THE ORGANIZATION OF REPLICONS

1985

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in: The Cell Division Cycle in Plants
Soc. Exp. Biol. Semin. Series Vol 26, pp 15-35
Cambridge University Press, Cambridge 1985

INTRODUCTION

This paper is a collection of ideas related to the concept of a unit of DNA replication, the replicon. The various levels of organization of chromatin and nucleus together ensure the correct regulation, both temporal and spatial, of the process of replication of all the DNA molecules, and their equal distribution to the two daughter cells during mitotic division.

The experiments with which we will illustrate this discussion are mainly taken from studies with the lower eukaryote Physarum polycephalum. This organism has a very high natural mitotic synchrony which allows many experimental approaches to the regulation of DNA replication that are impossible in other cell systems.

MOLECULAR STRUCTURE OF A REPLICON

Definition of a replicon

A replicon is a unit of replication of DNA. It may be detected by DNA fibre autoradiography. Its length is the distance between two neighbouring sites of initiation of DNA synthesis on the same DNA molecule (Bryant, 1982; Francis, Kidd & Bennett, this volume; Van't Hof, this volume). The length may also be measured by electron microscopy of replicating DNA molecules as the centre-to-centre distance between replication eyes (Evans, 1982). In sedimentation studies of newly synthesized DNA it is recognized as a relatively stable intermediate (see Van't Hof, this volume).

The size of replicons is variable between organisms (Francis, Kidd & Bennett, this volume). Small replicons are seen in Physarum with a size of 30-50 kbp (10-15 μm) of DNA (Funderud, Andreassen & Haugli, 1978a, 1978b, 1979). Large replicons with up to 600 kbp (200 μm) of DNA occur in Triturus cristatus-carnifex (Buongiorno-Nardelli, Micheli, Carri & Marriley, 1982). During early development, replicon sizes may increase as in Drosophila

where the average replicon size increases from 10 kbp (3 μm) to 40 kbp (14 μm)(Blumenthall, Kriegstein & Hogness, 1974). Average values for replicon size are generally between 50 and 100 kbp (15-50 μm)(Edenberg & Huberman, 1975). The knowledge of replicon size and the size of the genome of an organism allows one to calculate the number of replicons in a cell. As an example, such calculations are shown for the acellular myxomycete Physarum polycephalum in Table 1. One genome of DNA sequences (haploid G1 phase; C value) contains approximately 5600 replicons distributed over approximately 40 DNA molecules or chromosomes. For other examples see Bryant (1982) and Francis, Kidd and Bennett (this volume).

Initiation of DNA replication in S-phase

Each DNA molecule in a cell is duplicated in its entirety once, and not more than once, in each cell cycle. This suggests a very tight control over the initiation of replicon duplication. In one way or another the cell marks the site of initiation of DNA synthesis after DNA synthesis has begun, to prevent re-initiation of the same replicon within the same cell cycle (Wanka, 1984). The cell presumably also prevents initiation of 'late' replicons when the replication fork from an 'early' replicon has passed and already has duplicated the DNA sequence. However, in a normal cell cycle no replicon may be left unduplicated (but see Nagl, Pohl & Radler, this volume). Although the precise mechanisms are not yet clear, a number of recent observations in Physarum have given us some idea as to how these events are accomplished.

Funderud et al. (1978a) and Haugli, Andreassen & Funderud (1982), have described observations which suggest a model that they have called 'master initiation' (Fig. 1, stage 2). Physarum macroplasmodia are giant, multinuclear cells with a natural mitotic synchrony. DNA synthesis starts at about eight minutes after metaphase (Beach, Piper and Shall, 1980a). Bromodeoxyuridine (BUdR) was incorporated for a very short period at the very start of S-phase. The remaining new DNA was labelled with radioactive deoxyadenosine (AdR) and the DNA was isolated and sized by alkaline sucrose gradient centrifugation. Exposure of the BUdR to ultra-violet light fragmented the full-sized DNA to replicon size (30-65 kbp). In addition to early replicated DNA, late replicated DNA was also fragmented to replicon size. They concluded that apparently at the beginning of S-phase, short patches of BUdR-containing DNA had been synthesized at all the origins (Fig. 1, stage 2). After this initiation, only the early replicons had continued DNA synthesis

