

Mechanisms regulating S phase progression in mammalian cells

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1. ABSTRACT

Cell proliferation demands that identical genetic material is passed to daughter cells that form during mitosis. Genetic copies are produced during the preceding interphase, when DNA of the mother cell is copied exactly once. While few processes in biology are regulated with this precision, the fundamental importance cannot be understated as defects might compromise genetic integrity and ultimately lead to cancer. Replication of the human genome in diploid cells occurs during S phase of the cell cycle. Throughout this ~10h period, about 10% of replication units – replicons – are active at any time, even though all potential initiation sites – origins – are established before the onset of S phase. Crucially, the mechanisms that regulate origin selection and define a structured replication programme remain to be defined. We review recent progress in understanding the structure and regulation of S phase and develop a model that we believe best describes the S phase programme in human cells.

2. INITIATING DNA REPLICATION

The sheer complexity of the replication process is evident from the size of the genetic complement in human cells – proliferating human cells have a diploid (2n=46) genome of roughly 6x10⁹bp DNA. Inevitably, this demands that the duplication process proceeds from numerous points – about 50,000 are used in each cell cycle – that are scattered, ideally uniformly, throughout the genome (1,2). Such synthetic initiation points, termed origins of DNA synthesis, are of fundamental importance in defining the efficacy of the replication process as they provide targets for binding of the replication machinery and a molecular function called replication licensing, which ensures that DNA is replicated once and only once during each cell division cycle (3).

2.1. Replication origins and initiation of DNA synthesis

Studies on the budding yeast *S. cerevisiae* provided an early paradigm for eukaryotic replication

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origins (2,4,5). In this simple, unicellular eukaryote replication origins are defined genetically and function outside their normal chromosomal context as autonomous replicating sequence (ARS) elements. Each element with replicator activity contains an ARS consensus sequence, which serves as a binding site for the origin recognition complex (ORC). The ORC complex directs assembly of pre-replication complexes (pre-RCs), which also contain assembly factors Cdc6 and Cdt1 and the likely replicative helicase, the Mcm2-7 complex. This pre-replication complex (pre-RC) is assembled during G1 phase of the cell cycle and serves to direct a single round of DNA synthesis at the appropriate time of the cell cycle, when activating Cdk/cyclin complexes, together with other replication factors such as Cdc45, drive assembly of the replication machinery at the target pre-RCs. Mcm2-7 complexes are incorporated into the competent synthetic machinery and as a result are displaced from the pre-RC once synthesis proceeds. Crucially, as further assembly of pre-RCs is precluded at this time of the cell cycle, the displacement of Mcm complexes from origins during synthesis ensures that each pre-RC can perform only a single initiation event during each cell cycle.

This basic molecular machinery is preserved in all eukaryotes. However, key features of the initiation process are very different from this simple case in cells from multi-cellular eukaryotes. Perhaps the most profound difference is the mechanism by which sites are selected for the initiation of DNA synthesis (2,5,6). Notably, there is little compelling evidence to support the existence of genetically defined replicator elements that allow identification of replication origins in higher eukaryotes. Instead, the replication machinery is believed to assemble at genomic locations that are defined by a combination of epigenetic features, which include genome structure, chromatin status and complex architecture of chromosome folding in nuclear space. A small number of origins have been defined with molecular precision but these appear to be the exception rather than the rule. Of these, the human lamin B2 origin is perhaps the best-characterised (7). The lamin B2 origin is located in an intergenic region of 1.2 kb, which lies between the 3' end of the lamin B2 gene and the promoter of the adjacent TIMM13 gene. This region operates efficiently as a replication origin when transferred to ectopic chromosomal loci (8) and activity depends on an AT-rich ~70 bp OPR (origin region that is shown by DNase footprinting to be protected *in vivo*) and adjacent CpG island. While single copy integrants maintain replication origin activity in 13 out of 16 clones analysed, altering the structure of the ectopic origin emphasises the sensitivity of origins to complex epigenetic regulation, which is apparent from the variable origin efficiency at different integration sites as a result of chromosomal position effects (8).

2.2. Initiation as a key determinant of mammalian S phase

The density of potential replication origins will define the efficiency of S phase. Even in budding yeast it is known that not all potential origins are used in every cell cycle (9,10); some origins are very efficient but others work in a minority of cycles. The redundancy of replication

origins is especially obvious in metazoan cells, where pre-RC complexes are loaded onto chromatin in >10 fold excess relative to the number of sites where DNA synthesis is activated.

As origins appear to operate stochastically, key insights into the behaviour of individual origins have relied on single molecule analyses, on isolated DNA fibres. Use of DNA fibres to monitor replicon structure and double labelling, with BrdU and IdU, to monitor replicon structure in consecutive cell cycles, was first described by Jackson and Pombo (11). This work provided compelling evidence that structurally stable replicon clusters generate DNA foci that represent both structural and functional sub-chromosomal units (Figure 1). Using double labelling, the same cohort of replicon clusters was seen to replicate at the onset of S phase in different cell cycles. This observation defined the concept of a structure programme of replication of DNA foci, which was later confirmed and extended by Sadoni *et al* (12). In the active clusters, individual replication zones were seen to be activated with good efficiency, but with low spatial resolution (Figure 1; 11). Similar conclusions were later reached by Okumura and colleagues (13), who used biotin-dUTP and digoxigenin-dUTP in consecutive cycles to demonstrate that while individual foci were labelled at the same time of S phase in consecutive cycles the precision of initiation was such that only 30% of replicons were activated from origins that lay within 10kb while 50% were 10-50 kb apart.

Stretched DNA fibres have also been used by Bensimon and colleagues to analyse the structure and activation of replicons at defined gene loci (14,15). In these very elegant studies, DNA FISH (using fragmented probes to generate recognition 'bar-codes') is combined with double CldU and IdU labelling to define the structure of active replicons (Figure 2). These experiments make a number of key points:

- 1 – At a specific locus, assuming the selected locus is typical of any selected at random, sites where DNA synthesis can begin are not specific but appear to be selected at random from within zones that typically measure ~10kb in length (14). These zones represent possible origins within regions that appear to contain a number of potential origins within a replication licensing group. One technical caveat warrants consideration here. DNA fibres labelled with halogenated precursors are only detected if DNA is first denatured, typically using acid or nuclease treatment. Following indirect immuno-labelling, the resultant signal is usually visualised as tracks of spots (see Figures 1&2), which define the zones of incorporation. Because of this appearance, and the fact that local DNA sequence can also influence the spreading properties of DNA fibres, it is difficult to make measurements with exceptionally high precision. At some locations, this limitation might give the impression that a single initiation sites might appear to extend over 5-10kbp when many sites from different cells are compared. That being so, it is also clear from biochemical studies on cell populations that many loci can activate DNA synthesis over broad initiation zones.

