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Site-specific initiation of DNA replication within the non-transcribed spacer of *Physarum* rDNA

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ABSTRACT

***Physarum polycephalum* rRNA genes are found on extrachromosomal 60 kb linear palindromic DNA molecules. Previous work using electron microscope visualization suggested that these molecules are duplicated from one of four potential replication origins located in the 24 kb central non-transcribed spacer [Vogt and Braun (1977) *Eur. J. Biochem.*, 80, 557–566]. Considering the controversy on the nature of the replication origins in eukaryotic cells, where both site-specific or delocalized initiations have been described, we study here *Physarum* rDNA replication by two dimensional agarose gel electrophoresis and compare the results to those obtained by electron microscopy. Without the need of cell treatment or enrichment in replication intermediates, we detect hybridization signals corresponding to replicating rDNA fragments throughout the cell cycle, confirming that the synthesis of rDNA molecules is not under the control of S-phase. The patterns of replication intermediates along rDNA minichromosomes are consistent with the existence of four site-specific replication origins, whose localization in the central non-transcribed spacer is in agreement with the electron microscope mapping. It is also shown that, on a few molecules, at least two origins are active simultaneously.**

INTRODUCTION

Although various methods have been introduced to investigate the mechanisms of chromosomal DNA replication, the nature of eukaryotic replication origins is not yet elucidated. In the yeast *Saccharomyces cerevisiae*, autonomously replicating sequences (ARS) were proposed to correspond to origins. To analyze their chromosomal activity, two-dimensional (2D) gel methods were developed (2,3) and it was then demonstrated that most ARS function as replication origins (4–6). Although the initiation of replication occurs at specific sites in *S.cerevisiae*, the existence of such defined sites remains controversial in other organisms, as illustrated by the amplified dihydrofolate reductase (DHFR) locus in Chinese Hamster Ovary (CHO) cells. An origin of bi-directional replication was mapped 17 kb downstream of the gene by detecting the transition between lagging and leading strand synthesis or by measuring the length of nascent DNA strands (7,8). However, 2D gel analysis of this locus indicates that its replication is initiated from multiple sites located over a 55 kb region encompassing the origin mentioned above (9). Other studies involving these different methods (reviewed in 10) reinforce this conflict between two concepts for replication initiation: specific origins of bidirectional replication or complex broad regions involving multiple sites, with perhaps complete absence of specificity.

These studies have been carried out on a variety of loci and in different organisms. In this context, rRNA genes represent an interesting locus since they are highly conserved and are present in high copy numbers, allowing 2D gel analysis of replication initiation in complex genomes. Electron microscope (EM) data suggested that initiation takes place at fixed sites within rDNA non transcribed spacer (NTS), as deduced from observations of replicating DNA molecules from *Physarum, Tetrahymena, Xenopus* larvae and sea urchin embryos (1,11–13), or from chromatin spreads of *Drosophila* embryos and yeast (14,15). Site-specific initiation of rDNA replication was confirmed by 2D gel analysis in yeast: rRNA gene repeats are replicated from a subset of the ARS elements (~1 in 5) located upstream of each transcription unit (4,16,17). In contrast, results suggesting that initiation occurs without sequence specificity in rDNA of *Xenopus laevis* early embryos, and at multiple sites throughout the 31 kb NTS of rRNA gene clusters in human cells were recently published (18,19). Yet, the tandem repeat organization of the rRNA genes complicates interpretation of 2D gel results; in particular origin usage is ill-defined. In yeast, it is not known if the same subset of replication origins is activated at every cell cycle (4) and in animal cells, it is not clear whether multiple origins are used in some of the repeats, or if a single initiation event occurs for each repeat at different initiation sites (19).

Physarum polycephalum rRNA genes are located on extrachromosomal molecules, as is the case for *Tetrahymena* and *Dictyostelium* (20–22). These molecules, present in about 150 copies per haploid genome (23), constitute 60 kb linear palindromes (Fig. 1A; 24,25). The highly organized but simple structure of *Physarum* minichromosomes implies that each of these molecules acts as a replicon. It also made possible an alignment of replicating *Physarum* rDNA molecules and a precise mapping of replication initiation sites (1). It was deduced that replication forks proceed bidirectionally from four potential

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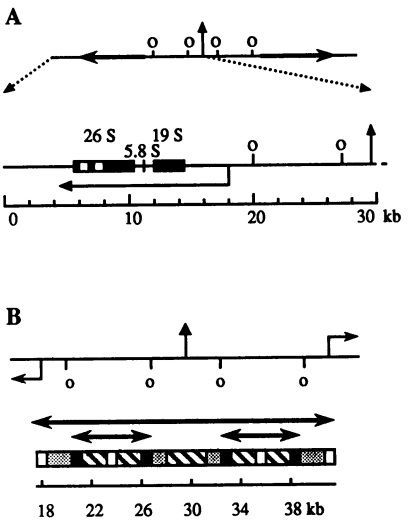

