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DNA Replication in *Physarum*

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The natural synchrony of the nuclear cycle within the plasmodium of the myxomycete *Physarum polycephalum* provides an opportunity to study, without the need for any cell treatment, the complex and transient patterns of replication intermediates generated during chromosomal replication. In this review, we focus on the parameters of replication kinetics from the synthesis of Okazaki fragments to the chromosome-sized progeny molecules and summarize data suggesting that their appearance at some specific loci is precisely programmed and intimately linked to the transcriptional activity of the cell.

STRUCTURE OF S PHASE

The Plasmodium

In one phase of their life cycle, the myxomycetes develop a particular multinucleated cell type, the plasmodium, that results from nuclear divisions in the absence of cell division (Rusch 1980). Deposited on a filter paper, small plasmodia cultivated in shaken liquid cultures fuse spontaneously into a single cell (5–10 cm in diameter) that contains more than 10^8 diploid nuclei (Fig. 1A). Being in a common cytoplasm, these nuclei divide synchronously every 10 hours.

Their division is characterized by the persistence of the nuclear envelope throughout the mitotic process. This "closed" mitosis should not be seen as a loss of capability for cell division, but rather as the result of a developmental process, since in the amoebal, haploid vegetative phase of *Physarum*, the uninucleated cells divide by a conventional mitosis in which the nuclear envelope disintegrates in prophase. Thus, the *Physarum* genome encodes different cell types, with the plasmodial stage particularly suitable for DNA replication studies.

The Synchronous Nuclear Cycle

The synchrony of mitosis within the plasmodium was first described by Howard in 1932 and was extended to the synchrony of S phase by the

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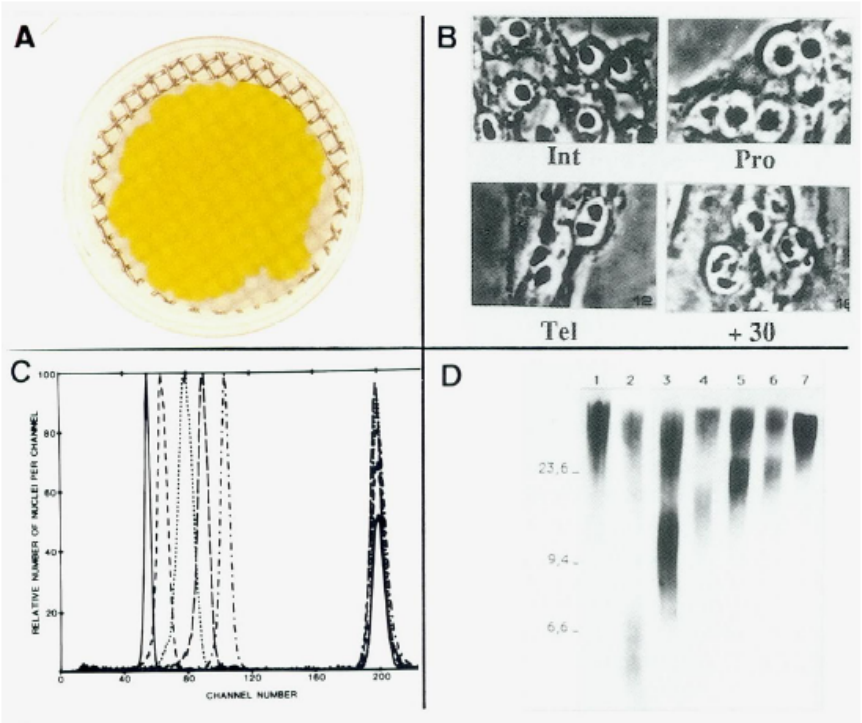

