Replicational organization of three weakly expressed loci in Physarum polycephalum

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INTRODUCTION

Eukaryotic genomes are organized into multiple replicons that fire only once per cell cycle and at a specific time in S phase, resulting in a complete replication of the chromosomes and a defined temporal order of replication of the genes (1).

Despite rapid progress in our understanding of the regulation of DNA replication as a once per cell cycle event (2–5), difficulties in defining replication origins genetically have precluded a detailed analysis of the replicons in metazoan (6,7). Although it is apparent in mammalian cells that the temporal order of replication of the genes is closely linked to their pattern of expression (8,9), the molecular basis for early replication in S phase of the active genes is undefined and our understanding of the functional interactions between replication and transcription is rudimentary.

It is only in the yeast Saccharomyces cerevisiae that the replicational organization of a complete genome has been elucidated (10,11). Furthermore, replication origins have been precisely defined only in this organism, leading to the identification of trans-acting factors, implicated in the once per cell cycle activation of the chromosomal origins (12,13). Importantly, these trans-acting factors are conserved and play a similar role in DNA replication in metazoan cells (14–16).

However, in S.cerevisiae, the early and late replicating portions of the genome are transcribed to the same extent (10), and the replication origins appear to be distributed randomly with respect to expressed genes. This organization of replication origins has been recently questioned in Schizosaccharomyces pombe. In both yeasts, the proportion of convergent, divergent or tandemly oriented genes is similar, and very close to what can be expected by chance (25, 25 and 50%; 17). Gomez and Antequera (18), by localizing a subset of S.pombe origins, produced evidence for a pronounced bias for initiation sites of DNA replication located within divergent promoter regions rather than in convergent 3’ ends of genes.

A biased distribution of replication origins would be consistent with the observation in mammalian cells that promoter-associated CpG islands are enriched within isolated newly synthesized DNA strands (19). Similarly, CpG dinucleotide runs are preferentially found in cross-linked DNA fragments immunoprecipitated with antibodies against human Orc1 and Orc2 proteins (20). However, the presence of mammalian replication origins has been reported within intergenic regions of convergently transcribed genes, such as the hamster DHFR and GNAI3 loci, as well as within promoter regions, such as the human lamin B2-ppv1 and PRKDC-MCM4 loci (20–23). Therefore, it remains uncertain whether replication origins are preferentially associated with transcriptional promoters in eukaryotic cells.

We previously studied the replicational organization of five abundantly transcribed genes within the slime mold Physarum polycephalum, and found an efficient bidirectional replication origin within the promoter region of each gene (24–26). To determine whether these results are biased by the fact that only abundantly transcribed genes have been studied so far (two
actin genes, two histone H4 genes and a profilin gene), we analyze the pattern of replication of three single copy genes transcribed at low levels within the plasmodium (27,28).

The cDNA clones for the three red genes examined in this study (redA, redB and redE; regulated in development), were isolated from a subtracted cDNA library prepared from developing cultures of P. polycephalum (27,28). Northern blot analysis indicated that all three genes are maximally expressed during the development of uninucleate amoebae into multinucleate plasmodia; none of the genes are expressed in amoebae and their mRNAs are of low abundance in total RNA from macroplasmodia. The sequences of the cDNA clones do not give any clues to the functions of these genes during development, although redB is related to invertebrate sarcoplasmic calcium-binding proteins (27).

We found that one of these three genes (redA) contains a replication origin in its 5’ proximal region. The firing of this origin takes place in mid-S phase, in a broad temporal window so that, despite being tightly linked to an origin, the replication of redA is not precisely defined temporally. The two other genes (redB and redE) are replicated very early in S phase, yet instead of containing an origin, they coincide with termination sites of DNA replication. Thus, not all the promoters correspond to replication origins in Physarum.

**MATERIALS AND METHODS**

**Strains and cultures**

The Physarum strains M3CIV and TU 291 were used in the present study and in our previous work on DNA replication. The methods for obtaining synchronous plasmodia and detecting cell cycle stages were as previously described (25,29).