Figure 1. rDNA organization in *Physarum*. (**A**) A 60 kb rDNA palindrome is represented: the vertical arrow indicates the symmetry axis whereas the horizontal arrows depict the two divergent transcription units separated by a large central NTS (24); positions of the replication origins (o) are according to reference 1. A 30 kb half molecule is enlarged; the 13.4 kb primary transcript is represented as a horizontal arrow, the black boxes indicate the position of the 19S, 5.8S and 26S RNA units, the latter containing two introns (white boxes) (25). (**B**) The structure of the central NTS is depicted (adapted from 26). A series of motifs is reiterated four times: bold horizontal arrows under the map underline the symmetries inside the palindrome. Blocks of short direct repeats (stippled boxes) and inverted repeats (hatched boxes) are shown; the few sequences which are not composed of repeats appear as black boxes when they are identical and white ones otherwise.

replication origins located in the central NTS, only one being active on a given molecule. Interestingly, further studies indicated that these four regions constitute nearly identical sequences in inverted orientation (Fig. 1B; 26). However, EM observations of four different origins may in fact correspond to an initiation from multiple sites within a broad region. To test this possibility, we undertook a 2D gel study of rDNA, as it allows the analysis of a much higher number of molecules than is feasable by EM. We conclude that *Physarum* rRNA genes are replicated from site-specific origins, in agreement with results obtained by EM visualization.

MATERIALS AND METHODS

Strains and cultures

Experiments were carried out on strain TU291, which is derived from the Wisconsin I natural isolate (27). Synchronous macroplasmodia were grown as previously described (28). Defined stages of the cell cycle were deduced from the time of mitosis, as seen under a phase contrast microscope of ethanol-fixed smears.

DNA preparation, endonuclease digestion and 2D gel electrophoresis

Total genomic DNA was prepared as described elsewhere (28). Fifteen µg of total DNA was digested with 150 U of restriction enzymes (Boehringer, Mannheim) for 2 h at 37°C.

Neutral–neutral 2D gel electrophoresis was carried out essentially as previously described (2). The first dimension was performed on a 0.4% agarose gel, submitted to either 1 V/cm or 0.5 V/cm for 24–60 h at room temperature. A 1% agarose gel run in a cold room at 3 V/ cm for 15–20 h was used for the second dimension.

Southern hybridization

Following electrophoresis, DNA was transfered onto nitrocellulose membrane by using a VacuGene apparatus (Pharmacia).

Hybridization was carried out in a 65°C Hybaid oven (Schleicher and Schuell) for 20 h with a solution containing 10% sulfate dextran, 200 µg/ml salmon DNA, 3 × SSC, 0.5% SDS and 5× Denhardt's buffer. Filters were washed to high stringency (0.1 × SSC, 0.1% SDS for twice 15 min as final washes) and autoradiographed.

For quantitation of the signals, a MolecularDynamics 400A PhosphorImager and the ImageQuant software were used. The conditions of measuring were as previously described (18).

Hybridization probes

DNA probes were derived from four pBR322 plasmids provided by Richard Braun (Bern, Switzerland). After endonuclease digestion, the fragment of interest was isolated on agarose gel, purified and [^{32}P]dCTP labeled by random primed reaction (NEN).

Plasmids inserts and derived probes are depicted in Fig. 2B: (i) pPHR 116 plasmid contains a 5.4 kb *Bam*HI–*Hind*III fragment located 3 kb from the end of the molecules. We derived two probes from this plasmid, probe 1 which consists in the complete insert, and probe 1a corresponding to a 3.0 kb *Bam*HI–*Eco*RI fragment. (ii) From pPHR102 plasmid we derived probe 2, a 5 kb *Hin*dIII fragment including 3′ part of the 26S, the 5.8S and 5′ part of the 19S genes. (iii) pPHR103 insert is a 2.3 kb *Sal*I fragment corresponding to the 5′ external transcribed spacer. We used either the downstream 0.9 kb *Sal*I–*Pst*I or the upstream 1.4 kb *Pst*I–*Sal*I fragments, referred to probes 3a and 3b, respectively. (iv) pPHR117 insert which is repeated twice per half molecule is a 0.6 kb *Mbo*I fragment located respectively 2.6 and 7.8 kb upstream of the transcription initiation site, in the replication origin regions. Probe 4 contains the complete insert.

