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DNA Replication in Plants

Edited by
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Chapter 1

FUNCTIONAL CHROMOSOMAL STRUCTURE: THE REPLICON

Jack Van't Hof

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I. INTRODUCTION

Historically, plant breeding, classical plant genetics, and plant cytogenetics have established many fundamental rules of inheritance for both plants and animals. Similarly, contemporary work on plants is making leading contributions in genetic engineering, transformation, gene expression, somatic cell hybridization, and the use of sophisticated culture procedures to clone, develop, and propagate cells and plants, as well as adding to our knowledge of concepts applicable to plants and animals. Two basic biological processes essential to both classical and molecular plant research are cell division and chromosomal DNA replication. Should either of these two processes fail to function all experiments, whether classical or molecular, also would fail. Yet, despite their importance, too little is known about cell division and chromosomal DNA replication in higher plants. The objective of this chapter, and indeed, of the entire book, is to encourage more research in these fields.

The review is a synopsis of the current knowledge of chromosomal DNA replication in higher plants. It attempts to unfold the story from its historical beginnings, highlighting areas of ongoing research where questions are posed and answers are still forthcoming, and presents a short summary suggesting where future work with higher plants can contribute to the general principles governing how chromosomes are made and how replication of DNA is tied to functions peculiar to plants.

The information presented is from plants and from other organisms. Work with nonplant systems is well advanced and evidence from bacteria, protozoa, simple fungi, and mammalian cells offers helpful clues about how to approach similar problems in plants.

II. PLANT CHROMOSOMAL DNA REPLICATION

A. Plant Chromosomes Have Very Long DNA Molecules

Eukaryotic chromosomes viewed at metaphase are neatly and reliably structured. They are the classical hallmarks for species identification; their numbers, dimensions, and shape are commonly used by cytogeneticists and cytotaxonomists alike. More than 40 years ago it was recognized that simple treatments such as chilling changed the structure of plant metaphase chromosomes so predictably that the constrictions produced served as markers for species identification.[1,2] Metaphase chromosomes, seen with an electron microscope, are a mass of nucleoprotein fibers ranging in thickness from 100 to 200 Å.[3] Evidence from *Drosophila*,[4] yeast,[5] and a basidiomycete fungus, *Schizophyllum*,[6] indicates that the fibrillar structure is the package in which the DNA duplex molecules are compacted, one molecule per chromatid. In higher plants, assuming uninemy is characteristic of all species, the length of a single DNA duplex molecule can be enormous and the difference in length between species is great. *Trillium grandiflorum* S., for example, has about 9 pg or 284 cm of DNA per chromatid, while *Arabidopsis thaliana* with the same number of chromosomes has only 0.04 pg or 1.2 cm per chromatid.[7,8]

B. Plant Chromosomal DNA Is Replicated Simultaneously at Multiple Sites

Clues about how plants replicate long chromosomal DNA molecules were gathered over 20 years ago, in the early days of high-resolution autoradiography.[9,10] Cells, labeled with tritiated thymidine at a given time in S phase and subsequently viewed at metaphase, had clusters of silver grains scattered along the length of the chromatids. This finding established two rules governing the replication of chromosomal DNA. First, it showed that chromosome DNA is replicated simultaneously at many but not at all sites. Second, it showed that, at a given time within a given diploid chromosomal complement, segments of different chromosomes are replicated coordinately.

Additional rules governing chromosomal DNA replication awaited the development of

new methods with improved resolution. A silver grain produced by the β-ray emitted from tritium has an average diameter of 1 μm. A grain of this size above a metaphase chromosome covers 29 to 37 mm of DNA or about 9×10^7 base pairs (bp).[11] A better look at the DNA hidden beneath a silver grain was achieved by autoradiography of tritium-labeled isolated DNA fibers.[12] DNA fibers from lysed nuclei spread on the surface of a glass microscope slide could be viewed by light microscopy.[13-15] This simple procedure improved the resolution of DNA molecules 30,000-fold. A silver grain of 0.8 to 1 μm in diameter located above a DNA fiber covers roughly 3×10^3 bp. Application of DNA fiber autoradiography to replicating plant chromosomes was delayed because there was no simple quick procedure for isolating undamaged nuclei from plant tissue. This needed procedure now exists and it provides clean, undamaged nuclei that give long, undegraded molecules of chromosomal DNA.[16-18]

C. Three Replicon Properties of Plant Chromosomal DNA Seen by DNA Fiber Autoradiography

Taylor applied the term "replicon" to replication units of eukaryotes.[19] The term, first introduced by bacteriologists in reference to the replication unit of the bacterial chromosome,[20] emphasizes the similarities of replication units despite differences in phylogeny. The term serves this purpose provided it is recognized that replicons of bacteria and those of higher organisms have one fundamental difference. In bacteria, the entire chromosome constitutes a single replicon but the chromosome of eukaryotes has a multitude of tandem replicons along its longitudinal axis.[13,21-23] All replicons have three properties: an origin where replication begins, two replication forks that diverge from the origin in opposite directions while forming new daughter DNA chains, and a replication rate (chain elongation rate) determined by the speed at which a fork moves while copying the parental molecule. Evidence for these three properties is seen in DNA fiber autoradiograms of replicating plant DNA.

