Volume 17 Number 2 1989 Nucleic Acids Research

Replication timing of 10 developmentally regulated genes in *Physarum polycephalum*

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Received October 25, 1988; Revised and Accepted December 9, 1988

ABSTRACT

We have tested the hypothesis which stipulates that only early-replicating genes are capable of expression. Within one cell type of Physarum - the plasmodium - we defined the temporal order of replication of 10 genes which were known to be variably expressed in 4 different developmental stages of the Physarum life cycle. Southern analysis of density-labeled, bromodesoxyuridine-substituted DNA reveals that 4 genes presumably inactive within the plasmodium, were not restricted to any temporal compartment of S-phase: 1 is replicated in early S-phase, 2 in mid S-phase and 1 in late S-phase. On the other hand, 4 out of 6 active genes analysed are duplicated early, with the first 30% of the genome. Surprisingly, the two others active genes are replicated late in S-phase. By gene-dosage analysis, based on quantitation of hybridization signals from early and late replicating genes throughout S-phase, we could pinpoint the replication of one of these two genes at a stage where 80-85% of the genome has duplicated. Our results demonstrate that late replication during S-phase does not preclude gene activity.

INTRODUCTION

The duplication of eukaryotic chromosomes is achieved through a complex process involving thousands of replication units which are activated in a non-random temporal order(1,2). A high number of active genes have now been shown to be replicated early during S-phase(3-11). On the contrary inactive transcription units(6,7), heterochromatic segments of chromosomes(12) or even inactivated X chromosome in female mammals appeared to be preferentially replicated late in S-phase(13). Based on these observations, models linking the specific expression of genes to the temporal order of replication have been proposed(6,14). In short, early replication would be a prerequisite for gene expression whereas late replication would preclude gene activity. Consequently, the chronology of replication would be the highest level of gene control and would necessarily be strictly defined within one cell lineage and variable in the time course of the differentiation process(5,6,14).

We are using the slime mold Physarum polycephalum as a model system for studying the chronology of replication within the eukaryotic genome.In one stage of its life cycle, Physarum develops as a large multinucleated cell, called a plasmodium, which contains as many as 10^8 nuclei(15). Being in a common cytoplasm, these nuclei behave synchronously and divide

every 10 hours .They then immediately embark on a 3 hour synchronous S-phase as previously demonstrated by flow-cytometry(16). This provides a unique situation where DNA replication studies do not require artificial synchronization or cumbersome sorting of a random population of cells. Interestingly, the relation between the timing of replication of genes and their expression is also apparent in the naturally synchronous plasmodium of Physarum. Previously, we have visualized on plasmodial chromatin spreads a set of active genes which are replicated early in S-phase in the form of transcription units located on nascent replicons(17). Next, as a test-case, we determined the timing of replication and the transcriptional activity of the 4 members of the Physarum actin multigene family . We found that 3 of the 4 loci are replicated early whereas the fourth locus is late(18). Subsequent analysis of 233 actin cDNA clones revealed that all these cloned mRNA originated from two of the early replicated loci(19). Moreover,a probe specific for an actin sequence from the late replicating ardA locus did not hybridize to RNA on Northern blots, suggesting an inactivity of this actin sequence(19), which, as revealed by sequencing(20), is a *bona fide* isocoding actin gene . It had also been shown that the two histone H4 genes of Physarum are duplicated in early S-phase(21).

In this study, we determined the timing of replication within the plasmodium of 10 developmentally regulated genes of Physarum in order to further compare the chronology of replication of active and inactive genes.Much to our surprise,we found that 2 genes expressed within the plasmodium are replicated late in S-phase. These results are not compatible with the current concept which stipulates that gene expression is restricted to the early replicating compartment of the genome (5,6).

MATERIAL AND METHODS

Cultures

Two Physarum strains (Tu291 and M3CIV) were used in this investigation. Both are diploid derivatives of the Wis1 natural isolate and therefore have a common genetic background. The Tu291 strain has been previously utilised for most DNA replication studies in Physarum (18,21). The DNA content of the nuclei is slightly reduced in the M3CIV strain. A flow-cytometric analysis had indicated that this was due to a reduction of late-replicating DNA (16). So far, however, all the genes we have analysed were present and had equivalent replication timing in both strains.