RESULTS

In this communication, we analyzed rDNA replication in *Physarum* by the neutral–neutral 2D gel technique of Brewer and Fangman (2). The rationale of this method is based on differential electrophoretic properties of replicating DNA fragments according to their mass and also their shape. As depicted in Figure 2A, restricted linear fragments migrate along a diagonal while replicating fragments are retarded depending on the extent of their replication and the number of replication forks they contain.

First, we asked whether this technique could be applied to rRNA genes using total *Physarum* DNA extracted by standard procedures. Indeed, except in yeast, 2D gel studies on initiation of rDNA replication were carried out with DNA preparations enriched in replicating molecules (18,19). In *Physarum*, there exists ~300 copies of rRNA genes, in a genome size of 6×10^8 bp per diploid nucleus. The natural synchrony of the cell cycle (see below) cannot improve the analysis, as rDNA synthesis is not under the control of S phase (29,30), so that at a given moment of the cell cycle only a few rDNA molecules are actively replicated. In fact, as shown in Figure 2C, the 2D gel analysis of a total DNA preparation allows detection of easily recognizable

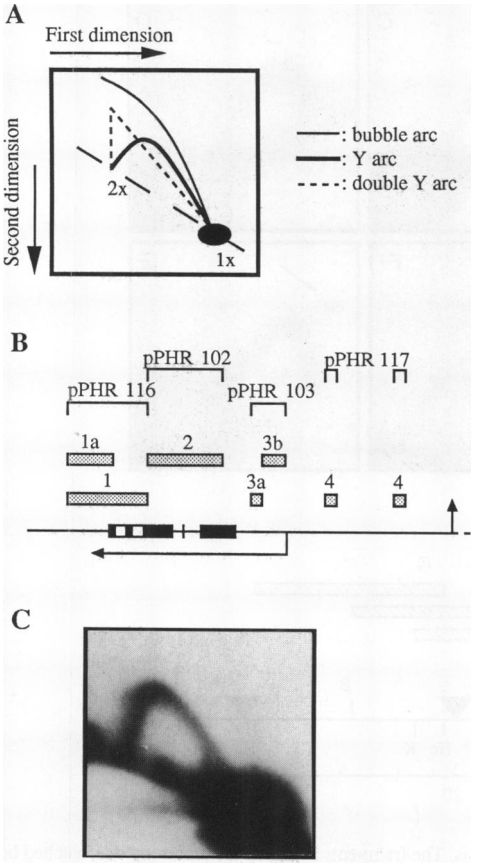

Figure 2. Experimental conditions. (**A**) During neutral–neutral 2D gel electrophoresis of Brewer and Fangman (2), the linear DNA fragments migrate along a diagonal and are separated from the replication intermediates. The bubble arc (plain) is generated by two replication forks initiating from an origin located close to the fragment center, the Y arc (bold) indicates that the fragment is passively replicated by one fork originating outside the fragment, and the double Y arc (dashed) results from two forks converging inside the fragment. (**B**) The extent and the location of the probes (stippled boxes) are depicted with respect to the rDNA unit, with the pPHR plasmids from which they are derived (see Materials and Methods). (**C**) Detection of replication intermediates was assayed at rDNA loci. A 2D gel analysis was carried out on total DNA extracted from a G2 phase plasmodium and digested with *Hin*dIII. The intragenic probe 2 was used for hybridization. After autoradiography, a typical Y signal is observed.

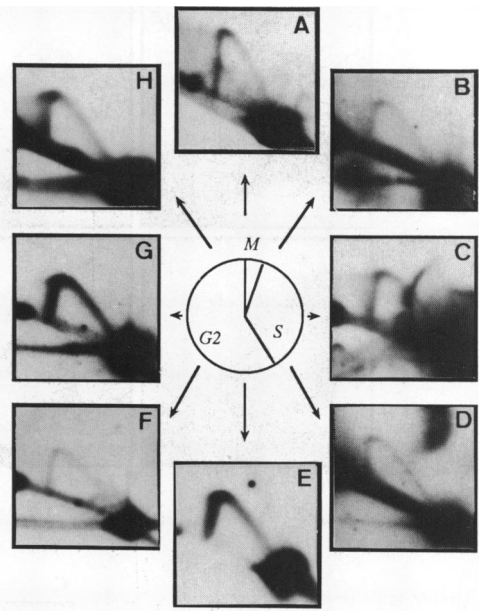

Figure 3. 2D gel analysis of rDNA replication throughout the cell cycle. For all panels, DNA was digested with *Hin*dIII and hybridized with intragenic probe 2. DNA samples were extracted from plasmodia at different stages of the cell cycle, as represented in the center of the figure (M = 30 min mitosis; S = 3 h S-phase; G2 = 5–7 h G2-phase). The letters mentioned on the autoradiograms refer to the cell cycle stage (0 time is the end of mitosis): (**A**) –30 min (prophase), (**B**) + 30 min, (**C**) + 1 h, (**D**) + 2 h, (**E**) + 4 h, (**F**) + 5 h, (**G**) + 6 h, (**H**) + 7 h. A simple Y arc is detected throughout the cell cycle.