Table 1A Levels of Chromatin Organisation in a Nucleus of Physarum polycephalum

	Nucleus (4C)	Genome (1C)	DNA Molecule	Loop/ Replicon	30 nm Turn	Nucleosome	Base Pair of DNA
Chromosomes	80	40	1	-	-	-	-
Molecules	160	40	1	-	-	-	-
DNA Loops	22 500	5 600	175	1	-	-	-
Nucleosomes	6.4×10^6	1.6×10^6	40×10^3	230	6.5	1	-
Base Pairs (bp)	1.1×10^9	280×10^6	7.0×10^6	40×10^3	1.1×10^3	172	1
30 nm Fibre (length)	11.0 mm	2.8 mm	68 µm	390 nm	11 nm	-	-
11 nm Fibre (length)	70.0 mm	18.0 mm	440 µm	2.5 µm	71 nm	11 nm	-
DNA Helix (length)	370 mm	90 mm	2.3 mm	13 m	365 nm	57 nm	0.33 nm
Linear Condensation of DNA	(72 000 X)	(18 000 X)	2100 X	190 X	33 X	5 X	1 X
DNA (g)	1.1×10^{-12}	280×10^{-15}	7.0×10^{-15}	40×10^{-18}	1.1×10^{-18}	170×10^{-21}	1×10^{-21}
DNA (D)	740×10^9	185×10^9	4.6×10^9	26×10^6	725×10^3	115×10^3	660
References	-	A,B	A	B,C	D,E	D,E	E

4C DNA content: diploid nucleus at G2-phase or metaphase.
1C DNA content: genome complexity, haploid nucleus at G1-phase.

Nucleus	Structure and Dimensions		References
Nucleus	Sphere with a diameter of 5000 nm	Association of approximately 80 chromosomes with nuclear matrix and envelope.	A
Chromosome	Rod, length 1100 nm, diameter 450 nm.	Loops of 30 nm fiber chromatin on scaffold. Condensed (see dimensions) in metaphase. No chromatids visible in metaphase chromasomes.	A
DNA loop	DNA loop, diameter 70 nm.	Attached loop of 30 nm chromatin fiber. Contracted to super-solenoid in metaphase (see dimension). Possibly identical to replicon and/or transcriptional domain.	F,G
30 nm Fiber	Solenoid, diameter 30 nm, pitch 11 nm.	11 nm fiber with 6 to 7 nucleosomes per turn. 'solenoid' could contain superbeads.	D,G
11 nm Fiber	Linear chain, diameter 11 nm.	Chain of nucleosomes, 'beads-on-a-string'. Potentially active chromatin?	G
Nucleosome	Disk, dimensions 11 x 11 x 6 nm.	Approximately 172 base pairs of DNA + 8 core histones (H2A + H2B + H3 + H4) + histone H1.	E,G
DNA (B-DNA)	Alpha helix, diameter 2 nm, pitch 3.4 nm.	Approximately 10.5 base pairs of anti-parallel DNA per turn of helix.	E,G

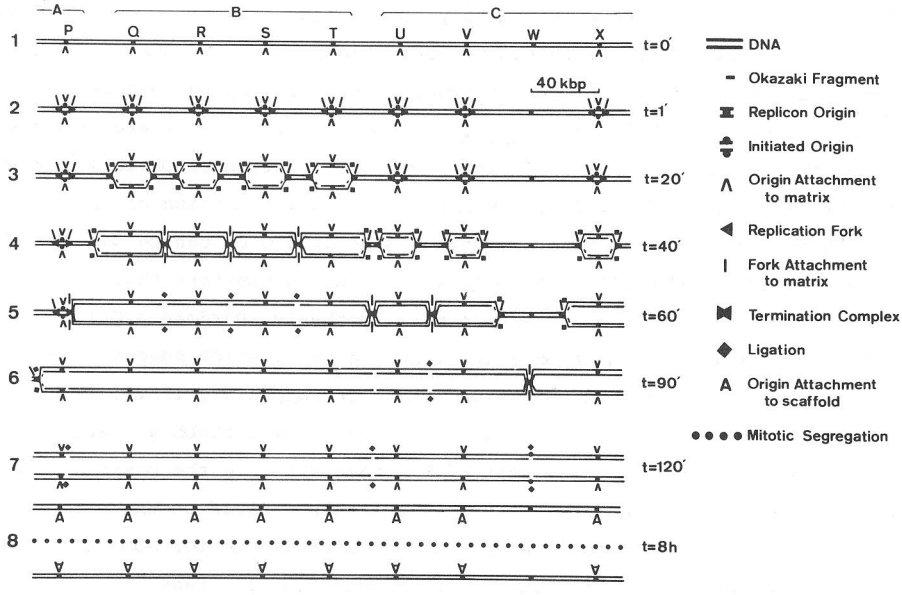
Underlined data were used for the calculations and are from references cited.

REFERENCES
A = Mohberg (1977, 1982); B = Holt (1980); C = Evans (1982); D = Matthews & Waterborg (1984);
E = Matthews & Bradbury (1982); F = Adolph & Kreisman (1983); G = Butler (1984)

bidirectionally, while all the other replicons were held in an initiated state (Fig. 1, stage 3) until a second signal allowed these to continue chain elongation (Willie & Kauffman, 1975).

