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Chromosoma Focus

Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci

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Abstract. According to the current paradigm, replication foci are discrete sites in the interphase nucleus where assemblies of DNA replication enzymes simultaneously elongate the replication forks of 10–100 adjacent replicons (each ~100 kbp). Here we review new results and provide alternative interpretations for old results to show that the current paradigm is in need of further development. In particular, many replicons are larger than previously thought – so large that their complete replication takes much longer (several hours) than the measured average time to complete replication at individual foci (45–60 min). In addition to this large heterogeneity in replicon size, it is now apparent that there is also a corresponding heterogeneity in the size and intensity of individual replication foci. An important property of all replication foci is that they are stable structures that persist, with constant dimensions, during all cell cycle stages including mitosis, and therefore likely represent a fundamental unit of chromatin organization. With this in mind, we present a modified model of replication foci in which many of the foci are composed of clusters of small replicons as previously proposed, but the size and number of replicons per focus is extremely heterogeneous, and a significant proportion of foci are composed of single large replicons. We further speculate that very large replicons may extend over two or more individual foci and that this organization may be important in regulating the replication of such large replicons as the cell proceeds through S-phase.

This review is dedicated to the memory of Professor J. Herbert Taylor, whose pioneering studies of chromosomal replication using ^{3}H-thymidine autoradiography formed the foundation on which the studies described herein were constructed.

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Introduction

This review article grew out of discussions between two of the authors (R.B. and J.A.H.) regarding interpretation of some of the experiments performed by Ma et al. (1998). These discussions took place in two contexts – during thesis committee meetings for Hong Ma (R.B. was Hong Ma's thesis advisor, and J.A.H. was a member of her thesis committee) and during meetings of the Buffalo DNA Replication Group, which sponsors discussions among scientists interested in DNA replication who work in Buffalo, N.Y. When D.D. visited the laboratory of J.A.H. for 2 months during the spring of 1999, he, too, joined in the discussions. All three of us learned a great deal from our interactions and debates. This review article represents our attempt to share some of what we learned with the rest of the world.

In addition to acknowledging the stimulating atmosphere created by our colleagues in the DNA replication field in Buffalo, we also wish to give credit to the review article by Liapunova (1994), which first brought to our attention the important experiments of Yurov and Liapunova (1977), who were the first to demonstrate that mammalian replicons can be very large, in some cases greater than 1 Mbp.

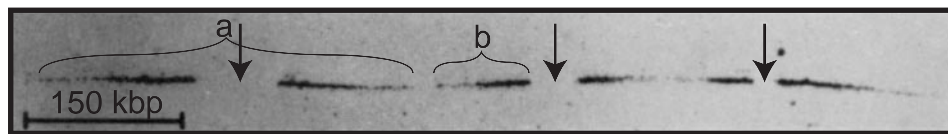

Fig. 1. Example of tandemly arranged replicons with origins firing at approximately the same time. Chinese hamster ovary cells were grown in Joklik-modified MEM supplemented with nonessential amino acids and 7% fetal calf serum (Life Technologies). 5-Fluoro-2′-deoxyuridine (FdUrd, an inhibitor of thymidylate synthetase; 0.1 µg/ml) and uridine (2.5 µg/ml) were added 12 h before the start of labeling. When 2×10^5 cells/ml had been reached, ^{3}H-thymidine (51 Ci/mmol; 100 µCi/ml) was added for 30 min. The pulse was terminated by removing the radioactive medium and replacing it with pre-warmed medium containing cold thymidine (5 µg/ml) for 45 min. Then the cells were harvested and processed for fiber autoradiography as described (Huberman and Tsai 1973). This figure is a modification of Plate IIIa in Huberman and Tsai (1973). The *arrows* show the deduced positions of replication origins. The *brace* labeled *a* shows the grain tracks generated by the two forks that moved outward from a single origin that fired prior to the start of the hot pulse and then continued through at least a portion of the cold chase. The *brace* labeled *b* shows the grain tracks produced by a single fork that was active during the entire hot pulse and at least a portion of the cold chase. Grain track patterns similar to *a* and *b* were used by Yurov and Liapunova (1977) to estimate the sizes of large replicons

The major lesson that has emerged from our discussions is that the "textbook" description of the relationships between replicons and replication foci is in need of revision. It appears that, in fact, the range of possibilities is considerably greater than previously imagined. Some replicons are so large that most of S-phase is required to complete them, while others are so short that they can be completed in less than an hour. Some replication foci may contain just one replicon while others appear to contain many. Whatever the number of replicons per focus, however, recent data suggest that replication foci are a reflection of fundamental, stable properties of chromatin structure, valid in both interphase and metaphase.

Evidence for replicon clusters

In this section, we summarize the evidence on which the classic view of replicon clusters and replication foci is based. According to this classic view, each replication focus represents a cluster of small (<~200 kbp) replicons, which complete replication in a small portion of S-phase (45–60 min).

DNA fiber studies

Many of the features of eukaryotic DNA replication, including bidirectional replication from irregularly spaced origins, were first characterized in experiments (reviewed in Edenberg and Huberman 1975; Hand 1978) in which DNA molecules labeled in vivo with ^{3}H-thymidine were stretched out on filters or microscope slides and then autoradiographed (exposed to a photographic emulsion sensitive to the β-particles given off by ^{3}H). Wherever ^{3}H-thymidine had been incorporated into the DNA molecule, a track of silver grains was generated in the overlying emulsion, and the density of silver grains in those tracks was proportional to the specific activity of the ^{3}H-thymidine. Thus, by intentionally altering the specific activity of the ^{3}H-thymidine during an experiment, a researcher could infer the direction of DNA replication fork movement from the corresponding change in grain density in the final autoradiogram. An example is shown in Fig. 1. In this case, Chinese hamster ovary cells were first exposed to ^{3}H-thymidine at high specific activity for 30 min and then chased with cold thymidine for 45 min. During the cold chase, the specific activity of the dTTP inside the cells gradually declined, leading to gradients of grain density that declined in the direction of replication fork movement. Based on this information, it seems straightforward to conclude that the DNA molecule in Fig. 1 was replicated by forks from three origins spaced 200 and 300 kbp apart from each other (arrows in Fig. 1) that had fired before the start of the high specific activity pulse. Similar measurements by many investigators on numerous additional DNA molecules in a variety of mammalian species led to the generalization that mammalian replicons are heterogeneous in size but that most fall into the range of 30–450 kbp with the most frequent sizes in the range 75–150 kbp. Furthermore, the appearance of tandem arrays of neighboring replicons that had initiated replication at similar times (like the array in Fig. 1) suggested that replicons may frequently be arranged in groups with all of the replicons in each group firing at similar times in S-phase (reviewed in Edenberg and Huberman 1975; Hand 1978).

Today similar experiments on extended DNA fibers can be carried out by in vivo labeling with a halogenated analog of thymidine such as 5-bromo-2′-deoxyuridine (BrdUrd) and then detecting the labeled DNA segments with fluorescent anti-BrdUrd antibodies. This modern approach obviates the need for the multi-month exposure times required for ^{3}H autoradiography and provides higher resolution. Using such an approach with similar measurement criteria, Jackson and Pombo (1998) recently reached similar conclusions with regard to replicon size and grouping.

Fiber autoradiography and fiber fluorography can also be used to study rates of replication fork movement. Results from many investigations (reviewed in Edenberg and Huberman 1975; see also Jackson and Pombo 1998) indicate that movement rates of mammalian replication forks, like replicon sizes, are heterogeneous. Fork movement rates vary according to species, cell type and time within S-phase but generally fall within the range 0.3–6 kbp/min. Even for cells of a single species at a single time during

S-phase (Housman and Huberman 1975), and even for individual cells at a single time during S-phase (Yurov 1980), replication fork rates are heterogeneous. For example, rates of fork movement within single human diploid fibroblasts can range from 0.6 to 3.6 kbp/min (Yurov 1980). It is important to note that, in these single cell measurements, fork movement rates at different forks operating at the same time within one cell were measured. Thus in this case the variability cannot be attributed to S-phase stage but appears to be characteristic of different replicons within the same nucleus.

Even within single replicons, variability of fork rates appears to be a frequent phenomenon (example in the rightmost replicon of Fig. 1). In a comparison of the rates of sister replication forks moving away from single origins in cells from the rodents *Bandicota bengalensis* and *Nesokia indica*, Dubey and Raman (1987a) noticed that a significant portion (~40%) of the sister forks moved at unequal rates (ratio of fast fork to slow fork ≥1.2).