replication intermediates: in addition to a main 1× spot corresponding to linear non-replicating fragments, a distinct Y arc is observed. These clear signals demonstrate that a 2D gel study of *Physarum* rDNA replication is feasible without any enrichment of DNA samples nor cell treatment.

The *Physarum* plasmodium is a giant cell containing up to 10^8 nuclei, all cycling synchronously. This allows a precise analysis of the cell cycle events without artificial synchronization. It has been shown that chromosomal DNA is replicated during the 3 h S-phase that immediately follows mitosis (there is no G1 phase). On the other hand, rDNA minichromosome duplication is loosely regulated: it begins in mid-S-phase and continues into the 5–7 h G2-phase, where >50% of the molecules are replicated; there is no rDNA synthesis during the first hour of S phase (29,30). To study the distribution of the replication forks at stages at which rDNA synthesis is arrested and when it resumes, we carried out 2D gel electrophoresis using DNA samples harvested at different points of the cell cycle (Fig. 3). We observed Y patterns at all stages, irrespective of the level of rDNA synthesis. Their different intensities seem to be due to variations in the quality of DNA preparations rather than a cell-cycle dependent modulation, as they were not reproducibly observed in duplicate experiments. These results indicate that replication forks are continuously present on the rDNA fragment, presumably transiently fixed on rDNA molecules in mitosis and early S phase. This suggest that the arrest of rDNA synthesis in early S phase is a result of an elongation blockade. The smoothness of Y arc patterns further suggests that resumption of rDNA duplication occurs from previous fork positions on rDNA molecules.

In order to map replication origins at rDNA loci, we then scanned the minichromosomes by 2D gels. The previous EM study of Vogt and Braun (1) suggested that there are four replication origins in the central NTS (Fig. 1). Therefore, we expected Y patterns in the coding regions and bubble patterns in the central NTS (Fig. 2A). Our results, summarized in Figure 4, show unambiguous and specific patterns which are consistent with this prediction.

In the transcription units, we analyzed four overlapping fragments B, C, D and E (see legend for a detailed description of restriction digests, fragment sizes and probes) and in all cases we obtained a simple Y arc (Fig. 4), demonstrating that the genes are passively replicated. We noticed the absence of double Y patterns, which would result from replication forks moving in opposite directions. A similar result was obtained when analyzing the replication of fragment A, which overlays the main part of the terminal NTS, shortened in its telomeric part (Fig. 4). Only a simple Y arc is detected (the hybridization signals seen on the left correspond to a partial digest). No replication fork barrier could be seen, whereas a polar barrier has been identified 3′ to the rRNA