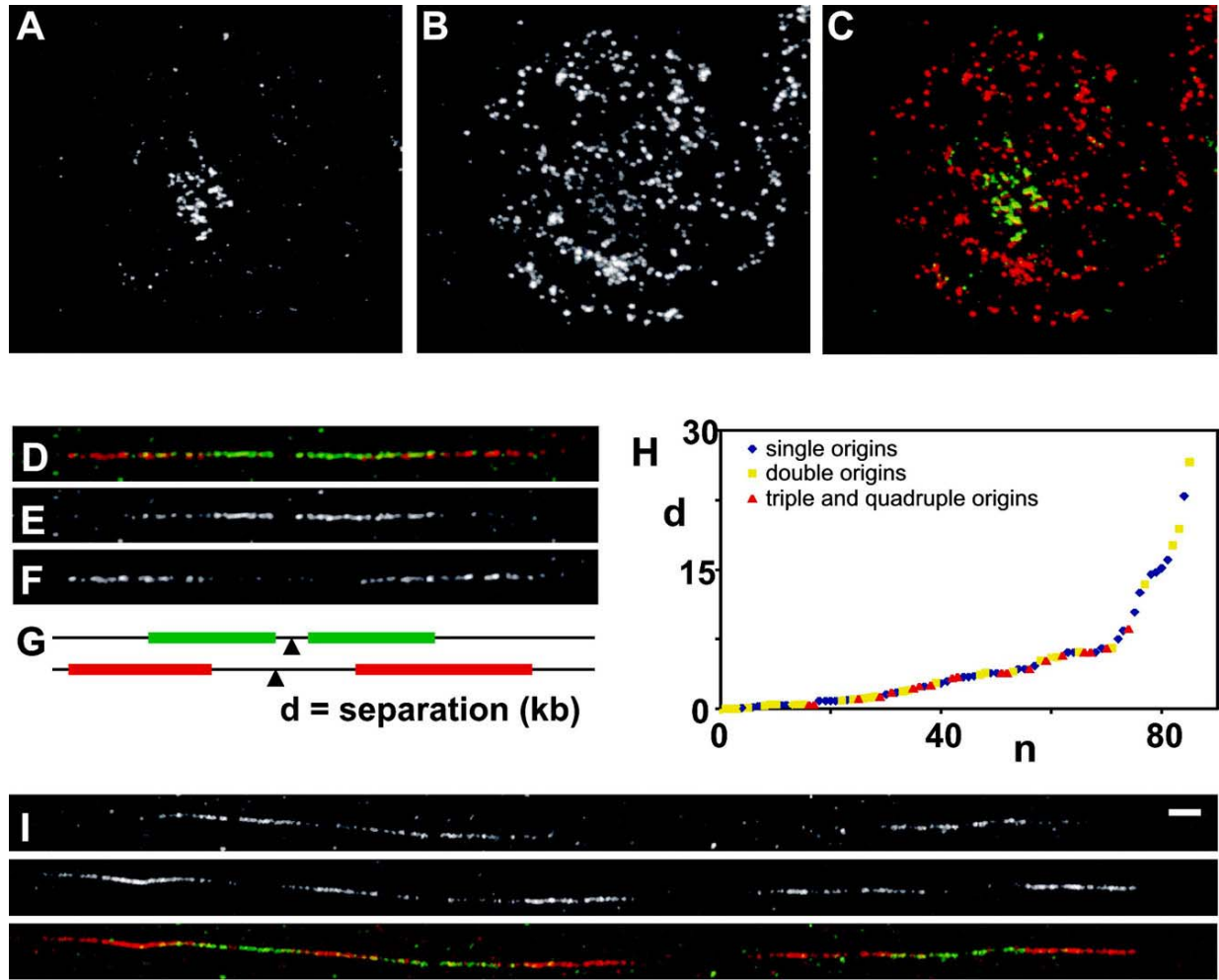


Figure 1. Replicon clusters are functional units of DNA synthesis. Human HeLa cells were synchronised with aphidicolin to block at the onset of S phase. Medium was replaced to remove aphidicolin and active replicons pulse labelled (20 min) with bromo-deoxyuridine (BrdU). Cells were grown in fresh medium for 4 days in order to resolve individual chromosome territories and the synchrony and pulse labelling protocol repeated using iodo-deoxyuridine (IdU). Sites of incorporation were visualised by confocal microscopy after indirectly immuno-labelling. (A-C) show an equatorial confocal section that contains a single BrdU-labelled territory (A in green) and a normal early S phase pattern labelled with IdU (B in red). A merged image (C) shows that the BrdU-labelled foci were also labelled with IdU, indicating that the same early replicating foci are activated in different cycles. (D-I) Samples were labelled with BrdU and IdU in consecutive cycles, after synchronising at the onset of S phase as above. Active replicons (eg D-F; D is a merge of the single channels in E and F) were visualised after indirect immuno-labelling of individual DNA fibres. In the first cycle, BrdU pulse-labelling was performed between 5-20 min after releasing cells into S phase; this leaves an unlabelled region that contains the replication origin (E). In the subsequent cycle, IdU pulse-labelling was performed 10-30 min after release (F). Overlapping double-labelled tracks define replicons that were active at the onset of S phase in both cell cycles. The labelled tracks of double-labelled replicons were measured and the boundaries of the tracks used to define sites of initiation (G). The location of initiation sites (ie active origins) in the two cycles was defined and the separation (d in kb) measured. Values of d for 85 well-defined double-labelled fibres are shown (H); analysis focussed on replicon clusters that contained 2 (squares) and 3 or 4 (triangles) active replicons though for comparison some isolated (diamonds) origins were also scored. While about $1/3^{\text{rd}}$ of active replicon initiate within $\sim 5\text{kb}$ in the two cycles (this represents the resolution limit of the technique) the majority are clearly activated at different but adjacent locations, indicating the plasticity of origin selection in these cells. Despite this stochastic element to the initiation process, the analysis of active replicons in replicon clusters showed that $\sim 90\%$ of replicons were active in both cycles (I), when S phase synchrony was performed with aphidicolin. (I; see ref 11 for further details). Bar in I represents 15kbp. A-C and I reproduced with permission from Rockefeller University Press.

2 – Though there are excellent examples where replication origins can be located close to or within promoters (5), more commonly the potential origins or

licensing groups are located in intergenic regions (14). This might imply that chromatin architecture restricts the loading of the ORC and Mcm complexes within transcribed

