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Low rate of replication fork progression lengthens the replication timing of a locus containing an early firing origin

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ABSTRACT

Invariance of temporal order of genome replication in eukaryotic cells and its correlation with gene activity has been well-documented. However, recent data suggest a relax control of replication timing. To evaluate replication schedule accuracy, we detailed the replicational organization of the developmentally regulated *php* locus that we previously found to be lately replicated, even though *php* gene is highly transcribed in naturally synchronous plasmodia of *Physarum*. Unexpectedly, bi-dimensional agarose gel electrophoreses of DNA samples prepared at specific time points of S phase showed that replication of the locus actually begins at the onset of S phase but it proceeds through the first half of S phase, so that complete replication of *php*-containing DNA fragments occurs in late S phase. Origin mapping located replication initiation upstream *php* coding region. This proximity and rapid fork progression through the coding region result in an early replication of *php* gene. We demonstrated that afterwards an unusually low fork rate and unidirectional fork pausing prolong complete replication of *php* locus, and we excluded random replication timing. Importantly, we evidenced that the origin linked to *php* gene in plasmodium is not fired in amoebae when *php* expression dramatically reduced, further illustrating replication-transcription coupling in *Physarum*.

INTRODUCTION

DNA replication is a key step of cell cycle that ensures the complete duplication of genomic DNA prior to mitosis. Over the past 40 years, it has been evidenced that eukaryotic genomes replicate accordingly to an invariant temporal order (1,2). This has first been shown in the Myxomycete *Physarum polycephalum*. Indeed, taking advantage of the natural synchrony of several million nuclei within a single plasmodium, the authors have carried out pulse-labeling experiments and showed that sub-fractions of replicating DNA are the same through successive S phases (1,3). More recently, the visualization of *in vivo* labeled replication foci within single cells strongly suggested that replicons remain associated within the same clusters throughout consecutive cell cycles (4). Cytogenetic analyses of metaphase chromosomes also showed an invariant pattern of replication banding (5) and density shift experiments validated these results at the level of individual genes by defining their timing of replication (6).

In addition, replication timing and transcriptional status of genes have been correlated in many organisms. Indeed, active genes are often found to replicate early whereas inactive genes replicate later (6,7). Genome-wide analysis in human cells and in *Drosophila* confirmed the connection between early replication timing and transcriptional activity (8–11). However, this link is more obvious for large domains rather than at a small scale (12) and was not seen at all in budding yeast (13). It was also shown that the temporal program of gene replication could change during cell differentiation or development, reinforcing therefore the concept of a co-ordination between replication and transcription (14). Studies of the profilin genes in *Physarum* and the immunoglobin heavy chain locus in mammalian cells have clearly demonstrated that, during differentiation, replication of these loci is altered by a change in the pattern of origin activation (15,16).

Nonetheless, recent reports suggest that replication timing is not strictly defined. Indeed, in mammalian cells, molecular combing of DNA molecules associated with FISH analyses showed that redundant origins fired randomly with no timing preference (17). Stochastic firing of origins was also described in fission yeast (18), although it was not confirmed by genome-wide analyses (19). In human cancer cells, studies of chromosomes 21–22 replication using micro-arrays analyses and FISH have demonstrated that a fixed timing of replication could not

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be assigned to large set of DNA sequences (i.e. they were found to replicate early as well as late). This led the authors to propose a 'pan-S-phase' pattern as opposed to the classical fixed pattern of replication timing (20).

We have previously demonstrated by *in vivo* incorporation of bromodeoxyuridine that active genes are replicated early in the naturally synchronous plasmodium of *P. polycephalum* (21). However, we also found that the highly expressed *php* gene is late replicated in plasmodia (21,22). Here, we used neutral bidimensional agarose gel electrophoresis (2D-gel) method (23) to determine whether *php* gene late replication comes from its association to a late firing origin or from its long distance from an early firing origin. Surprisingly, replication forks were found on the locus at the onset of S phase and could be detected through half of S phase. We demonstrated that this observation could not be explained by random replication timing among nuclei of a plasmodium but rather by a very slow progression of the forks enhanced by fork stalling upstream the gene. Importantly, we also showed that the coding region of *php* is actually early replicated because of its proximity to a replication origin activated at the onset of S phase. Here again, active transcription is thus related to early replication. Furthermore, our results also demonstrated that the origin is developmentally regulated in correlation with the *php* gene activity and reinforced the concept of replication-transcription coupling in *Physarum*.

MATERIALS AND METHODS

Strains and cultures

We used M3CIV and TU291 strains of plasmodia. They were routinely grown in shaken liquid cultures as multinucleated microplasmodia that are not synchronous to each other. Five centimeter diameter synchronous plasmodia were obtained by coalescence of microplasmodia as previously described (24). As plasmodium nuclei lack for G1 phase, monitoring of the 3 h S phase was made by mitosis detection on smears observed under phase contrast microscope. We used plasmodia after the second or third mitosis, indifferently. Once at the stage of interest, plasmodia were harvested and frozen in liquid nitrogen. We used LU352 strain of amoebae that were grown as described (25).

Cytometry analysis

Nuclei were isolated from one plasmodium as previously described (26). Nuclei were fixed with three volumes of ethanol and stored at −20°C. The number of nuclei within the sample was evaluated by measuring optic density (260 nm) of nuclei aliquot after lysis with 2 M NaCl, 5 M urea (27).

Typically, 10^7 nuclei were washed twice in isolation medium, then digested with 0.1 mg RNaseA for 30 min at 37°C and stained with 0.1 mg propidium iodide for 30 min at 37°C. Internal control for overlaying the curves were carried out in duplicate experiments in which isolated nuclei from different cell cycle stages were mixed during the washing step. Samples were analyzed with a FACSortTM (BD Biosciences, San Jose, CA, USA).

Drug treatment

For hydroxyurea (HU) treatment experiments, one half of the plasmodium was placed on 2 ml culture medium as control, while the other half was placed on 2 ml culture medium supplemented with 50 mM HU. The targeting of the drug in nuclei was estimated to ~15 min, thus treatment durations reported in the text and Figure 6 should be supplemented of 15 min to get the actual treatment duration. For instance, by placing the plasmodium on HU medium from +15 to +60 min after the beginning of S phase, the drug effect was estimated to ~30 min, from +30 min to +60 min.

DNA preparations

Macroplasmodial DNA was obtained from isolated nuclei and was embedded in agarose plugs as previously described (28).

Microplasmodia were pelleted (500 g, 5 min) and their nuclei were isolated (26). Pellet of nuclei was resuspended in 50 mM Tris [pH 8], 50 mM NaCl, 25 mM EDTA, lysed with 1% sarkosyl and digested with proteinase K (200 μg/ml) overnight at 45°C. CsCl and ethidium bromide were added at a final concentration of 915 mg/ml and 1 μg/ml, respectively. The gradient was centrifuged 6 h at 70 000 rpm at 20°C with a Beckman NVT90 rotor in a Beckman ultracentrifuge LE-80. The DNA band was withdrawn with a syringe and dialyzed against 10 mM Tris [pH 8], 1 mM EDTA (TE) at 4°C during 3 days.

