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Mapping of a *Physarum* chromosomal origin of replication tightly linked to a developmentally-regulated profilin gene

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ABSTRACT

We compared the pattern of replication of two cell-type specific profilin genes in one developmental stage of the slime mold *Physarum polycephalum*. Taking advantage of the natural synchrony of S-phase within the plasmodium, we established that the actively transcribed profilin P gene is tightly linked to a chromosomal replication origin and is replicated at the onset of S-phase. In contrast, the inactive profilin A gene is not associated with a replication origin and it is duplicated in mid S-phase. Mapping by two-dimensional gel electrophoresis defines a short DNA fragment in the proximal upstream region of the profilin P gene from which bidirectional replication is initiated. We further provide an estimate of the kinetics of elongation of the replicon and demonstrate that the 2 alleles of the profilin P gene are coordinately replicated. All these results were obtained on total DNA preparations extracted from untreated cells. They provide a strong evidence for site specific initiation of DNA replication in *Physarum*.

INTRODUCTION

Chromosome duplication is a highly complex process in the eukaryotic cell. Many replicons, unevenly distributed along a replicating chromatid, are simultaneously active at anyone time in S-phase (1). Nonetheless, it is likely that only a subset of the potential replication origins are utilized in one S-phase. This is best seen by comparing the frequency of replication origins in two developmental stages like in *Drosophila* embryos and in *Drosophila* cell-lines (2). There exists, however, a definite temporal order of replication of the genome within a particular cell-type (3–5). How the replication origins active within a cell are selected and how this selection is related to the transcriptional activity is a central question in the organization of the eukaryotic genome. Varying the replication origins fired within a chromosomal domain might lead to changes in the timing of replication of a gene, the polarity of its replication and eventually its localization within a constraint DNA loop attached to a nuclear scaffold (6–8).

Until recently, the replication origins of eukaryotic viruses were the only ones accessible to mapping methods. It has been generally observed, as in the case of the simian virus 40 (SV40) or the polyoma virus, that core sequences are absolutely required for origin activity, but that auxiliary sequences containing binding sites of previously defined transcription factors could act as enhancers (9, 10).

In contrast, *chromosomal* origins of DNA replication are poorly defined in eukaryotic cells, except for recent progress in *Saccharomyces cerevisiae*. In yeast, this progress is a direct consequence of a new approach using two-dimensional (2-D) gel electrophoresis to study the topology of replicating restriction fragments (11, 12). Using these techniques, compelling evidence for defined replication origins in yeast has been provided (13, 14). It has been shown that only a subset of the Autonoumously Replicating Sequences (ARS) previously defined for their activity in a plasmid are real origins in the chromosomes (15). Binding of transcriptional factors to sequences flanking the 11 bp consensus sequence of the ARS might be another important element in defining a yeast replication origin (16, 17). This resembles the organization of the viral origins and could be a way to coordinate replication and transcription activities during the cell cycle.

Due to a lack of sensitivity, the 2-D gel analysis cannot be applied to single-copy loci of mammalian cells. A number of reports based on various methods have nevertheless suggested the presence of a defined replication origin at about 2 kb upstream of the c-*myc* gene (18–20).

In order to overcome the paucity of replication intermediates derived from a single copy gene in cellular DNA, amplicons have been studied in organisms with a higher genome complexity than yeast. In *Drosophila*, multiple replication origins were detected in the autosomal chorion gene cluster by the 2-D gel analysis (21, 22). However, they appear to be 'preferred initiation sites' as the restriction fragments containing these origins were also frequently replicated passively from an external origin during the amplification of the third chromosome cluster. In the case of the dihydrofolate reductase (DHFR) amplicon in Chinese ovary cells, conflicting results have been reported. Based on DNA strand specificity of the Okazaki fragments (23) or on PCR mapping of the nascent DNA strands (24), it has been suggested that DNA replication initiates in a very defined 450 bp fragment situated 17 kb downstream of the DHFR gene. However, a study of the replication intermediates by the 2-D gel procedure

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suggested, on the contrary, a random initiation taking place over 28 kb of DNA in the same downstream region of the gene (25). In recent reports, 2-D gel analysis failed to reveal specific initiation sites of DNA polymerization either in *Drosophila* embryos (26) or on autonomously replicating plasmids in human cells (27), sustaining the notion that replication origin might not be strictly defined in higher organisms. These data are reminiscent of the lack of sequence specificity of exogenous DNA fragments replicated in *Xenopus* eggs (28) and cast doubts on the nature of replication origins in complex genomes.

We are using the slime mold *Physarum polycephalum* as a model system for studying DNA replication by taking advantage of a naturally synchronous cellular context (29). In one stage of their life cycle, the myxomycetes develop as a multinucleate cell, the plasmodium, in which up to 10^8 nuclei initiate DNA replication every 10 hours in near perfect synchrony (30). The 3 hours long S-phase is biphasic in the plasmodium, with a rather constant rate of DNA synthesis during the first 90 min (at about 5000 kb/min/nucleus) that then slows-down for the next 90 minutes (at about 1250 kb/min/nucleus) (30). The replicons are heterogeneous in size with a mean-size of about 35 kb (31). Within a replicon, the elongation is bidirectional with equal rate for each fork at about 0.6 kb/min/fork (32). Taken together these results indicate that the complex genome of *Physarum* (3×10^8 bp) could contain as many as 18 000 replicons, and that 4 000 of these could be activated at the onset of S-phase (33).