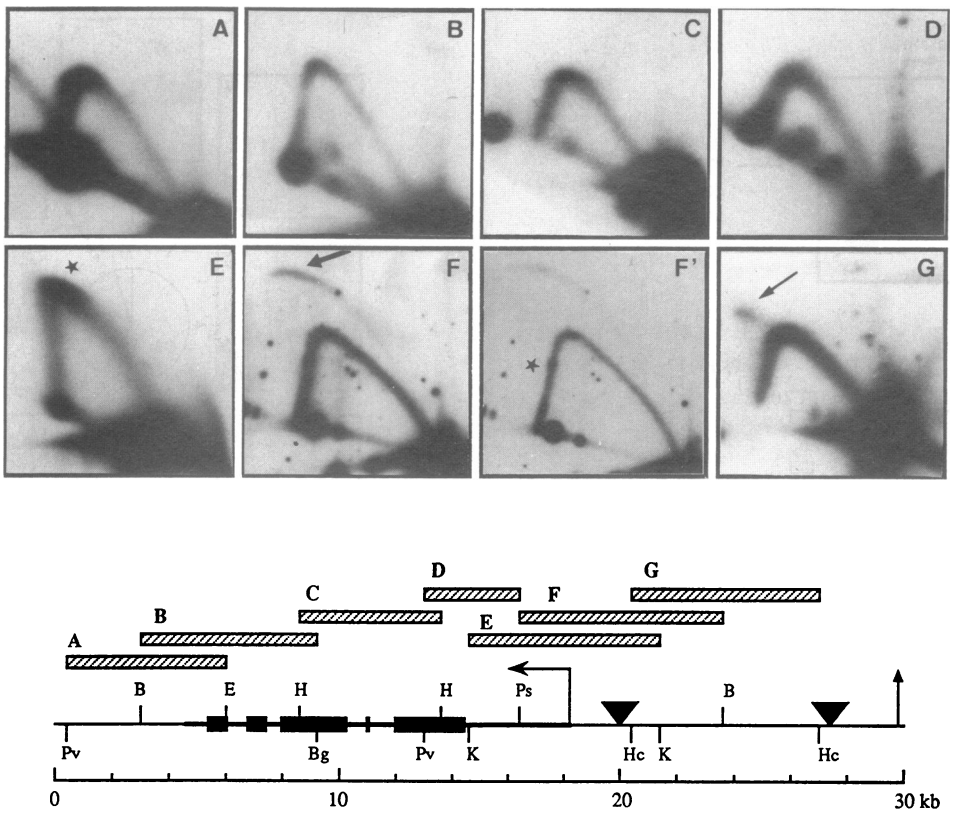

Figure 4. Mapping of replication origins within rDNA minichromosomes by 2D gel electrophoresis. The fragments analyzed are represented as hatched boxes, with letters refering to the corresponding panel; panel F′ is a lower exposure of the hybridization presented on panel F. Sizes, restriction endonucleases and probes used were as follows: (**A**) 5.5 kb *Pvu*II–*Eco*RI (probe 1a), (**B**) 6.1 kb *Bam*HI–*Bgl*II (probe 1), (**C**) 5.0 kb *Hin*dIII (probe 2), (**D**) 3.4 kb *Pvu*II–*Pst*I (probe 3a), (**E**) 6.8 kb *Kpn*I (probe 4), (**F**) 7.0 kb *Pst*I–*Bam*HI (probe 3b) and (**G**) 6.4 kb *Hin*cII (probe 4). The map indicates the location of the ribosomal genes (bold line with black boxes), the transcription polarity (horizontal arrow), and the restriction sites that were utilized (Ba = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; Hc = *Hin*cII; Hn = *Hin*dIII; K = *Kpn*I; Ps = *Pst*I; Pv = *Pvu*II). The vertical arrow depicts the center of the molecule at 30 kb (see scale). The origins (triangles) are as deduced from panel F, the only one for which a bubble arc is observed (thick arrow), and from panel G for which a double Y pattern is detected (thin arrow). Star in panels E and F′ underlines the slowing-down of replication fork near the transcription initiation site.

genes in many organisms (16,17,19,31,32), arresting the forks moving opposite to the transcription direction. Even after a longer exposure, we did not detect any bubble arc indicating that initiation events occur in fragment A. Therefore, we can exclude the presence of a replication origin 3′ to the rRNA genes, except in the terminal third of the fragment (33). We also analyzed complete telomeric fragments (data not shown) and we obtained diffuse patterns, probably due to the heterogeneity in length of the terminal NTS (34). Nevertheless, the simple patterns that we detected in the coding regions are compatible with replication forks moving from the transcription units towards the extremities of the minichromosome, as proposed from EM results. This progression is smooth as shown on Figure 4. The only place where we obtained some evidence for a stalling of replication forks is at the boundary of the central NTS and the coding region (fragments E and F). In fragment E, an accumulation of replication intermediates can be seen at the inflection point of the Y arc (star), that co-maps with the transcription initiation site region. This was further confirmed by detection of a faint spot (star) within the Y arc observed in fragment F (see low exposure, panel F′). These patterns, which are reproducible and not detected elsewhere (see other 2D gels), demonstrate the presence of a slow-down of the replication fork 5′ of the rRNA genes.

In the central NTS, analysis of fragment F shows a fully developed bubble arc (thick arrow) together with a strong Y arc. This composite signal can either mean that initiation takes place in a broad region or that it occurs at a site-specific replication origin which is not always used. In order to distinguish between these two possibilities, we analyzed the flanking fragments E and G where we no longer observed a bubble arc. This proves that there is a site-specific initiation of DNA synthesis in the middle of fragment F.

Similarly, another bubble arc is expected closer to the center of the molecule. Yet, because of the complex palindromic structure of the NTS and the lack of specific probes and appropriate restriction sites, we could not observe directly the activity of this second set of origins. Therefore, we analyzed fragment G, located in the central NTS between the two EM-mapped origins. We did detect a faint double Y signal (thin arrow) in addition to the major Y arc. This double Y pattern does not appear as a diffuse triangular region that is formed when replication forks collide at random in a fragment. This is reinforced by the absence of a termination signal in the overlapping fragment F. In addition, the high position of the spike (as compared to the Y arc) reveals that it is mostly composed of replication intermediates symmetrically branched rather than asymmetrically branched; this indicates that the two replication forks meet roughly in the middle of fragment G. Thus, this double Y pattern indicates indirectly that the potential origins closer to the symmetry axis are functional and that, in rare cases, two adjacent replication origins are simultaneously active on a given molecule.