Amoebal DNA was obtained from isolated nuclei and purified on CsCl equilibrium gradients as described (15).

DNA digestions and electrophoreses

For 2D-gel analyses, 10 μg of synchronous plasmodial DNA and 30 μg of asynchronous microplasmodial or amoebal DNA were digested with restriction enzymes. We used respectively 600 U for efficient digestion of plasmodial DNA embedded in agarose plugs and 300 U for DNA extracted from microplasmodia and amoebae.

2D-gel analyses were performed as previously described (26). Digestion of DNA before the second dimension was adapted from (29). Briefly, after the first dimension, the lane of interest was sliced off and rinsed twice in 10 mM Tris [pH 8], 0.1 mM EDTA. The DNA was digested with 3000 U of restriction enzyme overnight, followed by two additional incubations with 2000 U during 2 h. The lane was then rinsed with TE and with electrophoresis buffer before inclusion in an agarose gel for the second dimension.

DNA from plasmodia were denatured and analyzed on alkaline gel as previously described (26), except that alkali treatment was made on the agarose plugs.

After electrophoreses, agarose gels were transferred onto a nylon membrane (Gene Screen Plus, Perkin Elmer) (26).

RNA extraction and northern blot

RNA was extracted from plasmodia by solubilization in guanidium isothiocyanate and centrifugation onto a CsCl

cushion as described (30). RNA samples were analyzed by northern blot as previously described (24).

Hybridizations and probes

php probe derived from a 720 bp EcoRI-PstI fragment corresponding to the partial 5′ end of *php* cDNA (accession number X64708, nucleotides 9–728). *proP* probe is the 960 bp PvuII-PstI fragment derived from a genomic clone (accession number M38038, nucleotides 1358–2318). *ardC* probe is the 979 bp HindIII-XhoI fragment derived from a genomic clone (accession number X07792, nucleotides 20 to 999). Agarose gel purified fragments were [α-^{32}P]dCTP labeled by random priming with Radprime kit (Invitrogen).

Hybridizations were performed in Church buffer (0.5M sodium phosphate buffer [pH 7.2], 1mM EDTA, 1% bovine serum albumin, 7% sodium dodecyl sulfate) at 65°C overnight (31). The membranes were prehybridized for 1 h in Church buffer and hybridizations were initiated by adding heat-denatured probe and 0.1 mg/ml heat-denatured salmon testes DNA. Washes were performed at 65°C in five successive bathes of 40 mM phosphate buffer [pH 7.2], 1 mM EDTA and 1% sodium dodecyl sulfate. Hybridization signals were obtained and quantified by storage phosphor imaging (Molecular Dynamics 400A) and ImageQuant software.

RESULTS

Replication of *php* locus is not scheduled

To follow the replication of *php* gene encoding a subtilisin-like protease, we carried out kinetic analyses. Plasmodia were harvested at specific time points through the 3 h of S phase. DNA samples embedded in agarose plugs were digested by restriction endonucleases, resolved in 2D-gel electrophoresis and hybridized with a specific cDNA probe. Surprisingly, in disagreement with our previous reports (21,22), we found that the 6.7-kb EcoRV-EcoRI fragment encompassing *php* gene exhibited prominent Replication Intermediates (RIs) during the first hour of S phase (Figure 1A). A transition from a bubble arc to a Y arc was observed 5 min after the beginning of S phase (+5′) and indicated that initiation takes place within the fragment (see subsequently). At + 10 min (+10′), the RIs were essentially composed of Y-shaped molecules. This partial Y arc persisted up to + 60 min and was significantly detected until + 90 min, when about 75% of genomic DNA synthesis is completed. Quantifying hybridization signals evidenced the broad temporal window of *php* locus replication. Indeed, replicative arcs represented ~20% of the total hybridization signal from + 5 min until + 60 min, decreased to ~8% at + 90 min and lowered to <1% as late as + 120 min.

To rule out that this large temporal window of replication is due to a lack of synchrony of our plasmodia, we carried out flow cytometry analyses (Figure 1B). Nuclei were isolated at specific time points of the cell cycle and DNA content of each population of nuclei was measured after nucleic acid staining with propidium iodide. Figure 1B shows that each population exhibited homogeneous pattern of DNA content and that this latter increased synchronously from 2C to 4C as nuclei progressed in S phase. Therefore the synchrony of nuclei within a plasmodium is a property of the whole S phase.

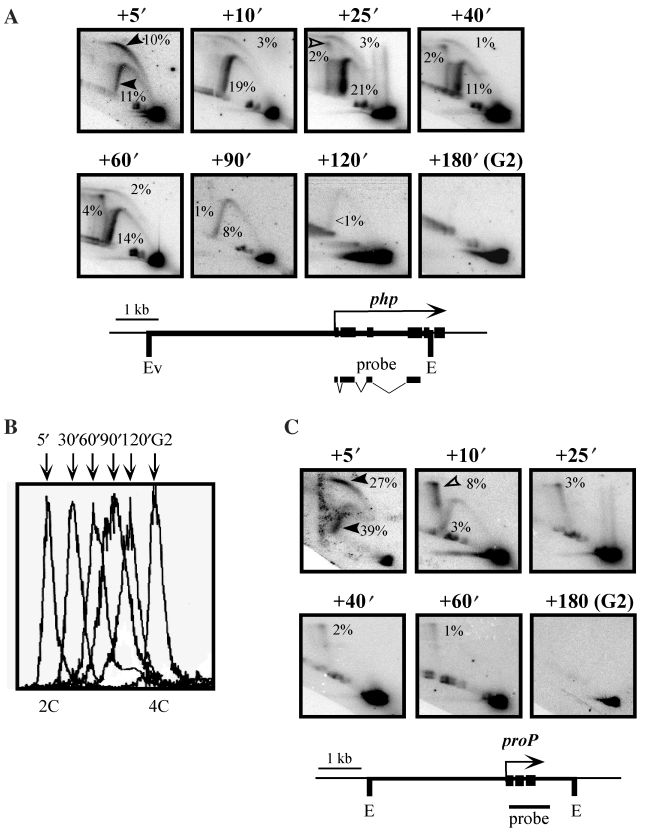


Figure 1. Contrasted replication timings at *php* and *proP* loci. (**A**) Kinetic analysis of replication pattern at the *php* locus. 2D-gel analyses of DNA samples extracted at successive time points of S phase are shown. The 6.7 kb EcoRV (Ev) - EcoRI (E) fragment containing *php* gene (black arrow) was studied (black boxes are for exons, scale and probe are indicated). Replication intermediates (black arrowheads) and signals corresponding to joint DNA molecules (open arrowheads) were quantified and expressed as a percentage of total hybridization signals. (**B**) Flow cytometry analysis of plasmodium nuclei throughout S phase. Nuclei were isolated from plasmodia at different time points in S phase and DNA was stained with propidium iodide. DNA content was measured. (**C**) Kinetic analysis of the replication pattern at *proP* locus. The same DNA samples as in A were analyzed by re-probing the blots with *proP*. A map of the 4.8 kb EcoRI fragment containing *proP* gene is shown.