Figure 1 Synchrony of the nuclear cycle in the plasmodium of *Physarum*. (*A*) Macroscopic aspect of a plasmodium containing about 4 x 10^8 synchronous nuclei. Abruptly, in early S phase, an amount of DNA corresponding to 600 km of double-helix is synthesized per min in that cell. (*B*) Cell-cycle stage determined by phase-contrast microscopy on ethanol-fixed smears. Interphasic (Int) nuclei are characterized by a large, centrally located nucleolus, and condensed chromosomes are apparent in prometaphase (Pro), and a few minutes later in telophase (Tel). In the absence of G_1, the onset of S phase is morphologically defined by observation of telophase. Fusion of the prenucleolar bodies occurs in early S phase (+30). (*C*) Flow-cytometric analysis of the natural synchrony of S phase within a plasmodium. Internal standard chicken erythrocyte histograms overlap at channel 200. Lefthand peaks represent Hoechst-stained *Physarum* nuclei isolated at 5 min, 30 min, 1 hr, 2 hr, and 7 hr after the onset of S phase. From the positions of these peaks, the rate of DNA synthesis has been estimated at 5000 kb/min/nucleus in early S phase. (Reprinted, with permission, from Kubbies and Pierron 1983.) (*D*) Nascent-strand elongation of the LAV 1–2 replicon on alkaline gel. The growth of the replicon is perceptible in total DNA samples taken every 5 min in early S phase at 5, 10, 15, 25, 30, and 40 min post-telophase (lanes *2–7*). In contrast, only the parental DNA hybridized to the probe in the G_2-phase sample (lane *1*, above 23.6 kb). The kinetics of elongation of this particular replicon is estimated at 1 kb/min. The smearing of the nascent strands suggests a 5-min asynchrony within the 600-min-long nuclear cycle. (Reprinted, with permission, from Bénard and Pierron 1990.)

use of radioactive precursors (Nygaard et al. 1960; Braun et al. 1965). During this procedure, a remarkable feature of the nuclear cycle was discovered: the absence of a G_1 phase. Thus, the synchronous onset of S phase is morphologically defined by the observation of telophase (Fig. 1B). The 3-hour S phase is followed by a 6- to 7-hour G_2 phase, leading to the next mitosis (Fig. 1B).

The degree of synchrony has been estimated by flow cytometry of Hoechst-stained nuclei isolated at various time points after mitosis (Fig. 1C). Narrow and symmetrical histograms of DNA fluorescence were found, leaving no doubt that 99% of the nuclei enter into and exit from S phase simultaneously. This unique level of synchrony can be appreciated by measuring, at one time point, the heterogeneity in length of the 10^8 copies of a replicon. In Figure 1D, the kinetics of elongation of a specific replicon is measured at 1 kb/min up to a size of 30 kb, yet the dispersion of the nascent strands at a given time point is limited to about 5 kb, suggesting a 5-minute asynchrony within the 600-minute-long nuclear cycle, i.e., a 1% temporal window (Bénard and Pierron 1990).

Kinetics of DNA Synthesis

In *Physarum*, the C value is equal to 0.3 pg, defining a genome size about 20 times larger than yeast or one-tenth of the human genome (Mohberg et al. 1973). Nevertheless, renaturation kinetics indicate that two-thirds of the DNA is single-copy, the rest being composed of interspersed middle-repetitive sequences (Hardman et al. 1980). Incorporation studies (Braun et al. 1965) and flow-cytometric measurements (Fig. 1C) reproducibly indicate that within 5 minutes of the uncoiling of telophase chromosomes, a maximal rate of DNA synthesis of about 5000 kb/min per nucleus is reached and maintained for 90 minutes. At this point, 75% of the genome is replicated. It takes another 90 minutes to replicate the late-replicating quarter of the genome, due to a slowdown of DNA synthesis at about 1200 kb/min per nucleus. However, the mean size and the elongation rate of the replicons appear to be constant throughout S phase (Funderud et al. 1978b).

Structure of the Replicons

The replicons were found to be heterogeneous in size at 12–60 kb, with a mean size of about 35 kb, by sedimentation on alkaline sucrose gradients (Fig. 2A), electron microscopic observations, and DNA fiber autoradiography (Funderud et al. 1978a,b, 1979). The progression of the replica-