The analysis of grain arrays on an autoradiogram of labeled DNA fibers is summarized diagrammatically in Figure 1. The lines noted as A, B, and C represent DNA duplex molecules with origins (the "O's") spaced at regular intervals along their lengths. Each row of arrows immediately below each line indicates arrays of contiguous silver grains produced by the incorporation of tritiated thymidine into newly replicated DNA. The arrowheads give the direction of fork movement. The space between the arrowshafts of diverging forks represents DNA replicated before the radioactive pulse. Where no space exists between arrows and where the arrow has two heads, replication began during the pulse. In this instance, a single array of contiguous grains represents the movement of two diverging forks (line C; the 3 arrows located at the righthand side).

When two neighboring replicons start replication at the same time before the pulse, the movement of the four forks along the parental chains is recorded by four arrays of grains that appear as sequentially aligned nearly symmetrical pairs of labeled segments (Figure 1; the arrows beneath lines A and B). The length of the individual labeled segments is determined by the speed at which the forks traveled during the pulse, the pulse duration, and the time of replication initiation. A plot of the average length of each labeled segment of each pair of grain arrays expressed as a function of pulse duration produces a curve with an initial slope corresponding to the average rate of a single replication fork.[11]

Examples of this pattern of grain arrays and the increased length of labeled segments with longer pulse times are seen in Figures 2a and 2b. In Figure 2c are patterns of grain arrays produced by a step-down labeling protocol. In this case, the protocol consisted of a 60-min pulse of high specific activity tritiated thymidine (1 mCi/mℓ; ~60 Ci/mM) and then diluted by the addition of 1 mM thymidine to reduce the specific activity. Replicons beginning replication during the high specific activity pulse have high density grain arrays centrally

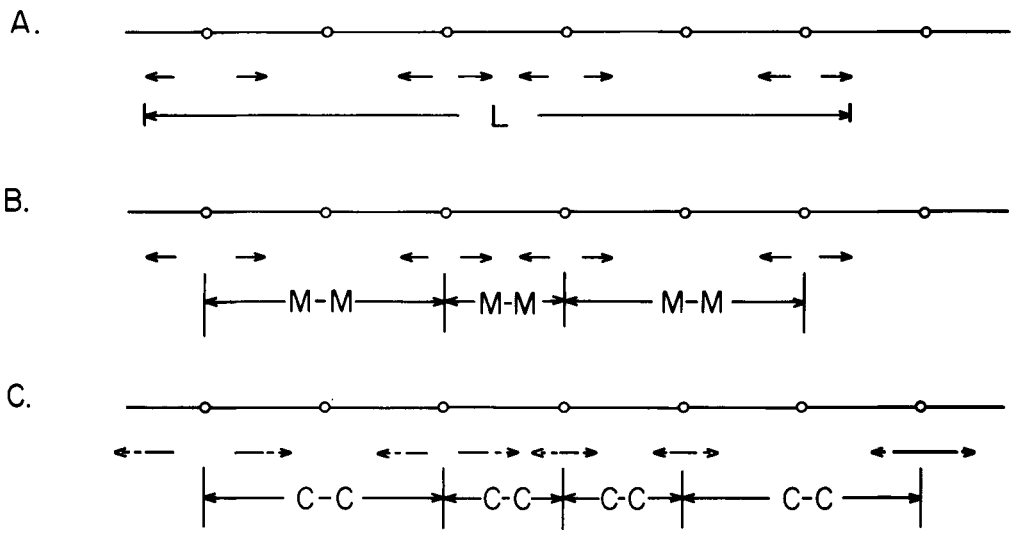

FIGURE 1. Diagrams depicting a DNA duplex molecule with equally spaced origins (the "O's") along its length (lines A, B, and C), and the pattern of labeled DNA segments sequentially aligned as pairs on autoradiograms where replication began before a tritiated thymidine pulse (below lines A, B, and the first two in the series under line C). The last three drawings under C show the expected pattern when replication began during the pulse. The arrows indicate the length of DNA replicated by a single fork and its direction of movement. M-M denotes the distance between the unlabeled central portions of two or more sequential pairs of labeled DNA segments; C-C represents center-to-center distances between grain density gradients produced by a step-down labeling protocol; L, a given length of DNA. (From Van't Hof, J. and Bjerknes, C. A., *BioScience*, 29, 18, 1979. With permission.)