Another indirect evidence of the activity of these origins is drawn from the comparison of bubble arc and Y arc intensities in fragment F, as explained in Figure 5A. If the two origins, a and a′, located near the transcription initiation start sites, were solely active, the replication of fragment F and its palindromic counterpart should give a ratio of one bubble arc to two Y arcs. On the other hand, if four origins were active, the fragments would be more often passively replicated, so that the total expected ratio should be one bubble arc to six Y arcs. In order to determine origin usage, we have quantified the relative amounts of the different hybridization signals by PhosphorImager technique on the filter shown in Figure 5B. The 1× spot represents 97% of the signals, which illustrates the low number of replicating rDNA molecules at a given stage of the cell cycle; this value is very similar to the 96% of linear rDNA molecules that were seen on EM (1). The relative amount of replicating signals is one bubble arc to nine Y arcs, which compares better with the model in which four replication origins are active. The lower than expected bubble arc to Y arc ratio can be interpreted either as a preferential loss of bubble-shaped replication intermediates or as a lower usage of origins closer to the genes than the distal ones (see Discussion).

In Figure 6, we summarize results obtained on mapping of *Physarum* rDNA replication origins in three different studies. In the EM study (1), the alignments of bubble-containing rDNA molecules indicated that there are two origins per half molecule, localized in the central NTS. Next, the origins located near the promoter region, when placed in a plasmid, were shown to initiate replication *in vitro* (35). Finally, our 2D gel analysis confirmed these earlier results. The bubble arc seen in fragment F shows that there is an origin in its center while the Y arcs obtained with fragments E and G delimit the border of the origin site, according to the rule that an origin situated in the terminal third of a fragment will not be detected (33). The second replication origin has been positioned symetrically to the first one we mapped with respect to the middle of the fragment G, for which a double Y arc was detected. As depicted in Figure 6, results obtained with these different methods are in good agreement.

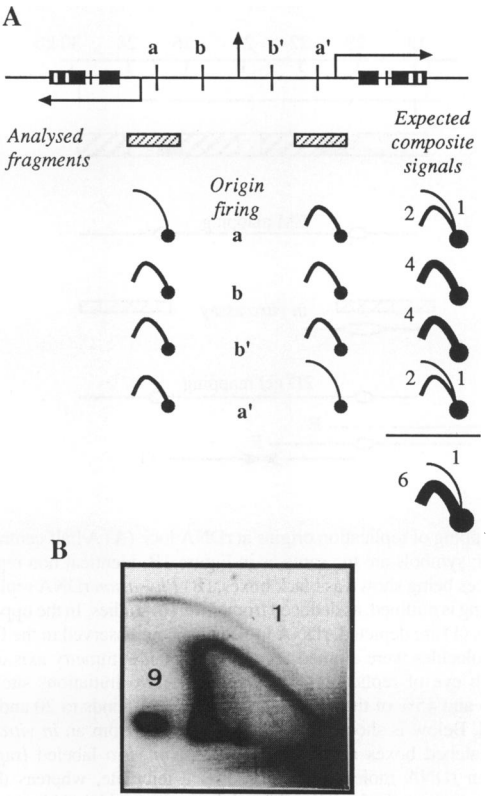

Figure 5. Quantitation of relative bubble arc and Y arc intensities. (**A**) A rDNA palindrome is represented with four potential replication origins a, b, b′, a′ (other symbols are the same as in Figure 1). Below is illustrated the fact that, depending on the number of origins which are activated, different ratios between bubble arc and Y arc are expected in the composite signal obtained for the 7.0 kb *Pst*I–*Bam*HI fragments (shown as hatched boxes and corresponding to fragment F in Figure 4). When one origin is fired inside a fragment its mirror counterpart is passively replicated, which generates a composite signal (right). Since the origin-containing fragment is replicated by two forks, it is duplicated twice as fast as the fragment replicated by a single fork, so that the bubble arc has an intensity twice weaker than the Y arc. If only the two origins a and a′ were active, addition of respective composite signals would give a ratio of one bubble arc to two Y arcs. If the two external origins were also active, the total ratio should be one bubble arc to six Y arcs, as indicated on the figure. (**B**) A *Pst*I–*Bam*HI digest was subjected to 2D gel analysis and probed with probe 3b. Following autoradiography, the signals were quantified by storage phosphorimaging. Background was measured above the bubble arc and then substracted from the signals. The measure of each arc intensity was obtained from integration of the corresponding area : 1× spot = 96.9%, bubble arc = 0.3% and Y arc = 2.8%. The ratio of bubble arc to Y arc is one to nine, which is closer to the one expected for four origins.