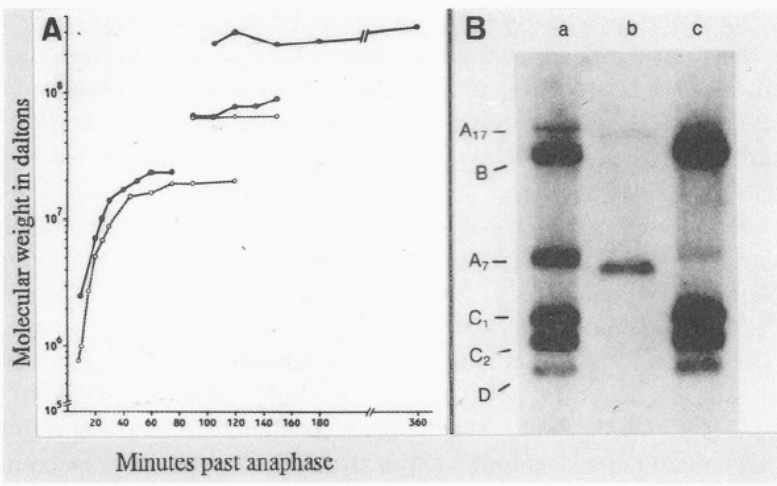

Figure 2 Structure of S phase in *Physarum*. (*A*) DNA chain growth as seen by sedimentation on alkaline sucrose gradients. Open circles correspond to the peak DNA molecules pulse-labeled at various time points in S phase, closed circles depict molecules labeled at the onset of S phase. A constant increase during the first 40–60 min of S phase, at a rate of about 1–2 kb/min/replicon is followed by a two-step increase that reveals clustering of the early activated replicons. (Reprinted, with permission, from Funderud et al. 1978b [copyright Cell Press].) (*B*) Temporal order of replication at the four unlinked actin gene loci. Following a BrdU treatment during the first 40 min of S phase, the replicated heavy–light (lane *c*) and unreplicated light–light (lane *b*) DNA fractions were isolated on cesium chloride gradients and their content in actin genes was compared to total DNA (lane *a*). Filter hybridization indicates that the *Hin*dIII restriction fragments containing the actin *ard*B gene, the two alleles of the *ard*C gene (C1 and C2), and the *ard*D gene have replicated, whereas the two *Hin*dIII fragments that define the *ard*A locus have not. This order of replication is invariant within the plasmodium. (Reprinted, with permission, from Pierron et al. 1984.)

tion forks, in which Okasaki fragments of 200 bp primed by short oligoribonucleotides (Waqar and Huberman 1975) are involved, has been evaluated at 1.2 kb/min/replicon in independent investigations (Funderud et al. 1978b; 1979; Hunt and Vogelstein 1981). Within a replicon, replication is bidirectional, with equal rates on both forks. This was shown by photolysing DNA that had been substituted with bromodeoxyuridine (BrdU) for 30 seconds at the onset of S phase. Following a chase, the nascent strands were cut into two equal pieces by UV irradiation, indicating that the BrdU-substituted origins are centrally located within the growing replicons and providing evidence for initiation limited to only

one site in every replicon (Funderud et al. 1978a). This conclusion was strengthened by the observation of discrete replicating eyes in the electron microscope (EM), at a mean frequency of 1 bubble every 525 kb of early S phase DNA (Funderud et al. 1979; Haugli et al. 1982). However, a conflicting EM observation of clusters of microbubbles as the most abundant replication intermediates in early S phase of *Physarum* has also been reported (Hardman and Gillespie 1980). In this latter case, larger bubbles were also observed, but were 10 times less abundant. It was concluded that microbubbles were transient precursors of the more extended bubbles (Hardman and Gillespie 1980). From the comparison of the two sets of data, we suggest an alternative interpretation.

Macrobubbles Versus Microbubbles in *Physarum*

In observations by N. Hardman, only 1% of the DNA molecules contained large bubbles. The mean size of these DNA molecules, as inferred from measurements of distances between successive clusters of microbubbles, should be in the range of 100 kb. If so, this corresponds to 1 large bubble every 10,000 kb of DNA, much too low a number as compared to observations by F. Haugli at the same stage in S phase. Considering this lack of extended bubbles, it is tempting to speculate that microbubble clusters are generated by a partial reassociation of the parental DNA within large bubbles. This would explain that the size of the microbubble clusters and their center-to-center distances coincide with the size and the distribution of the growing replicons seen by F. Haugli and his collaborators. This interpretation would also account for comparable numbers of replicons in the two studies, rationalize the frequent and puzzling single-stranded nature of the microbubbles, and explain their absence from DNA extracted from G_2-phase or mitotic plasmodia. Finally, it is of note that microbubbles, occasionally described as the most abundant replicating structures by observation of naked DNA (Hardman and Gillespie 1980; Micheli et al. 1982), have never been observed on the corresponding chromatin spreads either in blastoderm embryos of *Drosophila* (McKnight and Miller 1977) or in early S phase of *Physarum* (Pierron et al. 1982).