**Northern blot analysis**

Total RNA was extracted from G2-phase plasmodia as described by Chirgwin et al. (30). For each sample, 10 µg of RNA was loaded in a 1.1% agarose gel (1× MOPS, 18% formaldehyde) and electrophoresed in appropriate buffer (1× MOPS, 8% formaldehyde). The gel was blotted with 20× SSC and hybridized as described by Bénard et al. (25).

**Bromodeoxyuridine (BrdUrd) incorporation**

Density-shift experiments were done with plasmodia treated at 10 min before mitosis until harvested in S phase as described by Pierron et al. (31). Plasmodia were frozen in liquid nitrogen and the Light-Light (LL) and Heavy-Light (HL) DNA fractions were purified from nuclei isolated as described by Bénard and Pierron (24,26). The actin C (ardC) gene was detected with a DNA probe derived from the 3’ untranslated part of the gene as described by Hamelin et al. (34).

**DNA isolation**

For gene dosage analysis, isolated nuclei were treated with proteinase K and RNase A, and the DNA was purified by phenol–chloroform extractions.

For density-shift experiments, ∼150 µg of digested BrdUrd-substituted DNA was diluted to 10 ml with TE (10 mM Tris–HCl pH 8, 1 mM EDTA), mixed with 9.15 g of solid CsCl and adjusted to a final concentration of 1 µg/ml of ethidium bromide. Following centrifugation in a vertical NVT90 rotor at 60 000 r.p.m. for ≥6 h, the LL and HL DNA fractions were picked up with a syringe under UV light and dialyzed extensively against TE.

For two-dimensional gel analysis, isolated nuclei were embedded into low melting agarose to preserve the integrity of the replication intermediates as described by Bénard et al. (25,29).

**Gene dosage analysis**

Following hybridization of the Southern blot, the intensity of each hybridization band was measured by storage phosphor-imaging using the Molecular Dynamics 400A and ImageQuant.

In each pairwise comparison, the ratio obtained in G2 phase was set to 1. The timing of replication of the restriction fragments was then deduced from the deviation in the S-phase time points of the G2-phase abundance ratio.

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis was carried out as originally described by Brewer and Fangman (32). In-gel digestion was as described by Friedman and Brewer (33), with the following minor modifications: the digestion lasted ~20 h at 37°C and ~200 U enzyme/µg of DNA were added three times onto the gel slice of interest.

**Hybridizations and probes**

DNA was blotted onto Nylon GeneScreen Plus (NEN). Hybridization and washes were as described by Bénard et al. (25). Purified cDNA inserts were used as specific probes and labeled with [32P]dCTP by random labeling. The cDNA inserts were removed from the pBluescript vector by EcoRIV–SacI digestion for redA (674 bp cDNA from 800 nt mRNA; EMBL accession no. Y18123) and redB (655 bp cDNA from 800 nt mRNA; accession no. Y18124). For redE (1100 bp cDNA from 1300 nt mRNA; accession no. AJ297387), SacI–KpnI digestion was used. The profilin P (proP) and the H4-1 genes were detected with a 0.9 and a 0.6 kb genomic DNA fragment, respectively, as described by Bénard and Pierron (24,26).

**RESULTS**

**Abundance of red genes mRNAs in macroplasmodia**

The red genes were isolated on the basis of a maximal expression during differentiation of a culture of haploid amoebae into plasmodia (27). No transcripts corresponding to these genes were found in total RNA from amoebae; mRNA levels increased progressively with the percentage of cells committed to differentiate and dropped to a low level in macroplasmodia (28). In Figure 1, we estimated by northern blot analysis the abundance in the plasmodium of the red gene mRNAs compared with the mRNAs of three abundantly transcribed genes coinciding with replication origins. This experiment confirmed the red genes as a class of genes with an apparent low level of expression. The redB and redA transcripts were about 100 times less abundant, and the redE transcript was about 1000 times less abundant, than the mRNA of the actin ardC gene that was used as a reference (Fig. 1).
Timing of replication of the red genes

We initially used in vivo BrdUrd incorporation to determine whether the red genes are contained within early or late firing replicons in the 3 h long S phase of the naturally synchronous plasmodium. Typically, a plasmodium is deposited on BrdUrd-containing growth medium at the onset of S phase until harvest. The unreplicated LL and replicated HL DNA fractions are separated in CsCl gradients and analyzed by Southern blotting and hybridization.