Moreover, in previous studies we were able to pinpoint the replication timing of single copy DNA sequences within a 5–10 min period during S phase (26,28,32,33). Therefore, as an internal control of DNA samples used for analysis of *php* replication kinetics, we re-hybridized the same blots with a probe derived from *proP* gene that replicates at the onset of S phase (26). We detected in the 4.8 kb EcoRI fragment containing *proP* gene RIs at +5 min, with a level of about 65% of the hybridization signal (Figure 1C). This demonstrated that much more molecules containing *proP* gene were engaged in replication than in the case of *php* gene at this time point.

In contrast, only a faint signal about (or "~") (3%) was detectable at +10 min and no replicative signal could be detected later on, in agreement with reported results (26). The size difference between *proP* and *php* containing restriction fragments could not explain these contrasted replication patterns. Therefore, direct comparison of the two loci indicated radically different temporal windows of replication: *proP* gene is replicated in less than 10 min whereas it takes ~90 min to replicate the *php* gene-containing fragment. We also detected X-shaped molecule signals for both loci (see open arrowheads in Figure 1A and C) after the forks have reached both ends of the fragment (i.e. from +25 min to +60 min for *php* and from +10 min to +40 min for *proP*). Such molecules correspond to transient post-replicative joint DNA molecules involving sister chromatids (33). These X-shaped molecules had a maximum of intensity at +10 min for *proP* and +60 min for *php*. The delay in X-DNA apparition in the *php* gene-containing fragment is consistent with a later period of replication.

We observed that, in contrast to *proP* and other loci (26,28,32,33), the timing of replication of *php* locus is extended. This unexpected long period of replication of *php* locus may be explained either by slow progression of replication forks or by different replication patterns among the millions of nuclei contained within a single plasmodium.

An early firing origin is located in the promoter region of *php* gene

To distinguish between these possibilities, we first wanted to map the replication origin of the *php* gene-containing replicon (Figure 1A) and to determine the fork position on the locus early in S phase. We thus performed a series of 2D-gels to analyze different restriction fragments of DNA extracted 5 min after the onset of S phase. After probing with *php* probe, we compared RI patterns in overlapping fragments for deducing the localization of replication origin (Figure 2A). We found a bubble arc in fragment a, and a bubble to Y arc transition in fragments b-c1, indicating the firing of a bidirectional replication origin in these fragments. We located the origin at the middle of fragment a, since it exhibited the most developed bubble arc (see the schematic extending bubble above the map in Figure 2). Consistently, the extent of the Y arc was more important in fragments b and c1 in which the origin would be less centered. Only Y arcs were detected in fragments c2, d and e2, in agreement with an outside position of the origin. In the case of fragment e1, due to the origin position close to its extremity and due to its size, no bubble arc could be detected and only a nascent Y arc was revealed. These results are consistent with an origin positioned at the 5′ side of the gene. The observation of a partial Y arc when analyzing the origin-containing fragment b strongly suggests that the origin is efficiently fired. Otherwise, a complete Y arc would be observed in addition to the bubble arc, as a result of passive replication of the locus in some nuclei (34).

Early replication of *php* gene by rightward moving fork and pausing of leftward moving fork

In order to follow the progression of replication forks, we compared the RI patterns shown in Figure 2A with those obtained from similar analyses performed with DNA prepared at +10 min (Figure 2B). A slow evolution of fork distribution was revealed by the different mean positions of RIs along the replicative arcs. Indeed, at +10 min, for fragment a, the maximal density of RIs was found at the end of the bubble arc. We also detected a faint terminal portion of a Y arc. For fragment b, the bubble arc was then essentially converted in a Y arc, as a result of fork movement within the fragment. Similarly the major position of RIs had moved along the Y arc for fragments c1 and e1, revealing the homogeneous displacement of replication forks. Knowing the origin position in a context of apparently smooth velocity for both forks, we could deduce which replication fork came out first of the restriction fragment. The downstream position of the origin in fragment b implied that the rightward moving fork reached first the end. Importantly, as at +10 min the bubble arc had almost disappeared in fragment b, and as fragments c2 and d were almost free of replication forks, we thus conclude that *php* gene is replicated in early S phase.

At +25 min, the fork movement was again evidenced by a change of RI mean position in the 2D-gels (Figure 2B). Interestingly for fragment b, we also detected a spot (star) close to the intersection of the Y arc with the diagonal of linear molecules that corresponds to accumulation of RIs of a 2X size, like observed in the kinetics (Figure 1A). This pattern differed from those obtained at +5 and +10 min where steady progression of RIs along the bubble and the Y arc could be detected. Such RI accumulation at +25 min indicated a stalling of the leftward moving fork close to the upstream EcoRV site (see the striped rectangle above the map). This stalling did not correspond to an arrest of the fork but rather to a slowing down. Indeed, the spot marked by the star for fragment b analysis spread on most of terminal portion of the Y arc for the shorter fragment c1. The accumulation of RIs at the apex of the Y arc for fragment e1 confirmed the fork stalling and allowed to map pausing at the middle of the fragment. We also noticed that replication forks go through the stalling region since RIs were found on the last part of the Y arc for fragment e1.

Thus, in agreement with kinetic analyses shown in Figure 1A, we observed a low mobility of replication forks through 21 kb surrounding *php* gene (Figure 2). The slow removal of replication forks from the restriction fragments is enhanced by fork stalling upstream the gene, while the coding region is rapidly replicated. Our results also indicated that, within a plasmodium, the collection of replication forks progresses concomitantly at *php* locus and argued against a randomly timed replication.

The *php* locus is replicated by a single origin

We have shown that a replication origin close to *php* gene is fired at the onset of S phase; however, we still observed on kinetics a particularly prominent Y arc (~20% of the