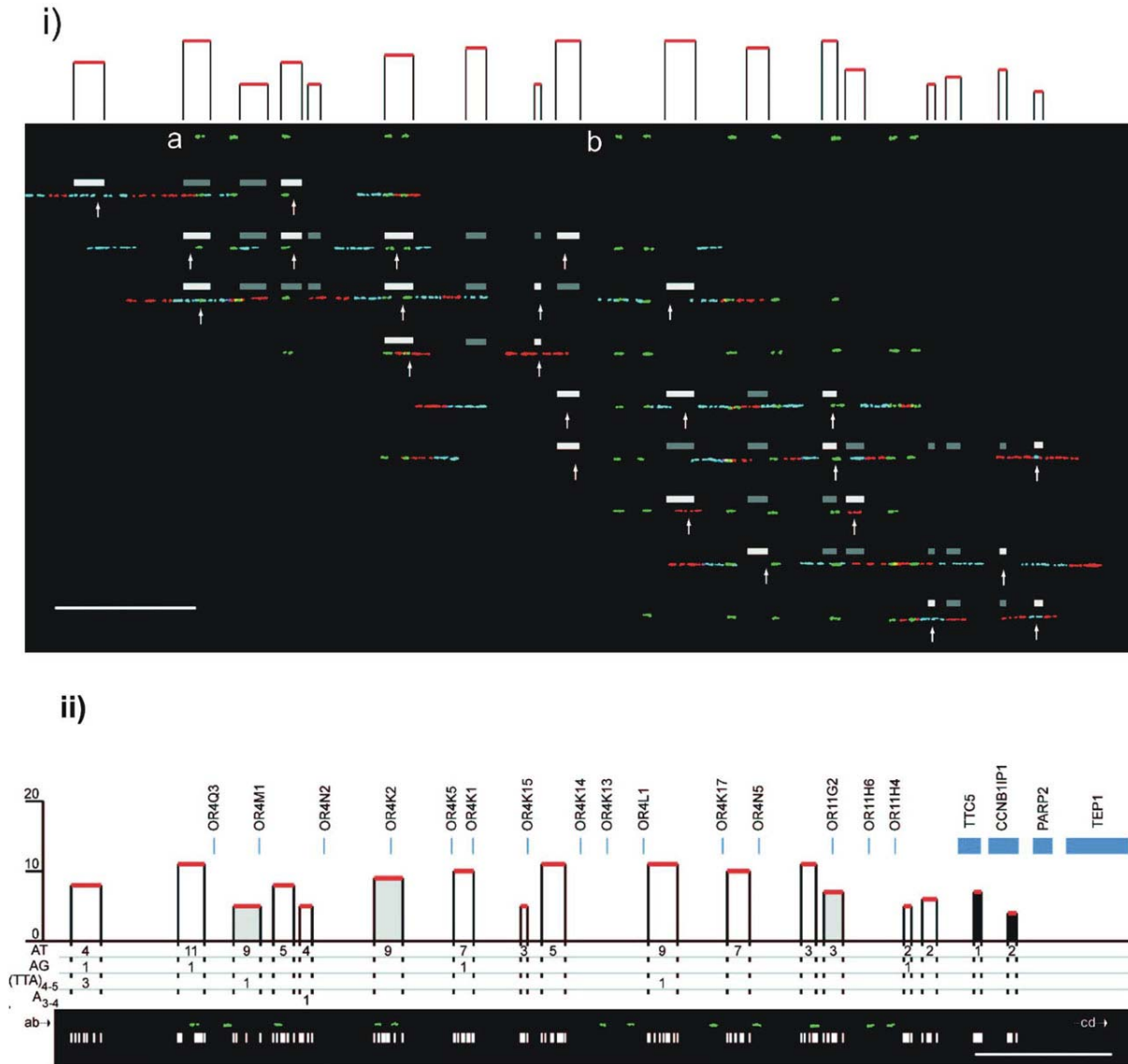


Figure 2. Using DNA fibres to analyse the redundancy of replication origins and synchrony of origin activation. Normal human primary keratinocytes were cultured and pulsed labelled (20 min) consecutively with IdU and chloro-deoxyuridine (CldU). DNA was purified and single molecules spread on a glass surface by ‘DNA combing’. Stretched DNA fibres covering ~1Mb of chromosome 14q11.2 (i) were selected after labelling by fluorescent *in situ* hybridisation (using fragmented probes to generate recognition ‘bar codes’; shown green) and indirect immuno-fluorescence to identify regions of IdU (shown blue) and CldU (shown green) incorporation. Panel i) shows a gallery of replicating fibres, which are aligned using the FISH signals. Replicon structures are designated using IdU and CldU signals to define active origins (white arrows) and growing forks. Note that in many fibres adjacent origins are activated at similar times, though this is not always the case. Active origins are localised to replication initiation zones (white blocks), which represent licensing groups. The structure of these potential replication origins is shown in (ii) and aligned relative to the position of known genes and sequence motifs that might contribute to origin function. Note that only a fraction (about 1/3rd) of potential origin clusters is active in each replicating fibre; the inactive origins appear to be suppressed by an active origin interference mechanism. Reproduced from (14) with permission from The American Society of Cell Biology.

regions of the genome or alternatively that active transcription tends to displace any pre-RCs that are loaded within transcribed domains.

3 – During initiation, adjacent active origins tend to be activated at similar times (Figure 2). In almost all

cases the sister forks from active origins grow at similar rates (11,15) until synthesis of the intervening DNA is complete; only in rare instances are origins within replicon clusters seen to fire at substantially different times. This coupling of fork speed at individual origins, and even between adjacent origins within replicon clusters, appears

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to be an intrinsic property of the replication machinery and may relate to the functional coupling of synthetic replisomes within individual factories (15). However, the rate of synthesis shows some level of plasticity, which allows replication fork rate to accelerate in regions between widely spaced adjacent origins. This implies the existence of homeostatic regulation to accommodate the wide variation in replicon size and origin activation efficiency and at the same time ensure that replication is performed within the required cell cycle period (15). The deterioration of sister fork coupling in cells from Werner's Syndrome patients hints at the complexity of the replication process and molecular mechanisms that might contribute in coupling activities at sister forks (16).

4 – Only about 1 in 3 of the potential initiation zones function in each cell cycle (14). In the primary human cells used in this study, the distance between central positions of the licensing clusters in the chosen locus is, on average, 40.6 \pm 20.7 kb (min = 14.3 kb; max = 93.1 kb) with a mean inter-origin spacing of 113 \pm 66.4 kb. Activated origins appear to be selected at random so that different combinations of active origins are seen in different cells. Moreover, once an initiation zone is used to activate synthesis, the local adjacent zones show a lower frequency of activation. This leads to the concept of origin interference, and adds a spatial or architectural component to the initiation process.

2.3. Possible roles for excess potential replication origins

It has been known for some time that higher eukaryotes have evolved with a 10-20 fold excess of potential replication origins, defined by those that are used or not used in a particular cell cycle (17). This excess includes both supernumerary pre-RCs within licensing groups that provide active origins and also within licensing groups that are not used in a particular cell cycle (14; Figure 2). As, intuitively, this would seem to be extremely wasteful, we might assume that the over-abundance of potential origins has some important physiological role. What might that be? A number of possibilities are worthy of consideration. First, if initiation of replication occurs stochastically, but with modest efficiency, it might be necessary to have an over-abundance of potential targets in order to activate the required density of origins to perform synthesis during the desired time frame. Second, if ORCs are deposited at random on DNA the over-abundance might ensure that there are no excessively long spaces between adjacent origins which would compromise the efficiency of S phase. In addition, over-abundance might be required to ensure appropriate licensing of different classes of chromatin – eg euchromatin and heterochromatin – if the licensing machinery is able to assess relatively open, euchromatin, with comparative ease. Third, latent potential origins might operate to provide secondary sites of initiation if for any reason the forks from the active origins fail to complete synthesis – for example during replication on a damaged template.

2.4. Mechanisms of origin interference and suppression

The use of origin activation mechanisms that utilise an excess of pre-initiation complexes, which

function as potential origins, raises the question of whether a specific mechanism is used to regulate origin activation and suppression. An alternative hypothesis would be that active origins are selected from a pool of potential origins using a stochastic mechanism, and that local adjacent pre-RCs (potential origins) are displaced passively during fork progression. In fact early observation on labelled DNA fibres are not consistent with the latter view, as even in replicon clusters with large inter-origin spacing the majority of active origins are seen to be synchronised temporally and the activation of later initiation events within the active clusters occurs only rarely (11,14,18).