This explanation of their observations suggests that within a very short interval all origins are initiated. It is thought that this initiation changes the structure of the DNA at these sites in such a way that no re-initiation can occur within the same cell cycle (Wanka, 1984). As will be discussed below, the replicon origins or sequences very near them,

Fig. 1. A schematic model of DNA replication in the Physarum cell cycle. Replication of three clusters of replicons (A-C) containing replication origins (P-X). Stages 1-8 exist at the time indicated, relative to the beginning of S-phase, immediately after mitosis. (1) t = 0 min: DNA prior to the start of S-phase and in G2-phase. (2) t = 1 min: Master initiation of all active origins at start of S-phase, start of DNA synthesis in cluster B. Origin W is an inactive origin due to matrix detachment. (3) t = 20 min: Okazaki synthesis and ligation in cluster B, start of synthesis in cluster C. (4) t = 40 min: termination in cluster B, Okazaki synthesis in cluster C. (5) t = 60 min: ligation in cluster B, termination in cluster C except at origin W, termination of replicon Q at initiated origin P, start of synthesis in cluster A. (6) t = 90 min: ligation in cluster C, termination at origin W, synthesis in cluster A. (7) t = 120 min: ligation of new, cluster sized DNA. (8) t = 8 hours: mitotic segregation of chromosomes.



may be attached to the nuclear matrix. The interaction between origin and matrix could change irreversibly upon initiation, for instance by formation of the presumed 'eye' and doubling of the attachment structure. The regulation of the initiation of replicons may also require soluble proteins that are made during only a very small time interval of the cell cycle.

Physarum is the only organism so far which suggests 'master initiation' of replicon origins. The conventional view that replication of the origin is part of the complete replicon duplication and is therefore sequential, as replicon synthesis itself, in S-phase (see below), may still be valid for other organisms although no experimental support for this view is known.

DNA synthesis and termination in S-phase

DNA synthesis is, at least partially, a discontinuous process. DNA is synthesized by DNA polymerase from nucleoside triphosphates using the opposite strand of the DNA as template. In many cells the 'leading' strand synthesis is continuous (Kornberg, 1980). The 'lagging' strand can only be synthesized discontinuously, since DNA polymerase can only add to a 3' terminus. It seems however that in Physarum, both strands are synthesized discontinuously (Holt, 1980; Evans, 1982); this may also be so in higher plants (Bryant, 1982).

The smallest discrete pieces of new DNA are 'Okazaki fragments'. These pieces of DNA are approximately 200 bp long and are primed by a short sequence of RNA. Their synthesis proceeds bidirectionally away from the origin at an average rate, in Physarum, of 600-1200 bp per minute per fork (Funderud et al., 1979; Beach et al., 1980a); similar rates are observed in many other eukaryotic cells (Bryant, 1982). Initiation of Okazaki fragments may precede the separation of the DNA strands and formation of the fork structure. Microbubbles with an average size of 500 bp can be detected in carefully prepared replicating DNA. They occur in clusters that have been interpreted as sites of Okazaki pre-fork synthesis (Hardman & Gillespie, 1980), although the data from SV40 virus DNA replication suggest that priming occurs after fork formation (Hay & De Pamphilis, 1982).

In Physarum, approximately 2% of the microbubbles were observed as single eyes, separated by tens of kilobasepairs. On the basis of their distribution in the DNA it is possible that these could be the early-initiated origins of 'late' replicons that are waiting for some signal that will allow them to continue chain elongation (Fig. 1, stage 3).

Termination of DNA synthesis in a replicon apparently occurs randomly. Specific termination sites or sequences have never been observed. Termination will thus generally occur approximately halfway between adjacent origins, when replication forks meet (Fig. 1, stage 4). The molecular process of termination is unknown. Presumably the replication complexes dissociate, a repair-like process fills the gap and ligase joins the replicon-sized DNA (Fig 1, stage 5). The synthesis of DNA from Okazaki pieces to the replicon size of approximately 40 kbp in Physarum is complete in 40 to 50 minutes for those replicons that are initiated at the beginning of S-phase. The increase in the size of this newly synthesized DNA then stops for about 30 minutes. It then increases stepwise to the size of a replicon cluster: three or four replicons. After a further interval until nearly the end of S-phase, two hours after mitosis, it finally reaches full size DNA (Fig. 1, stage 7). Condensation of newly synthesized DNA to intermediate and full length fragments seems to be a rather slow process compared with the rate of chain elongation. The ligation of new DNA may continue into G2-phase (Funderud et al., 1978a, 1978b, 1979); this has also been observed in higher plants (Van't Hof, this volume).

The temporal organization of S-phase

The order in which replicons are duplicated in S-phase, is fixed. This was shown in Physarum by radioactive labelling and density labelling of replicating DNA at identical times within the S-phase of two successive cell cycles. All the labelled DNA was double-labelled (Holt, 1980; Evans, 1982). Mixing experiments with nuclei and cytoplasm from different phases of the DNA replication process showed that cytoplasmic, cell-cycle-specific factors are essential for the correct progression of replication (Wille & Kauffman, 1975; Wille, 1977; Holt, 1980).