located with trailing arrays of fewer grains on either side. The two grain density gradients trace the movement of diverging replication forks during the time the specific activity of tritiated thymidine was reduced. These patterns, noted by the arrows in Figure 2c, offer visible evidence of bidirectional DNA replication in a higher plant.

The distance between replicon origins, i.e., replicon size, is determined in three ways. One method is to measure the distance between the centrally located unlabeled segments of two symmetrical pairs of grain arrays positioned side-by-side on the same molecule (M-M in Figure 1; arrows in Figures 2a and 2b); another is to measure the distance between the centrally located highly dense regions of two adjacent replicons labeled by the step-down protocol (C-C in Figure 1; arrows in Figure 2c). A third method uses a half-replicon calculation.[17]

The multiplicity of replicons per chromosome in eukaryotes is the basis of another feature of replication: that tandem replicons replicate DNA in clusters.[24,25] The number of replicons per cluster can vary. In plants, up to 18 simultaneously active replicons constitute a cluster.[26]

D. Hierarchical Organization of Temporally Ordered Plant Chromosomal DNA Replication

The foregoing cytological and molecular evidence indicates that replication of plant chromosomal DNA is temporally ordered and has a hierarchical organization. The hierarchy consists of three units. The elementary unit is a single replicon and it is a member of a secondary unit, a cluster. The third unit, the family, consists of one or more clusters. A family is operationally defined as a group of clusters that replicate DNA at a given time during S phase.[27] Evidence that replicon families are activated sequentially comes from several sources. First, at the cytological level, the grains seen above pulse-labeled chromosomes are produced by clusters of replicons that are members of a particular family. In barley, for instance, the late-replicating heterochromatin represents one family, while the