In nuclei assembled using *Xenopus* egg extract, it has been proposed that ATR/Chk1 regulate the sequential activation of early and late replication origins (19,20) by suppressing the activation of late origins during early S phase. This is potentially exciting with respect to S phase regulation (21), though it is worth reflecting on the relevance of these cell free experiments to somatic cells and the fact that key experiments were performed by altering the natural balance of components involved in synthesis. Whether checkpoint proteins such as ATR and Chk1 support checkpoint function and also contribute to S phase regulation is another question. The accepted function of these proteins is to ensure the preservation of genomic integrity under conditions of replicative stress. Hence, if DNA is damaged during S phase, activation of the intra-S phase checkpoint maintains replication fork stability and blocks initiation of latent replication origins (22,23). During the damage response, activation of Chk1 leads to phosphorylation of Cdc25, which prevents downstream activation of the Cdk/cyclin complexes that are required to activate latent origins. Perhaps, by maintaining the physiological balance of this pathway, Chk1 could contribute to the regulation of DNA synthesis in the absence of replicative stress (24).

In somatic mammalian cells, loss of Chk1 function correlates with a slight (20-30%) increase in global replication (25,26), consistent with Chk1 contributing to the regulation of DNA synthesis. However, to complicate matters, recent experiments have suggested that the effect of depleting Chk1 activity is to reduce the global rate of replication fork progression by about 50% (27) while increasing the density of active origins by 2-3 fold (18). These observations appear to support the general model that the ATR/Chk1 pathway controls the activation and suppression of replication origins to regulate the density of active origins throughout S phase of somatic vertebrate cells (28).

Even so, it is important to identify key differences between the studies on embryonic (*Xenopus*) and somatic (mammalian) systems. In the embryonic system, assembled nuclei have simple chromatin architecture, no transcription and a very rapid cell cycle, with an S phase of ~30 min and correspondingly short replicons of ~15kb (29). While replicon clusters are activated at different times during this short S phase (29), it is unknown if this is of physiological significance or – more likely – reflects the stochastic nature of activating

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replicon clusters in this system. In clear contrast, somatic mammalian cells have been shown to have well-established S phase programmes with specific regions of the genome replicated at different times (11,12). Progress through the programme can be correlated with the spatial architecture of the active replication sites (30,31) and concomitantly correlated with the assembly of different classes of chromatin at different times during synthesis (32,33).

In the *in vitro* *Xenopus* system, the temporal activation of replicon clusters is lost if ATR function is inhibited using caffeine (20). In human cells, in contrast, S phase timing is preserved when ATR function is compromised, with super-activation of latent origins being restricted to regions of the genome that are already engaged with active replication factories (18). Hence, while caffeine treatment might be expected to override Chk1-dependent suppression of late replicons in an early S phase cell (19) the late origins of somatic human cells are not activated because they are unable to access active replication sites. This implies that the temporal programme of replication factory assembly plays a dominant role in defining which potential origins can be activated at different times of S phase.

2.5. Checkpoint proteins function to regulate initiation during normal S phase

Experiments described above suggest that Chk1 contributes to the efficacy of replication at active replication forks and couples defects in synthesis at active forks to the local activation of nearby latent origins. This implies that even during a normal S phase, a Chk1-dependent system continually senses the status of active replicons within replication factories and monitors the number of active forks to ensure progress of the synthetic process. The molecular mechanism for this is unclear, though it may not be unreasonable to propose some links to the role of ATR/Chk1 during the checkpoint response (22). When DNA is damaged, for example by irradiation with uv light, damage induced perturbation of fork progression is thought to result in uncoupling of the leading and lagging strand synthesis and alteration in the amount of RPA bound to DNA at the compromised forks. The altered structure of these forks then signals to ATR/ATRIP and activates Chk1. Activated Chk1 operates on CDC25 proteins to prevent subsequent initiation events.

Throughout S phase, mammalian nuclei appear to have mechanisms that maintain the balance of replication by altering the density of active origins if the rate of DNA synthesis is changed (15,27,34). For example, human cells with Chk1 function depleted by RNAi or specific inhibition display a ~50% decrease in the rate of replication elongation and corresponding >2 fold increase in origin density (27). This ensures that the duration of S phase is maintained. Speculating on a model to account for this, we are attracted to the notion that mammalian cells have an innate means of balancing fork rate and origin density using a mechanism that is related to the checkpoint function. Perhaps replication forks with only slightly different rates of elongation have sufficiently different amounts of a replication protein – such as RPA – to allow

activation/suppression of different origin densities. As a hypothetical model, we might compare optimal and reduced rates of synthesis; the optimal rate might have more uncoupling of leading and lagging strand synthesis and therefore more associated RPA, which in turn would activate a feedback loop to suppress additional origin activation. Though mechanistically similar to the checkpoint regulation system, this process would appear to be spatially restricted to operate at the level of individual replication factories.

An important feature of this model is the provision of latent origins, which are suppressed during normal synthesis but serve to rescue replication if for any reason the active forks might fail (35,36). In fact, it has been known for many years that immediately prior to S phase, replicons have ~10-20 fold excess of potential replication origins – defined by Mcm2-7 complexes – which are clustered into licensing groups (see above). The redundant Mcm complexes are displaced from chromatin during replication, but prior to being displaced these complexes appear to provide a rescue function if active forks are aborted because of DNA damage. This means that under normal conditions only a minority of potential origins is actually used, and that a mechanism must operate to limit origin activation so that once the required number of origins has been activated the use of proximal potential origins is suppressed.

3. THE S PHASE PROGRAMME

The replication phenotype of Chk1-deficient avian cells (DT40 and primary fibroblasts) alludes to a possible role for Chk1 in regulating S phase progression in higher eukaryotes. This appears to be consistent with the observed regulatory role for ATR and Chk1 during *Xenopus* development (21,28) but contrary to the completely normal pathway of S phase progression in human cells with altered Chk1 function (18). This difference between somatic human and avian cells raises interesting questions concerning the evolution of S phase regulation and possible complementation of the different regulatory proteins in different organisms. Perhaps intriguingly, a fascinating recent paper describing S phase progression in fission yeast has shown that loss of the S phase checkpoint function (provided by Cds1 kinase activity – which is homologous to human Chk2 protein and compliments some intra-S phase Chk1 activity) results in a profound loss of structure of the S phase programme in this organism (37).