Although some well-characterized exceptions have been demonstrated (34, 35), most of the active genes studied so far were found to be duplicated early in S-phase. This has been visualized on Miller's spreads of early S-phase chromatin in the form of genes transcriptionally active on nascent replicons (36). One remarkable feature of these transcription units was their location at the center of the replication bubbles, suggesting a tight linkage between early replicating genes and replication origins in *Physarum*. As this could represent some unrecognized aspects of the organization of the eukaryotic genome, we undertook a molecular analysis of some of these genes. We recently demonstrated a close association between a gene replicated at the onset of S-phase and a replication origin by measuring the size of the nascent strands of the replicon (37). In this report, we analyzed the pattern of replication of 2 genes of *Physarum* encoding profilin isoforms. The expression of these 2 genes, which are 60% identical at the nucleotide level, was shown to be mutually exclusive during the *Physarum* life cycle. The *pro*A gene is expressed in the ameba whereas the *pro*P gene is expressed in the plasmodium (38). We demonstrate that only the profilin P gene, which is active within the plasmodium, is tightly linked to a chromosomal replication origin. This replicating origin is contained within a defined DNA fragment smaller than 1 kb that also contains the transcription initiation site of the gene.

MATERIAL AND METHODS

Strains and cultures

The experiments reported in this paper were conducted on two diploid strains (M3CIV and TU291) derived from the same natural isolate (Wisconsin 1). A remarkable feature of the M3CIV strain is the very frequent restriction fragment length polymorphism (RFLP) that is observed when one compares allelic genes (39). Hence, the two profilin genes studied in this work are polymorphic in genomic DNA digested either by HindIII or EcoRI. This gives us the possibility of comparing the pattern of replication of alleles. The TU291 strain is the result of crossing haploid amebal progeny of the M3CIV strain. It is observed that alleles are less frequently polymorphic in that strain and, as an example, neither of the two profilin genes are polymorphic in genomic DNA digested either by HindIII or EcoRI (34). These 2 strains were used in all previous studies on DNA replication in *Physarum* (30–37).

Synchronous plasmodia were grown as previously described (37). As there is no G1 phase in the nuclear cycle of the plasmodium, observation of mitosis under a phase contrast microscope allows for a morphological definition of the onset of S-phase. Telophase and/ or separation of the newly-formed nuclei was taken as time zero of S-phase. About 200 μg of DNA was extracted from a single synchronous macroplasmodium (5 cm of diameter).

DNA extraction

DNA was extracted essentially as described (37) with minor modifications that improved the quality of the DNA preparations as seen on denaturating gels. We added 0.25 M Sucrose to the homogenization buffer (10 mM CaCl2, 0.1% Nonidet-P40, and 10 mM Tris-HCl, pH 8.4). In addition, Proteinase K (0.2 mg/ml for 3 h at 37°C) was added and after its removal by phenol-chloroform extractions, RNase A digestion was subsequently carried out (0.1 mg/ml, 30 min, 37°C). DNA samples were then precipitated by ethanol and resuspended in TE. They did not require any further treatment to be readily digested by restriction enzymes.

Crude DNA extracts from small pieces harvested every 5 minutes from a single plasmodium were obtained as previously described (37) by solubilization in a solution containing 0.2 mg/ml of Proteinase K, 0.45 M EDTA, and 0.5% Sarkosyl at 50°C for 1 h. They were briefly kept at 4°C until used in alkaline gel electrophoresis.

Gel electrophoresis

For gene dosage analysis, DNA preparations digested to completion with EcoRI were electrophoresed in 0.8% agarose gel in Tris-Acetate-EDTA buffer (TAE) overnight at room temperature at 1 volt/cm.

For alkaline gel electrophoresis, DNA extracts were first denaturated for 30 min at room temperature in alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2% Ficoll and 0.025% bromocresol green). They were loaded on gels containing 0.6% agarose in 100 mM NaCl and 1 mM EDTA that were submerged in electrophoresis buffer (30 mM NaOH, 1 mM EDTA), pre-electrophoresed for 30 min at 1 volt/cm and then run at room temperature for 14–18h. Gels were then neutralized in 0.5 M Tris-HCl, pH 7.6, stained with Ethidium bromide (EtBr) and photographed.

2-D gels analysis was carried out essentially as described in (11). Approximatively 10 μg of DNA was digested for 2–4 h at 37°C and then resolved on a 0.4% agarose gel in Tris-Borate-EDTA buffer (TBE) that was subjected to electrophoresis at 0.5 to 1 volt/cm for 36–60 h. The duration of electrophoresis depended on the size range of interest. The gel was stained with EtBr (0.3 μg/ml) and photographed to record the mobility of lambda HindIII digested restriction fragments run in parallel lanes. Following excision, the lane of interest was included in a second dimension gel poured at right angle and containing 1% agarose and 0.3 μg/ml EtBr in TBE. Electrophoresis in the second

dimension was performed at 4°C for about 24 h at 3 volts/cm in the same buffer.

Southern hybridization

After ethidium bromide staining and photographing, the gels were submerged for 15 min in 0.25N HCl for DNA depurination and then prepared for transfer onto nitrocellulose by standard denaturation and neutralization treatments. Transfer was for 90 min using a Vacugene transfer apparatus. After baking, the position of lambda DNA restriction fragments on the nitrocellulose filter was detected under UV light and marked with a pencil. Prehybridization and hybridization were carried out as described. Occasionally 10% dextran sulfate was included in the hybridization solution (34).