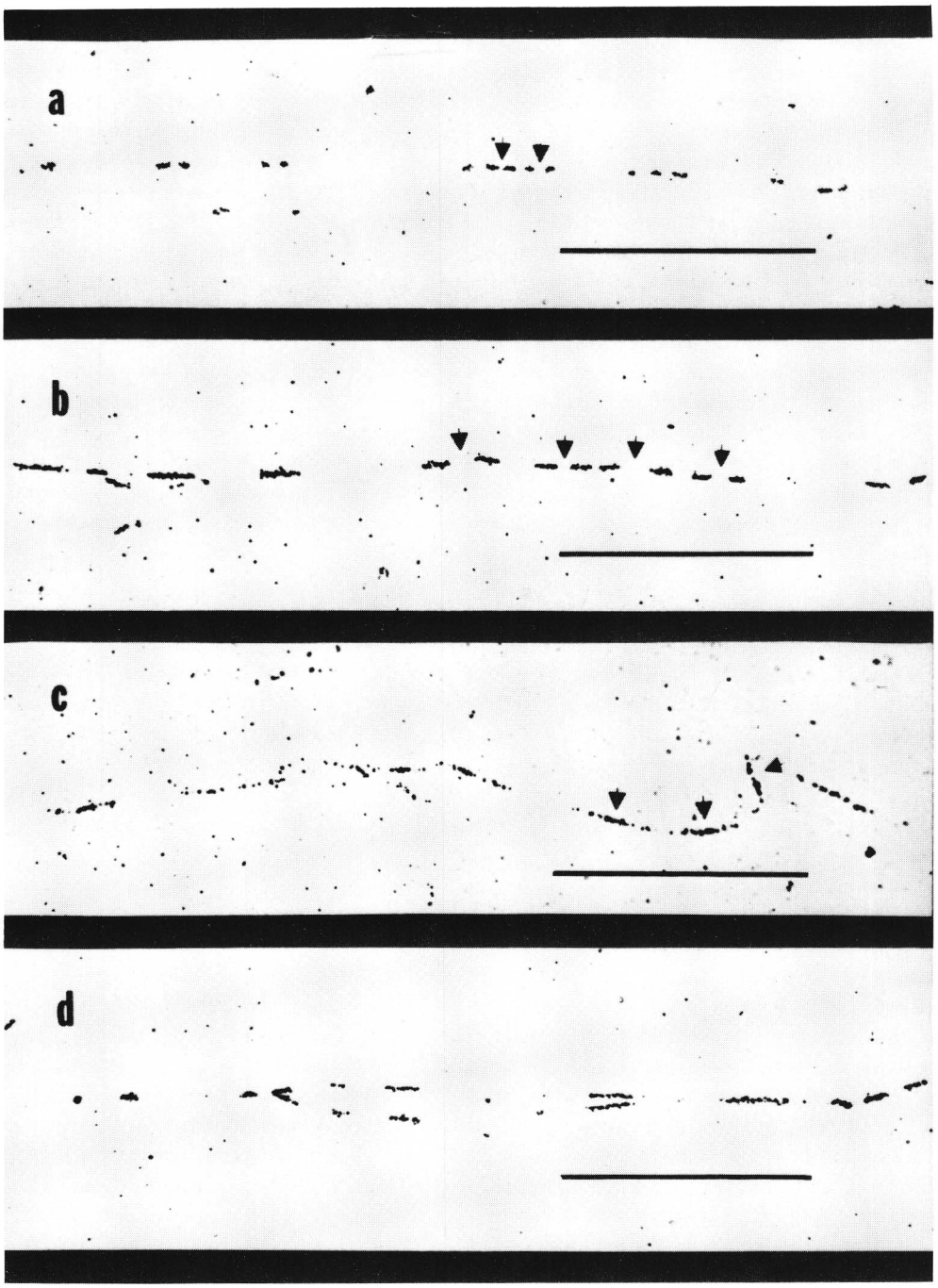

FIGURE 2. Tritiated-thymidine-labeled chromosomal DNA segments on fibers from *Crepis capillaris* root-cap cells grown at 23°C and pulsed for 30 min (a), 60 min (b), and (d). The labeled fiber in (c) is of *Arabidopsis thaliana* after a step-down protocol of 60 min high specific activity and then low specific activity by dilution with 1 m*M* thymidine for 120 min. The arrows note the putative positions of the replicon origins. In (d), is an example of partially separated duplex molecules. The unlabeled segments were replicated before the radioactive pulse. The bar scale is 100 μm. (From Van't Hof, J. and Bjerknes, C. A., *BioScience*, 29, 18, 1979. With permission.)

earlier-replicating euchromatin represents another.[28] Autoradiograms of *Drosophila* polytene chromosomes show that while one region on the chromosome may be active and another not, replication of the active regions occurs sequentially.[29] Also, in *Drosophila,* replication is temporally ordered within as well as between chromosomes.[30] Finally, the temporal replicative order of a segment of the X chromosome of *Drosophila* remains unchanged when translocated to an autosome.[31] This result is taken by the author to confirm the notion that control of replication time resides within the segment itself and that this control is independent of surrounding chromosomal loci. The size of the translocated segment may be important, however, since more recent data from mouse cells show that the time of replication of a small fragment containing a translocated gene is determined by the replicon domain into which it is inserted.[32]

Calculations based on data from DNA fiber autoradiograms show that replicon clusters, and hence replicon families, replicate sequentially.[27,33] In higher plants the temporal order of replicon families contributes to the duration of S phase.[18,27] Plants with large genome sizes have more replicon families and a longer S phase (also see Chapter 3).

Analysis of DNA from lower plants and mammalian cells by isopycnic centrifugation provides further evidence that DNA replication is temporally ordered.[34-36] Molecules replicated during early S phase of one cell division cycle are replicated at the same time during the next cell cycle. Likewise, DNA replicated at the end of S phase also is replicated at the end of S phase of the subsequent cycle.

At the gene level, the evidence for temporally ordered DNA replication is mixed, presumably because some genes or gene families are distributed among several replicon families and others are not. Replication of the rRNA genes of HeLa cells,[37] yeast,[38] and mouse erythroleukemia cells[39] does not exhibit a temporal order. On the other hand, in *Physarum,*[40] kangaroo rat,[41] and pea[42] cells, most of the rRNA genes are replicated in the late S phase, while Chinese hamster cells[43] replicate most of their rRNA genes in early S phase. Replication of low copy genes is ordered temporally. This was shown in yeast genetically by mutation rates[44] and by molecular techniques in mouse,[32,39,45] Chinese hamster,[46] human,[46] *Physarum,*[47] and pea cells.[42]