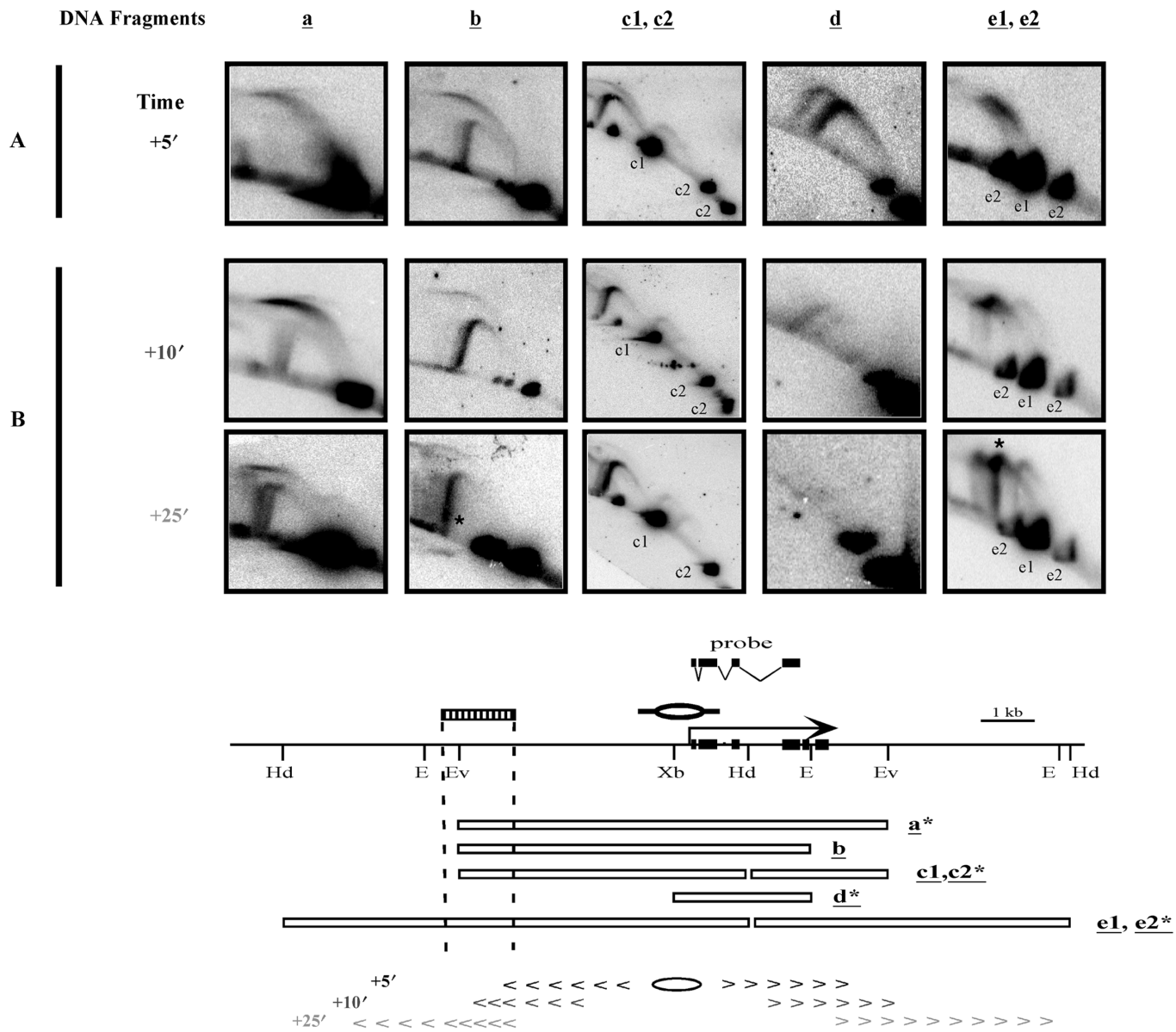


Figure 2. Origin and replication forks mapping at *php* locus in early S phase. Overlapping restriction fragments were analyzed by 2D gels. **a:** 8.2 kb EcoRV fragment; **b:** 6.7 kb EcoRV-EcoRI fragment; **c1:** upstream 5.5 kb HindIII-EcoRV fragment; **c2:** downstream polymorphic 2.5–2.7 kb HindIII-EcoRV fragments; **d:** 2.6 kb XbaI-EcoRI fragment; **e1:** upstream 8.9 kb HindIII and **e2:** polymorphic downstream 6.2–12.0 kb HindIII fragments. (**A**) Blots were obtained with DNA samples extracted from a plasmodium harvested 5 min after the onset of S phase. (**B**) Blots obtained with DNA samples proceeding from plasmodia at +10 and +25 min are shown. Scale and probe are indicated above the map. Hd = HindIII, E = EcoRI, Ev = EcoRV, Xb = XbaI. Star represents a Restriction Fragment Length Polymorphism. Scheme under the map shows the progression of replication forks on the locus as deduced from RI patterns obtained upon 2D-gel analysis. The deduced position of the origin is reported on the map (see the schematic bubble structure above the map). The stalling zone, corresponding to accumulating RIs (stars) is represented as a striped rectangle.

signal) at +25 min after the onset of S phase (Figures 1A and 2B). The fact that this Y arc was never completed strongly suggested the conversion of bubble-containing fragments to single fork-containing fragments as a result of fork progression. To further confirm our assumptions and to rule out that these RIs originated from other origins activated in the vicinity of *php* locus, we determined the direction of replication fork movement at *php* locus in early S phase by using an adaptation of the 2D-gel method (29).

In this approach, DNA contained in the agarose lane from the first dimension was digested again before it was submitted to the second electrophoresis. The resulting RI patterns depend on the polarity of replication forks in the shortened fragments (Figure 3). We used a plasmodium at +20 min in S phase, when a strong intensity of RI signals was found. Following HindIII digestion, three restriction fragments were obtained, two of them (H2 and H2*) resulting from a restriction fragment length polymorphism. We observed Y arcs for the three fragments

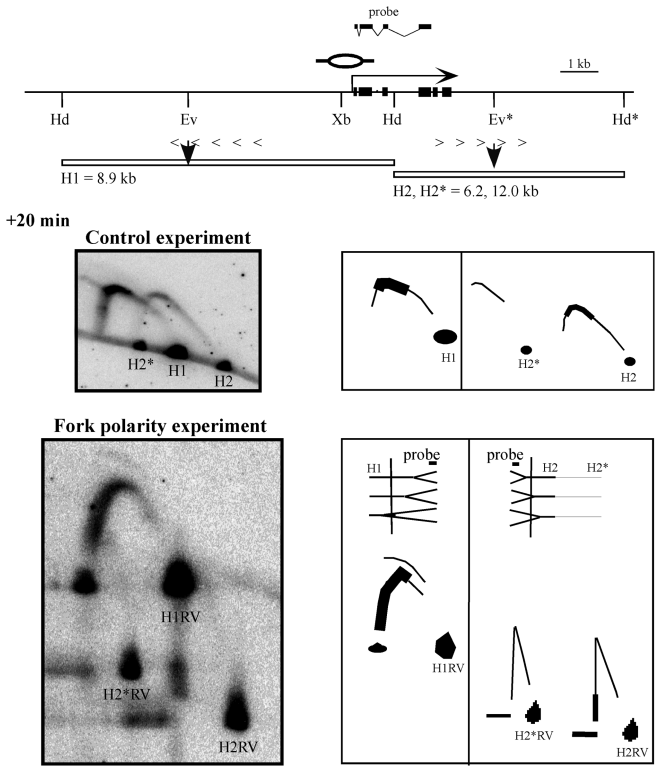


Figure 3. Homogeneous replication fork direction at *php* locus. DNA preparation obtained from a +20 min plasmodium was restricted with HindIII and submitted to a first electrophoresis. The lane of interest was excised and DNA was digested in the gel with EcoRV before the run of the second dimension. The resulting fragments are shown. Symbols are the same as above. Upper frame: control experiment shows the RI pattern obtained for the upstream 8.9 kb fragment HindIII fragment (H1) and for the polymorphic 6.2–12.0 kb downstream fragments (H2-H2*) at this stage of S phase. Lower frame: to analyze the fork polarities, the DNA was re-digested with EcoRV after the first run. Probe hybridized with an upstream 5.5 kb EcoRV-HindIII fragment (H1RV) and two downstream polymorphic 2.5–3.0 kb HindIII-EcoRV fragments (H2RV, H2*RV). Right: interpretative schemes of RI patterns obtained in the control experiment and in the fork polarity experiment.