As an example, in Figure 2A, a blot of EcoRI-digested LL and HL DNA fractions following BrdUrd incorporation during the first 45 min of S phase was hybridized with a mixture of radioactive probes complementary to proP, a gene known to replicate at the onset of S phase (24), and to the redA, redB and redE genes. The hybridization pattern shows that the EcoRI fragments corresponding to proP (as expected), redB and redE are density shifted, and therefore replicated early in S phase. In contrast, the redA gene is found in the unreplicated LL DNA fraction, showing that it replicates later than the other red genes.

This temporal order of replication was confirmed by ‘gene dosage’ experiments that have the advantage of not requiring any treatment of the plasmodia (i.e. no BrdUrd incorporation). A Southern blot is performed with DNA samples harvested at different stages of the synchronous cell cycle. The blot is hybridized with specific radiolabeled DNA probes: redA, redB and proP in Figure 2B. In G2 phase, all three genes are replicated, providing a standard for normalizing hybridization values in a DNA sample containing two copies of each gene (2/2/2). At one time point in S phase, if one gene replicates earlier than the others, the relative copy number is changed (1/2/1) and the abundance ratio of the hybridization signals varies (31,35). Using this principle, we determined that redB replicates in the interval 0–30 min of S phase, like the early-replicating proP gene, whereas redA replicates mainly during the 60–90 min interval (Fig. 2B). These results confirm the results of the density-shift experiments (Fig. 2A and data not shown), indicating that the BrdUrd incorporation had no gross effects on the temporal order of replication of the genome.

To gain insights into the organization of the replicons encompassing the red genes, we then determined the structure of the replication intermediates over these loci by two-dimensional gel electrophoresis (32).

Replication organization of the redB gene

We scrutinized four overlapping restriction fragments (A–D) spanning a 16 kb region of the early-replicating redB locus by two-dimensional gel analysis (Fig. 3). Experiments were carried out with DNA samples extracted after 5, 10 and 15 min in S phase in order to monitor fork propagation. In the 5 min DNA sample (Fig. 3, left), replication intermediates were detected in the two external A and D restriction fragments but not in the two central B and C fragments, indicating partial replication of the locus by invading forks originating from both sides. Accordingly, in the +10 min DNA sample (Fig. 3, center), a rightward-moving fork has progressed beyond the

Figure 1. Comparative analysis of mRNA abundance from the red genes and from abundantly transcribed genes in the plasmodium. Multiple lanes of a single northern blot, each containing 10 µg of total RNA from a G2-phase plasmodium, were hybridized with probes corresponding either to the actin ardC, profilin proP and histone H4-1 genes or to the red genes. As a control for probe efficiency, each northern strip was co-hybridized with a Southern blot lane containing 3 µg of DNA and restricted such that DNA fragments of sizes between 5 kb (EcoRI digest, proP probe) and 10 kb (EcoRI digest, H4-1 probe) were recognized (not shown). Measurement of signal intensities by PhosphorImaging on DNA blot revealed that probe efficiency was within a range of 2. Signals on northern blot were normalized to these values to determine the relative mRNA levels shown in the histograms. Exposure was for 1 day for all samples except for redE, which required 10 days. Sizes of the mRNAs extend from 480 nt for H4-1 to 1.4 kb for ardC.