Patterns of DNA Chain Growth

Using the size of the genome and the mean size of the replicons, the kinetics of DNA synthesis at 5,000 kb/min/nucleus, and the elongation rate of 1.2 kb/min/replicon, one can calculate that 4,000 of the 18,000

replicons are activated at the onset of S phase. Joining of the early activated replicons takes place by steps (Fig. 2A) that provide the first indication of their clustering (Funderud et al. 1978b). The early S-phase clusters are composed of 3–4 replicons that are completed by 40 minutes in S phase, but that fuse only at 60 minutes. A second transition in the maturation of the progeny molecules occurs at 120 minutes and further suggests a clustering of the replicon clusters. These very defined steps in the elongation of the DNA chains suggest that the same initiation sites are used in the many nuclei of a plasmodium. The invariant temporal order of replication implies that these sites are also used in consecutive S phases.

TEMPORAL ORDER OF REPLICATION IN *PHYSARUM*

Invariant Replication Timing of DNA Sequences

The first demonstration of a defined order of replication within eukaryotic chromosomes came from labeling replicons in two consecutive, synchronous S phases of the same plasmodium. In one cycle, the late-replicating DNA was labeled by [^{3}H]thymidine incorporation and, after a chase, the replicating DNA of the following S phase was density-labeled by BrdU. The tritiated late-replicating DNA was not density-shifted and therefore not replicated in the early compartment of the ensuing S phase but became denser in late S phase, demonstrating a temporal order of replication of the eukaryotic genome (Braun et al. 1965).

Studies of the timing of replication of specific genes confirmed the programmed replication of the genome. By standard density-shift experiments (Fig. 2B), it was shown that three of the four unlinked actin gene loci had replicated at 40 minutes in S phase. Measurements of the relative intensity of the hybridization bands from DNA extracted at different time points of the cell cycle on a standard Southern blot provide a control experiment, i.e., a "gene dosage" analysis, that further establishes that three actin gene loci have replicated at 8–10 minutes of S phase and that the fourth actin gene is replicated at 80–100 minutes of S phase (Pierron et al. 1984). This invariant temporal order of replication was extended to the two histone H4 genes and to a set of genes that are duplicated early or late (Jalouzot et al. 1985; Pierron et al. 1989b). On the basis of a thorough gene dosage analysis, it was recently observed that the two alleles of the tubulin *altB* gene replicate concurrently in early S phase whereas, surprisingly, the two alleles of the *altA* gene replicate in a very

broad temporal window in mid S phase, with each allele having its own timing of replication. It was concluded that the control of early replication is stringent, whereas the replication schedule in mid S phase would be relaxed (Cunningham and Dove 1993). These results contrast with the apparent strict timing of the late-replicating genes (Pierron et al. 1984, 1989b). This suggests diversified late-replication patterns, whereas early patterns are constant and precisely defined temporally (Diller and Sauer 1993; Bénard et al. 1996).

Chronology of Replication and Gene Expression

The functional importance of the early-replicating DNA has been recognized for some time in *Physarum*. Inhibition of DNA replication at the onset of S phase had drastic effects on the transcriptional activity and little effect when carried out in the second half of S phase. It was inferred that the early S phase newly replicated DNA is rapidly transcribed and that the late DNA is relatively inactive (Rao and Gontcharoff 1969; Fouquet et al. 1975). To better define the relationship between genes and replicons in early S phase, a direct visualization was conducted on chromatin spreads (Pierron et al. 1982). Observation of nascent replicons undergoing active transcription provided direct evidence for a rapid activation of the newly synthesized genes (Fig. 3A). In contrast, no transcripts were found between replicating eyes, demonstrating a preferential transcription of the early replicons. Finally, the most striking feature of the majority of these early-replicating genes was their location at the center of the nascent replicons, indicating a close proximity, if not a coincidence, of these genes with their replication origin.

This enrichment of the early replicons in active genes is further confirmed by comparing the pattern of expression and the replication timing of 18 genes. The 5 genes that are inactive within the plasmodium were not confined to one compartment of S phase; 1 was found to be early, 2 replicate in mid S phase, and 2 replicate very late (Pierron et al. 1989b). Among the 13 active genes, 10 replicate very early in S phase and 1 replicates in mid S phase (the tubulin *altA* gene), whereas 2 genes encoding abundant mRNA were found both by BrdU incorporation and by gene dosage to be duplicated late in S phase: at 100–120 minutes for the LAV 3-2 gene (Pierron et al. 1989b) and later than 90 minutes for the *php* gene, a single-copy gene whose two alleles replicate late (Bénard et al. 1992).