S phase in *S. Pombe*, at ~75min under normal growth conditions, is much shorter than the equivalent cell cycle period in mammalian cells. Even so, Meister *et al* (37) were able to show that like mammalian cells, fission yeast have a structured S phase, which can be broken down into 4 distinct intervals using morphological features (number, size, nuclear location) to describe the structure of the replication factories. An elongated S phase with the same characteristic patterns is seen when wild type cells are treated with hydroxyurea, to induce replicative stress. However, when stress is induced in cells that lack

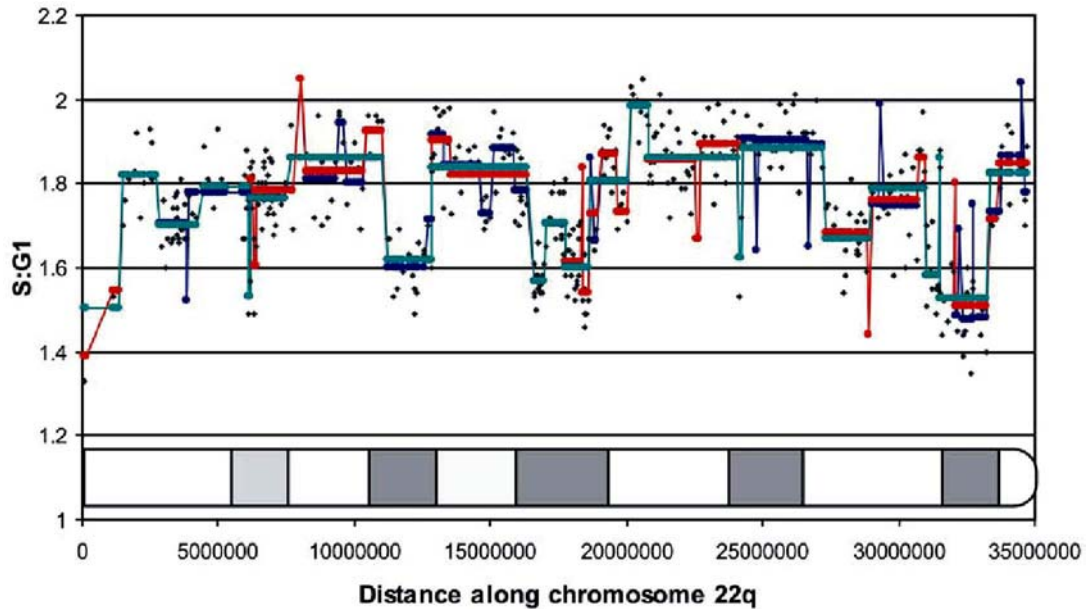


Figure 3. Genome-wide analysis of replication to demonstrate timing within the S phase programme. This analysis compares the optimal segmentation of replication timing across human chromosome 22q (top) with the chromosome banding pattern (below; R bands are shown white and G bands in grey tones). DNA was isolated from unsynchronised lymphoblastoid cells in G1 and S phase. Purified DNA from each cell population was fluorescently tagged, probed to a 22q tile path array and the signal ratios determined – sequences with ratios close to 2:1 represent loci that replicate early in S phase and those with ratios nearer to 1:1 replicate late in S phase. Raw timing data (points) were clustered into timing zones using a segmentation analysis. See Woodfine *et al* (44) for further details. Reproduced with permission from Oxford University Press.

expression of the intra-S checkpoint kinase Cds1 the replication factories appear to be dismantled. Moreover, even in the absence of damage the replication foci of *cds1* cells assume a novel organisation, which suggests that Cds1 activity might contribute to the spatio-temporal organisation of replication in wild type cells.

In a similarly intriguing recent report (38), the budding yeast checkpoint kinase RAD53 (the fission yeast Cds1 homologue) was shown to couple the cell division cycle to the circadian cycle. In this study, yeast cells were grown under nutrient-limited conditions than mimic growth in the wild. Under these conditions, cells rhythmically oscillate between glycolysis and respiration, with a period of 4 to 5 hours. During this metabolic cycle, the cell division cycle is constrained to the reductive phase, with DNA replication occurring only during periods of glycolysis. The observation that a checkpoint kinase serves to synchronise the cell cycle and metabolic cycle may provide insight into the evolution of the regulatory networks that, at the same time, control DNA synthesis and preserve genome integrity.

3.1. Spatial nuclear architecture and S phase progression

While some function of the checkpoint proteins, notably ATR and Chk1, might operate to regulate S phase progression in *S. pombe* (37), *Xenopus* embryonic development (28) and even somatic avian cells (18), this seems not to be the case in mammalian cells, where S phase progression appears to involve alternative or additional

mechanisms (18). In fact, almost nothing is known about the molecular mechanisms of S phase progression in mammalian cells. Few basic facts are robust. Notably, it has been known for many years that early replication of gene rich chromosomal R bands occurs before heterochromatic G band replication takes place (39). Hence, while there are some exceptions, most transcribed genes replicate in early S phase and late replicating DNA contains few active genes (Figure 3). This temporal organisation also correlates with a spatial architecture of characteristic early, mid and late S phase patterns (30), which reflect the structure and location of euchromatin and heterochromatin in mammalian cells (Figure 4). Physiologically, a strict temporal programme, which in some regions of the genome can be reprogrammed during cell commitment and differentiation (6,40), may be important in maintaining epigenetic programming if replication timing correlates with maintenance or inheritance of different classes of chromatin (32,33).

In somatic human cells, S phase will typically take ~10 hours. Throughout S phase, the average replicon size is ~150 kbp. With an average synthetic rate of 1.5 kbp/min/fork, this means that synthesis of a typical replicon will complete in ~1 h (11,15). The analysis of nascent replication intermediates, DNA fragments that contain replication bubbles and the appearance of duplicated DNA foci at different times of S phase confirm that defined chromosomal loci have a tendency to replicate at preferred times during S phase (41-43). An elegant recent study from Joyce Hamlin and colleagues (42) emphasises this point. In