Figure 2. Temporal order of replication of the red genes. (A) BrdUrd density-shift analysis. Following in vivo BrdUrd incorporation during the first 45 min of S phase, LL and HL EcoRI-digested DNA fractions were purified on a CsCl gradient and analyzed by Southern blotting. Four different radioactive probes, one of which corresponds to the early replicating proP gene as a control, were mixed in the hybridization solution. The EcoRI fragments containing proP, redE and redB are enriched in the HL DNA fraction and therefore replicated early, whereas redA is not replicated by 45 min in S phase. (B) ‘Gene dosage’ analysis. A Southern blot of EcoRI-digested synchronous DNA samples was hybridized with a mixture of the redA, redB and proP probes. The intensity of the hybridization bands was measured by PhosphorImaging. In each pairwise comparison, the ratio of the G2-phase DNA sample was set to 1.0 and values for the other time points were normalized to this value (see histograms). The proP gene was included in this experiment as an indicator of early replication. Histograms of relative values indicate that proP and redB are both replicated in the first 30 min of the 3 h long S phase, whereas redA replicates in mid-S phase (mainly in the 60–90 min interval).
center of the A fragment, as revealed by the Y-arc apex on the two-dimensional gel. Consequently, this fork must have entered the B fragment where, indeed, a partial arc of simple Ys is apparent (Fig. 3, center). Similarly, the downstream fork detected at 5 min in S phase in the D fragment has progressed up to the center of this fragment at +10 min (Fig. 3, center). It is likely that this leftward-moving fork is generating the replication intermediates detected in the C fragment at +10 min (Fig. 3, center). These results confirm that forks are entering into the locus from nearby origins that must be activated at the onset of S phase to generate such an early replication of the locus. If these origins fire on the same DNA molecules, replication forks should converge over the redB gene region and produce a termination signal on the two-dimensional gels. The patterns observed after 15 min of S phase (Fig. 3, right) exemplify such a situation. Both the rightward- and leftward-moving forks have progressed as seen by evolution of the Y-arc signals in the A and D fragments. As a result, a termination signal, in the form of a spike extending leftward from the apex of the Y arc, is appearing in the C fragment (see arrow, Fig. 3). This reveals that a fork collision site is closely associated with the redB gene.

**Polymorphism of replication at the redB locus**

To confirm the presence of forks with opposite polarities within the C fragment (HincII–HincII, Fig. 3), we utilized a modification of the two-dimensional gel electrophoresis technique. This modification includes an in-gel digestion of the replication intermediates after the first dimension to separate, during the second dimension, the structures generated by forks running in either direction (33). In Figure 4 (left), HincII-digested DNA at +15 min was used to run a two-dimensional gel with a secondary in-gel KpnI digestion. The resulting pattern clearly showed two arcs, corresponding to replication intermediates generated by the rightward (arc 1) and leftward (arc 2) moving forks (see schematic representation). Surprisingly, the termination signal is only associated with arc 2. Therefore, arc 1 defines the frequency with which the fork originating from the upstream origin traverses the fragment unimpeded by a converging fork. This suggests that the downstream origin does not always fire. In contrast, the termination signal (spike 3, Fig. 4) associated with arc 2 reveals that when a fork is generated by the downstream origin, it frequently encounters a fork originating from the upstream origin traverses the fragment unimpeded by a converging fork. Consequently, this termination site can be mapped close to the center of these fragments, i.e at the 3′ end of the redB gene. These results define precisely the passive, early replication of the redB gene at +15 min onwards in the 3 h long S phase, from flanking origins activated at the onset of S phase.
overlapping the gene and its 3′ untranscribed region. The location of this origin, ∼2 kb downstream of the gene, was refined by additional two-dimensional gels using overlapping EcoRV and BglII fragments (data not shown). It is of note that, in this KpnI profile, the Y arc underlying the bubble arc is complete. Such a composite pattern suggests that this fragment is actively replicated in some nuclei of the plasmodium from the internal origin and passively replicated in others by forks arising from a nearby, early firing origin. At whatever time-point in S phase we looked, no bubble arc could be detected in the 7.5 kb HindIII fragment spanning the promoter region of the gene. Figure 5A shows a partial arc of Y structures at the onset of S phase. However, probing the same +3 min DNA preparation for the overlapping 8.5 kb EcoRI fragment that extends one more kb on the 5′ side of the gene resulted in a clearly detectable bubble arc above the apex of an incomplete Y arc (Fig. 5A). This reveals another early firing origin asymmetrically located within the EcoRI fragment, such that the bubble structure is too rapidly converted into a simple Y to be seen following restriction by HindIII. The distance between the two origins flanking redE is ∼9 kb.