Chromosomal replication bands

Additional evidence for functional grouping of eukaryotic replicons comes from studies on the relationships between replication time during S-phase and the banding patterns that can be detected in mammalian metaphase chromosomes after application of appropriate staining protocols (reviewed in ISCN 1995). The most commonly used protocol employs Giemsa stain; bands that appear dark with this technique are called G-bands. Application of other staining protocols results in bands with intensities opposite to those of the Giemsa technique. Bands appearing dark under these conditions are referred to as R (reverse) bands. The number of detectable bands depends on the staining technique employed and on the degree of condensation of the chromosomes being stained. The International Standing Committee on Human Cytogenetic Nomenclature recognizes low-, medium- and high-resolution banding patterns with 400, 550 and 850 bands, respectively (ISCN 1995).

Various techniques permit DNA replicated at a particular time during S-phase to be detected in the context of metaphase chromosomes so that its position can be correlated with standard banding locations. The results of experiments with these so-called “replication banding” methods consistently demonstrate that R-bands replicate in early S-phase while G-bands replicate in late S-phase (reviewed in Drouin et al. 1994). It is interesting to note that replication banding can also be detected in both cold-blooded vertebrates (Schmid and Guttenbach 1988) and plants (Sparvoli et al. 1994), in whose chromosomes G- and R-bands are not evident.

When well-extended metaphase chromosomes are studied using replication banding techniques, it is sometimes possible to detect as many as 1200–1300 bands (Drouin et al. 1990). Even at this high level of resolution, all bands appear to fire either in early or in late S-phase. With the synchronization protocol employed in these experiments, no bands were detected that replicated in both portions of S-phase (Drouin et al. 1990).

Taken together, these observations also lead to the conclusion that replicons are likely to be arranged in functional groups. At 1200–1300 bands per haploid human genome, the average band would contain 2.5 Mbp. Each band completes replication in half of S-phase (about 4 h), and human replication fork movement rates range from 0.6 to 3.6 kbp/min (Yurov 1980). Therefore, the largest replicons that could possibly exist within a human chromosomal replication band would correspond to the amount of DNA that could be synthesized by two forks moving at these rates for a full 4 h: between 290 kbp and 1.7 Mbp. Thus, each replication band must contain at least 2–9 replicons. If any of the replicons within the band should complete replication within less than 4 h, the number of replicons in the band would have to be even larger.

Replication foci

First observation

The evidence we have discussed so far makes a strong case for the existence of groups of adjacent replicons that fire at similar times in S-phase. None of this evidence, however, requires that the replication forks of the individual replicons within a group be clustered together in the nucleus. If DNA undergoing replication were decondensed to the same extent as 30 nm chromatin fibers (packing ratio ~40:1), then two adjacent origins spaced 100 kbp from each other along a DNA molecule could be as far apart from each other as 0.75 μm – easily resolvable by the light microscope. The first evidence suggesting that the replicons in a cluster may replicate in much closer proximity to each other came from studies by Nakamura et al. (1986), who observed that, when mammalian cells entering S-phase were labeled with BrdUrd for increasing lengths of time and then stained with anti-BrdUrd antibodies, replication appeared to start at ~126 discrete sites within the nucleus. A similar view of discrete, BrdUrd-labeled sites within a mammalian nucleus is shown in Fig. 2. Nakamura et al. (1986) observed that during the first hour of S-phase, the number of sites (also called replication foci, granules or domains) remained roughly constant, but each site became brighter and appeared to grow larger, first appearing globular and subsequently appearing to form “novel ring-like or sometimes horseshoe-shaped structures.” After about an hour, new small foci appeared in different positions, and the process repeated itself until the mature and replicating sites became so crowded that they were no longer resolvable. Based on the brightness of the apparently mature, ring-like regions compared with the brightness of mitochondrial DNA, Nakamura et al. (1986) deduced that each mature region contained at least 1 Mbp of DNA – in other words, at least ten replicons of 100 kbp.

That each of the mature regions studied by Nakamura et al. (1986) seemed likely to contain multiple replicons could also be inferred from the sizes of the mature re-

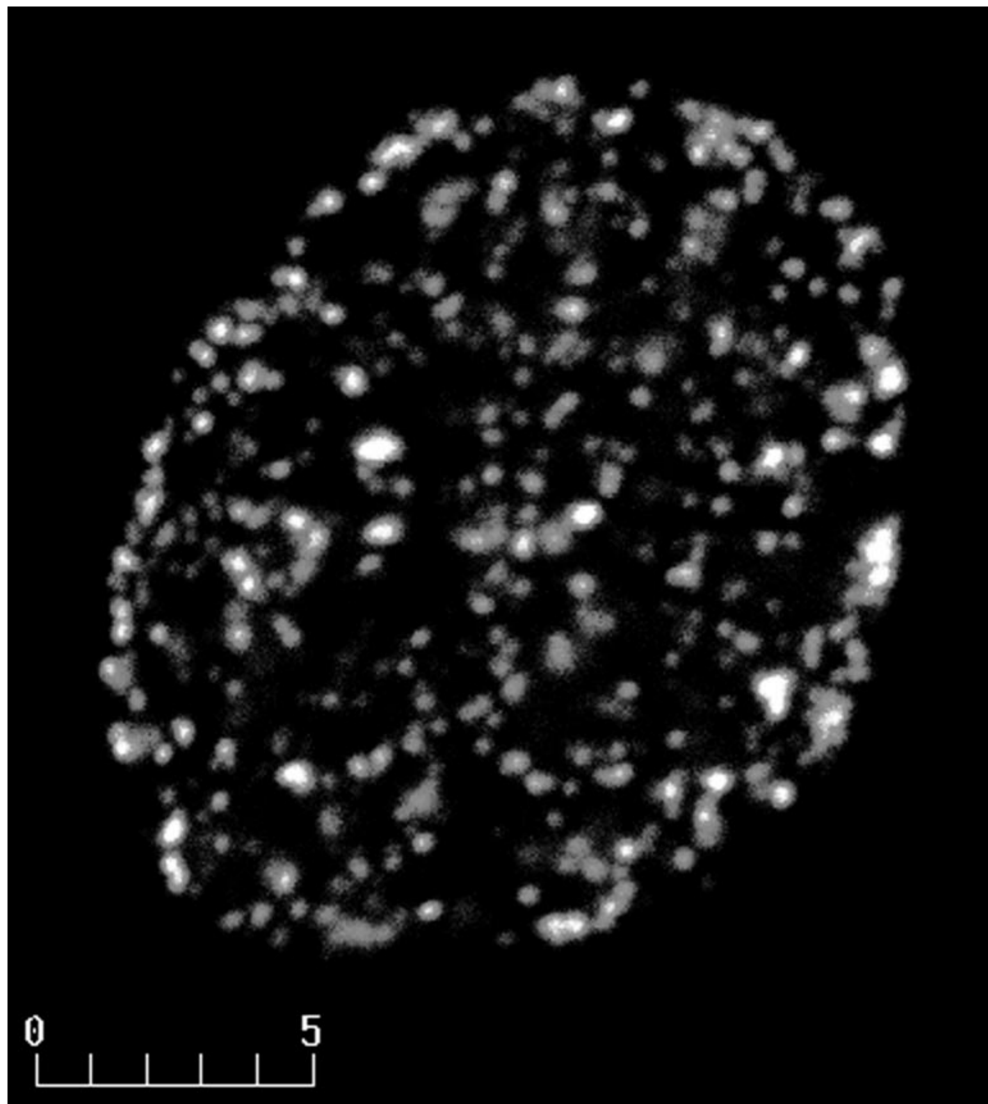

Fig. 2. Normal human diploid fibroblast cells (NHF-1) were grown on coverslips and pulsed for 5 min with 5-bromo-2′-deoxyuridine (BrdUrd). The image is a 0.5 μm section obtained by laser scanning confocal microscopy using Texas Red secondary antibodies following reaction with anti-BrdUrd monoclonal antibodies. The *bar* represents 5 μm in 1 μm divisions. This image is modified from Fig. 3A of Ma et al. (1999)

gions (≥1 Mbp), the apparent time required to reach maturation (~60 min) and the average bidirectional replication fork movement rate (1.5–3.4 kbp/min; Housman and Huberman 1975; Jackson and Pombo 1998) in early mammalian S-phase. These data suggested that the maximum size replicon capable of being completed within 60 min would be 90–200 kbp. Thus, each mature region ought to contain at least five such replicons.