E. Plant Chromosomal DNA Replicates Stepwise

Replication occurs semidiscontinously through defined intermediates in dicotyledonous plants (see Chapter 2). The first step is the polymerization of deoxyribonucleotides to form Okazaki fragments of up to 300 bases. These small fragments are detectable by velocity sedimentation through an alkaline sucrose gradient. In pulse-labeling experiments, radioactivity appears first in small-sized fragments of 4 to 5S, and then it is seen in fragments of 25 to 35S.[48-50] The formation of the 25 to 35S fragments constitutes the second step. These larger fragments correspond in size to plant replicons seen by fiber autoradiography indicating that replicon-sized DNA is produced by the ligation of many Okazaki fragments. Evidence for the first two steps in replication in higher plants comes from work with embryonic cells,[48] cells of the radicle,[49] and cells grown in suspension culture.[50] Pretreatment with 5-fluorodeoxyuridine, an inhibitor of DNA replication, does not interfere with the two-step pattern.[50] Experiments with *Vicia*[48] and soybean[50] show that even after a 5-min pulse much of the radioactivity sediments with molecules larger than Okazaki fragments. The coincidence of these larger radioactive fragments with those of 4 to 5S is evidence that replication occurs semidiscontinuously via a leading and a lagging strand just as in bacteria.[51]

The third step in the replicative process is the joining of nascent chains of adjacent replicons. This step can be delayed until most of the genome is replicated.[26,52-56] The delay is due to semidiscontinuous bidirectional replication of replicons that function in clusters. Upon completion of chain elongation, the replication forks of each replicon in a cluster converge and, as visualized by autoradiography, appear to fuse producing a single long

thread of cluster-sized nascent DNA. Velocity sedimentation in an alkaline sucrose gradient, however, shows that replicon-sized nascent molecules remain unjoined long after replication ceases. For example, pea cells that are labeled in S phase and subsequently arrested in G2-phase have nascent molecules of replicon-, cluster-, and chromosomal size.[26] These cells completed DNA replication, i.e., they have a 4*C* amount of nuclear DNA, and yet they still have breaks between nascent replicons and clusters. Also, in synchronized pea root cells, Okazaki fragments ligate soon after replication starts, forming replicon-sized molecules, but the joining of these nascent replicons is delayed until cells reach the end of S phase or during G2 phase.[56]

The final joining of nascent replicons also occurs stepwise, going from replicon-size (~18 megadaltons single-stranded DNA) to an intermediate of about 72 megadaltons, and then on to another larger intermediate of approximately 300 megadaltons.[56] Similar-sized intermediates are seen in mammalian cells[52-54] and in cells of *Physarum*.[55] These larger-sized intermediates are groups of nascent replicons within a cluster. Once the larger intermediate cluster-sized molecules are joined, a complete uninemic strand of chromosomal DNA results. The entire process, beginning with the ligation of nascent replicons, and ending with the joining of clusters, is called chromosomal DNA maturation.

F. Chromosomal DNA Maturation Is Influenced by the Size of the Thymidine Pool

Two experiments with pea cells, one using cells synchronized by 5-fluorodeoxyuridine treatment and another using asynchronous untreated cells, show that exogenously supplied thymidine accelerates the joining of nascent replicons and the maturation of chromosomal DNA.[57,58] Accelerated maturation occurs even if the replication fork rate is reduced. The augmentation of joining nascent replicons by excess thymidine suggests that the K_m value of enzymes responsible for chain elongation differs from that of enzymes involved in the joining of nascent replicons.[59]

Experiments with T4 phage-infected *Escherichia coli* offer a view of how endogenous thymidine triphosphate concentrations may vary from one intracellular locale to another. In *E. coli*, a three- to four-fold concentration gradient of DNA precursors increases towards the replication fork sites.[60] When converging replication forks of the phage meet, the enzyme complexes are removed, ending the concentration gradient and leaving a gap where they met. Later on the gap is sealed and ligated, producing a gap-free phage chromosome.

The idea that chromosomal DNA maturation requires increased endogenous deoxythymidine concentration is supported by evidence that enzymes responsible for deoxythymidine production are maximally active in late S phase, a time when maturation occurs. This finding was reported several years ago for mammalian cells[61,62] and cultured plant cells.[63] Recent work with suspension cells of soybean and *Brassica napus* confirms these earlier results[64] (see Chapter 2).

G. Replicon Termination Sites

Replicon termination sites in higher organisms have not been found.[21] Termination sites do exist, however, in *E. coli*,[65] in *Bacillus subtilis*,[66] in animal viruses,[67] and in certain plasmids.[68] In each case, as the replication fork approaches the terminus, the rate of polymerization decreases and the fork is either temporarily retarded at the site or slowly traverses it, giving the impression of being stalled. Eventually, the terminus sequence is replicated, producing a complete nascent replicon. The nucleotide sequence surrounding the replication terminus of the plasmid R6K is known.[69] It is a 215-bp fragment of undistinctive properties that probably acts as a binding site for one or more host specified proteins.