To test whether the promoter region of the redE gene, located at the mid-point between the two flanking origins, coincides with a termination site of replication, an early S-phase DNA sample (+8 min) was digested by BclI. Due to a polymorphism of restriction sites in the diploid plasmodium, the allelic BclI fragments of the locus were separated on two-dimensional gels. The promoter region of the gene is centrally located in the shortest 5 kb BclI fragment, whereas the 10 kb fragment of the other allele should encompass both the putative termination site and the upstream origin (see map in Fig. 5B). Hybridization of the BclI digest with the redE probe resulted in a conspicuous termination signal, extending upwards from the apex of the

Pattern of replication of the redE gene locus

Like redB, the redE gene is flanked by two origins activated at the onset of S phase. The nearest replication origin is positioned downstream of the gene, as revealed by a prominent bubble arc (Fig. 5A), within the 6.5 kb KpnI fragment

Figure 5. Replicon organization at the redE locus. (A) The restriction map depicts the 16 kb region under study. The position of the gene and the polarity of transcription are indicated. Restriction sites are as follows: E, EcoRI; K, KpnI and H, HindIII. The cell cycle stage of the synchronous DNA sample is indicated. A replication origin on the 3′ side of the gene is evidenced by the prominent bubble arc in the 6.5 kb KpnI fragment at +5 min. In the overlapping 7.5 kb HindIII fragment, only a partial Y arc is seen at the onset of S phase. However, probing the 8.5 kb EcoRI fragment, which extends only 1 kb more on the 5′ side, resulted in a distinct bubble arc (arrow within the frame), above the apex of a partial Y arc. This demonstrates the presence of another early firing origin upstream of the redE gene. (B) The scheme depicts the location of the two initiation sites flanking the redE gene and the predicted replication events if the two origins fire on the same chromatids. The 5 and 10 kb allelic fragments of a BclI digest are separated on the two-dimensional gel (see fragments a and b in the restriction map; the polymorphic BclI site is marked B*). The two-dimensional gel pattern reveals a termination signal (arrows within the frame) for each of the two alleles, indicating merging of forks within the promoter region of the gene.
simple Y arc, for the shortest allele. This demonstrates the merging of forks originating from the simultaneously activated flanking origins onto the 5′ proximal region of the gene. The pattern observed for the 10 kb BclI fragment is characterized by a deficit in short replication intermediates which is compatible with the presence of an origin within the fragment and, as a consequence, short-lived replication intermediates of a small size, and then a distorted simple Y arc with a termination signal extending from the apex. This indicates that both alleles are replicated simultaneously and have a similar replicon organization. The absence of bubble-like structures in this blot further demonstrates that the flanking origins are site specific rather than components of an initiation zone of DNA replication. Therefore redE is, like redB, embedded into a cluster of early firing replicons.

The redA gene coincides with an origin of replication

BrdUrd density-shift and gene dosage experiments indicated that the redA gene is replicated in mid-S phase. We studied the pattern of replication of an 8.5 kb BamHI fragment in which the promoter region of the redA gene is centrally located (see restriction map in Fig. 6), at different time points in S phase. These two-dimensional gel experiments revealed that the redA gene coincides with a replication origin but also that it is replicated in a remarkably broad temporal window. In Figure 6, replication intermediates are detected over a 1.5 h period of time; they are more abundant in mid-S phase (+40 to +90 min) and then decrease progressively. The slow replication of redA is evident when compared with fork progression within a 10 min interval at the redB locus (Fig. 3), or at early replicated abundantly transcribed loci (25,29).

The redA origin is the first Physarum DNA replication origin detected that does not fire at the onset of S phase. The presence of bubble-arc structures between +40 and +90 min suggests a low level of temporal control of the origin which is not, however, significantly activated during the earliest time points in S phase (+15, +25, +35 min in Fig. 6). The absence of replication intermediates at these early time points is consistent with the lack of the downstream EcoRI fragment in the HL bromosubstituted fraction at 45 min in Figure 2.