Changes during S-phase

Subsequent experiments employing BrdUrd or other fluorescent labels have amply confirmed the major conclusion of Nakamura et al. (1986) – that replication takes place at discrete sites in the nucleus – but have raised questions about some of the details. Use of single- or double-color pulse labeling with variable chases between pulses, instead of or in combination with continuous labeling, and the use of laser scanning confocal microscopy or epifluorescence microscopy with sensitive CCD cameras plus techniques for removing interfering signals from neighboring focal planes have permitted development of a more detailed understanding of nuclear replication patterns during S-phase. All studies to date demonstrate that early S-phase replication sites are distributed throughout the nucleoplasm (with the exception of regions occupied by heterochromatin or nucleoli) as relatively discrete sites. The estimated number of sites varies considerably. While many reports estimate 100–350 sites in early S-phase (Nakayasu and Berezney 1989; van Dierendonck et al. 1989; Fox et al. 1991; Manders et al. 1992; Neri et al. 1992; O’Keefe et al. 1992; Hassan and Cook 1993; Manders et al. 1996), some of these measurements are likely to be underestimates due to analysis by epifluorescence microscopy of a single focal plane or to use of segmentation approaches following three-dimensional confocal microscopy, which grossly underestimate the number of sites (see Ma et al. 1998). Using an improved segmentation program, Ma et al. (1998) recently estimated over 1000 individual replication foci in early S-phase following a 5 min pulse of mouse 3T3 fibroblast cells, and Jackson and Pombo (1998) arrived at a similar number in HeLa cells using a

direct counting method following extensive spreading of the samples. Interestingly, some studies have indicated that in very early S-phase (e.g., the first 30 min) the number of foci is much smaller than later in early S-phase (van Dierendonck et al. 1989; Fox et al. 1991; Neri et al. 1992). Whether this corresponds to a special subpopulation of replication foci or results from asynchronous initiation within a larger population of early firing foci is an important question that deserves further attention.

All recent studies also indicate that replication patterns change in mid- and late S-phase. The number of replication foci associated with euchromatic regions in the nucleoplasm gradually drops to zero, while the number of foci associated with heterochromatic regions in the nucleoplasm, around the nucleoli, and at the nuclear membrane increases. The number of resolvable foci in mid- and late S-phase is generally smaller than in early S-phase, but the sizes of the mid- and late S-phase foci are frequently larger. Furthermore, many of the large *pulse-labeled* mid- and late S-phase foci have a ring- or horseshoe-like appearance (Nakayasu and Berezney 1989; O'Keefe et al. 1992), greatly resembling the ring- and horseshoe-like "mature regions" detected by Nakamura et al. (1986). This observation suggests that the large mature regions reported by Nakamura et al. (1986) may, in fact, have corresponded to mid- or late S-phase replication sites, which Nakamura et al. (1986) failed to resolve from early S-phase foci owing to their use of continuous labeling.

There is minor disagreement regarding the precise sequence of patterns in mid- and late S-phase. In some experiments, foci appear at the nuclear and nucleolar peripheries before they appear at internal heterochromatin blocks (Nakayasu and Berezney 1989; Manders et al. 1992; O'Keefe et al. 1992; Hassan and Cook 1993; Manders et al. 1996; Ma et al. 1998; Wei et al. 1998), while in other studies the heterochromatin at the nuclear periphery is the last to be labeled (van Dierendonck et al. 1989; Fox et al. 1991; Neri et al. 1992; Ferreira et al. 1997). While we do not know the reasons for these differences, the use of different cell lines and synchronization procedures offers one possible explanation. The important point, agreed on by all investigators, is that euchromatic regions in the nucleoplasm tend to replicate before heterochromatic regions, wherever those regions may be located. In mid-S-phase, overlap between these patterns is frequently detected. The nucleus shown in Fig. 2 is an example. In addition to the multiple, discrete foci distributed throughout the internal portion of the nucleus (typical of early S-phase euchromatic replication), there is also a concentration of foci near the heterochromatin-rich nuclear periphery (typical of mid-S-phase labeling, under these conditions). The apparent contradiction between the detection of overlap of early and late patterns in these replication foci experiments and the lack of overlap in the high-resolution replication banding experiments (Drouin et al. 1990) can probably be explained on the basis of the different synchronization protocols employed. The protocol employed for the replication banding experiments blocked replication at the transition between R- and G-band labeling (between early and late S-phase), and it permitted completion of R-band replication before G-band replication commenced (Drouin et al. 1990).

Adjacency of replicons within each focus

The phenomenon of replication foci provided strong support for the concept of replicon clusters but did not prove that the replicons within each cluster are adjacent to each other on chromosomal DNA molecules. Evidence for the adjacency of the replicons within individual foci comes from more recent experiments (Sparvoli et al. 1994; Jackson and Pombo 1998; Ma et al. 1998), which show that the signals from individual replication foci persist undiminished (except at the first mitosis, when signal strength is reduced by half) when the BrdUrd pulse label is followed by growth in the absence of BrdUrd through the subsequent phases of the cell cycle and then for numerous generations. The number of foci per daughter cell is decreased by approximately half at each generation after the second, consistent with semiconservative segregation of labeled chromatids to daughter cells. If non-adjacent replicons, even on the same chromatid, contributed significantly to individual replication foci, then, at each cell division, the processes of mitotic recombination (chromatid segregation and sister chromatid exchange) would be expected to reduce the signal within individual foci and to increase the total number of foci. This is not observed (Sparvoli et al. 1994; Jackson and Pombo 1998; Ma et al. 1998). Thus, it is most likely that, in the case of foci containing multiple replicons, those replicons are adjacent to each other on the same chromosomal DNA molecule.

Relationship to chromosomal replication bands

Consistent with the calculations that the average high-resolution replication band contains ~2.5 Mbp of DNA (see above) while the average replication focus contains only ~1 Mbp (Ma et al. 1998), the experimental results show that, when short pulse labeled early S-phase replication foci are chased into mitosis, multiple discrete spots are displayed on the metaphase chromosomes (Jackson and Pombo 1998; Ma et al. 1998). While none of the spots spans the width of the metaphase chromosome and forms a complete band, the individual replication foci labeled in early S-phase are often very close together in band-like array (see Ma et al. 1998). Longer periods of labeling (2 h) do generate complete replication bands (Zink et al. 1999). Moreover in the study of Ferreira et al (1997), labeled regions appear to form continuous chromosomal bands that correspond to R-bands following a pulse of only 15 min during early S-phase. Similar variation from band-like arrays of individual replication foci (Sparvoli et al. 1994) to apparently continuously labeled replication bands (Ferreira et al. 1997; D. Dimitrova and D. Gilbert, personal communication) has been reported for late S-phase. These variations in

packing of replication foci into bands are likely a reflection of the methods used for preparation of chromosomes, i.e., the relative degree of chromosomal swelling and/or the system of microscopy used for detection, where oversaturation of the signal or lower resolution would give the appearance of a continous banding pattern. Evaluating the data as a whole, however, we conclude that replication foci throughout S-phase are the basic subunits of chromosomal bands.

Conservation throughout the eukaryotic kingdom

Although the studies discussed so far have concentrated on mammalian cells, it is important to note that the phenomenon of replication foci appears to be a universal feature of eukaryotic DNA replication and nuclear structure. All features discussed so far, including changes in pattern of foci during S-phase consistent with later replication of heterochromatin and stability of foci through multiple cell generations, have been detected in pea root cells (Sparvoli et al. 1994). Although not as extensively studied, discrete replication sites have also been detected in yeast cells (Pasero et al. 1997).

Higher order chromatin structure

Additional evidence for a stable, conserved replication focus architecture

We noted above that, when replication foci are chased after labeling, spots with the approximate brightness and size of the pulse-labeled foci persist in all phases of the cell cycle for many generations. Additional evidence for a conserved structure for replication foci comes from observations on the lack of correlation between length of pulse label and focus size (Ma et al. 1998). It is important to emphasize that replication foci are heterogeneous in size. While the large majority of sites range between 0.3 μm and 0.5 μm in diameter, a smaller population of sites extend to over 1 μm (Ma et al. 1998; see example in Fig. 2). What is striking and surprising is that these dimensions do not change significantly when pulse time is increased from 2 to 30 min. The heterogeneous distribution of replication focus sizes remains roughly constant over this range of pulse times, but the signal strength per focus increases in proportion to pulse time (Ma et al. 1998). This phenomenon is not simply a consequence of attempting to make measurements near the resolution limit of light microscopy. The resolution limit (about 0.2 μm) is well below the dimensions of most replication foci.