The synchronous cultures (macroplasmodia;5-6 cm in diameter) were grown on Whatman filter paper according to published procedures (22). The second and third synchronous mitosis generally took place about 15 and 24 hours after feeding as judged by phase contrast microscopy.

Isolation of BrdUrd-Substituted DNA

Cultures were treated in mitosis (about 10 min before the onset of DNA replication) until harvested in S-phase with a mixture of BrdUrd (100 µg/ml), fluorodeoxyuridine (5 µg/ml) and uridine (100 µg/ml) (2,18) . After a BrdUrd pulse, about $3x10^8$ nuclei were isolated from a single macroplasmodium essentially as in (23).The DNA was then extracted and purified as described in (24).To separate the newly replicated DNA from the unreplicated fraction of the genome, 150 µg of isolated DNA were digested with either HindIII or EcoRI and then diluted to 9 ml in 10mM Tris-HCL, pH 8.0/1mM EDTA. After addition of 11g of solid CsCl and 20 µg of ethidium bromide,the restricted DNA was centrifuged 60h at 40,000 rpm in a Beckman 50 Ti rotor. The light-light (LL) and heavy-light (HL) DNA bands were then visualised under UV-light and withdrawn separately with a syringe and a 21 gauge needle. The two fractions were desalted by dialysis and finally ethanol precipitated.

Hybridization probes:

The 10 cDNAs used in this study had all been cloned in the Pst1 site of pBR322. Bulk preparations of the different plasmids were digested with Pst1 and the inserts purified by standard methods (25) after agarose gel electrophoresis . The length of the cDNA inserts is as follow: LAV1-1: 860 nt ; LAV1-2: 800 nt ; LAV1-3: 370 nt ; LAV1-4: 480 nt ; LAV1-5: 560 nt ; LAV3-1: 600 nt ; LAV3-2: 550 nt ; LAV3-3: 850 nt (26); LAV2-1: 1040 nt (27); LAV5-1: 650 nt (28).

Hybridization analysis:

The LL and HL restricted fractions of the genome were electrophoresed on 0.7% agarose gel. The relative concentration of the two fractions was adjusted as a function of their complexity. Since one third of the genome is replicated during the first 40 min of S-phase (16),we loaded twice as much LL DNA as HL DNA on the gel after a 40 min BrdUrd pulse. Equal amounts of LL and HL DNA were loaded after a 60 min BrdUrd pulse, a time corresponding to 50% of genome replication. As a 90 min pulse corresponds to 75% of genome replication, we loaded the LL and HL DNA in a 1 to 3 ratio.

After blotting onto nitrocellulose the LL and HL DNA were hybridized with a ^{32}P-labelled cDNA insert for 20 hours under standard conditions (0.45 M NaCl/0.045M sodium citrate pH7, 68°C, 10% dextran sulfate). Probes were labelled by either nick-translation(29) or random -labelling (30) to a specific activity of 1 to $8x10^8$ cpm/ug with ^{32}P-dCTP. The presence of GC tails in the cDNA inserts which hybridize to repetitive sequences in the Physarum genome rendered highly stringent washes of the filters mandatory. The two final washes were for 5 min at 70°C , in 15 mM NaCl, 1.5 mM Sodium citrate pH 7 and 0.1% Sodium dodecyl sulfate.Northern-blot hybridizations were performed as previously described (26).

Gene dosage analysis

Following DNA extraction at various time-points in S-phase, equivalent amounts of S- and G2-phase DNA samples digested with EcoRI or HindIII were electrophoresed on 0.7% agarose gels. After blotting onto nitrocellulose filter, hybridizations were performed with two ^{32}P-labelled cDNAs known to detect genes replicated in different comparments in S-phase.The two cDNAs were labelled in a single random-priming reaction in such a way that the resulting hybridization signals were of similar intensities. This was required for comparing the copy number of both genes within the linear order of response of the X-ray films. The autoradiographs were scanned with a Chromoscan 3 Joyce-Loebl densitometer attached to a computer equipped with the Joyce-Loebl data system software. The timing of replication of two restriction fragments was deduced from the deviation in S-phase of the ratio of their hybridization signals obtained in G2-phase (18).