The complete Y arc underneath the bubble arc suggests that the redA origin does not fire in every nucleus of the plasmodium. However, when overlapping HindIII or EcoRI fragments spanning the downstream region of the gene (see Fig. 6) were analyzed by two-dimensional gels, simple Y structures were generated (data not shown), indicating that initiation sites are confined within a region that overlaps the 5′ region of the gene as schematized in Figure 6. The decrease of the ratio of bubble- to Y-arc structures in S phase indicates a switch from active replication at +40–50 min to passive replication later on in S phase. The redA gene is, thus, mainly replicated during a broad temporal window (+40 to +90 min), during which ≥40% of the 3 × 10^8 bp of the Physarum genome is duplicated (36).

CONCLUSIONS

We previously established that five abundantly transcribed genes were replicated at the onset of S phase in the synchronous plasmodium of Physarum, from efficient origins located within their promoter regions. In this study, we analyzed the pattern of replication of three weakly expressed red genes and found diversified patterns. The 5′ proximal region of the redA gene coincides with a replication origin firing in mid-S phase. The two other red loci replicate early from flanking origins, demonstrating that in Physarum, as in other eukaryotic cells, not all the promoters of transcribed genes correspond to replication origins.

The redA promoter region coincides with an origin of DNA replication

The replication of the redA gene differs in several ways from the uniform pattern of replication of the abundantly transcribed genes. First, although clearly transcribed within the plasmodium (Fig. 1), redA was not found to be replicated early (Fig. 2). Secondly, the replication schedule of the gene is not well defined temporally. This is best seen by the persistence of
replication forks on an 8.5 kb fragment throughout a large temporal window on two-dimensional gels (Fig. 6). A gradual desynchronization of the nuclei after the onset of S phase could explain these results. However, the level of synchrony was found to be constant throughout S phase in the plasmodium by flow cytometric experiments (36).

From the redA two-dimensional gel patterns, it cannot be determined at first sight whether there is a progressive activation of the origin within the different nuclei of a plasmodium or whether the replication forks are frozen over the locus. However, there are no apparent pause sites in the BamHI fragment in Figure 6 as would result from fork stalling. Furthermore, although the percentage of replication intermediates as measured on two-dimensional gel is relatively constant during the 40–90 min interval (Fig. 6), their characteristics are changing with a decrease of the bubble-arc to Y-arc ratio (compare +40 and +50 min with +70 and +90 min time points), suggesting that replication forks are dynamic rather than frozen on this locus. In addition, the gene dosage experiment in Figure 2 indicated ongoing replication of the gene during that period of time (see proP/redA abundance ratio).

These results, although surprising, are not unprecedented. It has been previously shown by gene dosage experiments that within the same plasmodium, one allele of the weakly expressed α-tubulin actA gene completed replication in a large interval between 40 and 80 min in S phase, while the other allele was found to replicate earlier and more rapidly between 20 and 40 min in S phase (35). It was hypothesized that the more relaxed time schedule of replication of one of these alleles might result from a long distance between the gene and its replication origin and a random variation in the fork rate movement along the template. Our results at the redA locus demonstrate that even genes coincident with an origin can be replicated slowly in mid-S phase. It is therefore possible that a relaxed timing of activation is an intrinsic property of the redA origin. This assumption is confirmed by the recent observation of another Physarum origin that fires at a low level during a 60 min interval starting at the onset of S phase in the plasmodium (M.Bénard and G.Pierron, unpublished data).

The redE and redB genes are flanked by early origins

In contrast to redA, the redB and redE promoter regions are not preferred initiation sites of DNA replication. Their early replication within the plasmodium is due to flanking origins activated at the onset of S phase. At the redE locus, as shown by two-dimensional gels, both origins are firing simultaneously at the onset of S phase and are separated by ~9 kb (Fig. 5).