Replication foci and chromosome territories

An entirely independent line of investigation – into the structure of chromosome territories – has also led to the suggestion that chromatin domains with the dimensions of replication foci (0.4–0.8 μm diameter) may be fundamental units of chromosomal architecture. Numerous observations (reviewed in Cremer et al. 1993) had suggested that individual mammalian chromosomes occupy distinct spaces in nuclei, called “chromosome territories,” and do not intermix. However, these experiments had all been carried out on cells that had been fixed and then subjected to DNA denaturation and other potentially disruptive steps of the fluorescent in situ hybridization (FISH) procedure. To find out whether chromosomes occupy distinct territories in unfixed, living cells, and, if so, what the structure of those territories might be, Zink et al. (1998) labeled the DNA of living human normal diploid and aneuploid cancer cells by microinjection of a fluorescent DNA precursor and then followed the fluorescent patterns as the cells continued to grow and divide in culture. The effective duration of labeling was not known, but the results suggest that label was incorporated over significant portions of single S-phases. Due to semiconservative DNA replication and random chromatid segregation, after a few generations most cells contained only a few (or even just one) labeled chromatids, and these could be seen to occupy distinct intranuclear regions. Furthermore, due to the absence of interfering signals from the other 45 chromosomes, details of chromatin structure could be perceived with unusual clarity by fluorescence microscopy of the living cells. Such microscopy revealed that the chromatid territories seemed to consist of chains of “beads” on strings, and the beads had diameters of 0.4–0.8 μm, similar to the diameters of replication foci. The authors refer to these beads as “subchromosomal foci” and suggest that they may correspond to the stable replication foci observed by others (Sparvoli et al. 1994; Jackson and Pombo 1998; Ma et al. 1998).

Essentially identical results were obtained when DNA was labeled with halogenated thymidine analogs (Zink et al. 1998, 1999). This procedure had the disadvantage that the labeled cells had to be fixed and treated with appropriate fluorescently labeled antibodies before the label could be detected, but it offered the advantage that the time of labeling could be controlled. When cells were labeled for 2 h with iododeoxyuridine (IdUrd), chased for 4 h and then labeled for another 2 h with chlorodeoxyuridine (CldUrd), IdUrd labeling took place during early S-phase and CldUrd labeling occurred during late S-phase in doubly labeled cells. When such doubly labeled cells were examined at metaphase, the positions of IdUrd-labeled and CldUrd-labeled foci corresponded to early replicating R-bands and late replicating G-bands, respectively. After several generations, when chromatid territories had segregated from each other, it could be seen that both the early IdUrd subchromosomal foci and the late CldUrd subchromosomal foci had similar sizes. Furthermore, even when labeling was extended to 16 h, the size distribution of subchromosomal foci was maintained (Zink et al. 1998). These experiments therefore seem to carry the observation of Ma et al. (1998) of pulse length independent replication focus size to new extremes. Zink et al. (1998, 1999) also observed that the early and late foci occupied distinct subdomains within chromatid territories and did not significantly overlap with each other.

Ma et al. (1999) have recently demonstrated that chromosome territories, like replication foci, remain intact after most conditions of extraction used in the preparation of nuclear matrices. However, extraction with the combination of RNase A and 2.0 M NaCl (but not RNase A and 0.65 M ammonium sulfate) leads to simultaneous disruption of both chromosome territories and replication foci. This observation suggests that a common subset of proteins is required to maintain both chromosome territories and replication foci.

Taken together, these observations suggest that replication foci are stable cell cycle-independent elements of chromatin structure in addition to representing intranuclear sites of DNA synthesis during S-phase.

Evidence suggesting that replicon clusters and replication foci may be more heterogeneous than previously suspected

In this section we present both new evidence and new interpretations of old evidence suggesting that the classic view of replicon clusters and replication foci is only partially correct. The range of replicon sizes is broader than previously thought. The number of replicons per cluster/focus is variable, and a significant proportion of replication foci are likely to contain just one replicon.

Uncertainties in replicon size measurements

As noted above, one of the lines of reasoning previously used to calculate the number of replicons per replication focus is based on estimates of replicon size from DNA fiber autoradiographic studies (Nakamura et al. 1986). As we have emphasized from the beginning (Huberman and Riggs 1968), these estimates are inherently inaccurate. The major reason for the inaccuracy of autoradiographic measurements of fiber replicon size is the extreme heterogeneity of such sizes. The smallest eukaryotic replicons have been detected by electron microscopy (Blumenthal et al. 1973) or two-dimensional gel electrophoresis (Hyrien and Mechali 1993), not fiber autoradiography. At ~10 kbp, they are well below the resolution level of the fiber autoradiographic technique (~30 kbp). The largest replicons so far detected by fiber autoradiography are more than 1 Mbp (Yurov and Liapunova 1977; Liapunova 1994), which is longer than many of the fragments into which mammalian chromosomal DNA is broken when it is prepared for fiber autoradiography (Huberman and Riggs 1966; Liapunova 1994). In other words, the measured range of replicon sizes is greater than 100-fold, and at both the small and large extremes it exceeds the range in which accurate measurements can be made by fiber autoradiography. Under these conditions the average replicon size measured by fiber autoradiography may bear little resemblance to the true in vivo average replicon size. Instead, the average replicon size measured by fiber autoradiography may simply reflect the range of sizes in which measurements of replicon size by fiber autoradiography can best be made.

Another indication that previous measurements of average replicon size have been misleading comes from the discovery that measurements of average replicon size give different results depending on the length of the pulse label used in the experiment. Independent experiments in the United States (Stimac et al. 1977) and in Russia (Liapunova 1994) both demonstrated that average replicon size appeared to be directly proportional to the length of the labeled DNA segments in tandem arrays of replicons like that in Fig. 1.

Of course, the average size of the replicons in a cell should be independent of the duration of pulse label or the length of DNA labeled. To explain this contradiction, Stimac et al. (1977) suggested that longer labeled DNA segments make it easier for the eye to span a gap and confidently conclude that two labeled segments are on a common underlying DNA molecule. In other words, the longer the segment of labeled DNA, the larger the replicons that would be included in the measurements. Liapunova (1994) offered an alternative explanation. She suggested that longer labeled segments would make it easier to distinguish between signals from parallel, co-aligned DNA molecules and signals from individual DNA molecules. When several molecules are co-aligned in a bundle (perhaps formed during drying on the surface of the microscope slide), their signals may add together and create the appearance of shorter inter-replicon distances. A third possible explanation is that smaller replicons may not be noticed because of being initiated and completed within the high specific-activity or low specific activity portion of the longer pulse. This effect would become more severe with longer pulse times. For example, all five of the small replicons in Fig. 3 appear to be capable of firing and terminating within a single hour. Thus, these five small replicons would probably be undetectable in experiments using pulse lengths of an hour or more.

Probably all three effects (unintentional exclusion of molecules with large unlabeled gaps, inclusion of measurements on molecular aggregates, and failure to resolve small replicons at longer pulse times) contribute to the phenomenon of proportionality between lengths of labeled segments and replicon sizes. The important point is that this variability in replicon size measurement means that it is likely that none of the previously reported average mammalian replicon sizes is correct.

Although previous conclusions about *average* replicon size are likely to be incorrect, it seems likely that at least some of the *individual* measurements are correct. In fiber autoradiography experiments occasionally two daughter molecules – still held together by replication forks – are sufficiently separated from each other that each sister can be resolved (example in Fig. 3). Under these conditions, one can be confident that the pattern of low and high grain density tracks on one daughter molecule is correct if it is identical to the pattern on its sister molecule (as in Fig. 3). Coincidence of the two patterns implies that each sister molecule is single and intact and is not the artifactual consequence of random molecular co-alignment. If a short pulse was used (as in Fig. 3), then one can also be relatively confident that small rep-

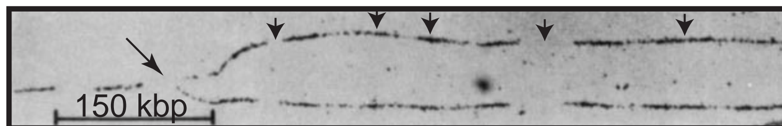

Fig. 3. Example of separated sister DNA molecules replicated by multiple small (<150 kbp) replicons. The *vertical arrows* show the deduced positions of origins. The *longer angled arrow* shows the position of one of the replication forks holding the sister molecules together. HeLa cells were grown as in Fig. 1 but without the addition of FdUrd or uridine. ^{3}H-thymidine (51 Ci/mmol; 100 μCi/ml) was added for 15 min. Then the medium was replaced with pre-warmed medium containing ^{3}H-thymidine at a lower specific activity (15 Ci/mmol; 100 μCi/ml). The remaining procedure was as in Fig. 1. This image is modified from Plate IXd of Huberman and Tsai (1973)

licons were not excluded. Figure 3 displays a pair of sister molecules in which the distances between origins (marked by short arrows) are all less than 150 kbp.