RESULTS

Southern analysis of the LAV genes of Physarum

The Physarum life cycle offers a great variety of different cell types which are easily propagated in the laboratory. Physarum can be cultured as uninucleated haploid amoeba which under certain circumstances fuse in pairs to form a diploid zygote. These diploid cells differentiate into large multinucleated plasmodia by intranuclear mitosis in the absence of cell division. Under unfavorable conditions, a plasmodium can either form resistant cysts (spherules) or initiate another differentiation program (sporulation) which culminates with the formation of haploid spores (31). Differential screening of cDNA libraries established from poly(A+) RNA from these 4 distinct differentiation stages of Physarum

Table 1. Comparative patterns of HindIII and EcoRI restriction fragments hybridizing with the LAV cDNA probes in strains Tu291 and M3CIV.

	HindIII fragments,kb		EcoRI fragments,kb	
cDNA clones	TU 291	M3CIV	TU 291	M3CIV
LAV1-1	10	21;10	1.2	1.2;1
LAV1-2	2.8	2.8	9.6;9.2	9.6;9.2
LAV1-3	17	17;12	25	nd*
LAV1-4	12;6	12;6	16;13;7;4	16;13;7;4
LAV1-5	3.8	nd	4.8	4.8
LAV3-1	6.6	nd	4	4;1.8
LAV3-2	25	25	20	20
LAV3-3	25	25	2.7	2.7
LAV2-1	15	nd	9;6.6	9;6.6
LAV5-1	13	nd	13	13;11

* nd=not determined

allowed for the isolation of cDNA probes specific for developmentally regulated genes (26,27,28).

In this communication , we established the timing of replication, within the plasmodium, of genes which are either expressed in the plasmodium (26) (LAV1-1; 1-2; 1-3; 1-4; 1-5; 3-2) , or apparently not expressed in the plasmodium but readily active in the amoeba (26) (LAV 3-1; 3-3) , during spherulation (27) (LAV 2-1) or during sporulation (28) (LAV 5-1). One gene (LAV 3-2) is expressed both in the amoeba and the plasmodium but the amount and the size of the mRNAs differ in these two developmental stages (see fig. 3a).

In Table1, the EcoRI and HindIII restriction patterns of these genes in the diploid strains Tu291 and M3CIV are summarized . It can be noted that many of these genes show a simple restriction pattern in the Tu291 strain, suggestive of single copy genes. However, there exists frequent restriction fragment length polymorphism in the M3CIV strain. HindIII and Eco RI restriction fragment length polymorphism was previously shown to affect the 4 actin loci in the M3CIV strain but only one actin locus of the Tu291 strain(32,18). As predicted from the common genetic background of the two strains, the allelic actin sequences found in Tu291 represented a subset of the M3CIV alleles (32,18). This is also the case for the genes analysed in this communication(Table 1).

Chronology of replication of the LAV genes of Physarum

We subdivided the 3 hour S-phase of Physarum into four compartments by extracting DNA after in vivo BrdUrd incorporation for either 40, 60 or 90 minutes . About 1/3 of the genome is duplicated during the first 40 minutes of S-phase, about one half after 60 min and 3/4 after 90 min (16). By analysing the segregation of hybridisation signals from the cell -type specific cDNA clones between the LL and HL fractions of these preparations we deduced

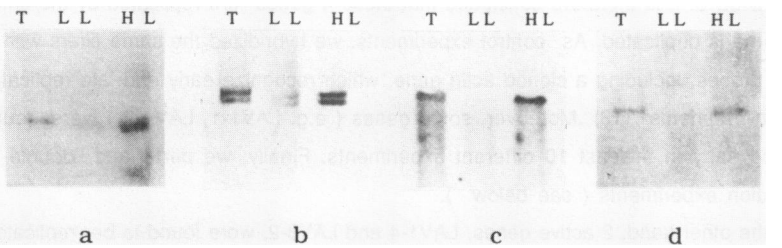

Figure 1 : Early replication in S-phase of four genes which are active within the plasmodium of Physarum: this is deduced from the preferential hybridization of ^{32}P-labelled cDNAs to the HL DNA after in vivo bromodeoxyuridine incorporation for the first 40min in S-phase. T=EcoR1 digested Total DNA; LL= EcoR1 digested non-replicated DNA; HL= EcoR1 digested DNA replicated during 0-40min in S-phase . a) LAV1-1 cDNA b) LAV1-2 cDNA c) LAV1-3 cDNA d) LAV1-5 cDNA.