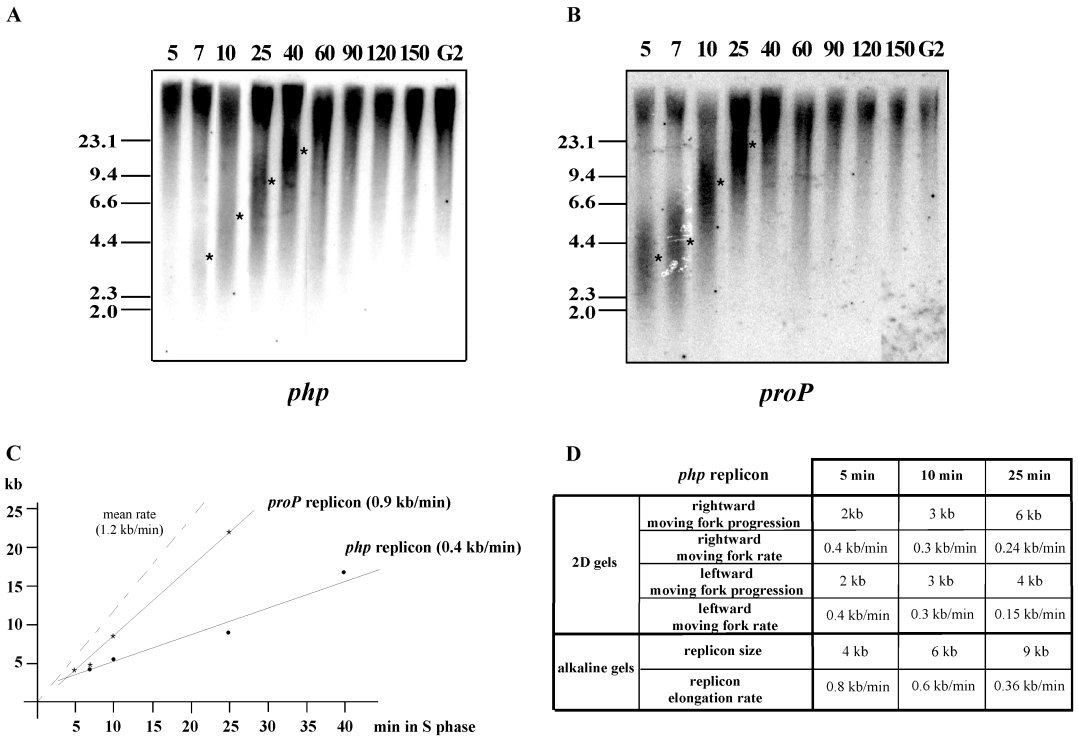
(see control experiment in Figure 3), with an accumulation of RIs at the apex of the Y arc for H1 fragment. This corresponds to the stalling of replication forks close to the EcoRV site at this stage of S phase (Figure 2B). The second digestion in the gel was performed with EcoRV. For each resulting fragment, we found only one derived pattern (see fork polarity experiment and interpretative scheme in Figure 3). For the upstream fragment H1-RV, a faint bubble arc and the end of a Y arc were observed, which implied that forks moved leftward. The spot appearing on the left at a size of 2X corresponds to forks stalling close to the upstream EcoRV site, since digestion with EcoRV converted stalled forks into linear fragments. Rightward moving forks replicated the downstream polymorphic fragments H2. At this stage of S phase, part of the RIs has gone beyond EcoRV site so that the resulting fragments were linear. Shorter RIs formed the vertical end of the Y arc originating from H2-RV 1X spot.

These results showed that fork directions are identical among the population of nuclei and also that replication forks have the same polarity for both alleles. Importantly, fork direction is not the same in the HindIII fragments: in the H1 upstream fragment we detected leftward moving forks, while in the H2-H2* downstream ones we detected rightward moving forks (see the arrowheads under the map in Figure 3). This fork divergence confirms the presence of a replication origin coinciding with the 5′ region of the gene. Therefore this analysis reinforces our previous data and rules out the possibility of a distant origin whose firing would produce forks reaching the EcoRI-EcoRV fragment 20 min after the onset of S phase. Since we have shown that the persistence of *php* RIs could not be the consequence of multiple initiation events among the nuclei, it was most likely due to a slow elongation rate of replication forks.

Slow elongation of *php* replicon in early S phase

To test this hypothesis, we measured the elongation rate of the *php* replicon by analyzing the growing of nascent strands by alkaline gel electrophoresis (Figure 4A). The natural synchrony of the plasmodium allows detection of the nascent strands of a single-copy replicon (24). After probing with *php* cDNA, short single stranded RI (stars) was observed from stages +7 to +40 min (Figure 4A). At later stages, our electrophoresis procedure did not allow their separation from parental DNA. Although RIs were seen on 2D-gel at +5 min (Figure 1A), they were not detected by alkaline gel electrophoresis at this stage due to lesser sensitivity of the latter method. We measured the mean size of *php* RIs to calculate the mean rate of replication fork progression (Figure 4C). Interestingly, the small size of *php* RIs at earliest stages confirmed that the replication origin is close to the coding region. Furthermore, we measured a slow increase from 4 kb at stage +7 min to 17 kb at stage +40 min, that corresponds to an average rate of 0.4 kb/min/replicon. Comparison with the rate of elongation of *proP* replicon was performed by re-hybridization of the blot with *proP* gene (Figure 4B). A stronger signal was obtained for nascent strands especially at earlier stages, suggesting a more acute firing of *proP* origin. *proP* nascent strands were detected slightly earlier, at +5 min, and had a size of 4 kb. The largest nascent strands that could be separated from parental DNA in these conditions were seen at +25 min with a size of 22 kb. Plotting the nascent strand lengths against time in S phase (Figure 4C) revealed an average rate of 0.9 kb/min/replicon for *proP* replicon. Although the rate of elongation of *proP* replicon has been evaluated to be more than twice greater than the one of *php* replicon, this value is in agreement with the canonical mean of 1.2 kb/min/replicon that has been calculated for *Physarum* (35). We thus conclude that *php* replicon is characterized by an unusually slow progression of replication forks.