What attracts interest here is the similarity between the action of replication forks in pea prior to the onset of chromosomal DNA maturation and the action of replication forks of bacteria, viruses, and plasmids when they encounter a terminus sequence. The stalling of

fork movement as it approaches a known terminus is analogous to the delayed ligation of nascent replicons in pea. This similarity raises the possibility that the delayed joining of nascent replicon-sized DNA chains in pea results from terminus-like sequences spaced at intervals throughout the genome.

H. Rate of Replication Fork Movement in Higher Plants

The rate of replication fork movement in higher plants ranges from 5.8 to 10 μm/hr.[27] This corresponds to 0.3 to 0.5 kilobases (kb) per min. Fork movement in plants at 23°C is slower than that of other eukaryotic cells. Mammalian cells, for instance, have replication fork rates of 0.5 to 5.0 kb/min. The highest fork rates are seen in bacteria, where forks move at a rate near 100 kb/min.[51]

Fork movement in higher plants is temperature sensitive. It doubles from 0.3 kb/min at 15° to about 0.6 kb/min at 35°C in root meristem cells of sunflower.[70] The average replicon size remains unchanged over this temperature range, but the duration of S phase shortens[71] as a consequence of a faster replication fork rate.

A change in the rate of fork movement is associated with floral induction in shoot apices.[72] The rate is accelerated 1.7-fold within 30 min from the start of the photostimulus, i.e., from the moment the light regime is changed from a short-day to a long-day exposure. This is a particularly interesting finding, since it suggests that plants have photo-inducible factors that are quickly released and cause, either directly or indirectly, accelerated chain elongation.

III. ORIGINS OF REPLICATION

A. Prokaryotes

The origin of replication in bacteria provides a model to which that of eukaryotic cells can be compared. The nucleotide sequences of six different bacterial origins are known.[73] The minimal functional origin has 245 base pairs, 56% of which are (A + T). It contains two kinds of equal-sized regions, those that are conserved and those having a variable sequence. The variable sequences are viewed as spacer regions. There are 9 to 14 GATC sites in each of the 6 bacterial origins, and 8 of these sites are conserved in position in all 6. Methylation of the deoxyriboadenine nucleotide in one or more of the GATC sites within or near the origin is important for the origin to function.

Plasmids containing the unique 245-bp origin of *E. coli* can initiate replication when given any of three enzyme-priming systems.[74] The three systems are: primase alone, RNA polymerase alone, or primase plus RNA polymerase. The overall replication reaction has four stages: (I), prepriming in the presence of DNA and ATP and seven purified proteins: *dna* A, *dna* B, *dna* C, gyrase, single-stranded binding protein, RNA polymerase, and protein HU; (II), priming by the addition of primase and ribonucleotides to the prepriming complex; (III), elongation of the primed DNA by the addition of deoxyribonucleotides and DNA polymerase III; and (IV), amplification of DNA synthesis to produce larger nascent molecules. The complexity of the prepriming and priming stages argues that the sequence of a functional replicon origin has several binding sites for specific proteins and that the number of base pairs needed for these sites is no more than 245. This information from bacteria and that from viruses and yeast[75] should prove useful for future work with higher plants.

B. Higher Plants

The size of replicons in higher plants is 16 to 27 μm (48 to 81 kb)[27] or approximately 15×10^3 per pg of nuclear DNA. Therefore, there are about 15×10^3 origins per pg of nuclear DNA. Yet, in spite of their abundance, no plant origins have been isolated.

In pea, there are primary and secondary preferred sites for replication initiation and therefore, primary and secondary sequences that function as replicon origins.[76] The use of

a secondary site of initiation occurs when the polynucleotide strands of duplex DNA are cross-linked by photoactivated psoralen. The cross-linked strands are inseparable, thereby impeding replication fork movement for chain elongation and preventing unwinding at the origin itself. Replicating DNA, thus treated, initiates replication at a secondary site detectable by a shortening of replicon spacing to about half that of the untreated controls. The use of a secondary origin is accompanied by slower replication fork movement. These two responses, however, may not be related causally. For instance, in sunflower cells grown at low (10 or 15°C) or high (35°C) temperatures, replication fork movement is reduced, but replicon size remains unchanged.[70] If a slower fork movement induces the use of secondary origins, a corresponding reduction in replicon size is expected at the extreme temperatures.