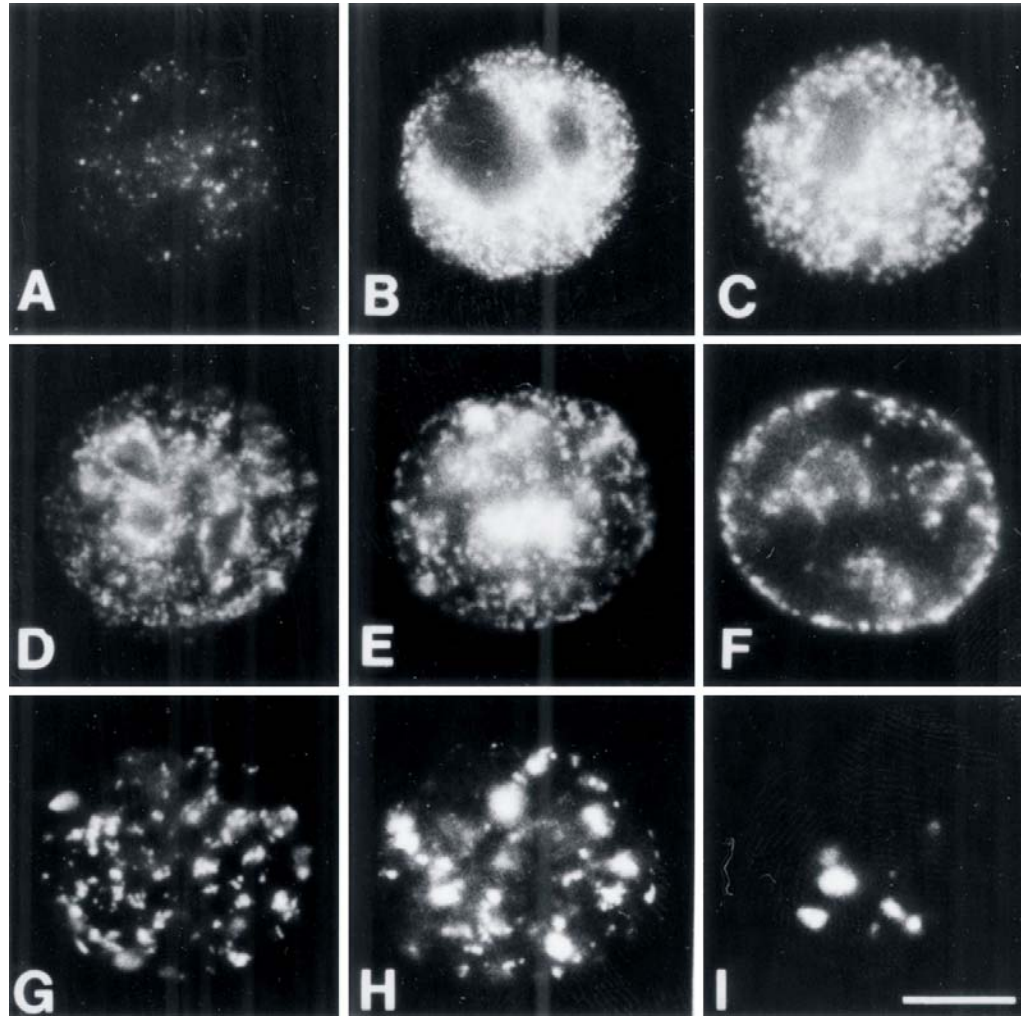


Figure 4. The spatial distribution of active replication during the S phase programme. During S phase, different classes of DNA elements are replicated at specific times. Chromatin that contains the majority of transcribed genes, within chromosomal R bands, is replicated over the first ~4h of S phase. During this period active sites of DNA synthesis are in discrete foci dispersed throughout the nuclear interior (A-C). At mid S phase, replication begins to switch to inactive chromatin, within chromosomal G bands. Patterns of replication foci that reflect the peripheral location of heterochromatin are seen (D-F). Finally, heterochromatic blocks of late replicating chromatin are duplicated within the nuclear interior (G-I). Images shown are replication sites labelled in permeabilised HeLa cells – using biotin-dUTP – that were fixed and indirectly immuno-labelled under conditions that preserve nuclear organisation. The bar is 5 microns. For more details see Hozak *et al* (49). Reproduced with permission from the Company of Biologists.

this study, DNA restriction endonuclease fragments with recently activated replication origins were isolated by trapping fragments with replication bubbles in agarose. A specific class of origins was shown to function at the beginning of S phase while others were activated only later. Notably, however, most early activated origins were not restricted to synthesis at the onset of S phase and in all cases studied the majority of potential origins were replicated passively from origins lying up or downstream. These observations confirm 2 key points: first, there are clearly regions of the genome that replicate preferentially at very early times of S phase and other that do not; and second, for all origins, activation appears to be stochastic,

so that any particular pre-RC (potential origin) will be used in only a minority of cells during a particular cell cycle.

This apparent plasticity of origin function and stochastic nature of their activation provides a plausible mechanistic explanation for the observed weak temporal precision that is seen when genome-wide approaches are used to analyse replication timing. Perhaps the simplest genome-wide strategy (44,45) used unsynchronised diploid human cells that were stained with the DNA binding dye Hoechst 33528 to quantify DNA. Cells in G1 and S phase were purified by flow cytometry, their DNA differentially labelled and then probed to DNA tiling arrays. DNA sequences are duplicated during S phase, so by comparing

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the amount of DNA in the two fractions at defined loci the number of cells in which a sequence has replicated could be determined. The resolution of this approach showed clear early and late replicating domains that correlated broadly with chromosomal R and G bands (Figure 3) but could not define timing transitions at the level of individual DNA foci. A similar conclusion was reached using a PCR-based analysis of replication timing using immuno-precipitation of nascent DNA (labelled with bromo-deoxyuridine) during different intervals of S phase (46). More recently, a strategy using synchronised HeLa cells with labelling over 2h intervals of S phase estimated that as many as 60% of origins might undergo replication throughout S phase (47). While for technical reasons this estimate was later refined to ~20% (48), it is nevertheless apparent that many genomic loci are able to engage replication at different times in different cells of a population.

3.2. A model of S phase progression – temporal activation of DNA foci

The genome-wide studies do appear to conflict with single cells studies, which clearly support models for a structured S phase programme (30). It has been known for many years that active sites of DNA synthesis contain groups of active replicons and that two organisational features contribute to this observation. First, the active synthetic centres contain groups of polymerase complexes within synthetic factories that have the appearance of discrete structures in both fixed (49) and living (50) cells. Second, the clusters of active replicons that contribute to individual active sites appear to remain associated throughout many cell division cycles (11,51). Notably, throughout S phase, the majority of replicons are seen to be activated in small groups – replicon clusters – that initiate DNA synthesis at very similar times. This implies that potential replication origins within chromatin domains of 0.5-1 Mbp are functionally equivalent and supports the idea that some structural feature of chromatin organisation is also a functional feature that contributes to the regulation of replicon activation.

There is some evidence that replicon clusters and the corresponding replication foci might play a fundamental role in regulating S phase progression. Within single cells, the same or very similar clusters of replicons are activated at the beginning of S phase in different cell cycles (11,51) and preliminary observations suggest that individual clusters are replicated at predictable times (12). The importance of nuclear organisation as a mechanism of regulating S phase progression has been suggested by the observation that throughout S phase the majority of newly activated sites lie adjacent to sites that were engaged in synthesis during the previous S phase interval (52). This suggests that the spatial architecture of chromatin foci might be a key determinant of S phase progression with successive activation of foci occurring following a 'next-in-line' principle (53). Though there is no information on relevant molecular mechanisms, it is clear that during progression factories are only present while engaged in synthesis, so that once synthesis begins to decay by fork fusion the components will be released and available for de novo assembly of new factories at adjacent sites (53). A

possible explanation for this might be that the growth of forks at the extremities of active clusters will cause perturbations in the structure of chromatin within adjacent foci, which are subsequently targeted for activation.

Though this is appealing, it is notable that a normal programme of assembly of S phase replication patterns is seen when cells are treated with aphidicolin and caffeine (54). This combination of inhibitors allows the initiation of replication so that factories are able to assemble even though aphidicolin will prevent significant levels of elongation - under condition where the intra-S phase checkpoint is inhibited by caffeine. This shows that extended synthesis, which might impact on the structure of adjacent foci, is not required for S phase progression and implies that the mechanism of progression could be dependent of the spatial architecture of the replication factories themselves. Notably, in metazoan cells, with the exception of *Chk1*^{-/-} DT40 cells (18), which have compromised checkpoint function, we are not aware of any situation in which specific classes of replication factory are active at inappropriate times of S phase. This implies an obligatory link between the patterns that are seen during sequential periods of the S phase programme and a regulatory mechanism that involves checkpoint function.

3.3. Are structure-function links defined by DNA foci?

If the structure of DNA foci plays a significant role in defining the architecture of the replication programme it is important to understand how individual foci are defined. In fact, very little is known about the structure of foci and the molecular principles that might allow stable structures to be established and maintained. There is some evidence that chromatin foci are maintained by epigenetic chromatin states. For example, the analysis of sub-chromosomal regions with interspersed gene islands (highly gene-rich regions) and gene deserts (regions with very few active genes) shows that the two chromatin classes are separated into discrete foci with chromatin that does not mix (55,56). If such specific examples define a general feature of chromatin organisation, it is not unreasonable to suggest that chromatin status might dictate the replication timing of foci with different chromatin epistates. Supporting this model, replication timing in yeast and notably the transition from euchromatin to heterochromatin replication (57) is defined by histone acetylation (58,59). In mammalian cells, modification of acetylation status (using the HDAC inhibitor TSA) can be shown to advance the replication timing of genes in line with the simple correlation that transcriptionally active genes are replicated early in S phase (30).