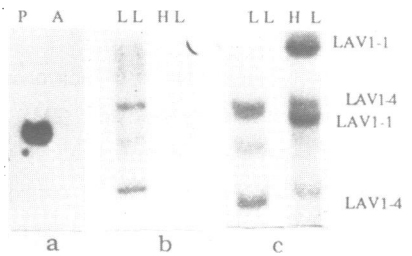

Figure 2 : Expression and replication timing of the LAV1-4 gene in the plasmodium of Physarum.

a) Hybridization of the ^{32}P-labelled LAV1-4 cDNA to plasmodial (P) and amoebal (A) poly (A+)RNA. Note the presence of a 1500 nucleotides long mRNA detected exclusively in the plasmodial RNA.

b) Non-early replication of the LAV1-4 gene as demonstrated by preferential hybridization of the LAV1-4 cDNA to the unreplicated (LL) fraction of HindIII digested Tu 291 DNA after BrdUrd incorporation for 0-40min in S-phase.

c) Late replication of the LAV1-4 gene as evidenced by double-hybridization experiment. The cDNA of the LAV1-4 gene and of the early-replicating LAV1-1 gene were P^{32} -labelled and hybridized to the LL and HL DNA fractions obtained after BrdUrd treatment for the first 90min of S-phase in the M3CIV strain. The two HindIII fragments of the LAV1-4 gene (12 and 6 kb) are enriched in the LL fraction whereas, as internal controls, the two HindIII LAV1-1 fragments (21 and 10 kb) are found in the HL fraction.

the timing of replication of the genes . Genes not replicated by 90 minutes in S-phase were assumed to replicate very late in S-phase.(See below)

First, we analysed the timing of replication of 6 genes selectively expressed within the plasmodium. Not surprisingly, most of these active genes (4 out of 6) are replicated early in S-phase. In Figure 1,we displayed the hybridization patterns of these 4 genes. It is obvious that all the respective cDNA hybridized preferentially to the HL fraction following a 40 min pulse of BrdUrd. It is therefore concluded that these 4 genes are replicated by the time 1/3 of the genome is duplicated. As control experiments, we hybridized the same filters with different probes, including a cloned actin gene, which recognize early and late replicating restriction fragments (18) .Moreover, some genes (e.g. LAV1-1, LAV1-2) were found to be replicating early in at least 10 different experiments. Finally, we performed double-hybridization experiments (see below).

On the other hand, 2 active genes, LAV1-4 and LAV3-2, were found to be replicated late in S-phase . These unexpected results are presented in Figures 2 and 3.

That the LAV1-4 gene is really expressed in the plasmodium is ascertain by the detection on a Northern blot of a relatively abundant mRNA of 1500 nucleotides (fig.2a, lane P).In contrast, no hybridization occurs with the amoebal poly(A+) RNA present on the same filter