In contrast, the origins flanking redB could not be directly detected. However, from the continuous progression of the forks towards the gene (Fig. 3), we can estimate the respective positions of these origins. The upstream fork can be mapped within the 9 kb PstI fragment A after 5, 10 and 15 min in S phase (see arrowheads of rightward moving fork in Fig. 3). Its progression rate is ~6 kb in 10 min. Assuming that the origin fires at the onset of S phase and that a similar rate of fork progression occurs in the first 5 min of S phase, this would add 3 kb to the distance traveled by the fork. A similar reasoning can be made for the fork entering the locus from downstream of the gene. We conclude that the replication of redB results from the convergence of forks produced by origins separated by 18 kb at most and located at approximately similar distances from the gene. Modification of the two-dimensional gel electrophoresis technique (Fig. 4) reveals the different efficiencies of the origins flanking the redB gene. Nevertheless when they both fire on the same chromatids, they produce a termination signal that is spatially and temporally defined (Figs 3 and 4), indicating a high level of temporal control for these two adjacent origins.

It had been shown in yeast that termination sites of DNA replication, with the noticeable exception of the replication fork barrier Y′ of the rRNA genes (37), were not DNA sequence specific (38). When a yeast chromosomal origin is deleted, a new site of fork merging is created, demonstrating that termination regions are simply determined by the positions of replication origins (39). Our results in Physarum are also in agreement with the absence of sequence-specific termination sites, since we are following freelwheelsing forks up to their site of merging at the redB and redE loci (Figs 3 and 5).

Coordinated firing of adjacent origins in Physarum

The prominent termination signal found in the promoter region of the redE gene (Fig. 5) reveals that both flanking origins often fire on the same chromatid. This indicates that there is no significant interference between two naturally occurring origins that are only 9 kb apart. The concept of origin interference within plasmids and chromosomes is derived from the study of experimentally juxtaposed origins (40–42). As an example, when the ARS1 and ARS501 chromosomal origins of S.cerevisiae were placed 6.5 kb apart, replication initiated from either one or the other origin, but very rarely from both origins on the same chromatid, i.e. they interfered (41). Similarly, the naturally occurring origins of the central spacer of the Physarum rDNA, which are also 6.5 kb distant, rarely fire simultaneously (43,44). The same is true of the three closely spaced origins at the urad4 locus of S.pombe (45). On the other hand, DNA fiber autoradiography and direct electron microscopy observations (46,47) have shown the physical clustering, in groups of 3–4 units (range 2–8), of early firing replicons in Physarum. This temporal clustering of replicons has also been seen by DNA fiber autoradiography in yeast (48) and mammalian chromosomes (49,50). Therefore, provided that they are not too closely spaced, adjacent origins have a high probability of firing simultaneously. Although it is not known whether there is a threshold distance for origin interference, to our knowledge, the origins flanking redE define one of the shortest replicons ever characterized in a transcriptionally active cell.

Why so many origins?

It has been pointed out many times that the number of replication origins in eukaryotic genomes exceeds the minimal number needed to achieve the replication of the DNA (51). Hence, in Physarum, the simultaneous activation of all the replicons would result in the shortening of the 3 h S phase to <30 min. In both the redB and redE loci, the inter-origin distance is even shorter than the mean size of the Physarum replicons at 35 kb. If only one of the two origins flanking the redE gene was present, the locus would still be replicated very early in S phase. It is therefore conceivable that these redundant initiation sites of DNA replication fulfill other functions such as modulation of transcription within the locus or a structural role in the chromosomes. A question that arises is whether
these origins, firing at the onset of S phase, correspond to promoters of abundantly transcribed genes yet to be discovered in the vicinity of the redE gene. This would support a working model in which strong promoters coincide with efficient, early S-phase replication origins, whereas weaker promoters are either passively replicated when flanked by more actively transcribed genes, as may be the case at the redD and redE loci, or correspond to weaker and/or later firing origins when contained within a less active chromosomal domain, as may be the case for redA. Such a model of genomic organization would be consistent with our results on the replication of genes transcribed at different levels in Physarum. It would also suggest an epigenetic selection of replication origins coupled to regulation of gene expression during eukaryotic cell differentiation (7).

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