We also compared these data with the evaluation of fork rates obtained from our 2D-gel analyses of *php* locus (Figure 2). Indeed, the mean position of the signal on the arc of RIs indicated the mean location of forks within the



	php replicon	5 min	10 min	25 min
2D gels	rightward moving fork progression	2kb	3 kb	6 kb
	rightward moving fork rate	0.4 kb/min	0.3 kb/min	0.24 kb/min
	leftward moving fork progression	2 kb	3 kb	4 kb
	leftward moving fork rate	0.4 kb/min	0.3 kb/min	0.15 kb/min
alkaline gels	replicon size	4 kb	6 kb	9 kb
	replicon elongation rate	0.8 kb/min	0.6 kb/min	0.36 kb/min

Figure 4. Slow elongation of *php* replicon. (**A**) DNA fragments extracted at various time points of S phase were denatured and submitted to an alkaline agarose gel electrophoresis. Hybridization with *php* probe revealed the nascent strands containing the *php* gene (stars). Their increasing size evidenced the elongation of *php* replicon. Size markers were HindIII fragments of Lambda phage DNA. (**B**) The same blot was re-hybridized with *proP*, showing the growing of nascent strands at this locus. (**C**) The mean size of growing nascent strands was evaluated by comparison with the size marker and plotted against time in S phase. The curves allowed the measurement of fork rate at each locus during this period of S phase. The mean rate curve corresponds to the mean rate of replicons in plasmodia as measured by (35). (**D**) Table comparing data on *php* replicon elongation obtained by 2D-gel and alkaline gel experiments.

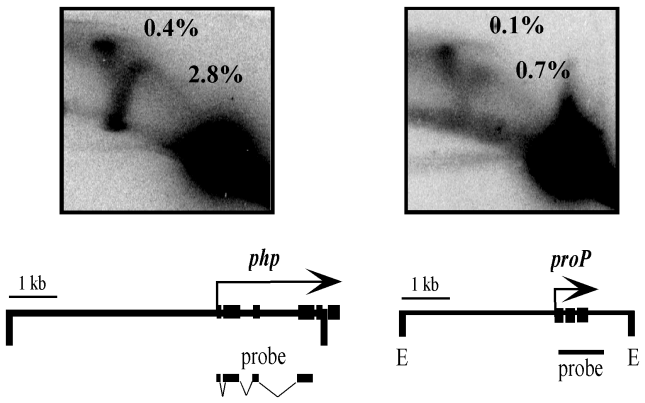


Figure 5. Longer life-span of *php* RIs as compared to *proP* RIs. DNA obtained from asynchronous liquid cultures of microplasmodia was restricted with EcoRI and EcoRV, submitted to 2D-gel, hybridized with either *php* or *proP* probe. Hybridization signals were quantified. The percentages refer to replication arcs hybridization signals with respect to total hybridization signals.

fragment of interest. As indicated in Figure 4D, alkaline gel and 2D-gel analyses gave consistent results. Both forks had covered each 2 kb as a mean after 5 min (i.e. at a speed of 0.4 kb/min/fork) and 3.0 kb after 10 min (i.e. 0.3 kb/min/fork). However, after 25 min the rightward moving fork had progressed over 6 kb (i.e. 0.24 kb/min/fork) whereas the leftward moving fork had progressed over less than 4 kb (i.e. 0.15 kb/min/fork) likely due to the stalling. Thus, the replication forks have an unequal rate. The accumulation persisted up to +60 min and implied that the replicon elongation is mostly unidirectional during this period.

php RIs have a long life span

In order to evaluate the life span of *php* RIs during the whole S phase, we studied *php* RI pattern in an asynchronous nuclei population prepared from microplasmodia where all replication events were represented. Our aim was to compare intensities of *php* and *proP* signals. We reasoned that, if the life span of *php* RIs was longer than the one of *proP* RIs, we would expect stronger signal intensity for *php* RIs since they were present during a longer period of S phase. On the opposite, a stochastic replication of *php* locus at a normal rate would not give any difference between signal intensities for *proP* and *php* loci in an asynchronous population.

Figure 5 shows a comparison of the replication pattern of *php* and *proP* loci obtained by 2D-gel analysis from DNA extracted from the same culture of exponentially growing microplasmodia. We could see on a single 2D-gel all the

RIs appearing at any moment of S phase, in addition to the prominent 1X spot of non-replicating molecules. A similar transition from a bubble arc to a Y arc was detected for both genes. However, we obtained about 3–4-fold more RIs at *php* locus, as compared to *proP* locus, meaning that the *php* RIs life span is longer (the experiment was repeated four times). Clearly, this asynchronous population of nuclei, like plasmodium nuclei, exhibited a not fully expanded Y arc, demonstrating the efficient activation of the *php*-linked origin. The higher intensity found for *php* as compared to *proP* locus rules out the hypothesis of a random replication timing of the locus.

Hydroxyurea blocks fork progression at different time points of S phase

To check that replication forks at *php* locus are *bona fide* moving forks, we inhibited DNA replication with HU, a drug that prevents replication fork elongation. Drug treatments were performed at successive periods of the S phase on half of each plasmodium and the other half was used as a control. We tested fork movement from the onset of S phase up to +25, 15–60, 60–90 and 120–150 min (Figure 6). 2D-gel analyses revealed delays of RI patterns for treated plasmodia as compared to the control, except for the latest period of treatment (120–150 min). Therefore, fork progression was impeded by drug treatment. Note that a bubble arc was still observed after a 60–90 min treatment, indicating that, in a non-negligible part of nuclei, both replication forks were active within the EcoRI-EcoRV fragment at least at +60 min. These results underline the delayed activation of the origin in a small proportion of nuclei, which is consistent with the faint bubble arc seen until +60 min on Figure 1. We conclude that, despite the long kinetics of elongation of *php* replicon, we detected on 2D-gels moving forks since they were sensitive to HU treatment. Therefore the slow replication of *php* locus is due to slow progression of replication forks rather than arrests randomly distributed along the replicon or fork collapsing.

The actively transcribed *php* gene is located in the vicinity of a developmentally regulated replication origin

The *php* gene has been previously described as developmentally regulated during the two alternative stages of growth, the diploid multinucleated plasmodium and the asynchronous haploid uninucleated amoebae (36). We used northern blot analysis to compare the steady state level of *php* mRNA in our strains of plasmodia and amoebae. We detected with *php* probe an abundant 1070 nt mRNA in total RNA from plasmodium (Pl in Figure 7A). In contrast, a weak signal could be detected in the amoebae sample only upon much longer exposure (Am' in Figure 7A). This indicated that *php* gene is highly expressed in the plasmodium. On the opposite, expression of *php* gene is almost extinguished in amoebae. Quantification and normalization against the constitutively expressed actinC gene mRNA (upper band in Figure 7A, Pl and Am) indicates a 1 to 500 ratio of *php* mRNA abundance between these two stages, confirming the developmental regulation of *php* gene expression.