Evidence for a higher proportion of large replicons than indicated by early measurements

All of the earliest and many of the more recent measurements of replicon size by fiber autoradiography or fluorography were carried out on molecules displaying apparent tandem arrays of replicons, like those in Figs. 1 and 3. In addition to the measurement problems noted in the preceding three paragraphs, the exclusive use of such tandem arrays automatically excluded any replicons that were so large that signals from an adjacent replicon could not be aligned with them. Yurov and Liapunova (1977) pointed out that such tandem arrays represented a minority, only about 15% (Liapunova 1994), of the total autoradiographic signals in their experiments with human and Chinese hamster cells labeled with a hot ^{3}H-thymidine pulse (30 or 60 min) followed by a warm chase (150 or 120 min, respectively). By measuring replicon size in this subpopulation of tandemly arrayed replicons after this labeling protocol (total effective labeling time of 3 h), they obtained an average replicon size of 390 kbp – significantly longer than detected by other investigators, presumably because of the longer labeling time (as described above). They noted that another 15% of signals came from solitary symmetrical figures similar to region “a” in Fig. 1 (except for the absence of signal from adjacent replicons), consisting of a single high grain density track (or double track with intervening empty space as in Fig. 1a) flanked on both ends by low grain density tracks. These solitary figures corresponded to bidirectional replication from single origins. The lengths of such regions (240–960 kbp; average about 450 kbp) provided minimum estimates of the sizes of these large replicons. Larger sizes might have been detected if the warm pulse time had been longer.

The majority of signals in the experiments of Yurov and Liapunova (1977) came from solitary asymmetric figures like region “b” in Fig. 1 (except for the absence of signal from adjacent replication forks). These solitary figures corresponded to replication by single forks during both the hot and warm pulses. It was not possible to say whether these forks represented unidirectional replication or single halves of figures like “a” (Fig. 1) with very large spaces between the two halves. The average size of these abundant figures was 330 kbp. If these figures represented halves of bidirectional replicons, then the average size of such replicons would have to be considerably greater than 660 kbp.

Yurov and Liapunova (1977) detected these large replicons because they extended their measurements to solitary – not tandem – figures (similar to “a” and “b” in Fig. 1) that had been ignored by other investigators. In addition, they employed an effective pulse time of 3 h, longer than employed by other investigators. As discussed above, the use of long pulse times can obscure the presence of smaller replicons that initiate and complete their replication during the pulse. It is unlikely, however, that fusion of small replicons contributed significantly to the apparent large replicons detected by Yurov and Liapunova (1977), because comparison of the lengths of the hot and warm tracks in each of their solitary figures suggested that each was the product of replication forks moving at constant speed. Fusion of smaller replicons would have produced irregularities in apparent rate of replication fork movement.

Thus the studies of Yurov and Liapunova (1977; reviewed in Liapunova 1994) provide strong evidence that many mammalian replicons are larger than previously suspected. What is not clear, however, is what proportion of the total replicon population is formed by such large replicons. Small replicons are more easily detected with short pulses (Fig. 3, for example), and larger replicons more easily detected by longer pulses. The smallest replicons are too short to be detected by fiber autoradiography, and the largest are comparable in size to the largest DNA fragments recoverable from cells under the conditions used for fiber autoradiography. As a consequence of these measurement difficulties, a reliable estimate of the proportions of mammalian replicons of various sizes is not yet available.

Despite this uncertainty about the proportions of small and large replicons, independent methods based on determining origin location, origin efficiency, replication fork direction(s) and replication timing have recently provided additional evidence for the existence of large replicons in mammalian cells. For example, analyses of fork directions in the human dystrophin gene reveal that it is organized into six replicons ranging in size from 170 to more than 500 kbp (Verbovaia and Razin 1997). In CHOC 400 cells, the dihydrofolate reductase (DHFR) gene and its flanking sequences, a segment totaling 220–260 kbp, are amplified about 500-fold, primarily as head-to-head and tail-to-tail tandem repeats (Looney and Hamlin 1987). Measurements of origin locations, origin efficiencies and fork directions by two-dimensional gel

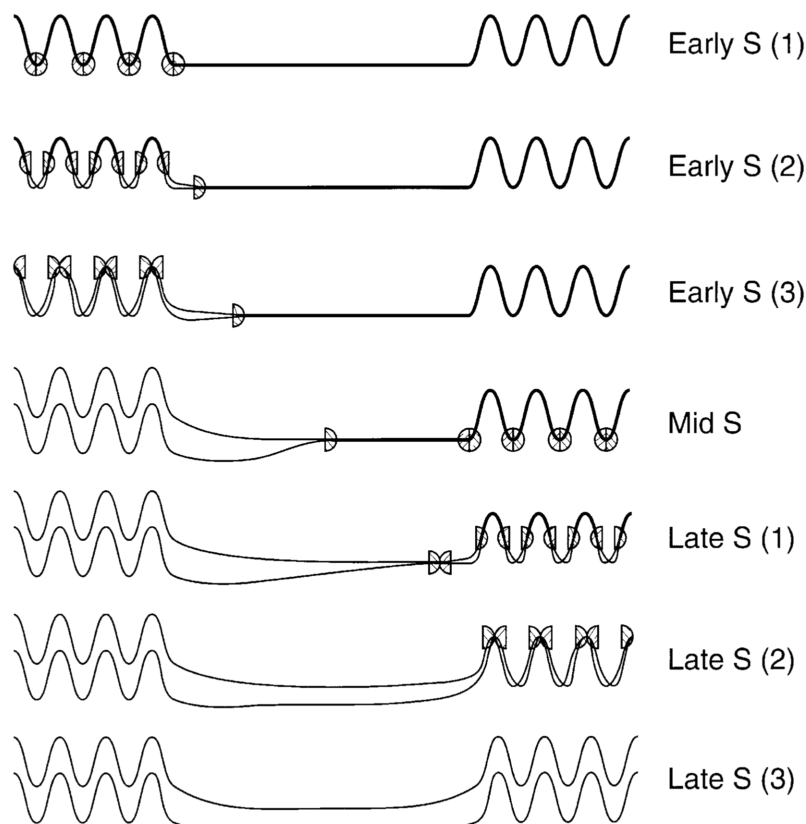

Fig. 4. A diagram that, in speculative fashion, attempts to explain the sequence of events during replication of the murine IgH region (Ermakova et al. 1999) or similar regions where an early replicating replicon cluster is joined to a late replicating cluster by a long segment of DNA that is replicated by a single fork. The unreplicated DNA is shown with a *thick line*; replicated portions are represented by *thinner lines*. The *small semicircles with internal diagonal lines* represent assemblies of enzymes needed for DNA synthesis at replication forks. Each semicircle contains sufficient enzymes for a single fork. See the text for additional details

electrophoresis show that within this region replication initiates primarily in a 55-kbp segment downstream of the DHFR gene (Vaughn et al. 1990; Dijkwel and Hamlin 1992). In any one S-phase initiations are estimated to take place in only about 15% of the repeat units (Dijkwel and Hamlin 1992; Dijkwel et al. 1994). The remaining repeat units are passively replicated by forks coming from active origins in flanking repeats (Dijkwel et al. 1994). Thus the effective replicon size for the DHFR amplicons appears to be (~240 kbp)/0.15 or ~1.6 Mbp. In Chinese hamster cells, the maximal bidirectional rate of fork movement is about 6 kbp/min (Housman and Huberman 1975). At this rate, these large DHFR replicons would be completed in ~4.5 h.

Ermakova et al. (1999) recently combined detailed replication timing analyses with measurements of fork direction to obtain evidence that, in most mouse cells, the immunoglobulin heavy chain (IgH) region is part of a large asymmetric or unidirectional replicon. The 400 kbp IgH region is bounded on one side by a region of at least 250 kbp that is uniformly replicated in very early S-phase and on the other by a region of at least 600 kbp that is uniformly replicated in late S-phase. The IgH region itself is replicated by a single fork travelling from the early replicating region to the late replicating region. The fork direction and timing assays suggest that both the early and late replicating flanking regions are replicated by forks from several small, synchronously firing origins. In other words, the flanking regions appear to be replicated by the mechanism first suggested by fiber autoradiography experiments – clusters of small, synchronously firing origins. The results indicate that the IgH-proximal origin in the early cluster sends its IgH-proximal fork through the IgH region, and that fork progresses until, in late S-phase, it meets a fork coming from the IgH-proximal origin in the late-firing cluster (approximately as shown in the diagram in Fig. 4).