Hybridization probes

DNA probes were digested free of vector sequences, purified from agarose gels and labeled by random priming (40).

The profilin A probe is a 860 bp BamHI-EcoRI fragment derived from a 4 kb EcoRI fragment inserted in pTZ19R (Pharmacia). It corresponds to the nucleotides 131 to 991 of the published sequence of the profilin A gene (38). In a EcoRI digest, this probe hybridizes to a single 4 kb in the TU 291 strain and to polymorphic alleles at 4 and 1.8 kb in the M3CIV strain. The profilin P probe is a 960 bp PvuII-PstI fragment corresponding to the nucleotides 66 to 1026 of the published sequence of the *pro*P gene (38). It hybridizes to a single EcoRI fragment of 4.8 kb of TU291 genomic DNA and to 2 allelic fragments of 4.8 and 5 kb of the M3CIV strain. These 2 probes do not cross-hybridize under stringent conditions (final washes at 70°C in 0.1×SSC, 0.1% SDS) (38).

RESULTS

Timing of Replication of the 2 profilin genes

As part of a study comparing the replication of 10 developmentally regulated genes (34), we determined the timing of duplication within the plasmodium of the two profilin genes of *Physarum*. We found that the profilin gene active within the plasmodium was replicated earlier than the non-expressed gene. This result was obtained by probing with cDNA probes the light-light and heavy-light DNA fractions isolated from a plasmodium that had been treated by a BromodeoxyUridine (BudR) pulse for the first 40 min of S-phase. At this stage where about one third of the genome had duplicated, hybridization revealed that only the *pro*P gene (LAV1−5 cDNA) has replicated. In a similar experiment, we provided circumstancial evidence for the duplication of the *pro*A gene (LAV3−1 cDNA) between 40 and 60 min after the onset of S-phase, a stage corresponding to 50% of genome replication.

In the present work, we confirm and extend these results by carrying out a direct comparison of the timing of replication of the two profilin genes by gene dosage analysis. The method consists of measuring the relative copy number of the two genes at different stages of S-phase by filter hybridization (33−35). The strain TU291 and the restriction enzyme EcoRI were chosen in this experiment on the basis that the 2 profilin genes were both contained within unique DNA restriction fragments of similar sizes (4.8 and 4 kb for the *pro*P and *pro*A genes respectively). DNA preparations extracted at specific stages of S and G2-phase were digested with EcoRI, electrophoresed on a 0.8% agarose gel and blotted. A double-hybridization was then performed with a mixture of the two profilin genomic probes, labeled in such a way that the resulting hybridization signals were of similar intensities. This was required for comparing gene copy number

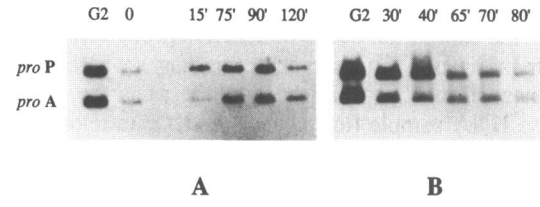

Figure 1. Timing of replication of the two profilin genes of *Physarum* as determined by gene dosage analysis. DNA preparations extracted at different time-points of the nuclear cycle in strain TU291 were digested with EcoRI, electrophoresed on 0.8% agarose gel and blotted onto nitrocellulose. The blot was probed with a mixture of 2 genomic fragments, each specific of a profilin gene. These 2-DNA fragments were mixed and labelled in a single random priming reaction in such a way that the hybridization signals of the 2 genes had similar intensities in G2-phase, a cell cycle stage where there are 2 copies of each gene in the genome. This allows us to compare the timing of replication of the *pro*P gene (4.8 kb EcoRI fragment) and of *pro*A (4 kb fragment) by visual inspection. Numbers on the upper line indicate at which time points of S-phase (in minutes) the DNA samples were extracted with respect to the onset of DNA replication (zero time corresponds to telophase). G2-phase DNA sample was extracted 2 hours after completion of S-phase. A) Visual inspection indicates that the profilin P gene replicates during the first 15 min of S-phase, whereas *pro*A replicates between 15 and 75 min in S-phase. B) This second blot scales-down the replication of the profilin A gene between 40 and 65 min in S-phase. This is deduced from the fact that at 65 min both genes are replicated (similar hybridization intensities) whereas 30 and 40 min after the onset of S-phase, only *pro*P was duplicated (unequal hybridization signals).

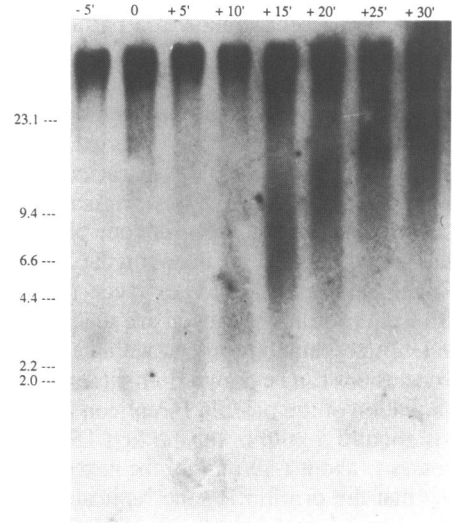

Figure 2. Kinetics of elongation of the profilin P gene replicon. Crude DNA samples, obtained by solubilization of plasmodial fragments harvested exactly every 5 min from a single plasmodium, were denatured and electrophoresed on a 0.6% alkaline agarose gel. Following hybridization with the profilin P gene, the mean size of the nascent strands of the replicon was estimated approximately at 2.5, 5, 9, 12, 18 and 22 kb at 5 min intervals from +5 to +30 min in S-phase. Lambda HindIII digested fragments were used as DNA size markers. Timing of sampling is indicated in min with respect to the onset of S-phase (zero time is telophase). A single exposure (4 days) is presented despite over-exposure of the latest samples in S-phase. This illustrates the relative weakeness of hybridization signals in very early S-phase.