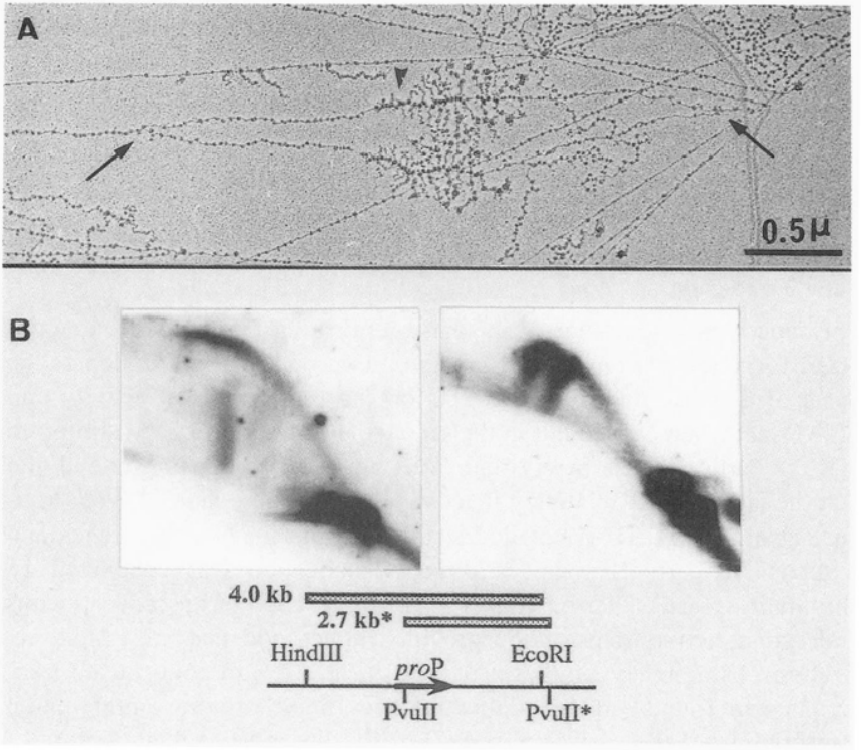

Figure 3 Tight linkage between genes and replication origins in *Physarum*. (*A*) Visualization at 15 min in S phase of a newly replicated gene at the center of a growing replicon. Assuming a bidirectional fork-rate movement, the presumptive location of the origin of replication coincides with the gene. Arrows indicate replication forks, arrowhead marks the center of the replicon. (Reprinted, with permission, from Pierron et al. 1982.) (*B*) Coincidence of a profilin gene and a site-specific replication origin. (*Left*) Two-dimensional agarose gel electrophoresis analysis of replication, on total DNA extracted at 5 min in S phase, of the 4-kb *Hin*dIII–*Eco*RI fragment that contains the profilin P gene. The distinct transition from a bubble arc to a Y arc reveals the presence of a bidirectional, site-specific origin slightly asymetrically located. (*Right*) Replication of the *Pvu*II fragment that covers the 3′ side of the gene (see map). Owing to a variable restriction site (*asterisk*), the two alleles are shown to be coordinately replicated, implying a simultaneous activation of the allelic origins. The Y-arc pattern excludes the origin from the 3′ side of the gene. (Reprinted, with permission of Oxford University Press, from Bénard and Pierron 1992.)

MAPPING OF REPLICATION ORIGINS IN *PHYSARUM*

Chromosomal Origins

In these studies, the early-replicating active genes that were examined were most likely closely associated with a replication origin. A linkage analysis, based on the assumption that the shortest nascent strands of a replicon detected by hybridization with a cDNA probe contain both the gene and the replication origin, confirmed a close association between five genes that have been studied and their respective origins (Bénard and Pierron 1990, 1992; Bénard et al. 1996). These replication origins can be further defined by two-dimensional gel analysis. In one case, the LAV 1-2 developmentally regulated gene, a composite pattern of replication intermediates was obtained, suggestive of two relatively inefficient origins, located about 10 kb apart, on both sides of the gene. These origins would be simultaneously activated, generating forks converging and stalling on the gene (Diller and Sauer 1993). This complex pattern could not have been predicted from the analysis of the nascent-strand elongation of that replicon shown in Figure 1D. In the four other cases, two-dimensional gel analysis established the presence of an efficient, site-specific origin closely associated with the promoter region of the genes (Fig. 3B). These results confirm the EM observations of replication-transcription coupling at early-replicating loci (Fig. 3A). The proximity of an origin to a gene was shown for a developmentally regulated profilin P gene (Bénard and Pierron 1992; Diller and Sauer 1993), the two unlinked and constitutively expressed actin genes, ardB and ardC (Bénard et al. 1996), and a cell-cycle-regulated histone H4 gene (G. Pierron et al., in prep.). It seems likely that these site-specific origins are genetically determined. This view is reinforced by the analysis of the replication of the rDNA of *Physarum*.

