase. Whether this condensation concerns only the immediately replicated chromatin is not clear. We can therefore assume that a focus, as seen by fluorescence microscopy, represents a small visible chromatin region consisting of both dispersed and condensed chromatin fibers, when DNA precursor pulses are longer than 5 min. Small patches of chromatin labeled only on their periphery may also appear as foci, when occuring within the thickness of an optical section. Moreover, to what extent replication sites visualized after shorter labeling periods are also represented by a diffuse signal which, obviously, is not taken into account in further image processing, remains to be determined.

REPLICATION STRUCTURES OR REPLICATION DOMAINS

Work on the nuclear matrix led to formulating ideas of a fixed and organized nucleoplasmic structure allowing replication and anchorage of nascent DNA. The nuclear matrix has been defined as a DNA depleted fraction of nuclei obtained by subsequent extraction with

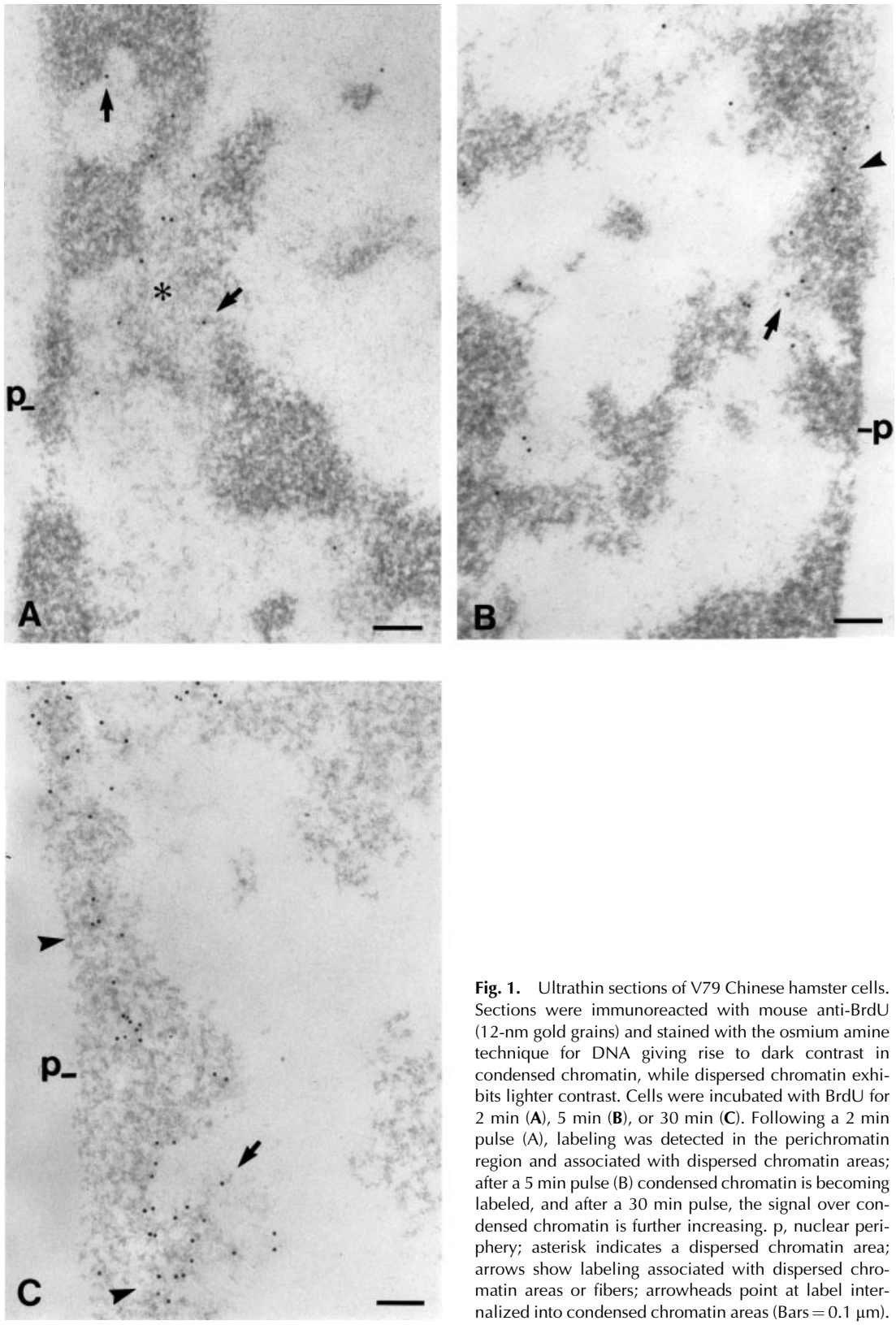

Fig. 1. Ultrathin sections of V79 Chinese hamster cells. Sections were immunoreacted with mouse anti-BrdU (12-nm gold grains) and stained with the osmium amine technique for DNA giving rise to dark contrast in condensed chromatin, while dispersed chromatin exhibits lighter contrast. Cells were incubated with BrdU for 2 min (**A**), 5 min (**B**), or 30 min (**C**). Following a 