within the linear order response of the X-ray films. As seen on Fig. 1A, the calibration of the radioactive probe resulted in very similar hybridization signals in cell-cycle stages where there exists two copies of each gene in the genome as is the case in the G2-phase DNA sample (left lane, Fig. 1A), in telophase (zero time) and in late S-phase DNA samples (75, 90 and 120 min later). On the contrary, a bias in the relative intensity of the hybridization signals is easily seen at a stage +15 min after the onset of S-phase, indicative of the early replication of the plasmodial specific *pro*P gene (Fig. 1A). On a second blot, we detected the replication of the *pro*A gene by a transition from unequal to nearly equal hybridization signals that took place between 40 and 65 min in S-phase (Fig. 1B). These data, obtained without any drug treatment of the plasmodium, are totally coherent with what we had found earlier by using BudR pulse treatments (34). In addition, they are now sufficiently precise to allow for an analysis of the replicons containing these 2 genes.

Replication of the plasmodial specific profilin gene

We recently described a method that gives informations on the kinetics of elongation of one replicon and on the distance between a gene and its replication origin (37). This method, which takes advantage of the high degree of synchrony within a plasmodium, is based on the detection of the nascent strands of a replicon by filter hybridization. Samples of DNA extracted during the period of S-phase where a gene is known to replicate are electrophoresed on denaturating gels in order to resolve single-stranded replication intermediates from the parental DNA template. After blotting, the elongation of the nascent strands of a particular replicon is studied by hybridization with a specific DNA probe. The size of the smallest nascent strands that can be detected with a single copy gene probe is indicative of the distance between the gene and its replication origin. As previously reported, this experiment can be done either on purified DNA extracted from a whole plasmodium or on crude DNA extracts originating from small pieces taken exactly every 5 minutes from one plasmodium (37). We carried out both types of experiment on the 2 profilin genes.

As seen on Fig. 2, even on crude extracts, nascent growing DNA strands of the profilin P replicon are readily detectable in early S-phase DNA preparations probed with the profilin P gene. Three main conclusions can be drawn from this experiment. First, the rate of elongation of the profilin P replicon in this and other experiments is about 0.5 kb/min for the first 15 min of S-phase and then increases to about 1 kb/min for the next 20 min (Fig. 2). We also show that the profilin P gene replicates in very early S-phase. This can be inferred from the fact that the *pro*P genomic probe hybridizes with the nascent strands of the replicon as early as 5 or 10 min after the onset of S-phase. At these stages, the mean size of the single stranded replication intermediates was about 2.5 and 5 kb respectively. This demonstrates, as a third conclusion, that the profilin P gene is tightly linked to a chromosomal origin of replication. We noticed that the hybridization signals were reproducibly weak in the earliest time points of S-phase. This could suggest an asynchrony in the plasmodium such that only a fraction of the nuclei had initiated DNA replication in these samples. However, in the samples taken 5 min later, the whole population of nascent strands showed a clear increment in size and no small replication intermediates could be seen. Therefore, other explanations, like an instability of the shortest nascent strands during the alkaline gel electrophoresis are more likely.

To further study the smallest nascent strands that could be

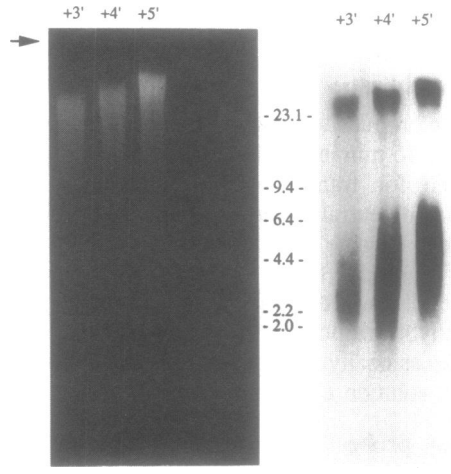

Figure 3. Nascent-strands of the profilin P replicon. Purified DNAs extracted from post-telophasic plasmodia were separated on alkaline agarose gel. The gel was then neutralized, stained with EtBr and photographed. These three DNA preparations were isolated from distinct plasmodia that were at about the same stage of the cell-cycle i.e 4 min +/− 2 min after the end of mitosis. From the observations made on smears under the phase contraste microscope, it was not known whether one was harvested significantly earlier than the others with respect to the onset of S-phase. They were nevertheless called +3', +4' and +5' to be distinguished one from the other. Following blotting onto nitrocellulose and Southern hybridization with the *pro*P probe, the size of the shortest nascent strands found in these 3 preparations was found to be very similar at about 2 kb. Lambda HindIII digested fragments were used as DNA size markers. The arrow denotes the position of the dwells of the agarose gel.