Evidence for frequent asynchrony in the firing of adjacent origins

Evidence for significant synchrony in the firing of neighboring origins within tandem arrays is apparent in many fiber autoradiographic experiments (reviewed in Hand 1978). For example, a certain amount of synchrony is evident in Fig. 1. All three origins (marked by arrows) fired before the start of the high specific activity pulse. At the same time, it is also obvious that synchrony was not perfect in Fig. 1. The origins appear to have fired one after the other in order from left to right. A more obvious example of asynchronous replicon firing is shown in Fig. 3, where two of the origins (first and fourth from the left) fired before the high specific activity pulse, while the other three origins (second, third and fifth from the left) fired during the high specific activity pulse. However, because the pulse length was only 15 min in this experiment, it is likely that all five origins fired within 30 min of each other.

Dubey and Raman (1987b) attempted to quantitate the degree of synchrony of origin firing in various tissues of the mole rat. By pairwise comparisons between

neighboring origins in tandem arrays of three to eight replicons, they found that the majority (55%–77%) of pairs fired within 30 min of each other, but a significant minority (23%–45%) did not.

Thus the generalization that neighboring replicons in tandem arrays fire synchronously is only partially valid. Perfectly synchronous initiations are rare, but imperfect synchrony (initiation within the same 30 min interval) is common. However, asynchronous initiations (greater than 30 min apart) take place about one-third of the time. The relationships between degree of synchrony and organization into replication foci are not yet clear.

Heterogeneity of focus size and brightness suggests a variable number of replicons per focus

As is evident from Fig. 2, replication foci differ from each other in both size and brightness. Ma et al. (1998) have used a spot-based segmentation algorithm to assist in quantitating the differences in size. Their measurements reveal a heterogeneous volume distribution with a predominance (~90%) of smaller and intermediate volumes (0.04–0.33 μm^3) and a small but reproducible population (~10%) of larger (up to ~0.8 μm^3) foci. Jackson and Pombo (1998) independently quantitated brightness per replication focus and found a range of tenfold or more. What could be the sources of these variations?

The brightness of each focus should be proportional to the amount of DNA synthesized in the focus during the pulse. Thus for those foci that are active during the entire pulse, brightness should be directly proportional to both the replication fork rate and the number of replicons in the focus. The distribution of brightnesses measured by Jackson and Pombo (1998) is heavily skewed to lower brightnesses, consistent with the skewed distribution of replicons per cluster measured by the same authors using fiber fluorography. In contrast, the distributions of replication fork rate frequencies measured by fiber autoradiography (Housman and Huberman 1975; Yurov 1980) are more symmetric. Thus it seems likely, as concluded by Jackson and Pombo (1998), that the major determinant of the variability of replication focus brightness is variations in the number of replicons per focus. In other words, a replication focus with a single active replicon of 200 kbp would be less intensely labeled than one with ten active replicons of 20 kbp each.

It is reasonable to think that the heterogeneity of sizes measured for replication foci is directly related to heterogeneity in the amount of DNA present within each focus, assuming a similar packing density for the DNA contained within each site. Since the average focus has a volume of ~0.2 μm^3 and contains ~1 Mbp of DNA (Ma et al. 1998), it can be calculated that the entire volume range of ~0.04–~0.4 μm^3 (which accounts for nearly the entire population of replication foci) corresponds to a range of DNA content for each site from ~0.2–~4 Mbp. Thus the largest foci contain much more DNA than even the largest replicons (up to 1.7 Mbp; see above) and therefore must contain more than one replicon. The smaller foci may contain variable numbers of replicons of variable size, with the constraints that the total mass of DNA per focus should be sufficient to account for focus size and the number of replicons per focus should be sufficient to account for focus brightness.

Some replication foci may contain just a single replicon

Several lines of reasoning lead us to consider that some replication foci may contain just a single replicon. First, as noted above, the simplest explanation of the large variations in replication focus size and brightness is that different foci contain different numbers of replicons. Second, if replication focus architecture is sufficiently flexible that different foci can have different numbers of replicons, then there is no obvious a priori reason why foci with just one replicon should not be possible. Third, the fact that current technology is sufficiently sensitive to detect single stretched-out DNA molecules labeled with BrdUrd (Jackson and Pombo 1998) suggests that there should be no problem in detecting single replicons inside nuclei, where the signal is expected to be more concentrated owing to chromatin compaction. However, since no one has yet demonstrated a single replicon in a replication focus, it cannot be ruled out that many single-replicon-containing foci are not detected under the conditions used for microscopy and image collection. Fourth, as the apparent average size of replicons has become larger due to expansion of possibilities for identifying and characterizing large replicons (see above), and as the apparent average number of foci per cell has become larger owing to use of more sensitive detection methods, the calculated need for large numbers of replicons per focus has diminished. In fact, if the average size of a mammalian replicon is 500 kbp rather than 100 kbp (see above), and the average number of foci per cell is ~10,000 (~1000 foci completing replication every 45 min during an 8 h S-phase; Ma et al. 1998), then the average number of replicons required per focus to replicate a 10 Gbp mammalian diploid genome is just two! Thus, although there are good reasons to think that, in many cases, replication foci are likely to contain multiple replicons (see above), it now seems possible that a significant portion of replication foci may contain single replicons.

The implications of pulse-chase-pulse experiments

The finding that certain antibodies raised against BrdUrd-containing DNA can discriminate between DNA labeled with CldUrd and DNA labeled with IdUrd (reviewed in Manders et al. 1992) has permitted sophisticated comparisons between the replication foci labeled within a single cell at different times during S-phase. This is accomplished with a pulse-chase-pulse protocol, in which a pulse with one of the halogenated thymidine analogs, say CldUrd, is followed by a chase of variable length, which is then followed by a second pulse, this time with the other analog (IdUrd in our example). The cells are then fixed and stained with modified antibodies

that fluoresce in different colors. For example, the anti-CldUrd antibody might produce green fluorescence, and the anti-IdUrd antibody might produce red fluorescence. Manders et al. (1992, 1996) and Ma et al. (1998) have used this approach with relatively short chase periods in an attempt to learn when one set of replication foci completes replication and a new set begins. The results show that, if the chase time is zero, there is essentially complete overlap (as expected) between the first (green) and second (red) pulse labels, but as the pulse length increases, the amount of overlap diminishes, reaching a low background level in less than 1 h.

Although the experimental results are simple and reproducible, they lend themselves to more than one interpretation. Here we present two alternative views. Due to uncertainties in measurements of proportions of replicons of various sizes (mentioned above) and to uncertainties about the relative intranuclear locations of specific DNA sequences, it is possible for each of these alternative interpretations to be both self-consistent and consistent with what is known about replicon size distributions and the arrangement of DNA in the nucleus. When more is learned about replicon size distributions and intranuclear DNA positions and their relationships to replication foci, it will become possible to distinguish between these alternatives. We also note that the two views presented here are not mutually exclusive and may provide equally valid interpretations of reality, albeit with different emphases.

Interpretation A: the brighter foci contain multiple replicons, and these replicons complete synthesis within 45 min

In a particularly thorough pulse-chase-pulse study, Ma et al. (1998) demonstrated that the brighter replication foci in mouse 3T3 fibroblasts in early S-phase behave like a homogeneous population with a replication lifetime of 45 min. That is, red and green signals were completely separated from each other when the chase time was longer than 45 min. Furthermore, the yellow signals (where red and green overlapped) displayed the same size constraints as the signals produced by simple pulses: varying chase time affected the amount of yellow signal but not the size distribution of yellow signals (Ma et al. 1998). This argues strongly that each replication focus is an independent structural unit that increases in signal intensity but not in size until it completes replication. In other words, absence of significant yellow after 45 min implies completion of replication in 45 min, not movement of DNA labeled in the first pulse away from DNA labeled in the second pulse. The latter possibility might well require an increase in replication focus size with increasing pulse time, which is not observed (Ma et al. 1998). This interpretation is equally consistent with the experimental results of Manders et al. (1992, 1996), who showed loss of most yellow overlap signals in less than 60 min in V79 Chinese hamster cells.