At the present time, the mechanisms that link the structure of DNA foci and their replication timing are a matter for speculation. Understanding any potential links is hindered by deficiencies in our knowledge of foci structure. We do not, for example, understand how the boundaries of foci are established or if the chromatin within individual foci is stably defined or temporally dynamic. An impressive body of literature has described the behaviour of genomic elements such as nuclear scaffold and matrix attachment elements, locus control regions and domains

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insulators (reviewed in 60), but how these elements correspond to the architecture of DNA foci *in situ* is unknown. For example, perhaps the best candidate for boundary elements that contribute to the definition of independent chromatin domains are defined by insulators that contain CTCF binding sites (60). Intriguingly, these sites have been shown to be hot-spots of cohesin interaction, which suggest that these sites might assume special structural properties (61). Even so, the average genomic spacing of CTCF binding sites is only ~25 kb, making them at least 20 fold more frequent than boundary domains that would flank typical DNA foci.

4. STOCHASTIC MODEL OF S PHASE

We have attempted to use the available literature, with particular emphasis on key experiments described above, to elaborate a model that provides a description of how mammalian S phase might be performed according to a temporal programme that maintains the required efficacy. The key points are summarised here:

1 – Sites of initiation of DNA synthesis do not contain a genetically defined replicator element, as exemplified by the budding yeast ARS element. Despite the absence of a genetic component in mammalian cells the machinery that defines the pre-replication complex is conserved. Pre-RC complexes, which are targets for initiation of replication, are established at many more genomic locations than are actually required to initiate efficient synthesis during each cell cycle. Only about 1/3rd of the DNA zones – or licensing groups – that contain potential origins are used in any one cycle and selection seems to be stochastic (based on probability), though dominant or preferred sites are seen at some loci (10). The distribution and selection process means that a minority of potential origins will be used to initiate synthesis in a particular cell during each S phase, so that the majority are replicated passively by forks that emanate from adjacent origins that lie up-stream of down-stream on the chromatin fibre.

2 – Replicons are activated in small groups within functional replicon clusters. These are believed, though without decisive formal proof, to be both structural and functional sub-chromosomal units, such that the active replicons within a specific DNA focus will be replicated within a discrete replication factory. Once the required density of initiation events has been activated any remaining potential origins are suppressed. The suppression mechanism is regulated by proteins that also serve to provide replicative checkpoint function. In the absence of replicative stress, these suppressed potential origins lie dormant until they are displaced from chromatin by a active elongation complex. However, if replicative stress, such as DNA damage, leads replication to be aborted the latent pre-RCs can be activated to complete synthesis of any unreplicated DNA within the active replicon cluster (36).

3 – The synthetic machinery within replication factories is stable while the factory is engaged in synthesis and disassembles as synthesis completes. For reasons that

remain unclear, the completion of synthesis from one factory appears to be spatially coupled to activation of synthesis within the nearest adjacent DNA foci. Reiteration of this process, using some form of ‘next-in-line’ principle, provides a mechanistic basis to explain some features of S phase progression.

The key molecular features of progression remain unknown and important questions remain to be resolved. For example, while it is known that all potential pre-RCs are established on chromatin before S phase begins it is not immediately obvious how/if potential origins that are activated at the onset of S phase differ from those that become activated later. In mammalian cells, early S phase will typically occupy 4h while each replication focus is active for about 1h. Hence, within the class of early origins that are active during early S phase there will be ~4 rounds of initiation before early synthesis is complete. There is some evidence that specific initiation preferences are seen (42) even though genome-wide analyses show that many sequences are replicated throughout early S phase. Clearly such observations do not support the idea that replication involves a ‘hard-wired’ activation programme that is the same in every cell of a particular cell type. To explain these data, we propose that the efficiency of origin selection during this phase of replication is based on probabilistic mechanisms that are linked to chromatin structure and related dynamic properties. In this regard, it is known that histone acetylation status will influence the stability of higher order chromatin fibres in a way that will determine the dynamic properties of chromatin within individual nuclear domains. Acetylation status is very dynamic during gene expression and clearly able to respond rapidly to changes in expression (62), so that gene domains that are transcriptionally active or in a quiescent of poised state will assume different temporal dynamics and structural ground-states.

Both the spatial organisation of DNA foci and the dynamic behaviour of the chromatin that they contain will inevitably influence the efficiency with which individual foci are able to interact with functional nuclear domains. The dynamic behaviour of this association will define the efficiency of the activation process. In considering this point it is important to recognise that nuclei are organised so that the chromatin within DNA foci occupies a chromatin compartment that is for the most part spatially distinct from the inter-chromatin domain. In proliferating mammalian cells the two compartments occupy ~50% of the nuclear volume, which can be imaged at very high resolution using electron spectroscopic imaging (63). This provides an elegant view of nuclear organisation, in which the chromatin rich domains, most of which measure 300-500nm – the same size as DNA foci visualised by light microscopy (51) – are dispersed within a reticulated inter-chromatin compartment (see 63 for excellent examples). Importantly, by perturbing the interaction of these compartments with mild hypertonic conditions, it has been shown that the key functional interactions occur within the interstitial zone where the two compartments meet (64). The need for regulatory elements that define the location of potential pre-RCs within DNA foci to interact with the

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replication machinery within the inter-chromatin compartment has the potential to be a rate limiting step in the assembly pathway – which might then define how some foci are activated if preference to others. Given the complex and dynamic nature of the epi-genetic status of chromatin within different foci it might be predicted that some foci, perhaps with very active genes, assume an open configuration that is targeted for replication at the onset of S phase in almost all cells. In contrast, other foci might be more variable in structure and hence be targeted in some cells at the onset of S phase and in others at later times. Local gene architecture and chromatin epi-states related to gene expression might also define how active origins are preferentially selected from a larger number of potential origins within different replicon clusters.

While such a stochastic model of origin selection might be plausible, an additional component will be needed to explain why only about 25% of the foci that are replicated during early S phase are activated at the onset of S phase. This begs the question: What restricts or suppresses the subsequent activation of other potential origins during this very early S phase period? While we have few ideas as to how this might be achieved, an obvious possibility is that the level of synthesis is limited by the concentration of a key ingredient in the assembly pathway. The chromatin bound initiation factor CDC45 might be a good candidate for a component that in principle could target active origins and limit the extent of activation. There are, however, circumstances that do not support this simple view. Notably, mammalian cells appear to have evolved regulatory mechanisms to ensure that S phase is performed within a relatively well-defined time interval of the cell cycle, so that cells in which the rate of fork elongation is reduced exhibit a compensatory increase in the local density of active origin (27,34). Importantly, such super-activation of origins is restricted to already engaged DNA foci and does not alter the replication timing programme, which in mammalian cells is unperturbed (18). These observations imply that cells have a mechanism of regulating the absolute amount of DNA synthesis (ie overall kbp/min/cell) based on the number of active forks and the rate of fork elongation.