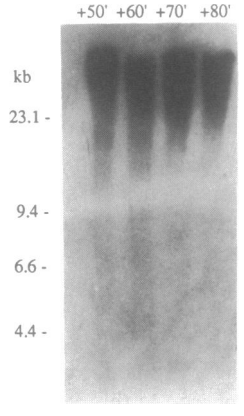

Figure 4. Absence of short nascent strands of the profilin A replicon in DNA samples in which this replicon is active. Synchronous mid-S-phase purified DNA preparations were run on a 0.6% alkaline agarose gel, blotted and hybridized with the *pro*A genomic probe. The activity of the replicon at these stages of S-phase has been inferred from two different type of experiments (see text). A unique hybridization signal is found at about 30 to 50 kb. This experiment demonstrates that, in spite of undegraded DNA preparations and high intensity of hybridization of the probe with the parental DNA, no small replication intermediates were detected.

detected with the profilin P probe, we repeated this experiment with purified DNA preparations extracted from individual plasmodia. This allowed us to analyze large amounts (5 − 10 μg) of denaturated DNA in each experiment. In Fig. 3, three different DNA preparations taken at the onset of S-phase are shown. It is obvious that the probe hybridizes with single stranded DNA

molecules as short as 2 kb. Since bidirectional replication is the rule in *Physarum*, this result demonstrates a colocalization of the gene and the replication origin. However, despite overloading of the gel and overexposure of the autoradiogram, no hybridization occurred with nascent strands smaller than 2 kb. This could mean that replication did not originate from the DNA fragment used as a probe or alternatively that the DNA samples were harvested a few minutes after the onset of S-phase and consequently were depleted in the shortest nascent strands of the profilin P replicon.

Replication of the profilin A gene in plasmodia

In constrast to what is observed with the profilin P replicon, we have never been able to detect the nascent strands of the profilin A replicon. Both the BudR incorporation data and the gene dosage analysis indicated a replication of the *pro*A gene during 40–60 min in S-phase. Consequently, we searched for replication intermediates in either crude DNA samples or on purified DNA samples covering that period of S-phase. In no case did we find single-stranded DNA molecules shorter than the parental DNA hybridizing with the *pro*A genomic probe. Trivial reasons, like degradation of the parental DNA or low level of probe labelling have been excluded by different controls (See Fig. 4). This result can be best explained by a long distance between the gene and its replication origin, such that by the time the replication fork reaches the gene, the replication intermediates are so large that, in our electrophoretic conditions, they are not resolved from the parental DNA (at a size of about 30 kb). This would indicate that the gene and its replication origin are at least 15 kb away on the genome. This contrasts with the tight linkage observed between the actively transcribed profilin P gene and its replication origin.

Mapping of the profilin P replication origin

In order to further documents the association between the profilin P gene and its replication origin, we analyzed the replication of the locus by the 2-D gel method of Brewer and Fangman (11, 14). The rationale of this method is based on the fact that restriction fragments that are branched as a consequence of a partial replication migrate in a very specific position in a two-dimensional gel electrophoresis.

Our previous results indicated that the replication origin should be located within the 4 kb EcoRI-HindIII restriction fragment (Fig. 5), in which the profilin P gene is centrally located. A 2-D gel analysis of a EcoRI-HindIII digested DNA preparation extracted in early S-phase and hybridized with the profilin P probe results in a distinct transition from a bubble to a Y arc. This demonstrates that the restriction fragment is initially replicated as a bubble, up to a point where one fork exits the fragment. Thereafter, this 4 kb fragment is replicated as a simple Y-structure which ultimately returns to the diagonal at twice the size of the monomer (8 kb). This result confirms that the replication origin of the profilin P replicon is found within the 4 kb EcoRI-HindIII restriction fragment that contains the gene. It also demonstrates that the replication is bidirectional in this locus. Finally, the replication origin is asymetrically positioned in this 4 kb restriction fragment, since one replication fork leaves the fragment before the other one. This transition occurs approximatively at the inflexion point of the Y arc, a zone corresponding to branched restriction fragments having three arms nearly equal in length. This suggests a replication origin at about 1 to 1.5 kb from one of the restriction sites. This would