Based on this interpretation, a significant portion of mammalian replicons should be smaller than ~150 kbp, the size of replicons that could be completed in 45 min at an average early S-phase bidirectional replication rate of 3.4 kbp/min (Jackson and Pombo 1998). The presence of larger replicons is compatible with this view of the experimental results if most of the larger replicons are in single-replicon foci, because single-replicon foci would be expected to give faint signals and may not have been detected by the threshold-based methods employed by Manders et al. (1992, 1996) or the direct visual method used by Ma et al. (1998) for this experiment. Indeed, since single-replicon-containing replication foci have yet to be identified, it is conceivable that all such foci, if they exist, may not be detectable by current methods.

This view of the data is also compatible with the distribution of large and small replicons suggested by the detailed analysis of replication of the murine IgH region by Ermakova et al. (1999). The IgH genes are part of a large (~400 kbp) segment of DNA that is replicated by a single fork, from one end to the other, during most of S-phase (see the generalized diagram in Fig. 4). This interpretation assumes that the replication focus containing that single fork would have been too faint to be included in the measurements (Manders et al. 1996; Ma et al. 1998). The 400 kb IgH segment is flanked on each side by segments containing multiple smaller replicons that complete replication in a small portion of S-phase (the data are compatible with completion within 45 min). It seems likely that each of these two multi-replicon flanking segments may have given rise to a single replication focus, which, due to its content of multiple replicons, would have given brighter signals and therefore would have been included in the study (Manders et al. 1996; Ma et al. 1998).

Interpretation B: some replicons complete replication in 45 min, but others do not

According to interpretation B, the disappearance of overlap between green and red foci after a 45 min chase is attributable to two different effects. The first is identical to that described above for interpretation A and accounts for the fact that in many cases after a chase of 30–60 min, green and red foci appear far from each other (Manders et al. 1996; Ma et al. 1998). The distances of these foci from each other suggests that the first-pulse foci that are far from second-pulse foci must have completed replication during the first pulse or during the chase, and the second-pulse foci that are far from first-pulse foci must have initiated replication during the chase or second pulse, as proposed by interpretation A.

The second effect proposed by interpretation B is different from interpretation A. The second effect is proposed to explain the fact that, in many other cases after chases of 30–60 min, green and red foci appear relatively close to each other (Manders et al. 1996; Ma et al. 1998), and the average distance between them appears to increase with chase time. Indeed, computer-based analysis by Manders et al. (1996) of the distances (in three dimensions) between green and red foci as a function of chase time suggests that *on average* red and green foci separate

from each other at a rate of ~0.5 μm/h when the chase time is varied from 0 to 65 min. Note that Manders et al. (1996) did not necessarily detect moving foci. Their data do not distinguish between continual motion of the first- and second-pulse foci with respect to each other and the transient appearance of new second-pulse foci at increasing (but fixed) distances from the first-pulse foci as chase time is increased. Since we are not aware of experimental observations suggesting movements of replication foci, we favor the second alternative, which is consistent with the possibility that some large replicons may extend across more than one replication focus. If this is the case, then some foci labeled during the first pulse would necessarily remain physically close to some foci labeled during the second pulse.

Note that the data of Manders et al. (1996) can also be explained by interpretation A, which would suggest that separate replication foci that overlap temporally in activity may frequently be close together spatially. The increased distance that Manders et al. (1996) measured may, therefore, be a consequence of new sites of replication that become spatially more distant as the chase time between the two pulses increases. Thus while juxtapositioning of replication foci offers the possibility of single large replicons extending across more than one replication focus, this possibility remains to be demonstrated experimentally.

Both interpretations A and B are based on the stability of replication focus structure summarized earlier in this review. Recall that the studies of Zink et al. (1998, 1999) suggest that individual chromosomes occupy discrete portions of the nucleus (chromosome territories) and consist of linear fibers with the appearance of strings of closely packed beads, and the beads have diameters typical of replication foci (0.4–0.8 μm).

Let us assume for purposes of discussion that these beads are, indeed, replication foci at the time when the DNA in them is replicated, and that they are stable elements of chromatin structure at all times. Exactly how DNA is packed within these beads/foci is not known, but one can imagine the DNA being packed in such a way that, even after pulses as short as 1 min, the newly labeled DNA would be distributed throughout the volume of the bead. At longer pulse times, the newly synthesized DNA would continue to be restrained within the volume of the bead, until all of the DNA in the bead had been replicated. This would explain the observed pulse-length independence of replication focus volume (Ma et al. 1998; Zink et al. 1998).

To illustrate how some large replicons might extend across two or more beads/foci, consider the diagram in Fig. 4, which generalizes the situation described by Ermakova et al. (1999) – the case of a large DNA segment separating an early replicating cluster from a late replicating cluster. It seems likely that the early replicating and late replicating clusters would each form their own beads and function as independent replication foci, but what about the long intervening DNA segment? We suggest that this segment may also form one or more beads with their own fixed structure. The results (Ermakova et al. 1999) indicate that the origin responsible for replicating the long segment is part of the early replicating cluster. Thus, if our supposition that the early replicating cluster and long segment form different beads is correct, the replicon of which the long segment is a part should extend into at least two adjacent beads, possibly into a third (the bead formed by the late-firing cluster).

Based on these considerations, the following scenario seems likely in the case of the IgH region described by Ermakova et al. (1999) and similar situations. A replication focus containing a cluster of small, early firing replicons completes replication in early S-phase (Fig. 4, early S). A replication fork at an end of the cluster continues on into the next replication focus. In this particular case, the proposed replication focus contains only half a replicon – just a single replication fork – and is active in mid-S-phase (Fig. 4, mid S) as well as in portions of early and late S-phase. In late S-phase, the adjacent replication focus containing a cluster of small, late-firing replicons initiates replication, and forks from the long segment and the late cluster meet and terminate (Fig. 4, late S).

This example (Fig. 4) deals with the case of replicons so long that a large portion of S-phase is required to replicate them completely. One can imagine, however, that there may be a limit to the length of DNA that can fit within a single bead/focus, and this length limit may dictate that all replicons requiring more than 45 min to complete their replication must extend across more than one bead. This would simultaneously explain the observation that a significant portion of second-pulse foci appears close to first-pulse foci (Manders et al. 1996; Ma et al. 1998) and the observation that replication foci possess pulse length-independent volumes (Ma et al. 1998) that are preserved during the cell cycle (Jackson and Pombo 1998; Ma et al. 1998). This interpretation can accommodate replicons of any size, because large replicons can be packed into multiple beads/foci. The only constraint is that, in the case of the brighter beads/foci, the amount of DNA and number of replication forks per focus must be consistent with completion of replication in that focus within 45 min (Ma et al. 1998).

In summary, we propose two models (which are not mutually exclusive) for replication foci. These models combine the properties of (i) an approximately 45 min time limit for replication at brighter foci and (ii) the likely presence of much larger replicons that take much longer than 45 min to complete replication. According to the first model, the larger replicons produce fainter signals and thus were not included in the measurements, suggesting a 45 min maximum time to complete replication in most foci. According to the second model, single large replicons may be packaged into two or more replication foci. Organization of large replicons into multiple foci may provide a means for organizing and coordinating their replication through S-phase.

Conclusions

We hope that the evidence we have reviewed has convinced the reader, as it has us, that it is no longer accu-

rate to describe replication foci as representing exclusively clusters of relatively large numbers of relatively small replicons. Indeed, many foci probably do contain large numbers of small replicons, but it also appears that some foci contain just one replicon. The data suggest that there is a continuum in the number of replicons per focus, ranging from one to ten or more, but the low numbers (one to five replicons per focus) predominate (Jackson and Pombo 1998). Whatever the number of replicons per focus, the data suggest that foci are stable chromatin structures that persist during the entire cell cycle, even in mitosis (Sparvoli et al. 1994; Ferreira et al. 1997; Jackson and Pombo 1998; Ma et al. 1998). Thus all chromatin fibers appear to be strings of structures with the same size and shape as the replication foci visualized by pulse labeling with a DNA precursor (Zink et al. 1998), and these structures may be stabilized by the same small set of proteins that stabilizes chromosome territories (Ma et al. 1999). Moreover recent data suggest that individual replication foci are further organized into even higher order chromatin domains or “nuclear replication zones” that are completely segregated spatially from similarly organized “transcription zones” (Wei et al. 1998).