A fixed temporal order in S-phase appears to be a general phenomenon. In yeast, functional origins (autonomously replicating sequences) ars, are activated in a specific temporal order during the cell cycle (Chan & Tye, 1983). Sister chromosomes of diplochromosomes replicate synchronously in identical patterns (Lau, 1983). Defined temporal groups or 'families' of replicons can often be distinguished; for example there are two in Arabidopsis thaliana and 10 in Physarum (Muldoon, Evans, Nygaard & Evans, 1971; Van't Hof, Kuniyuki & Bjerknes, 1978). This temporal organization results in Physarum in the observation that in the first quarter of S-phase little or no repetitive DNA is replicated (Evans, 1982). Generally,

autonomously replicating sequences (ars) from the chromosomal DNA of Saccharomyces cerevisiae (Struhl, Stinchcomb, Scherer & Davis, 1979; Beach et al., 1980b). The frequency of these sequences in the S. cerevisiae genome was found to be similar to the average spacing of replicons (Beach et al., 1980b; Chan & Tye, 1980); they contained similar short AT-rich blocks within a larger required sequence as do isolated non-chromosomal origins (Broach et al., 1982), and consequently, by analogy with the situation in prokaryotes, it was suggested that these special sequences may be eukaryotic origins of DNA replication. The ars assay in yeast also yielded active ars from other eukaryotic DNA, such as Physarum (Gorman, Dove & Warren, 1981), but not from E. coli DNA. Recent experiments (K. Maundrell & S. Shall, unpublished data) showed that out of a random selection of sequences from Schizosaccharomyces pombe, the expected number had ars activity in S. pombe. Testing all the sequences in the heterologous system of S. cerevisiae showed that less than half of the ars sequences in S. pombe were recognized as such by S. cerevisiae, while a certain number of the non-ars sequences from S. pombe showed ars activity in S. cerevisiae. This result suggests that a heterologous assay for ars activity may be of doubtful value for isolation of eukaryotic origin sequences.

Origins of DNA replication were isolated by us using a totally independent principle (Beach et al., 1980a). The protocol relied on isolating that DNA which was synthesized in the first few minutes of S-phase in Physarum. This DNA was density labelled with BUdR, isolated and analyzed by equilibrium centrifugation in caesium chloride density gradients. The analysis suggested that gentle shearing was probably breaking the DNA molecule selectively at the replication fork, thus freeing double-stranded, newly replicated DNA from the flanking unreplicated part of the molecule. We have not rigorously excluded the possibility of branch migration, but our evidence is that we obtained heavy-light hybrid molecules and not heavy-heavy ones expected as a product of branch migration. Sheared, newly initiated DNA thus consists of molecules of two size classes: bulk DNA with a molecular weight greater than 45 kbp, and newly replicated DNA whose size increases by 2.4 kbp per minutes from the beginning of S-phase, as expected for known fork rates in Physarum.

Our data further suggested that at the start of DNA replication there is a single initiation event simultaneously at approximately 3000 'origins' of replication (see Table 1), and that little or no further initiation occurs for at least 15 minutes.

A method for the purification, isolation and molecular cloning of this DNA has been developed. Subsequently, we have established that these newly initiated DNA fragments can be more readily isolated and cloned by tritium pulse labelling followed by controlled shear and isolation on a sucrose gradient. After 2 minutes of S-phase this procedure yields DNA fragments with an average size of about 3 kbp.

MACROSCOPIC STRUCTURAL FEATURES OF REPLICONS

The requirement for a structural organization of the nuclear DNA

In eukaryotic cells very large amounts of DNA are packaged within the very small volume of a nucleus. For an example, see Table 1. Once during every cell cycle the amount of DNA is doubled. The new DNA molecules are segregated equally over two daughter nuclei during mitosis. Apparently a minimal number of DNA strands are found at that point in the cell cycle to be entangled despite the fact that DNA replication requires the complete unwinding of all the DNA molecules down to the last helical turn. These highly complex processes that are interdependent, and also dependent on the transcriptional regulation of the genome lead one to expect a rigorous but flexible pattern of organization (Wanka, 1984).

Chromatin organization of DNA

Much is already known about the lower levels of organization of the DNA into chromatin. (For some recent reviews, see Matthews & Bradbury, 1982; Nagl, 1982; Butler, 1984; Matthews & Waterborg, 1984). In general, approximately 200 base pairs of DNA are associated with an octamer of core histones and one molecule of H1 to form nucleosomes. Most nucleosomes are organized in a fibre with a diameter of approximately 30 nm. This fibre is formed by the i eractions between the H1 molecules of the nucleosomes and by the interaction of the amino terminal regions of some of the core histones with the DNA of neighbouring nucleosomes. The packing of DNA into a 30 nm fibre gives a linear contraction of about 40 (Table 1).