2 min pulse (A), labeling was detected in the perichromatin region and associated with dispersed chromatin areas; after a 5 min pulse (B) condensed chromatin is becoming labeled, and after a 30 min pulse, the signal over condensed chromatin is further increasing. p, nuclear periphery; asterisk indicates a dispersed chromatin area; arrows show labeling associated with dispersed chromatin areas or fibers; arrowheads point at label internalized into condensed chromatin areas (Bars = 0.1 μm).

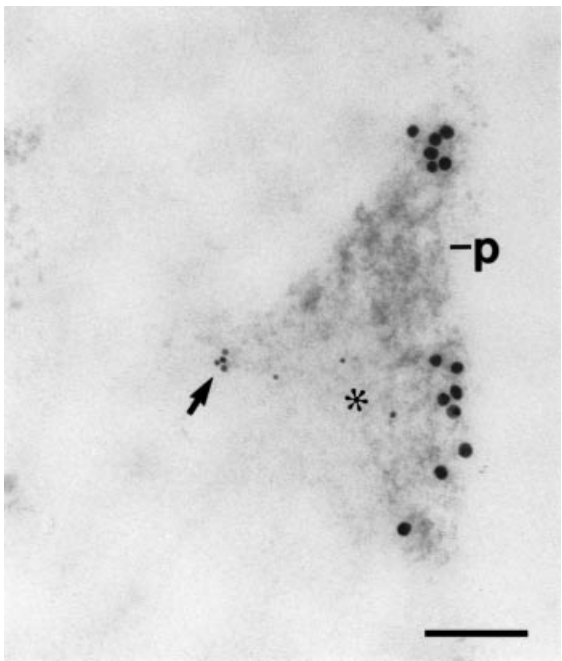

Fig. 2. Ultrathin section of a V79 Chinese hamster cell incubated with IdU for 20 min before a chase period of 10 min and subsequent labeling with CldU for 5 min. Sections were immunoreacted with different anti BrdU antibodies exhibiting specific affinity for the different halogenated precursors and stained with the osmium amine technique to specifically visualize DNA. IdU labeling (12-nm gold grains) corresponding to previously replicated DNA was confined to condensed chromatin regions (dark areas), whereas CldU labeling (6-nm gold grains) corresponding to nascent DNA was associated with dispersed chromatin areas and fibers exhibiting a lighter contrast and occurring in the border area of the condensed chromatin clump. p, nuclear periphery; asterisk indicates a dispersed chromatin area; arrow indicates labeling (nascent DNA) associated with dispersed chromatin (Bar = 0.1 μm).