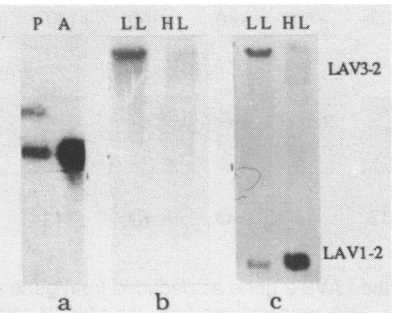

Figure 3: Expression and timing of replication of the LAV3-2 gene of Physarum.
a) Detection of specific transcripts within plasmodial (P) and amoebal (A) RNA preparations by hybridization with the LAV3-2 cDNA. Of the two plasmodial mRNAs of 870 and 1.000 nucleotides (lane P), note that the 870 nucleotides long mRNA is the only transcript found in the amoebal RNA (lane A).
b) Non-early replication of the active LAV3-2 gene as evidenced by preferential hybridization of the ^{32}P - labelled LAV3-2 cDNA to the LL DNA fraction after 60min of BrdUrd treatment (strain Tu 291, HindIII digest).
c) Late replication of the LAV3-2 gene as shown by double- hybridization experiment. The LL and HL DNA fractions obtained after 90min of BrdUrd treatment were hybridized with the cDNAs of the LAV3-2 gene and of the early replicating LAV1-2 gene as internal control. The 25kb LAV3-2 HindIII fragments is found in the unreplicated fraction whereas the 2.8kb LAV1-2 HindIII fragment is clearly enriched in the HL fraction (strain Tu291).

(Fig.2a,lane A). This confirms that the steady state level of the LAV1-4 mRNA varies notably during the development in Physarum (26).

BrdUrd incorporation studies consistently indicated a late replication of this active gene. As an example, we show a preferential hybridization of the LAV 1-4 cDNA to the LL DNA fraction after 40 min of BrdUrd substitution (fig.2b). We obtained a similar result for a 60 min BrdUrd treatment (data not shown) .Moreover, this holds true after 90min in S-phase (75% genome replication).In this case, in order to rule out eventual artefacts, we performed a double-hybridization by mixing the cDNA of the early replicating LAV1-1 gene with the LAV1-4 probe (Fig.2c). As expected, the 21 and 10 kb HindIII restriction fragments of the LAV1-1 gene (M3CIV strain) were found exclusively within the HL fraction whereas the 12 and 6 kb LAV1-4 fragments were comparatively enriched in the unreplicated fraction (Fig.2c). Therefore, the LAV1-4 gene is shown to be active and late replicating within the plasmodium of Physarum.

In addition, we found that the LAV3-2 gene also belongs to this class of eukaryotic genes. This is demonstrated in fig.3. Again Northern-blot analysis clearly indicates expression of the LAV3-2 gene in the plasmodium. Two mRNAs of 1 000 and 870 nucleotides are detected by hybridisation in the plasmodial poly(A+) RNA (Fig.3a,lane P). Interestingly, the steady-state

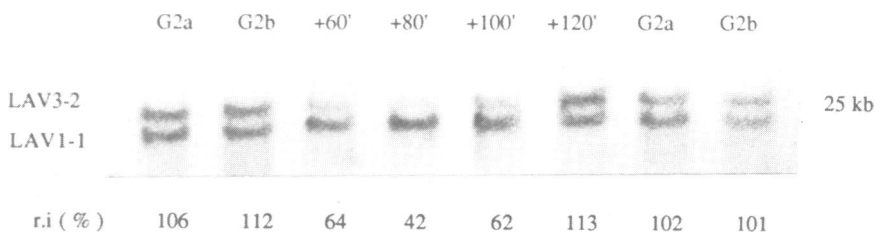

Figure 4: Late replication of the LAV3-2 gene as deduced from gene dosage analysis.
Samples of synchronous DNA preparations from the M3CIV strain, extracted in S and G_2 - phase were digested with HindIII, electrophoresed on 0.7% agarose gel and blotted.They were hybridized with a mixture of P^{32} -labelled cDNAs of the early replicating LAV1-1 gene and of the LAV3-2 gene. The 25kb LAV3-2 and the 21kb LAV1-1 HindIII fragments are presented. The hybridization signals were quantified by densitometry and the relative intensities (r.i) of the LAV3-2 to LAV1-1 signals calculated. As an internal control, duplicated samples from 2 different G_2-phase DNA preparations (G2a and G2b, isolated respectively 7 and 8 hours after mitosis) were loaded on each side of the S-phase samples . We assumed that there are 2 copies of each gene in the G_2-phase samples and in the 120 min extract (r.i of 106, 112, 102, 101 and 113% respectively) and 1 copy of the LAV3-2 gene for 2 copies of the LAV1-1 gene in the DNA extracted at 60,80 and 100 min after the onset of S-phase (r.i of 64, 42 and 62% respectively).This experiment indicate replication of LAV1-1 between 0-60 min and replication of LAV3-2 between 100 and 120min in S-phase.

level of both mRNAs differs in the amoebal poly(A+) RNA where the 1 000 nucleotides RNA is undetectable and the 870 nucleotides message is very abundant (Fig.3a,lane A).