DISCUSSION

Our analysis of *Physarum* rDNA synthesis by the 2D gel method of Brewer and Fangman showed that replication intermediates are found at all stages of the cell cycle (Fig. 3). This is consistent with previous studies based on incorporation of exogenous precursors, showing that rDNA duplication is not under the control of S phase (29,30). However, the finding of Y arcs during the first hour of S phase is apparently in conflict with these former data, which clearly demonstrated that there is no rDNA synthesis during this period. In fact, 2D gel detection of replication forks does not exclude that they are temporarily motionless. Taken together, these data suggest that, for the few molecules engaged in replication at the onset of S phase, elongation is impeded. Interestingly, this block would occur at a cell cycle period in which the nucleolus is disrupted and reconstructed; rDNA is then found in the prenucleolar bodies and is transcriptionally active (36). Further studies would be required to determine the exact relationship between the nucleolus structure and the progression of replication forks on rDNA molecules.

We next focused our analysis on the mapping of replication origins on rDNA minichromosomes, and we compared our data with the EM results (1). In the 2D gel method, we studied about 10^7 replication intermediates in each analysis, whereas 37 replicating molecules were observed in the EM; despite these different scales, the two methods lead to similar conclusions which reinforce each other. Indeed, 2D gel detection of a bubble arc only in fragment F (Fig. 4) demonstrates the activity of a site-specific, promoter-proximal origin. Interestingly, it corresponds to the site of initiation mapped by Vogt and Braun on the assumption that the eyes of only two molecules clearly fell outside of the more centrally located origin. Conversely, we could not provide direct evidence for this latter origin activity because of the repetitive structure of the central NTS, but we observed a

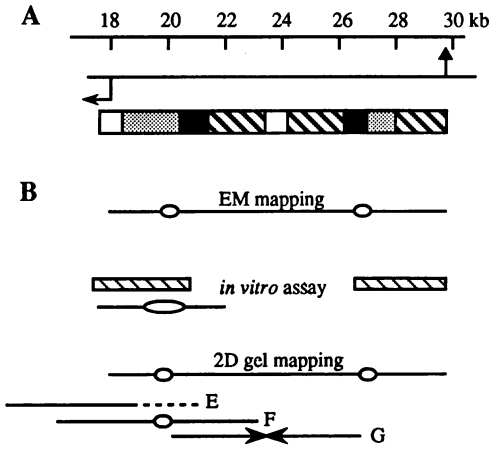

Figure 6. Mapping of replication origins at rDNA loci. (**A**) A half central NTS is represented; symbols are the same as in Figure 1B, identical non repetitive DNA sequences being shown as black boxes. (**B**) *Physarum* rDNA replication origins mapping is outlined, as deduced from different studies. In the upper part, the EM results (1) are depicted. rDNA molecules were observed in the EM; 37 replicating molecules were aligned according to their symmetry axis and the center of each eye of replication was measured. Two initiations sites were mapped at 33 and 45% of the molecules, which corresponds to 20 and 27 kb from one end. Below is shown the mapping deduced from an *in vitro* assay study (32): hatched boxes represent the earliest *in vitro* labeled fragments obtained when rDNA molecules are used as a template, whereas the eye indicates the region in which were confined the center of the bubbles observed in the EM after *in vitro* replication of a recombinant plasmid. At the bottom is represented the mapping as deduced from our 2D gel analysis (Fig. 4). First, the fully-developed bubble arc observed only in fragment F implies that there is a replication origin in the middle of the fragment (20 kb); the asymmetric position of this origin in fragment E precludes detection of a partial bubble arc (dashed line represents the part of the fragment in which an origin activity could not be detected). Second, the convergence of two replication forks in fragment G indicates that there is another replication origin located symetrically to the first one with respect to the center of fragment G (27.4 kb).