nucleases and high salt solutions [Nakayasu and Berezney, 1989; Martelli et al., 1998]. It appears, when visualized by electron microscopy, as a fibrogranular residual network composed of the lamina and intranuclear material containing some hnRNP proteins [Van Driel et al., 1995]. Such nucleoplasmic network has been reported to retain the ability to synthesize DNA in the form of foci [Nakayasu and Berezney, 1989]. Some activities such as DNA polymerase α and DNA primase have been detected in nuclear matrix preparations [Martelli et al., 1998]. In the residual DNA still remaining in nuclear matrix preparations after DNase treatment, some chromatin seems to be preferentially retained. However, nuclear matrix binding sites were reported to be randomly distributed without preference for replication origins [Ortega and DePamphilis, 1998] and obviously strongly depend on the extraction protocols [Donev, 2000]. Furthermore, an intranuclear structural matrix network like that seen in isolated nuclear matrix preparations has not so far been observed in situ in nuclei of intact cells. Moreover, recent reports analysing different features of the nuclear matrix suggest a possible artifactual nature of the nuclear matrix as a structural component of the nucleus [e.g., Hancock, 2000]. Fluorescence microscopic visualization of colocalization on replication foci of factors such as PCNA, DNA polymerase α, CAF1, RPA, or DNA ligase [Hozák et al., 1993; Krude, 1995; Cardoso et al., 1997; Kennedy et al., 2000] gave rise to an idea of a "replication factory" domain. This seemed to be supported by observations on HeLa cells of electron dense ovoid structures apparently attached to a nuclear matrix network after previous biotin-dUTP labeling and subsequent extraction of cells; PCNA and newly synthesized DNA seemed to accumulate in these structures which were named replication factories [Hozák et al., 1993, 1994]. However, such "replication factories" did not label for DNA polymerase α at the EM level [Hozák et al., 1993] and do not appear to be a recognizable structural feature in many eucaryotic cells.

As to the fine structural in situ investigations of replication site distribution in the nucleus, several laboratories have demonstrated that DNA synthesis predominantly occurs on chromatin fibers situated on the periphery of condensed chromatin areas [Sobczak-Thepot et al., 1993; Liu et al., 1995; Jaunin et al., 2000]. An electron microscopic in situ analysis [Jaunin et al., 2000] revealed that chromatin in Chinese hamster cells can be observed in three forms: condensed chromatin, dispersed chromatin areas, and individual dispersed chromatin fibers (Fig. 3). Dispersed chromatin areas and fibers belong to the periphery of condensed chromatin named the perichromatin region [Fakan, 1994]. In this domain, nascent DNA, DNA polymerase α, cyclin A, and PCNA were previously found [Sobczak-Thepot et al., 1993; Jaunin et al., 2000]. In addition, these observations show that nascent DNA and replication factors are not associated with special structural domains or do not appear as nuclear bodies (replication factories, see above). Perichromatin fibrils representing the in situ form of nascent hnRNA are often situated close to replication