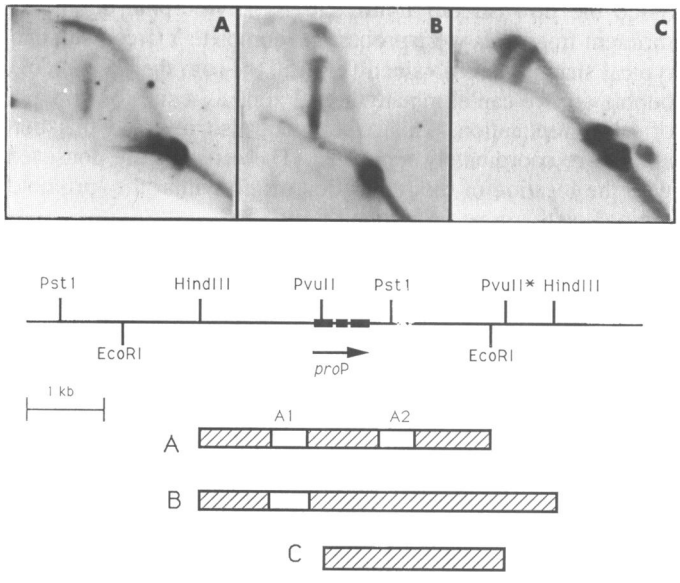

Figure 5. 2-D gel mapping of the profilin P-associated replication origin. Early S-phase total DNA was digested with the appropriate restriction enzyme(s) for 2 to 4 hours at 37°C and separated by 2-D gel electrophoresis according to Brewer and Fangman (11). After blotting and hybridization with the *pro*P probe, structure of the replication intermediates was analyzed. Non-replicating or fully-replicated DNA molecules appear as a spot which position on the diagonal depends on the size (1×) of the restriction fragment. Y-shaped arc generated by the presence of one replication fork within a fragment can be easily distinguished from a bubble arc (2 divergent forks within a fragment) on the following criteria : the Y-shaped arc ultimately reaches the diagonal at the position 2× (twice the size of the monomeric spot) and has a typical form. The bubble (initiating) structures are more retarded in the second dimension and migrate as a steeper arc which does not return to the diagonal. A discontinous pattern is obtained for fragment with an asymetrically located origin, resulting in a bubble-arc that becomes a Y-arc when one fork exits the fragment as it is the case in Fig. 5A and 5B. A restriction map of the profilin P locus is shown at the bottom. The arrow indicates the polarity of the 594 nt transcript. The gene contains two short introns (38). A) HindIII-EcoRI digest probed with the *pro*P genomic probe. Note that this 4 kb fragment is for a large part replicated as a bubble, up to a transition point where one fork exits the fragment, generating a simple Y-structure. This result is suggestive of an asymetrically situated replication origin. Considering the relative extension of the bubble arc, we located the origin between 1 to 1.5 kb of one or the other restriction site (zones A1 and A2). B) HindIII digest probed with the same probe. A clear Bubble to Y-arc transition is observed. The 3′ extension of this 5 kb fragment, as compared to the precedent one, does not result in an extension of the bubble arc as expected for replication initiation in the A2 zone. This favors A1 as the presomptive site for initiation of DNA polymerization. C) Replication of the gene and its 3′ side as a simple-Y. The same probe was used on a PvuII digest. Due to a polymorphic downstream site (PvuII*), two sligtly different restriction fragments were probed (2.5 and 2.8 kb). No bubble arcs were seen on that blot, ruling out the A2 zone as the initiation site of DNA replication. This experiment demonstrates that the two alleles are coordinately replicated. The polarity of the replication fork can be inferred from the fact that the Y-arcs are mainly superposed (similar retardation of the 2 fragments in the second dimension), suggesting that the replication fork replicates first the upstream non-polymorphic PvuII site.

place the origin either 5′ (zone A_1 in Fig. 5) or 3′ of the gene (zone A_2 in Fig. 5). In order to distinguish between these 2 possibilities, we probed a HindIII digest with the *pro*P gene. This fragment contains an extension on the 3′ side of the gene. In these conditions, the bubble arc was found to be shorter, and the Y arc relatively longer, suggesting that the asymetrical location of the bubble was accentuated (Fig. 5B). This favors A1 as the origin site. To definitively ruled out the A2 zone as the origin, we analyzed a PvuII digest by 2-D gel electrophoresis.

Since the downstream PvuII site is polymorphic, 2 slightly different fragments were probed and complete Y arcs, with their typical shape, were obtained (Fig. 5C). From the absence of a bubble arc we can eliminate the A2 zone as a site for initiation of DNA replication. This experiment also revealed that both alleles are coordinately replicated. These results are consistent with the location of the replication origin within the 'promoter region' of the gene (A1 zone of Fig. 5).

DISCUSSION

It had been previously demonstrated that the *Physarum* genome contains two profilin genes and that their expression is mutually exclusive (38). We also showed that they were replicated in different periods of S-phase in the plasmodium (34). In this report, we confirm that the profilin gene active within the plasmodium (*pro*P) is replicated at the onset of S-phase, whereas the inactive gene (*pro*A) is duplicated in mid S-phase.

In *Physarum*, there are more replicons activated at the onset of S-phase (about 4 000), than at any other time in S-phase (41). If the active genes tend to be linked to replication origins, as seen by direct observation on chromatin spreads (36) and demonstrated for the profilin P gene in this report and for another developmentally regulated gene in a precedent study (37), then there will be more active genes replicated early than late in the plasmodium. So far, 10 of the 12 active genes studied within the plasmodium were found to be replicated early (33, 34, 42), whereas the 2 others are replicated late in S-phase (34, 35). Whether these late replicating genes are also near replication origins remains to be seen. On the other hand, our data suggest that the profilin A gene, which is inactive within the plasmodium, is not associated with an origin (Fig. 4). Therefore, a model in which active genes and replication origins are often coupled emerges from our results.

This paper establishes that the initiation of DNA replication at the profilin P gene locus is confined to a definite DNA fragment immediately upstream of the gene. All the experiments reported in this study have been performed on DNA preparations extracted from untreated cells. Due to the natural synchrony of DNA replication within a plasmodium, it was not necessary to induce artificial synchronization that usually requires either drug treatments or a block in the cell cycle. Moreover, our experiments were carried out on DNA preparations in which no BudR was incorporated, and we did not enrich for a particular DNA fraction. Finally, we studied the replication of single copy loci that were not amplified.

The main features of the profilin P replicon in the plasmodium are summarized as follows. This replicon is activated at the onset of S-phase. The first indication came from a BudR density-shift experiment that suggested an early replication of the gene (0−40 min of S-phase) (34). This is confirmed and extented by a gene dosage analysis that restricted the replication of the gene to the first 15 min of S-phase (Fig. 1). Finally, the early replication can be deduced from hybridization of the gene probe with small replication intermediates that are present at the onset of S-phase (Fig. 2). The progressive increase in the mean-size of the replication intermediates seen in 5 min intervals within the cell cycle of a single plasmodium demonstrates that the replicon has a minimal size of at least 30 kb (Fig. 2 and data not shown). The kinetics of elongation of this replicon is in the range of the known fork movement in *Physarum* (31, 32). However, it might vary from 0.5 to about 1/kb min during the time course of the early S-phase (Fig. 2).