The secondary origins used in psoralen-treated pea cells are not used when DNA synthesis is inhibited by prolonged treatment with 5-fluorodeoxyuridine.[57] Mammalian cells, however, when subjected to 5-fluorodeoxyuridine, apparently *do* use secondary initiation sites spaced approximately 4 μm (12 kb) apart.[77] Nevertheless, a similar treatment of pea cells produces no change in replicon size and no evidence for initiation of replication using secondary origins even though fork movement is reduced. These results suggest that plant cells use secondary initiation sites only under unusual, but as yet unspecified, conditions.

Autonomously replicating sequences (*ars*-element) are possible candidates for origins of DNA replication of higher organisms.[78-80] An *ars*-element is a eukaryotic sequence that, when inserted into a yeast-*E. coli* vector, confers to it the ability to replicate autonomously, extrachromosomally in a yeast host. It is anticipated, though yet unproven, that the replicative phenotype in yeast corresponds to the same phenotype in cells from which the *ars*-element was obtained. When yeast *ars*-elements are tested in yeast their replication is temporal,[80] a finding that supports the notion that in yeast *ars*-elements are similar to, if not actually, replicon origins.

The sequences of two *ars*-elements containing chromosomal DNA sequences of tomato,[81] one of tobacco,[82] and two of rape[83] are known. Those of tomato are AT-rich (81 and 82%), contain numerous direct repeats and several inverted repeats, and have some features common to yeast *ars*-elements. The *ars*-element of tobacco is likewise AT-rich (73%), has two inverted repeats, three direct repeats, and a 32-bp AT repeat; the two from rape are very similar to the *ars*-element of tobacco. All five plant *ars*-elements have the yeast *ars*-elemental 11-bp consensus sequence (5′ A/TTTTATPuTTTA/T 3′). Whether these higher plant *ars*-elements are indeed origins of chromosomal DNA replicons awaits further experimentation. It must be shown that the yeast-selected sequences, when inserted into the chromosome of the plant from which it was obtained, function as constitutive replicon origins.

Evidence that *ars*-elements do represent nonyeast replication origins comes from work with *Tetrahymena*. Multiple copies of the rRNA genes (rDNA) in the macronucleus are linear, extrachromosomal molecules of about 20 kb with palindromic sequence symmetry.[84,85] Each half of the molecule contains the 17S, 5.8S, and 26S RNA genes centrally separated by a nontranscribing sequence. The origin of replication is traceable by electron microscopy to be less than 200 bp of the center of the palindrome in *T. pyriformis* and about 600 bp in *T. thermophila;* in both species the origin is located in the central nontranscribing sequences of the palindrome.[86,87] Plasmids containing the region, known to include the *T. thermophila* origin, function as *ars*-elements in yeast.[86,87] Other nontranscribing sequences, however, from the telomere of the molecule, a region not known to have an origin of bidirectional replication, also function as *ars*-elements.[88,89] The *ars*-elements from both regions have the A/TTTTATPuTTTA/T consensus sequence; there is one consensus sequence in the *ars*-element from the telomeric region and two in the *ars*-element from the *T. thermophila* rDNA origin.

On the one hand, these results with *T. thermophila* show that the *ars*-elemental phenotype expressed in the host yeast cell does select for replication origin activity of foreign (nonyeast)

DNA; on the other, they show that the sequences selected by yeast as *ars*-elements are not always those of a functional origin. Consequently, identification of sequences that function as origins in DNA of eukaryotes other than yeast requires at least two independent means of proof.

Moreover, the biological systems used to test for replicon origins require scrutiny. For example, though the yeast cell discriminately selects as *ars*-elements DNA templates with a certain consensus sequence, *Xenopus* eggs indiscriminately replicate prokaryotic, eukaryotic, and vector sequences.[90] The foreign DNA is replicated under the temporal control of the *Xenopus* host and the amount replicated depends on the size of the injected template. The *Xenopus* test system, therefore, offers no proof that the templates injected are specific sequences corresponding to replicon origins.

That the origin of replication is in the centrally located nontranscribing sequences of extrachromosomal rRNA in *Tetrahymena* is neither unique to this species nor to extrachromosomal DNA itself. Electron microscope studies of transcribing *Drosophila* chromatin during replication also indicate that replication is initiated in the nontranscribing segment of the rRNA genes.[91] Further, in *Xenopus* both electron microscopy and pulse labeling of synchronized cells show that the origin of replication is located on an *Eco*RI fragment containing the nontranscribing sequences.[92] We now await results from higher plants indicating that they too have replication origins in the nontranscribing spacers of the rRNA genes.