To allow replication of chromatin, the 30 nm fibre is dis-assembled into a 'beads-on-a-string' structure of nucleosomes on DNA. This causes an increase in the sensitivity of the DNA to nuclease digestion. Nucleosomes appear to remain associated with the DNA all through the process of replication, except in the immediate location of the replication fork. Re-packaging of the DNA with pre-existing and newly formed nucleosomes is

followed by a slow process of maturation. In the 30 minutes following DNA synthesis, the spacing of the nucleosomes, the 30 nm chromatin fibre and the low nuclease sensitivity of the chromatin are all restored (Matthews & Waterborg, 1984). This process of unfolding and reformation of chromatin causes a variation in the DNase I sensitivity of the chromatin through the cell cycle that can be observed in Physarum (Jalouzot et al., 1980).

Nuclear matrix and chromosome scaffold

Folding of the 30 nm fibre, the highest reproducibly recognizable chromatin structure, requires the interaction between DNA and other proteins such as those of the chromosomal 'scaffold' (Barrack & Coffey, 1982; Nagl, 1982; Butler, 1984; Wanka, 1984). This is a limited set of proteins, constituting approximately 3-4% of the total chromosomal protein, that are isolated from metaphase chromosomes after removal of histones and DNA by high salt and nuclease digestion. Together they form a three dimensional network that has the shape of the metaphase chromosome before the chromatin was removed. The structural integrity of this network depends on metallo-protein interactions involving Cu^{2+} and possibly Ca^{2+} ions and/or disulphide bonds between a subset of the proteins. The network has contractile properties that may require phosphorylation of scaffold proteins during mitosis (Barrack & Coffey, 1982). This contraction, in combination with contraction of the chromatin by phosphorylation of H1 (Matthews & Bradbury, 1982), yields the highly condensed metaphase chromosomes in which the linear compaction ratio of the DNA is increased to several thousand fold (Table 1). In mammalian cells this compaction ratio can reach, at metaphase, values of nearly 10,000 when 0.26 pg of DNA in 2 molecules, the average value for DNA in a human metaphase chromosome, i.e. 86 mm of DNA, is compacted into a 10 micron-long chromosome.

After mitosis a nuclear envelope with its associated pore complex lamina reforms around the two separated masses of telophase chromosomes. The chromatin in the new nuclei decondenses. Isolation of the 'scaffold' at this stage of the cell cycle, by high salt and nuclease treatment, yields a nuclear ghost or nuclear matrix which, just as the scaffold does during mitosis, retains the shape and structure of the nucleus. However, the protein composition is more complex, containing as much as 10-15% of the total nuclear proteins. It consists of the scaffold proteins, the proteins that form the pore and lamina structure at the nuclear envelope, and an abundance of minor structural, and probably also regulatory, proteins that are involved

in all the processes that occur localized on the nuclear matrix structure (Barrack & Coffey, 1982). In Physarum with its closed mitosis, the composition of the nuclear matrix proteins in interphase is virtually indistinguishable from that in mitosis (Wanka, 1984). Although the continuity between chromosomal scaffold and nuclear matrix has not been proven directly, due to the difficulties in isolating the intermediate stages at the beginning and end of mitosis, observations of prematurely condensed chromosomes, made by fusion of mitotic and interphase-cells in G1, S or G2 phase, supports this idea (Hanks et al., 1983; Wanka, 1984).

DNA association with the nuclear matrix

DNA is bound, as chromatin, to the chromosome scaffold and nuclear matrix structures in long loops of several tens of kilobasepairs. The size of these loops is identical on both structures, further supporting the idea of identity between scaffold and matrix (Butler, 1984; Wanka, 1984). Scanning electron microscopy shows these loops in human metaphase chromosomes as short stubby, radially orientated rods with diameters of approximately 70 nm and formed from a supertwisted 30 nm chromatin fibre, as judged after partial decondensation of the chromatin loop (Adolph & Kreisman, 1983; Hanks et al., 1983). These loops may be organized in groups that form the bands that can be visualized by staining of the metaphase chromosomes (Butler, 1984).

Removal of the chromatin proteins by high salt treatment allows the visualization of the DNA in the loop itself. Observation of an interphase nuclear matrix by fluorescence microscopy in the presence of ethidium bromide shows quite clearly a halo of DNA round the nuclear ghost structure. The diameter of this halo is dependent on the ethidium bromide concentration (Vogelstein, Pardoll & Coffey, 1980). This shows that all the DNA loops are separate supercoiled domains. Digestion of the loops by nucleases results transiently in the maximal size of the halo. This allows a determination of the loop size. The loops are generally about 50 to 100 kbp long but this value varies among different organisms (Buongiorno-Nardelli et al., 1982; Hyde, 1982; Butler, 1984).