From the hybridization of the profilin P gene probe with nascent strands of a size of 2 kb in three different DNA preparations that were extracted at the onset of S-phase (Fig. 3), we concluded that the replication origin was at most 1 to 1.5 kb away from the gene. The most distant location of the origin would only be compatible with a unidirectional replication of the locus. The analysis by 2-Dgels revealed very clearly that elongation is bidirectional in the profilin P replicon. This can be deduced from the transition from a bubble arc to a Y-arc that occurred in restriction fragments containing the origin (Fig. 5). The mobility of one fork is evidenced by its exit from the fragment, generating a Y-arc, whereas the mobility of the second fork is evidenced by the continual increase in the size and the topology of the simple-Y structures, resulting in the complete duplication of the fragment. Bidirectionality of replication reduces the maximal distance between the gene and the origin to about 0.5 kb.

On the other hand, the absence of hybridization of the probe to replication intermediates smaller than 2 kb on alkaline 1-D gel suggests that the replication did not originate from within the gene, but rather from its 5′ or 3′ region. Alternatively, one could postulate that our samples, harvested a few minutes after the onset of S-phase were depleted in the shortest nascent strands of the profilin P replicon. The 2-D gel analysis, however, revealed that nascent strands shorter than 2 kb were present in the DNA extracts studied by denaturating gels. This is demonstrated by short initiating structures, seen in the form of a bubble arc coming out of the 1×monomer size spot (Fig. 5).

To discriminate between the 5′ or 3′ side of the gene for the location of the origin, we compared the pattern of replicating structures derived from different restriction fragments. The results of these experiments are coherent and demonstrate that the origin is most likely located within a few hundred base pairs upstream of the transcribed region of the gene. However, considering that the elongation rate of the 2 forks is not necessarily constant, the origin cannot be located with a higher precision. We previously published 1494 base-pairs of the profilin gene locus, including 520 bp upstream of the PvuII site which is 64 bp downstream of the transcription initiation site (38). It is therefore likely that the initiation of DNA replication takes place within that sequence.

Our data are in clear contrast to what has been found using the same 2-D gel method on amplicons in higher organisms. In the chromosome III chorion gene amplicon of *Drosophila* or in the DHFR amplicon in CHO cells, whenever arcs were found on 2-D gels, they were composites in which the bubble arc was always weak as compared to the Y-arc, suggesting that the fragment under scrutinity was mainly replicated from outside origins and occasionnally from an inside origin. These data lead to the conclusion of multiple replication origins during amplification in *Drosophila* (21, 22) or of a random initiation over a zone of 28 kb in the DHFR locus (25). Random initiation is also supported by a series of experiments showing that any human sequence of more than 10 kb posseses an ARS activity in a plasmid constructed from an Epstein-Barr defectif virus, and that the initiation of DNA replication in the recombinant molecules is not restricted to a particular DNA sequence (27).

Our work, dealing with a single copy locus, compares better to what has been found in yeast where defined chromosomal origins are supported by genetical studies and by 2-D gel mapping (11−17, 43, 44). However, there exists considerable differences

in the size and in the organization of the 2 genomes. The *Physarum* genome is about 20 times larger than the yeast genome, yet the mean size of the replicons is similar in both organisms (45). As a consequence, the number of replicons is likely to be as different as 800 in *S. cerevisiae* and about 18,000 in *Physarum*. Although the *Physarum* genome encodes developmentally regulated genes (like the profilin gene family), it is likely that the number of genes is similar in the 2 genomes. As a consequence, the number of genes per replicon might be quite different in the 2 organisms and the relative positioning of the genes and the replication origins might differ as well.

It has long been known that the temporal order of the *Physarum* genome is invariant in the plasmodium (4). The timing of replication of specific genes is strictly defined (33, 34), and it has been shown that alleles replicate simultaneously. This has been implicit in all our experiments and confirmed for polymorphic alleles like an active actin gene (Ard C) (33) and the *pro*P gene in Fig. 5 (PvuII digest). This can be best explained by a rather rigid mechanism of DNA replication involving precisely defined replication origins as shown for the profilin P replicon in this study.

The localization of a replication origin upstream of a gene is comparable to the conclusions drawn from studies of the c-*myc* locus in human cells (18–20) and corroborates the demonstration of a tight linkage between chromosomal origins of replication and active genes as originally described in *Physarum* (36). This suggests a high density of controlling elements in particular regions of the eukaryotic genome. Our results on the replication of the profilin gene family in the plasmodium provide a frame work for further studies on the interactions between genes and replicons during the cell cycle and the development of an organism.