Both older fiber autoradiographic data and newer results obtained with modern replicon-mapping methods provide evidence for the existence and relative abundance of replicons much larger than previously considered – replicons so long that most or all of S-phase is required to complete them. It is equally clear that these large replicons co-exist in the same cell with smaller replicons, including some so small that only minutes are needed for their replication. Current methods do not allow determination of the relative abundances of replicons of different sizes, but it is probably a mistake to continue assuming that the average replicon size in mammalian cells is ~100 kbp. The average is likely to be larger.

The data show that many replication foci fire during early S-phase, while many others fire in late S-phase. Although these two sets of foci share similar sizes, they occupy different locations in the nucleus and on chromosomal bands. Thus there is an intimate relationship between replication foci and control of replication timing. Virtually nothing other than phenomenology is currently known about control of replication timing in mammalian cells; this is clearly an important area for future research.

Obtaining a full understanding of replicons and replication foci will also require learning more about the nature of mammalian replication origins, about relationships between replication and transcription, about the effects of transcriptional regulation on replication control and about the arrangement of DNA/chromatin in the interphase nucleus.

The studies we have reviewed point to extreme heterogeneity within the mammalian nucleus with regard to replicon sizes, replicon clustering and replication foci. The only way in which the full extent of this heterogeneity and its biological relevance will be revealed and understood is through numerous detailed studies at the level of small regions (a few adjoining replication bands, perhaps) within individual chromosomes. These studies will need to combine replicon and timing analyses (similar to the work of Ermakova et al. 1999) with combined FISH and BrdUrd labeling to identify the specific DNA sequences and replicons present in single replication foci. Much remains to be done, but the fascinating results obtained in the studies reviewed here suggest that the story will continue to be interesting and exciting as we continue to make progress and obtain a more detailed understanding of replicons in relationship to replication foci.

References

Blumenthal AB, Kriegstein HJ, Hogness DS (1973) The units of DNA replication in *Drosophila melanogaster* chromosomes. Cold Spring Harbor Symp Quant Biol 38:205–223

Cremer T, Kurz A, Zirb el R, Dietzel S, Rinke B, Schröck E, Speicher MR, Mathieu U, Jauch A, Emmerich P, Scherthan H, Ried T, Cremer C, Lichter P (1993) The role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harbor Symp Quant Biol 58:777–792

Dijkwel PA, Hamlin JL (1992) Initiation of DNA replication in the dihydrofolate reductase locus is confined to the early S period in CHO cells synchronized with the plant amino acid mimosine. Mol Cell Biol 12:3715–3722

Dijkwel PA, Vaughn JP, Hamlin JL (1994) Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain. Nucleic Acids Res 22:4989–4996

Drouin R, Lemieux N, Richer C-L (1990) Analysis of DNA replication during S-phase by means of dynamic chromosome banding at high resolution. Chromosoma 99:273–280

Drouin R, Holmquist G, Richer C-L (1994) High resolution replication bands compared with morphologic G- and R-bands. Adv Hum Genet 22:47–115

Dubey DD, Raman R (1987a) Do sister forks of bidirectionally growing replicons proceed at unequal rates? Exp Cell Res 168:555–560

Dubey DD, Raman R (1987b) Factors influencing replicon organization in tissues having different S-phase durations in the mole rat, *Bandicota bengalensis*. Chromosoma 95:285–289

Edenberg HJ, Huberman JA (1975) Eucaryotic chromosome replication. Annu Rev Genet 9:245–284

Ermakova OV, Nguyen LH, Little RD, Chevillard C, Riblet R, Ashouian N, Birshtein BK, Schildkraut CL (1999) Evidence that a single replication fork proceeds from early to late replicating domains in the IgH locus in a non-B cell line. Mol Cell 3:321–330

Ferreira J, Paolella G, Ramos C, Lamond AI (1997) Spatial organization of large-scale chromatin domains in the nucleus: a magnified view of single chromosome territories. J Cell Biol 139:1597–1610

Fox MH, Arndt-Jovin DJ, Jovin TM, Baumann PH, Robert-Nicoud M (1991) Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in mouse fibroblasts. J Cell Sci 99:247–253

Hand R (1978) Eucaryotic DNA: organization of the genome for replication. Cell 15:317–325

Hassan AB, Cook PR (1993) Visualization of replication sites in unfixed human cells. J Cell Sci 105:541–550

Housman D, Huberman JA (1975) Changes in the rate of DNA replication fork movement during S phase in mammalian cells. J Mol Biol 94:173–181

Huberman JA, Riggs AD (1966) Autoradiography of chromosomal DNA fibers from Chinese hamster cells. Proc Natl Acad Sci USA 55:599–606

Huberman JA, Riggs AD (1968) On the mechanism of DNA replication in mammalian chromosomes. J Mol Biol 32:327–341

Huberman JA, Tsai A (1973) Direction of DNA replication in mammalian cells. J Mol Biol 75:5–12

Hyrien O, Mechali M (1993) Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of *Xenopus* early embryos. EMBO J 12: 4511–4520

ISCN (1995) An international system for human cytogenetic nomenclature. S. Karger, Basel

Jackson DA, Pombo A (1998) Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. J Cell Biol 140:1285–1295

Liapunova NA (1994) Organization of replication units and DNA replication in mammalian cells as studied by DNA fiber autoradiography. Int Rev Cytol 154:261–308

Looney JE, Hamlin JL (1987) Isolation of the amplified dihydrofolate reductase domain from methotrexate-resistant Chinese hamster ovary cells. Mol Cell Biol 7:569–577

Ma H, Samarabandu J, Devdhar RS, Acharya R, Cheng P-C, Meng C, Berezney R (1998) Spatial and temporal dynamics of DNA replication sites in mammalian cells. J Cell Biol 143: 1415–1425

Ma H, Siegel AJ, Berezney R (1999) Association of chromosome territories with the nuclear matrix: disruption of human chromosome territories correlates with the release of a subset of nuclear matrix proteins. J Cell Biol 146:531–541

Manders EMM, Stap J, Brakenhoff GJ, van Driel R, Aten JA (1992) Dynamics of three-dimensional replication patterns during the S-phase analyzed by double labelling of DNA and confocal microscopy. J Cell Sci 103:857–862

Manders EMM, Strap J, Strackee J, van Driel R, Aten JA (1996) Dynamic behavior of DNA replication domains. Exp Cell Res 226:328–335

Nakamura H, Morita T, Sato C (1986) Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. Exp Cell Res 165:291–297

Nakayasu H, Berezney R (1989) Mapping replicational sites in the eucaryotic cell nucleus. J Cell Biol 108:1–11

Neri LM, Mazzotti G, Capitani S, Maraldi NM, Cinti C, Baldini N, Rana R, Martelli AM (1992) Nuclear matrix-bound replicational sites detected in situ by 5-bromodeoxyuridine. Histochemistry 98:19–32

O'Keefe RT, Henderson SC, Spector DL (1992) Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences. J Cell Biol 116:1095–1110

Pasero P, Braguglia D, Gasser SM (1997) ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei. Genes Dev 11:1504–1518

Schmid M, Guttenbach M (1988) Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 97:104–114

Sparvoli E, Levi M, Rossi E (1994) Replicon clusters may form structurally stable complexes of chromatin and chromosomes. J Cell Sci 107:3097–3103

Stimac E, Housman D, Huberman JA (1977) Effects of inhibition of protein synthesis on DNA replication in cultured mammalian cells. J Mol Biol 115:485–511

van Dierendonck JH, Keyzer R, van de Velde CJH, Cornelisse CJ (1989) Subdivision of S-phase by analysis of nuclear 5-bromodeoxyuridine staining patterns. Cytometry 10:143–150

Vaughn JP, Dijkwel PA, Hamlin JL (1990) Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell 61:1075–1087

Verbovaia LV, Razin SV (1997) Mapping of replication origins and termination sites in the Duchenne muscular dystrophy gene. Genomics 45:24–30

Wei X, Samarabandu J, Devdhar RS, Siegel AJ, Acharya R, Berezney R (1998) Segregation of transcription and replication sites into higher order domains. Science 281:1502–1505

Yurov YB (1980) Rate of DNA replication fork movement within a single mammalian cell. J Mol Biol 136:339–342

Yurov YB, Liapunova NA (1977) The units of DNA replication in the mammalian chromosomes: evidence for a large size of replication units. Chromosoma 60:253–267

Zink D, Cremer T, Saffrich R, Fischer R, Trendelenburg MF, Ansorge W, Stelzer EHK (1998) Structure and dynamics of human interphase chromosome territories in vivo. Hum Genet 102:241–251

Zink D, Bornfleth H, Visser A, Cremer C, Cremer T (1999) Organization of early and late replicating DNA in human chromosome territories. Exp Cell Res 247:176–188