Preparation of a nuclear matrix retaining all its DNA (Wanka, 1984) depends on the absence of shear and of endogenous nuclease activity during the high salt treatment of the nuclei. A standard protocol treats isolated Physarum nuclei with buffered 2 M NaCl followed by the isolation of the residual, rapidly sedimenting structure by sedimentation on top of a

cushion or as a pellet (Aelen, Opstelten & Wanka, 1983). Such a preparation gives maximally 90% of the DNA retained in the isolated matrix, and routinely 60% or more. Inclusion of polyethyleneglycol in such a protocol (Table 2) diminishes the shear forces on the DNA during the removal of the histones by 2 M NaCl, abolishes the shear forces on the DNA loops during sedimentation of the matrix surrounded by its halo, and suppresses endogenous nuclease activity. Routinely, more than 98% of the DNA is retained on such a matrix and the dependence of the halo size of a matrix, reswollen in a low ionic strength buffer, upon the concentration of ethidium bromide indicates the absence of nicking in the supercoiled DNA loops.

Preparation of nuclear matrix structures that retain all their DNA, allows the subsequent analysis of the DNA attached at or very close to the matrix. The loop DNA can be removed by nonspecific nucleases like DNase I or micrococcal nuclease or by restriction enzymes. The residual DNA is then isolated by sedimentation of the matrix (Table 2). In many organisms the residual DNA, 1% or less, does not differ from total, bulk DNA as judged by solution hybridization, although frequently, enrichment of satellite or 'ribosomal' DNA sequences on the matrix is observed (Wanka, 1984). Such enrichments could be real, i.e. these sequences could be consistently located in the DNA loops at or near the matrix, or they may depend on the sequence specificity of the nuclease used, since such satellites have only a limited sequence complexity.

So far hybridization studies have not been able to detect sequences that are common among the attachment sites of DNA loops to the matrix. This means that such a sequence does not exist or that it is too small to be detected by hybridization. Direct sequence determination of matrix DNA isolated from the G1/S boundary of mouse 3T3 cells has detected short blocks of repetitive sequences that were highly enriched on the matrix. These sequences contained features that may suggest the presence of replicon origins: AT-rich blocks and GC/AT switches with purine bias occur as in functional ars sequences; viral large tumour antigen recognition sequences are present, and the sequences display the possibility for secondary DNA structure (Goldberg, Collier & Cassel, 1983).

These structures, or short blocks of primary DNA sequence, may direct the binding of those DNA sites to specific proteins. In HeLa cells proteins have been detected that bind to DNA very tightly at sites spaced apart by approximately loop size distances (Barrack & Coffey, 1982; Bodnar et al., 1983). Many other proteins in the nuclear matrix have DNA binding

Table 2 Preparation of nuclear matrix DNA of Physarum

MACROPLASMODIUM

Homogenize in 0.25M sucrose 5mM $MgCl_2$ (or 10mM $CaCl_2$) - 10 mM Tris-0.1% Triton X100-pH 7.2

NUCLEI

+ homogenization buffer to 200,000,000 nuclei/ml.

+ 2 volumes 3M NaCl-15% PEG 6000-5mM EDTA-50mM Tris-pH 7.4

15 min on ice.

spin 1 min 3000 xg.

NUCLEAR MATRIX (contracted).

+ vortex in 0.5M NaCl-10% PEG 6000-5mM EDTA-20mM Tris-pH 7.4, at 2,000,000 per microliter.

+ 4 volumes 13.75mM $MgCl_2$-20mM Tris-pH 7.4

15 min on ice.

NUCLEAR MATRIX (swollen)

Digest 2 h 37 C with 0.5 Units Mbo 1 per µg DNA.

Optional: add 1 volume 4 M NaCl.

Spin 20 min 10,000xg through sucrose cushion

NUCLEAR MATRIX (digested)

Proteinase K digestion in 1% Sarkosyl.

Phenol extraction

Electrophoretic removal of slime

RNase A digestion

NUCLEAR MATRIX DNA

properties. Reconstitution of urea-dissociated matrix structures in the presence of bulk or matrix-associated DNA resulted in rapidly sedimenting structures that had bound DNA. However, no sequence specificity was apparent by solution hybridization (Mullenders, Eygenstein, Broen & Wanka, 1982).

In addition to DNA attachment to the internal nuclear matrix, DNA may be associated with the nuclear envelope, its pores and lamina. Such associations, e.g. of telomeric DNA sequences with the pore complex lamina (Franke, Scheer, Krohne & Janasch, 1981; Wanka, 1984), may play a role in recombination during meiotic prophase by allowing the pairing of homologous chromosomes. This association must be transient as the pore complex lamina dissociates during an open mitosis (Wanka, 1984).

Localization of DNA replication on the nuclear matrix

Radioactive labelling of newly synthesized DNA followed by autoradiography of nuclear halos or by sedimentation analysis for enrichment of label at the matrix during progressive detachment of loop DNA, has proved that DNA replication occurs in association with the nuclear matrix. The recent visualization of matrix DNA from S-phase HeLa cells, showed directly the enrichment for replication fork structures, as expected (Valenzuela, Mueller & Dasgupta, 1983).