double Y arc in fragment G in between the two EM-mapped origins (Fig. 4), and we quantified the relative intensities of hybridization signals in fragment F (Fig. 5). Together with the EM results, these data indicate that there is an efficient set of four replication origins located in the central NTS (Fig. 6). These results from two physical mapping methods are further supported by an *in vitro* assay of rDNA replication initiation (Fig. 6; 35); moreover, they are reinforced by the palindromic structure of the central NTS (26) in which the origin region is reiterated four times (Fig. 1). The origins are situated in the vicinity of the junction between series of 31 bp motif repeats and a more complex sequence of 900 bp (37); we did not find any obvious homology neither with the yeast ARS consensus sequence nor with sequences that are likely involved in rDNA replication of *Tetrahymena* (38). Our data also proves that initiation takes place at fixed sites (Fig. 4), as previously suggested by EM analysis. This result contrasts with the delocalized initiation described for rDNA in *Xenopus* embryos and human cells (18,19) and illustrates the possibility of two types of eukaryotic replication origins: site specific and diffuse. Interestingly, *Physarum* may contain both types: the LAV1-2 locus generated complex patterns when analyzed by 2D gels (39), while a discrete replication origin is closely linked to a profilin gene (28,39); hence, rDNA loci are the second example of site specific initiation in *Physarum*.

The replication fork propagation within rDNA minichromosomes occurs at a relatively constant rate. Only in the 5′ region of the primary transcript have we observed a slow-down of the replication forks (stars in Fig. 4), which may be caused by the proximity between a replication origin and the promoter of actively transcribed genes. Otherwise, the smoothness of our 2D gel patterns implies the absence of a RFB in the 3′ end of the rRNA genes, unlike in yeast where the genes are synthesized mostly unidirectionally as a result of a polar replication fork barrier (16,17) that was also found in *Drosophila*, *Pisum sativum* and human rDNA (19,31,32); the replication fork barrier prevents the replication of the coding region by the forks moving against the transcription direction. In yeast, the stalling of the replication forks was shown to occur independently of transcription (40); yet the barrier may be the result of a long-term selection, due to tandem organization of yeast rRNA genes which would allow a collision between the RNA and DNA polymerases (41). On the other hand, *Physarum* and *Tetrahymena* extrachromosomal rDNA structure may not require such a mechanism for replication and transcription coordination. In these organisms, EM studies have suggested that replication and transcription proceed in the same direction through rRNA genes (1,11). Our 2D gel analysis of replication initiation confirms these previous results, although we cannot exclude that there is a replication origin in the terminal kb of rDNA minichromosomes; however, this possibility is weakened by the absence of termination signals throughout the coding region. These studies favour the concept of replication origins located only in the central NTS.

Finally, the fact that several origins can be fired in *Physarum* rDNA raises the question of origin usage. Measurement of hybridization signal intensities gave a ratio of one bubble arc to nine Y arcs, which is lower than the one to six ratio expected if the four replication origins were equally activated (Fig. 5). This suggests that origins located near the transcription initiation sites are less often used than the more centrally located ones. Previous EM observations indicated that 2/3 of the eyes are most likely originating from the center-proximal origins (1). In such a case, the expected intensities of the Y arcs generated by the firing of each 'central' origins should be replaced by eight, as they are twice as active as a and a′ (Fig. 5A). The total ratio should be of one bubble arc to 10 Y arcs, which is consistent with our data (Fig. 5B). This different usage is surprising considering that the same sequences are involved. In fact, recent results obtained in yeast illustrated a similar context effect, since two identical ARS elements located at opposite sites in a circular plasmid were not equal: one was activated four times more frequently than the other (42). In *Physarum*, it was proposed that methylation level might regulate rDNA origin activity (43). In addition, the faint double Y pattern that we obtained in the central NTS (Fig. 4) demonstrates that more than one origin can be simultaneously active on the same molecule; however, this is not a frequent event, which explains why EM technique failed to detect it. The functioning of closely spaced origins was also studied in *S.cerevisiae* by inserting ARS1 on chromosome V 6.5 kb away from ARS501. In this case, the two origins are seldom active on the same DNA molecule, which suggests that the firing of one origin often precludes the activity of the other one, leading to the concept of origin interference (44). Our results, which show a low level of simultaneous firing of the two origins that are 6.6 kb apart (Fig. 4), suggest an interference between naturally occuring origins. On the other hand, the use of several origins may confer

an evolutionary advantage, as suggested by transformation of *Tetrahymena* with a plasmid containing a tandem repeat of rDNA replication origin region. An accumulation of linear rDNA molecules was observed, with many copies of the origin in tandem repeats obtained through homologous recombination, and was interpreted as a replicative advantage conferred by this spontaneous amplification (45). Another example of a high density of chromosomal origins has been recently described at the *ura4* locus in *S.pombe* where three ARS elements contribute to initiation in a hierarchical manner (46). Hence, initiation from several close replication origins, as shown for the first time in *Physarum* rDNA (1), could be relatively common in eukaryotic genomes and exemplifies the complexity of the replication process.

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