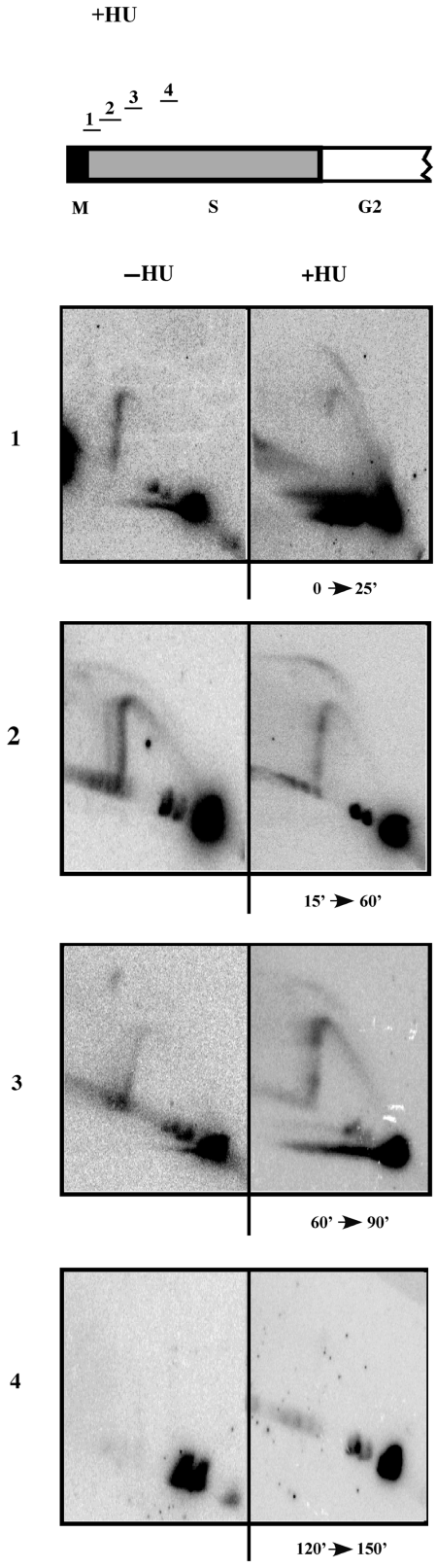


Figure 6. *php* RIs are sensitive to HU during half of the S phase. Plasmodia were cut in two pieces, one half used as control (−HU), the other half treated with 50 mM HU(+HU). The duration of the treatment is indicated below each frame. The control and the treated part were harvested at the same time, restricted with EcoRI and EcoRV, submitted to 2D-gel electrophoresis and hybridized with *php* probe.

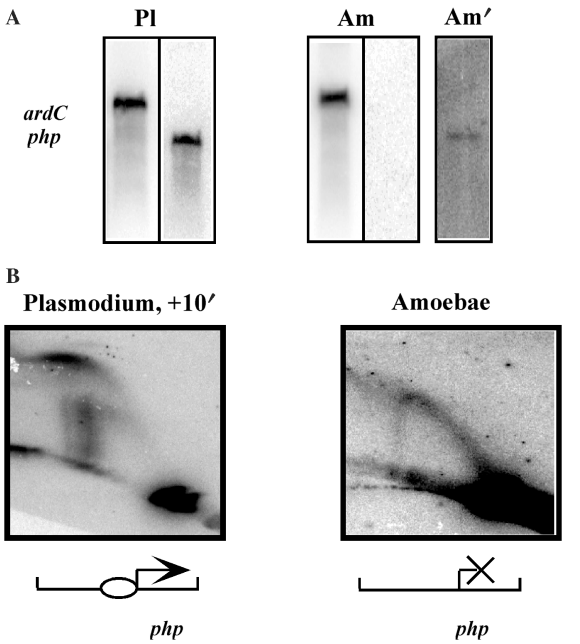


Figure 7. *php* origin is regulated during development. (**A**) Total RNA was extracted from either a G2 phase plasmodium or a liquid culture of amoebae. RNA samples of 10 µg each were analyzed by northern blot. Following hybridization with the actin probe *ardC*, a 1400 nt mRNA was detected in both plasmodial (Pl) and amoebal (Am) RNA after a 4 h exposure. Hybridization with *php* probe gives rise to a signal corresponding to the 1070 nt *php* mRNA in the plasmodium (Pl); in amoebae, *php* mRNA was only detected after a longer exposure (65 h, right panel Am'). (**B**) The replication of *php* locus in plasmodium and in amoebae was studied by 2D-gel. We used DNA plugs obtained from a +10 min plasmodium and 40 µg of total DNA of a growing amoebae culture.

Therefore, considering our previous results, showing a variation of origin usage in the case of developmentally regulated profilin genes *proA* and *proP* (15), we addressed the question of the *php* origin usage during development. We compared the replication pattern of the gene in plasmodia and amoebae by 2D-gel analysis (Figure 7B). In plasmodium, the 8.2 kb EcoRV fragment is replicated from an internal origin firing in early S phase and located at the center of the fragment, as deduced from the bubble to Y arc transition observed at +10 min (see above and Figure 7B). In contrast, DNA prepared from amoebae exhibited only Y arcs when the same restriction fragment was analyzed (Figure 7B). We previously showed that it is possible to detect a site-specific origin in this cell-type (15). Therefore, these data demonstrated that the replication origin evidenced in the promoter region of *php* gene in the plasmodium is inactive in the amoebae. We thus conclude that *php* replicon is developmentally regulated and that usage of the origin upstream the coding region is correlated with transcriptional activity of the gene.

DISCUSSION

Replicational organization and fork progression at *php* locus

We found that *php* gene was early replicated from a bidirectional origin located in its promoter region (Figure 2). However, the 2D-gel analysis of *php* locus also showed a surprisingly long life span of RIs that persisted for half of the S phase (Figure 1). It should be noticed that these kinetic data were obtained with plasmodia harvested in two consecutive cell cycles, showing the invariance of this feature over S phases.

This unusual pattern can be explained by a slow progression of replication forks on the locus in early S phase (Figures 2 and 4) and also by a stalling of the leftward replication fork from +25 min to +60 min (Figure 1). A slowing down of replication forks has been also found in *Physarum* upstream rRNA genes and downstream the histone H4-1 gene (32,37). In this latter case, it has been shown that forks are stalled in DNaseI hypersensitive regions (38). It is also possible that the DNA sequence of *php* locus might impede the replication forks. Indeed, particular sequence patterns such as trinucleotide repeats might reduce fork progression (39). In fact, several examples of replication stalling have been described in eukaryotic cells. For instance, in *Saccharomyces cerevisiae*, almost 1500 discrete sites were found to correspond to pauses caused by DNA sequences or by local protein-DNA complexes (40). These sites include tRNA genes (41), rDNA (42), centromeric and subtelomeric regions (43,44). Clearly, variation of fork rate is not a rare event and DNA replication does not seem to be a steady process.

Temporal order of replication

php RIs were found in restricted DNA fragments from plasmodia harvested in early S phase. Our previous studies showed that *php* gene is contained in a late replicating DNA fragment (21,22). This discrepancy is explained by the usage of different methods of analyses. In a first study, the replication timing of *php* locus had been analyzed by density-shift experiment following *in vivo* incorporation of bromo-deoxyuridine (21). The downstream allelic 6 kb and 12 kb HindIII fragments had been studied and were found clearly enriched in the heavy-light DNA fraction only after 90 min in S phase. Gene dosage analyses had confirmed these density shift experiments: the same HindIII fragments were found at 2 copies per genome only after 90 min. This led us to conclude at a late replicating locus (22). In the present work, 2D-gel technique favored detection of low amounts of RIs and allowed analyses of fork progression in a synchronous system. It revealed an ongoing and slow replication of *php* locus during most of the first half of S phase (Figures 1 and 2). As a result, completion of replication of *php*-containing HindIII fragments is achieved late in S phase.