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REFERENCES

1. Huberman, J.A. and Riggs, A.D. (1968) *J. Mol. Biol.*, **32**, 327–341.
2. Blumenthal, A.B., Kriegstein, H.J. and Hogness, D.S. (1974) *Cold Spring Harbor Symp. Quant. Biol.*, **38**, 205–223.
3. Taylor, J.H. (1984) *Mol. Cell. Biochem.*, **61**, 99–109.
4. Braun, R., Mittermayer, C. and Rush, H.P. (1965) *Proc. Natl. Acad. Sci. USA*, **53**, 924–931.
5. Hatton, K.S., Dhar, V., Brown, E.H., Iqbal, M.A., Stuart, S., Didamo, V.T. and Schildkraut, C.L. (1988) *Mol. Cell Biol.*, **8**, 2149–2158.
6. Cook, P.R. and Brazell, I.A. (1976) *J. Cell Sci.*, **22**, 287–302.
7. Adolph, K.W., Cheng, S.M. and Laemmli, U.K. (1977) *Cell*, **12**, 805–816.
8. Zehnbauer, B.A. and Vogelstein, B. (1986) *Bio Essays*, **2**, 52–54.
9. de Villiers, J., Schaffner, W., Tyndall, C., Lupton, S. and Kamen, R. (1984) *Nature*, **312**, 242–246.
10. DePamphilis, M.L. (1988) *Cell*, **52**, 635–638.
11. Brewer, B.J. and Fangman, W.L. (1987) *Cell*, **51**, 463–471.
12. Huberman, J.A., Spotila, L.D., Nawotka, K.A., El-Assouli, S.M. and Davis, L.R. (1987) *Cell*, **51**, 473–481.
13. Huberman, J.A., Zhu, J., Davis, L.R. and Newlon, C.S. (1988) *Nucleic Acids Res.*, **16**, 6373–6384.
14. Fangman, W.L. and Brewer, B.J. (1991) *Annu. Rev. Cell Biol.*, **7**, 375–402.
15. Dubey, D.D., Davis, L.S., Greenfeder, S.A., Ong, L.Y., Zhu, J., Broach, J.R., Newlon, C.S. and Huberman, J.A. (1991) *Mol. Cell. Biol.*, **11**, 5346–5355.
16. Palzkill, T.G. and Newlon, C.S. (1988) *Cell*, **53**, 441–450.
17. Marahrens, Y. and Stillman, B. (1992) *Science*, **255**, 817–823.
18. Ariga, H., Imamura, Y. and Iguchi-Ariga, S.M.M. (1989) *Embo J.*, **8**, 4273–4279.
19. Vassilev, L.T. and Johnson, E.M. (1990) *Mol. Cell. Biol.*, **10**, 4899–4904.
20. McWhinney, C. and Leffak, M. (1990) *Nucleic Acids Res.*, **18**, 1233–1242.
21. Delidakis, C. and Kafatos, F.C. (1989) *Embo J.*, **8**, 891–901.
22. Heck, M.M. and Spradling, A.C. (1990) *J. Cell Biol.*, **110**, 903–914.
23. Burhans, W.C., Vassilev, L.T., Caddle, M.S., Heintz, N.H. and DePamphilis, M.L. (1990) *Cell*, **62**, 955–965.
24. Vassilev, L.T., Burhans, W.C. and DePamphilis, M.L. (1990) *Mol. Cell. Biol.*, **10**, 4685–4689.
25. Vaughn, J.P., Dijkwel, P.A. and Hamlin, J.L. (1990) *Cell*, **61**, 1075–1087.
26. Shinomiya, T. and Ina, S. (1991) *Nucleic Acids Res.*, **19**, 3935–3941.
27. Krysan, P.J. and Calos, M.P. (1991) *Mol. Cell. Biol.*, **11**, 1464–1472.
28. Mechali, M. and Kearsey, S. (1984) *Cell*, **38**, 55–64.
29. Rusch, H.P. (1980) In Dove, W.F. and Rusch, H.P. (eds.), Growth and Differentiation in *Physarum polycephalum*. Princeton University Press, Princeton, pp. 1–8.
30. Kubbies, M. and Pierron, G. (1983) *Exp. Cell Res.*, **149**, 57–67.
31. Funderud, S., Andreassen, R. and Haugli, F. (1978) *Cell*, **15**, 1519–1526.
32. Funderud, S., Andreassen, R. and Haugli, F. (1979) *Nucleic Acids Res.*, **6**, 1417–1431.
33. Pierron, G., Durica, D.S. and Sauer, H.W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6393–6397.
34. Pierron, G., Benard, M., Puvion, E., Flanagan, R., Sauer, H.W. and Pallotta, D. (1989) *Nucleic Acids Res.*, **17**, 553–566.
35. Benard, M., Pallotta, D. and Pierron, G. (1992) *Exp. Cell Res.*, In press.
36. Pierron, G., Sauer, H.W., Toublan, B., and Jalouzot, R. (1982) *Eur. J. Cell Biol.*, **29**, 104–113.
37. Benard, M. and Pierron, G. (1990) *Exp. Cell Res.*, **186**, 299–305.
38. Binette, F., Benard, M., Laroche, A., Pierron G., Lemieux, G. and Pallotta, D. (1990) *DNA and Cell Biol.*, **9**, 323–334.
39. Schedl, T. and Dove, W.F. (1982) *J. Mol. Biol.*, **160**, 41–57.
40. Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, **132**, 323–334.
41. Pierron, G. (1986) In Dove, W.H., Dee, J., Hatano, S., Haugli, F.B., and Wolfarth-Botterman, K.E. (eds.), The Molecular Biology of *Physarum polycephalum*. Plenum Press, New-York, pp. 67–77.
42. Jalouzot, R., Toublan, B., Wilhelm, M.L. and Wilhelm, F.X. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6474–6479.
43. Linskens, M.H.K. and Huberman, J.A. (1990) *Nucleic Acids Res.*, **18**, 647–652.
44. Ferguson, B. M., Brewer, B.J., Reynolds, A.E., and Fangman, W.L. (1991) *Cell* , **65**, 507–515.
45. Kearsey, S. (1986) *Bio Essays* , **4**, 157–161.