A short pulse treatment with radioactive nucleotides during DNA synthesis, shows label exclusively localized above the nuclear ghost structure. This shows that all the DNA synthesized within the short period of the pulse, which can be given at any point within S-phase, is closely associated with the nuclear matrix. Very short pulses have suggested that replication occurs all through the nuclear volume and not exclusively near the pore complex lamina or the nuclear envelope, as some results have previously been interpreted (Wanka, 1984). Longer labelling gives a progressive appearance of label above the halo of DNA until by approximately 30 minutes equilibrium has been reached. By that time replicon size DNA has been synthesized (Barrack & Coffey, 1982).

Progressive detachment of loop DNA by DNase I digestion of nuclear matrix structures in cells, labelled continuously with ^{14}C-thymidine and pulse labelled with ^{3}H-thymidine at any point within S-phase, shows a gradual enrichment for newly replicated DNA at the nuclear matrix by the increasing ^{3}H/^{14}C-ratio (Dijkwel, Mullenders & Wanka, 1979; Wanka, 1984).

Pulse labelling at specific times in S-phase in Physarum, followed by a chase into G2-phase, allows the localization of a small patch

of newly replicated DNA relative to the matrix. DNA labelled at the start of S-phase displays a continuous increase in the $^{3}H/^{14}C$ ratio upon nuclease digestion. It must be located right at the nuclear matrix, and it must remain there in G2-phase (see next section). Pulse labelling at the time between initiation and termination of the 'early' replicons (Fig. 1) results, after a chase, in a $^{3}H/^{14}C$ ratio that initially increases upon nuclease digestion but subsequently decreases again. Such a patch must be located halfway between matrix and the end of the DNA loop. DNA labelled at the end of S-phase, i.e. near the time of termination of the last replicons, displays a continuous decrease in the ratio. The termination sequences must be near the end of the DNA loops (Aelen et al., 1983; Wanka, 1984).

In regenerating rat liver, but not in normal liver, a replication complex that contains DNA polymerase -α, can be isolated in association with the nuclear matrix. Such complexes may contain several replication forks and may be the explanation that replicon synthesis is observed in small clusters, e.g. three to four replicons per cluster in Physarum (Funderud et al., 1979; Barrack & Coffey, 1982; Smith & Berezney, 1983). The replication complex is not a permanent structure in the cell cycle. It forms just prior to the start of DNA replication (Fig. 1, stage 1)(Smith & Berezney, 1983), possibly just behind the replication fork (Dijkwel et al., 1979). Observation of prematurely condensed chromosomes made with S-phase cells has suggested that complex formation may change the interaction of the DNA with the scaffold proteins (Hanks et al., 1983).

Viral and plasmid replication may also be localized on the nuclear matrix. Replication of SV40 occurs in association with the nuclear matrix (Barrack & Coffey, 1982). The presence of a yeast ars, a functional origin, in a plasmid DNA causes the intra-nuclear replication of such a plasmid (Kingsman, Clarke, Mortimer & Carbon, 1979).

Permanent attachment of DNA to the nuclear matrix through the replicon origins

The loop size of DNA, measured by the size of a DNA halo around a nuclear matrix, is very clearly correlated with the size of replicons over the whole known range of replicon size from 20 kbp to 600 kbp. In Xenopus the size of the average replicon increases during embryonal development and the diameter of the halo increases in parallel (Buongiorno-Nardelli et al., 1982).

The size of the DNA halo remains unchanged throughout the cell

cycle. This indicates that attachment, which is more permanent than that exhibited by replication complexes, must determine the constant size of the DNA loops (McCready et al., 1980). Measurements of the number of permanent attachment sites per replicon vary around the value of one per replicon. McCready et al., (1980) observe in HeLa cell nuclear matrices (cages) one site per four replicons; Vogelstein et al., (1980) calculate one site per replicon from determination of the size of supercoiled domains by restriction nuclease digestion. Buongiorno-Nardelli et al., (1982) interpret their data on halo size as two attachment sites per replicon (reviewed by Wanka (1984)). Clearly more experimental evidence in systems with low variation in replicon or loop size is required to substantiate the idea that DNA is permanently attached to the nuclear matrix at one site per replicon.

Pulse-chase labelling at the start of S-phase in Physarum (see above) suggests that the site of permanent DNA attachment to the matrix is identical to a replicon origin (Aelen et al., 1983). DNA labelled at the beginning of S-phase remains after synthesis associated with or located near the matrix, irrespective of the length of chase. This suggests that the origin is at or very near to the permanent attachment point of the DNA loop to the matrix (Fig. 1). This conclusion is valid for the origins of the first temporal set of replicons of the S-phase of Physarum. The experiment does not prove that all origins, even origins of the first temporal family of replicons, are attached (Wanka, 1984). However, the observation that at the beginning of S-phase all origins are initiated in Physarum in the process called 'master initiation', may suggest the correctness of the observation for all origins. Isolation of origins from early and late replicons followed by the analysis of their location in the DNA loops relative to the nuclear matrix, may resolve this uncertainty.