In the same vein, if the *php* locus had been analyzed with microarrays, like DNA sequences from human chromosomes 21 and 22 (20), it is likely that it would be found to replicate both with early and late DNA and would be considered as a 'pan S phase' replicating locus. The lack of strict timing of replication at *php* locus raises the question of homogeneity of temporal order of replication among the millions of nuclei contained within a plasmodium. We argued that a random replication of *php* locus would give a similar intensity of RI signal towards 1X signal at *php* locus and *proP* locus in an

asynchronous population of microplasmodia. It is not the case, so that a long life span of *php* RIs is more consistent with our data (Figure 5). In addition, homogeneous replication fork progression was evidenced by the analyses of RIs obtained from DNA at specific time points of S phase (Figures 1 and 2). Therefore, our results rule out random replication timing among the plasmodium nuclei. In contrast, they illustrate a local variation of replication timing within a replicon, since we demonstrated that the *php* gene replicated early and surrounding DNA sequences replicated later.

Origin activation

Replication timing of DNA sequences depends also upon how and when replication initiation occurs. The question of the nature of eukaryotic origins has been largely debated and two models seem to emerge: either specific origins fire at a fixed timing with a given efficiency, like it has been described in budding yeast, or redundant origins fire stochastically, a model that correlates with many observations made in metazoan cells (45). Thus in *Xenopus* egg extracts, random initiation has been described and it has been proposed that the frequency of initiation events increases during the S phase in order to ensure the completion of genome duplication (46).

In our mapping experiments at *php* locus, transitions from a bubble to a Y arc were only detected in DNA fragments the most centered on the promoter region of *php* gene (Figure 2), which clearly demonstrated that replication initiates at a fixed origin linked to gene. Moreover, we did not find a mixture of bubble arc and complete Y arc throughout the S phase (Figure 1), indicating that the origin activation is efficient. Accordingly, the bubble arc signal did not result from a rare event, since it has also been detected in microplasmodia despite their asynchrony (Figure 5). Moreover, only the terminal portion of the Y arc was detected when analyzing this asynchronous population, showing the efficiency of origin firing. We also checked that no other origin was activated elsewhere within this locus in early S phase by determining replication fork directions (Figure 3). In addition, 2D-gel analyses of overlapping restriction fragments spanning 21 kb around the gene did not show other initiation events or termination during S phase (Figure 2; data not shown). These results argue for an efficient activation of a localized origin. Such origins have been described before in *Physarum* (26,28,32), indicating that stochastic firing is not the rule in this organism.

However, we detected a faint bubble arc signal from +10 min to +60 min (Figure 1). *In vivo* HU treatment from +60 to +90 min showed that forks forming this bubble arc were still active at +60 min since the drug treatment delayed replication pattern (Figure 6). These observations can be related to the low intensity of replication signals at the beginning of S phase, following 2D-gel and denaturing gel analyses (Figures 1 and 4). Altogether, these results suggest a delayed activation of the origin in a small part of the nuclei contained within a plasmodium or they reflect different replication patterns of the two alleles contained within each nucleus. Although our previous studies have clearly shown a concerted activation of allelic origins at other loci (28,32), such a different replication pattern between two alleles has been already described in *Physarum* (47). From a gene dosage analysis, the authors found that the 2 allelic *alt*B1 and *alt*B2 alpha-tubulin loci replicate synchronously in early S phase, while *alt*A locus replicates later. Remarkably, *alt*A2 allele replicates in a prolonged period of mid-S phase and asynchronously from *alt*A1 allele, which replicates earlier. In this view, for *php* locus, we can hypothesize simultaneous early activation of one allelic origin in all nuclei, while the other is activated progressively throughout the first hour of the S phase. Such distinct patterns of replication of the alleles were not obvious on 2D-gel. However individual quantification of allelic RI signals is not significant when allelic fragments are of a similar size. Furthermore, we did not find a restriction fragment length polymorphism that would allow unambiguous distinguishing of the replication timing of the two alleles. In these conditions, it is not clear whether *php* alleles are replicating exactly synchronously or not. Nonetheless a delayed activation of *php* origin certainly occurs in a non-negligible number of molecules.

Replication-transcription relationships

php origin is developmentally regulated and origin activation correlates with *php* transcriptional activity (Figure 7). Such a modulation of origin firing has been previously reported for *pro*A and *pro*P loci in *Physarum* (15) and has also been described in other organisms (16,48). These observations indicate that eukaryotic origins are at least in part epigenetically defined and suggest a strong correlation between replication and transcription. This relationship has been previously observed on chromatin spreads from early S phase plasmodia: electron microscope investigation showed a tight linkage between active genes and early firing origins (49). At the level of individual genes, we confirmed by 2D-gel mapping that efficient early firing origins are situated in the vicinity of abundantly transcribed genes. This was demonstrated for the constitutively expressed *ard*B and *ard*C actin genes, the developmentally regulated *pro*P profilin gene and the cell cycle regulated H4-1 and H4-2 histone genes (26,28,32).

In contrast, studies of weakly expressed genes revealed that they are replicated with different patterns. Inactive *pro*A profilin gene is passively replicated in mid-S phase (15,26). The weakly expressed *red*B and *red*E and topoisomerase II genes are replicated early in S phase since they are embedded in a cluster of early-activated replicons [(34), unpublished data]. Yet, in these cases, the genes are not coincident with an origin but with a termination site. Finally, the *red*A gene contains a replication origin in the promoter region, but this origin inefficiently fires in a large temporal window of mid-S phase (34).

Therefore, the association of an efficient early origin with a transcriptional promoter might be a unique property of highly expressed genes in *Physarum*. It remains to be determined whether the origins surrounding *red*B, *red*E and topoisomerase II genes could be coincident

with active genes. Likewise, the transcriptional status of the *php* locus is unknown; it would be of interest to investigate it in the region where replication forks are stalling. In metazoan, co-localization of active genes and origins has been often found and suggests that replication and transcription may share common regulation, perhaps as chromatin domain units (50,51). Several examples of origin specification in relation with transcription have been described at various loci like rRNA genes in *Xenopus* embryos (52), DHFR locus in hamster cells (53) and Hox genes in mouse cells (48). In addition, deletion of DHFR promoter results in a modification of replication initiation activity at this locus (53). This again suggests a coupling of these two nuclear activities.

In this light, we propose that the replication of highly expressed genes is strictly regulated in *Physarum*. This tight control would involve the location of the active genes close to very early firing replication origins. Lower expressed loci or non-coding regions would be under a more relax control, so that the replication timing would be less defined or the origin efficiency would be reduced. The replication organization of *php* locus may illustrate a transition in replication control stringency related with transcription level.

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