The mechanism by which such a balance might be achieved has not been explored. However, while speculating, we are attracted to the possibility that the natural structure of replication forks maintains a particular architecture with a specific amount of single-stranded DNA and associated proteins such as RPA (replication protein A). As the rate of elongation is perturbed so will be the organisation of leading and lagging strands and the amount of bound RPA. Chromatin-associated RPA is known to be a critical sensor of fork stalling on a damaged chromatin template – where single-stranded patches are thought to develop as a result of uncoupling of the leading and lagging strand synthesis. This process activates the replication checkpoint and while suppressing global initiation of synthesis, within previous inactive chromatin domains, allows novel initiation within already active domains to facilitate completion of their replication (18,36). A mechanism of this type could in principle regulate the

absolute amount of DNA synthesis during the unperturbed S phase in mammalian cells.

4.1 Is the replication programme really structured in time?

Despite the variability predicted by genome-wide studies there is good evidence that specific chromosomal loci are replicated at predictable times of S phase. Even more convincing than this are the distinct transitions in global patterns of synthesis that are seen during early, mid and late S phase. These patterns reflect the distribution of chromatin domains and the fact that replication of specific classes of chromatin (ie euchromatin, and different flavours of heterochromatin) is structured temporally so that the order of synthesis is strictly defined (30).

In view of this temporal precision, it is actually difficult to imagine that a purely stochastic mechanism with a limited capacity for synthesis could explain S phase progression and the clear structural transitions that are seen. Notably, if a process based entirely on probability was used one might expect to see some frequency of mid or late S phase replication factory assembly during early S phase. However, the characteristic spatial and structural features of replication factories at different times of S phase argue that this is not the case. Instead, a more likely model might incorporate a stochastic activation of potential origins within a specific sub-set of chromatin, which is defined by epigenetic criteria, and an additional spatial component that increases – perhaps dramatically – the probability with which down-stream initiation events are restricted to ensure the preferential selection of potential origins from within a particular chromatin compartment. The available evidence suggests that the spatial component is provided by some form of next-in-line principle (53), though crucially, it is unknown if this is based purely on the spatial organisation of DNA foci or involves the sequential activation of foci that are connected by their genetic continuity along the chromosomal fibre.

5. SUMMARY AND PERSPECTIVE

A summary of the key features discussed above is represented in the model shown in Figure 5. The central feature of the model is that structurally stable DNA foci represent functional units of DNA replication. These units contain multiple replicons that are activated and replicated in synchrony within dedicated replication factories. The DNA substrate and active machinery occupy discrete nuclear compartments so that the spatial architecture and dynamic behaviour of the compartments will define the ability and efficiency with which interactions can occur. Synthesis will occur at the interface of the two compartments and nascent chromatin will be displaced from the surface of factories to return to the chromatin domain as synthesis proceeds. Activation of synthesis appears to accommodate a particular threshold of assembly and is regulated by a process that monitors the absolute potential for DNA replication. Defects in this process, for example because of ongoing transcription or inappropriate structure of the local chromatin, may influence the local selection of potential origins and in some cases may lead to

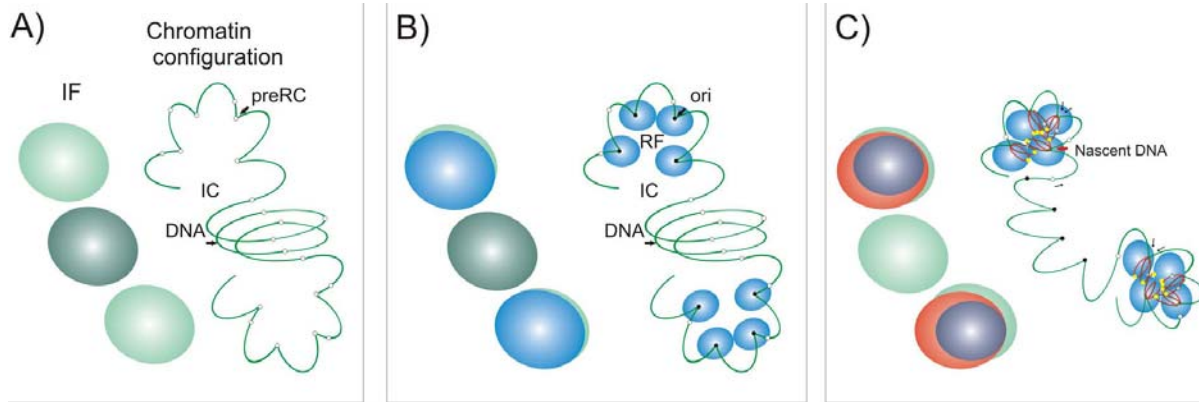


Figure 5. A model linking the architecture of replicon clusters to S phase progression. Potential initiation sites of DNA replication (pre-RC complexes – small open circles) are scattered throughout the chromatin fibre (line). In G1 phase (A), all potential replication complexes are established, some within an open chromatin configuration (equivalent to chromosomal R bands) and others in more condensed chromatin (equivalent to chromosomal G bands). Pre-RC complexes interact by chance with the replication machinery (blue circles; B) and initiate synthesis at a fraction of pre-RCs to define functional origins (small filled circles). Replication complexes are established more efficiently in open chromatin, where the high local density of engaged replication complexes leads to formation of replication factories (clustered blue circles; C), within the interchromatin compartment (IC). Active factories now form the regulatory unit of synthesis, where the function of latent potential origins (small open circles) is suppressed once the required rate of elongation is achieved. As synthesis continues, chromatin fibres are reeled into the active synthetic factory and nascent strands displaced from the factory surface. Eventually, the internal forks from adjacent replicons begin to terminate through fork fusion. At this time, the synthetic machinery is returned to a soluble pool as factories begin to disassemble, so providing material for assembly of new factories. In this model, the central DNA focus is altered in structure (C) because of changes that occurred during replication of the adjacent foci. This together with the high local concentration of replication components might increase the probability of activating synthesis within foci that lie adjacent to those that have recently completed the synthetic cycle. Large spheres on the left of each panel depict the structures that would be visualised by confocal microscopy: Grey - the structure of DNA foci that would be seen by prior labelling *in vivo* (for example with Cy3-dUTP); Blue – location of active replication complexes and factories; Red – the nascent DNA.

failure of the assembly/activation pathway, perhaps involving an assembly checkpoint. In mammalian cells, it appears that once a certain rate of synthesis is achieved any remaining potential origins within active DNA foci are suppressed and those remote from active factories remain dormant. As replication proceeds, the nascent replication forks begin to terminate and the replication machinery is disassembled from active factories and new factories begin to appear. Though the molecular mechanism is unclear (perhaps disassembly results in a high local concentration of key components) loss of activity within one factory appears to seed assembly at adjacent replicon clusters. The S phase programme continues by reiterating the process of activation of potential local origins and de novo assembly of new replication factories. In this way, as S phase continues, chromatin architecture, chromosome structure and nuclear organisation contribute to establish a spatially structured replication programme. Finally, while the literature described here provides a compelling intellectual framework for discussing the properties of S phase in mammalian cells it is clear that key molecular details are still missing. The elaboration of the missing mechanisms will be needed to fully appreciate how defects in S phase might contribute to genome instability phenotypes that have profound medical implications.

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