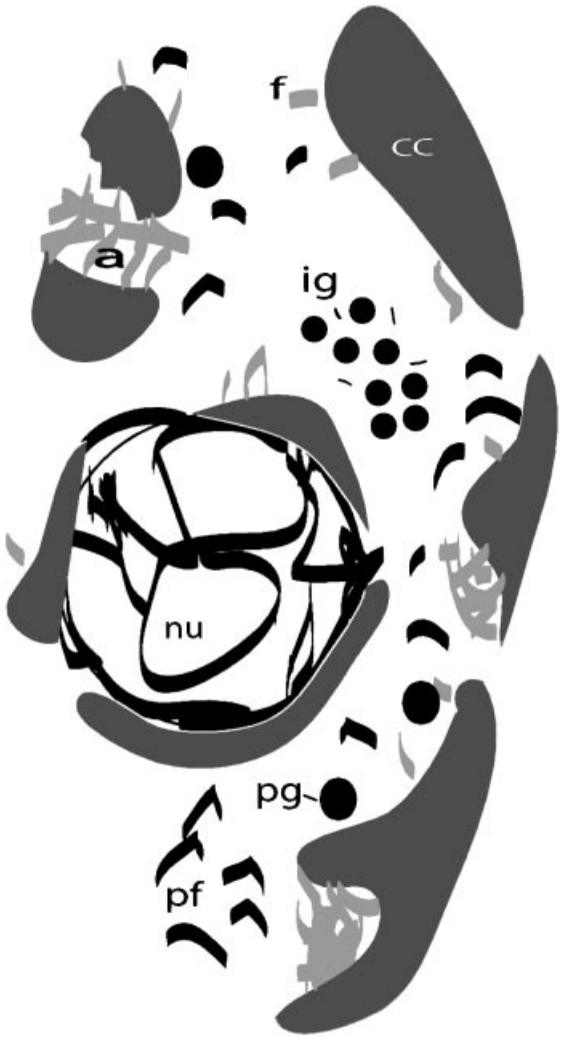

Fig. 3. Schematic drawing of a nuclear section showing the different domains as observed at the ultrastructural level. a, dispersed chromatin areas; cc, condensed chromatin; f, dispersed chromatin fibers; ig, interchromatin granules; nu, nucleolus; pf, perichromatin fibrils; pg, perichromatin granules. Replication sites occurring in dispersed chromatin areas (a) and fibers (f) within the condensed chromatin periphery coincide with the perichromatin fibril-containing regions, where transcription takes place. Newly replicated DNA moves from these sites towards the condensed chromatin interior. The internal nuclear domain excluding the chromatin, the perichromatin region, and the nucleolus can be designated as the interchromatin space. In this region, interchromatin granules involved in the spliceosome formation or storage of splicing factors are found.

sites. Electron microscopic analysis of spread active chromatin in *Drosophila* embryos showed a simultaneous occurrence of both DNA replication and transcription of the same genes [McKnight et al., 1977]. Whether both replication and transcription take place simultaneously within the same nuclear domains in situ, throughout the S-phase, still remains to be demonstrated using short pulses of DNA and RNA precursors. Although immunofluoescence microscopy provided contradictory results about colocalization of both functions, previous evidence suggests the recruiting of some transcription factors for stimulating DNA replication [Stagljar et al., 1999].

Several conclusions can be drawn from the above considerations. In order to catch replicating DNA at the sites of synthesis and therefore to localize replication sites without including too much of the previously replicated DNA in the final signal, it appears essential to perform very short labeling pulses of cells with DNA precursors. Moreover, one has to work on intact and non-permeabilized cells and to minimize as much as possible the specimen processing so as to avoid artifactual problems in analysing localisation of replication sites with regard to other nuclear domains. When studied at high resolution by means of ultrastructural methods, replication sites do not exhibit focal or dot-like pattern nor are they assembled in special structural domains. Do replication foci, revealed by fluorescence microscopic techniques as particulate signals, appear as such due to this method of visualization carried out on total cell preparations? In order to elucidate this point, it is essential to analyse replication sites by both in vivo labeling and fluorescence microscopic observations on living cells followed by transmission electron microscopic analysis of the same cells and nuclear domains. Furthermore, it is important to examine the possible occurrence of "replication factories" in a large variety of cells, since such structural nuclear compartments have, so far, been revealed rather occasionally in ultrastructural investigations [HeLa cells, Hozák et al., 1993, 1994]. In addition, although DNA replication and transcription both are localized within the perichromatin regions constituting the border zone of condensed chromatin areas, it is not clear yet whether these two processes frequently occur on the same DNA sequences during S-phase. Finally, since fine structural data strongly suggest that most DNA is internalized into condensed chromatin areas shortly after its replication, the question arises as to whether coding DNA sequences and the bulk of DNA are organized in the same way during the S-phase as well as within the condensed chromatin

domains during interphase. These points will be subjects of future investigations.

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