rDNA Replication

In *Physarum*, the rRNA genes are found on about 150 copies of a 60-kb linear palindromic molecule (Fig. 4B). It has long been known that these minichromosomes replicate throughout the cell cycle except during mitosis and the first hour of S phase. EM visualization of 37 replicating molecules by Vogt and Braun (1977) revealed a bidirectional elongation from either one or the other of two discrete initiation sites, slightly off-center at 33% and 45% of the molecules (Fig. 4A). Taking into account the palindromic structure of the molecules, it was concluded that only one of four potential origins was active on a given molecule. The detailed analysis of the central nontranscribed spacer structure strength-

ened this four-origins hypothesis, as it is composed of a sequence motif, reiterated four times in inverted orientations, at locations compatible with the origins mapped by EM (Ferris 1985). Recent data obtained by two-dimensional gel electrophoresis confirmed that there is a site-specific origin at 33% (Fig. 4C), and indirect evidence was found for a more efficient origin located at 45% (Bénard et al. 1995). A plasmid containing the origin located at 33% was shown to replicate in vitro in a cell-free extract, with the initiations being restricted to the origin region. This provides the first demonstration of the in vitro replication of a eukaryotic chromosomal origin (Daniel and Johnson 1989). All of these studies pinpoint the initiation site to a few hundred base pairs centered on the junction between a series of 31-bp repeats and a sequence of 900 bp that contains potential stem-loop structures (Fig. 4C). Although the role of these different structures in the initiation of rDNA replication is unknown, the presence of four site-specific origins in a region comprising a quadruplicate arrangement of a conserved sequence strongly supports the notion of origins defined by a specific *cis*-acting sequence.

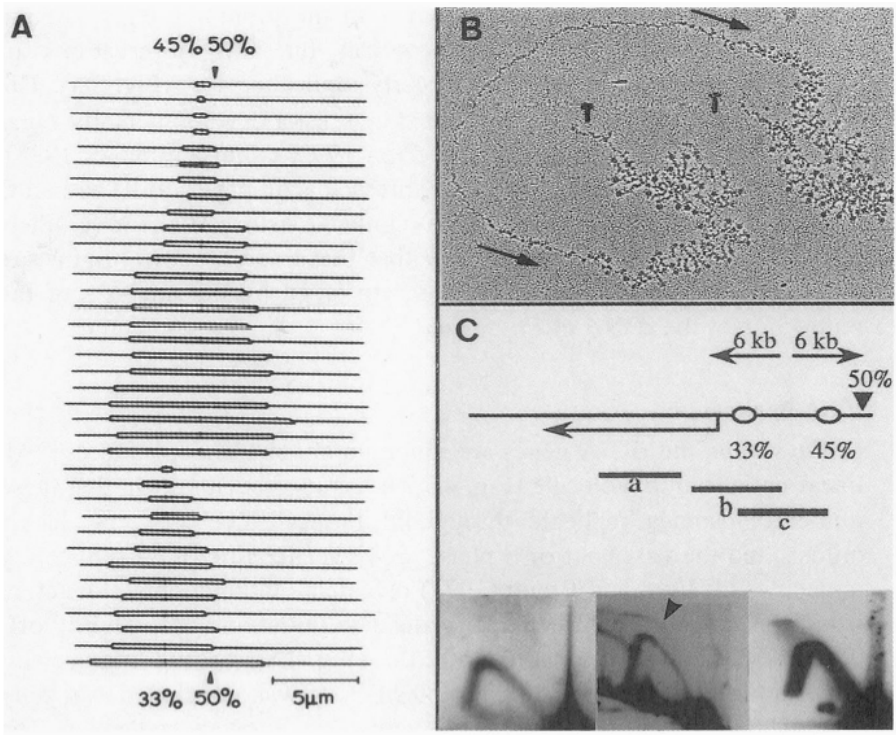

Figure 4 (*See facing page for legend.*)