The close proximity of rRNA genes to the origin of replication may be common to eu- and prokaryotes. In *B. subtilis,* a ribosomal operon, *rrnO,* located near the chromosomal origin, is viewed as the source of a transcript (16S and/or 25S RNA) that is covalently linked to DNA replicated at or near the origin.[93] In this instance, the bacterial requirement for RNA polymerase to begin replication is conveniently accommodated by a template near the origin, and the transcription product is provided conveniently near the initiation site. While similar arrangements may be rare in higher plants, the notion that rRNA transcripts participate in the initiation of DNA replication is worthy of consideration.

C. Chromatin Structure at the Replication Origins

Analysis of extrachromosomal rDNA of *Tetrahymena* suggests that the chromatin structure surrounding replication origins has a distinctive nucleosomal organization.[94] The central spacer regions of *Tetrahymena thermophila* and *T. pyriformis* have seven and five 200-bp nuclease-protected regions, respectively. These regions, presumably nucleosomes, are flanked by nuclease-sensitive sites whose location corresponds to that of replication origins and they are maintained in replicating and nonreplicating rDNA. The flanking nuclease-sensitive sites are also near the transcriptional initiation sites for rRNA. Consequently, they could play a role in both replication and transcription of the rRNA genes in a manner similar to that proposed for the *rrnO* operon in *B. subtilis*.[93] Besides being maintained in replicating and nonreplicating rDNA, the stable nucleosomal organization of the centrally located spacer region also is stable during transcription. In contrast, the nucleosomal organization of the transcription region of the ribosomal chromatin changes when the rDNA is transcriptionally active. In starved-cell nuclei the transcribed region shows a periodic accessibility to micrococcal nuclease,[95] while that of log-phase cells loses the periodic chromatin structure.[96] Thus, the stable chromatin organization of the centrally located spacer region may reflect a characteristic of neighboring sequences that contribute to the functional specificity of origins.

IV. STRUCTURAL ORGANIZATION OF REPLICON DOMAINS

Recently, visible evidence from mammalian cells shows that replicon clusters are organized in flower-like structures that appear granular when replication begins, change to larger

globular forms as replication proceeds, then to ring-like or horseshoe-shaped objects, and finally to rope-like structures.[97] It takes about 1 hr for each group of clusters of a given replicon family to complete these structural changes. The average number of participating clusters per replicon family is 126, and this number remains relatively constant as the cells proceed through S phase. Though these findings pertain to mammalian cells, they are in accordance with data from higher plant cells indicating that the duration of S phase is determined by the number of sequentially active replicon families.[27] Further, these findings show that the replicon cluster exists during replication as a structural entity somehow fixed to a proteinaceous element within the nucleus.

The nature of proteinaceous structures and the nature of the DNA bound to them is controversial.[98,99] The sources of controversy appear to be the tonicity of the salt solutions used to isolate nuclei and the means of extracting nuclei to produce what is commonly called the nuclear matrix. Workers in the field are aware of this problem and, though nucleoproteins are associated with replicating DNA,[97] the true nature of the nuclear matrix remains unclear. What is needed is a systematic analysis of factors associated with or responsible for the varying results. This approach will give a better idea about how to identify the proteins involved in the visible ring-like or horseshoe-shaped structures of replicating replicon clusters.[97]

V. GENERAL COMMENTS

The foregoing discussion provides ample evidence for the need of more work in nearly all of the fundamental aspects of chromosomal DNA replication in higher plants. Beside this need there are other fertile untapped areas of investigation for which higher plants are specially endowed by nature to yield new information and insights. One such field is naturally occurring polyploidy and polyteny. Polyploidy and polyteny are cytological indicators of failure of still-to-be-described mechanisms responsible for maintaining the rule that a chromosomal complement replicates but once per cell cycle, thereby conserving diploidy (see also Chapter 3). Such mechanisms are clearly important genetically, and they clearly involve chromosomal DNA replication. Another potentially productive area is the photoinductive factors that influence replicon properties of cells participating in the change from a vegetative to a floral meristem. Here again, the factors are unknown but they surely involve chromosomal DNA replication. Still another field is the coordinate control of nuclear and organelle DNA replication within a cell.[100] Is the replication of the chloroplast and mitochondrial genomes independent of nuclear control, and if so, why is there a positive correlation between nuclear ploidy level and the number of chloroplasts within a cell?[101] Finally, there is a need to develop plant-specific in vitro assay systems analogous to those developed for bacterial replicative functions. Such systems, using recombinant DNA technology, would be capable of giving unambiguous answers about nucleotide sequences suspected of functioning as origins and termini in higher plants.

ADDED IN PROOF

Since the submission of this manuscript (October, 1986), the occurrence of a specific origin and termini has been demonstrated in a higher plant.[102,103]

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