In a different model, permanent replication complexes are assumed to explain the permanent attachment of DNA loops to the matrix. DNA replication at the matrix starts at the DNA between two adjacent replication complexes that have met at termination of replicon synthesis in the previous cell cycle. The DNA between the complexes, the functional origin of the replicon, is duplicated and two loops of DNA are reeled through the complexes. After completion of replicon synthesis the origin is far away from the matrix at the top of the DNA loop. The initiation complex for the next S-phase is formed between two replication complexes when they meet at termination (Vogelstein et al., 1980; Barrack & Coffey, 1982). The observation by Smith and Berezney (1983) that the replication complex on the matrix is not stable

but reforms before every S-phase, does not support this model. Neither can it be reconciled with the observed pulse-chase labelling of DNA relative to the nuclear matrix at the beginning or at the end of the S-phase in Physarum.

The observation of increased replicon length and increased halo size during embryonic development, for instance in Xenopus (Buongiorno-Nardelli et al., 1982), in combination with the hypothesis that DNA is attached to the matrix at or near the origins of the replicons, suggests that the interaction between origin and matrix may be required for initiation. Such a system, in combination with 'master initiation' of origins, would be fail-safe since failure of initiation of an origin still leads to the correct duplication of all the DNA. If an origin has become detached accidently or as programmed during development, or has failed to initiate at the beginning of S-phase for some other reason, the replication fork of the neighbouring replicon continues chain elongation past the inactive initiation sequence until it meets another replication fork. Termination follows then as normal (Fig. 1, origin W).

ACKNOWLEDGEMENTS

J.H.W. thanks the European Molecular Biology Organization for an EMBO fellowship.

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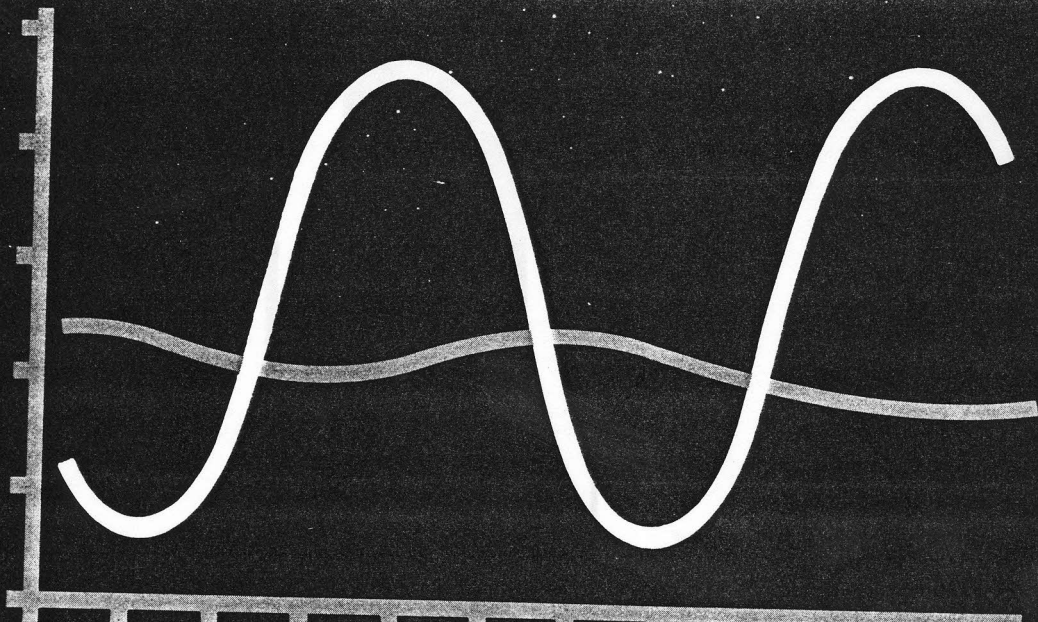

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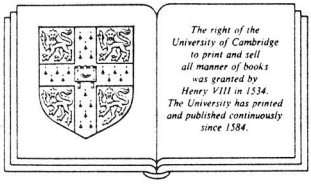

1985

CAMBRIDGE UNIVERSITY PRESS
Cambridge
London New York New Rochelle
Melbourne Sydney

Published by the Press Syndicate of the University of Cambridge
The Pitt Building, Trumpington Street, Cambridge CB2 1RP
52 East 57th Street, New York, NY 10022, USA
10 Stamford Road, Oakleigh, Melbourne 3166, Australia

First published 1985

Printed in Great Britain at the University Press, Cambridge

Library of Congress catalogue card number: 84-46159

British Library cataloguing in publication data
The cell division cycle in plants. - (Seminar series/
Society for Experimental Biology; 26)
1. Plant cells and tissues 2. Cell division
I. Bryant, J.A. II. Francis, D. III. Series
581.87'62 QK 725

ISBN 0 521 30046 0