CONCLUSIONS

Preliminary evidence for site-specific replication origins in *Physarum* is consistent with the wealth of data showing a precise pattern of growth of the nascent DNA strands and an invariant temporal order of replication. Considering that *Physarum* is evolutionarily distant from *Saccharomyces cerevisiae*, this tends to enlarge the base of organisms that rigidly control the initiation of DNA replication. It will be of interest to search for conserved sequences or structures within the replication origins of these two distantly related organisms. Due to a lack of resolution of the mapping of these origins and to an absence of obvious homologies, no consensus sequence has yet been established in *Physarum*. It may also be that the origin activity is dependent on the chromatin architecture of the promoter regions or the proposed association of these origins with the nuclear

Figure 4 Replication of the 60-kb linear palindromic rDNA chromosomes of *Physarum*. (*A*) Alignment of replicating rDNA molecules as seen in the EM. Bidirectional elongation from two discrete initiation sites at 33% and 45% was concluded by Vogt and Braun. Considering the palindromic structure of the molecules, they proposed the presence of four site-specific origins within the central nontranscribed spacer (CNTS). (Reprinted, with permission, from Vogt and Braun 1977.) (*B*) Visualization of an actively transcribed rDNA minichromosome on chromatin spread. Arrows depict polarity of transcription through the two divergent rRNA genes, illustrating the palindromic structure of the molecule and the extension of the 24-kb CNTS. (T) Telomeres. (Reprinted, with permission, from Pierron et al. 1989a.) (*C*) Neutral-neutral two-dimensional-gel analysis of rDNA replication. Scheme depicts half of a palindrome. Sequence analysis revealed that the CNTS is composed of a 6-kb motif of low DNA complexity, reiterated four times in inverted orientations. This motif begins with a series of 78 copies of a 31-bp repeat (TGAGACGGGGAAATTTCTTCAGCCGGTTGAA), continues with a more complex 900-bp sequence that contains potential stem-loop structures, and ends with multiple copies of a 300-bp repeat (Ferris 1985). Probing an intragenic 5-kb fragment (a) resulted in a Y-arc pattern consistent with the expected passive replication of the genes (*left* panel). In contrast, a distinct bubble arc (*arrowhead*) is obtained when a 7-kb fragment (b) that encompasses the presumptive origin at 33% is tested (*central* panel). The site-specificity of that origin is inferred from the absence of bubble arc in the overlapping fragment (c) (*right* panel). Because of a lack of appropriate restriction sites, no direct evidence for an origin at 45% could be obtained by two-dimensional gels; however, both the presence of a weak termination signal in the right panel and the ratio of bubble arc to Y arc in the central panel are consistent with the proposed four site-specific origins of Vogt and Braun (1977) at 33%, 45%, 55%, and 67% (Bénard et al. 1995).

matrix (Wanka 1991), emphasizing the importance of the functional organization of the nucleus in regulating simultaneously various genomic activities.

Indeed, one lesson learned from *Physarum* concerns the tight association between replication origins and active genes (Pierron et al. 1982). As a consequence, active genes are clearly overrepresented in the early-replicating fraction of the genome. Nevertheless, identification of late-replicating and active genes tends to rule out models in which the chronology of DNA replication of chromosomal domains would only be defined by the transcriptional status of the genes. It remains to be seen whether or not these late-replicating genes are linked to active origins.

Replication origins linked to genes have also been found in various loci in metazoan cell types, as for example, the *Drosophila* chorion genes (Heck and Spradling 1990), the human β-globin gene (Kitsberg et al. 1993), or the DNA puff II/9a in *Sciara* (Liang et al. 1993), illustrating that the same functional constraints are at work in shaping eukaryotic genomes.

PROSPECTS

Organisms having site-specific replication origins provide powerful tools for the elucidation of regulatory mechanisms, as exemplified in yeast and eukaryotic viruses. The plasmodium form of *Physarum polycephalum* is an abundant source of such origins, whose dissection will permit a better understanding of the interactions between genes and replicons at a molecular level. The recent development of stable DNA transformation of *Physarum* offers opportunities for assaying autonomous replication of the plasmodial origins (Burland et al. 1993). This would facilitate the definition of a *cis*-acting regulatory sequence and provide a straightforward analysis of their developmental regulation in the amoebal-plasmodial transition. Finally, the availability of discrete replication origins and the possibility of preparing protein extracts from synchronous cultures should stimulate the development of an efficient in vitro DNA replication system.

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