BrdUrd incorporation studies indicated that the 20 kb EcoR1 fragment containing the LAV3-2 gene was not replicated by 40 min in S-phase (not shown). This is also true for the 25 kb Hind III LAV3-2 fragment which is found in the unreplicated LL fraction after 60min in S (Fig.3b). Testing for the replication of the LAV3-2 gene by 90 min in S-phase, we performed another double-hybridization. As an internal control, the cDNA of the early replicating LAV1-2 gene (Fig.1) was mixed with the LAV3-2 probe. In this experiment, where the BrdUrd was incorporated in 75% of the genome, the hybridization clearly distinguished the 25 kb HindIII LAV3-2 fragment, which is found in the unreplicated LL fraction from the 2.8kb LAV1-2 fragment which is enriched in the replicated HL fraction (Fig.3c). We therefore conclude that both the LAV1-4 and LAV3-2 genes are not replicated by the time 75% of the genome has duplicated. We next wanted to determine how late in S-phase these genes were replicated . This was studied by gene dosage analysis.

Gene dosage analysis

Highly synchronous DNA preparations that can be obtained in Physarum permitted us to detect the replication of genes by measuring the relative copy number of two restriction fragments throughout S-phase . Originally, we demonstrated the effectiveness of this method

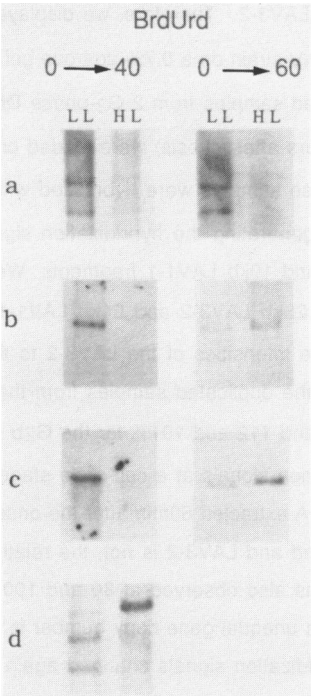

Figure 5: Timing of replication of 4 genes inactive within the plasmodium of Physarum.
After BrdUrd treatment for 0-40min (left) or 0-60min in S-phase (right), DNA was extracted, restricted and the LL and HL fractions were separated on CsCl gradients.
a) Hybridization of the spherulation specific LAV2-1 cDNA to EcoRI digested LL and HL fractions; strain Tu291. Note that the two LAV2-1 EcoRI fragment are not replicated by 60min in S.
b) Hybridization of the amoebal specific LAV3-1 cDNA to EcoRI digested Tu291 DNA. Note that the 4kb LAV3-1 fragment is enriched in the LL DNA for a 0-40min treatment and in the HL DNA for a 0- 60min treatment.
c) Hybridization of the amoebal specific LAV3-3 cDNA to HindIII (left) and EcoRI (right) Tu291 DNA. Preferential hybridization to the LL DNA (left, 0-40min) and to the HL DNA (right, 0-60min) demonstrates the replication of the LAV3-3 gene between 40 and 60min in S- phase.
d) Early replication of the sporulation specific LAV5-1 gene as shown by a double-hybridization. The LAV5-1 and LAV2-1 ^{32}P-labelled cDNAs were hybridized to EcoRI digested Tu291 DNA extracted after 40 min of BrdUrd treatment. The early replication of the LAV5-1 gene is deduced from the presence of the 13 kb LAV5-1 fragment in the HL fraction whereas the 9 and 6.6kb LAV2-1 fragments,as internal control, are found in the LL fraction .

by comparing the relative intensities of the hybridization signals from restriction fragments containing the early and late replicating Physarum actin genes (18).

In the present work, we chose to compare the relative intensities of the LAV3-2 and LAV1-1 genes since our BrdUrd incorporation studies indicated an early replication of

LAV1-1 and a late replication of LAV3-2. Therefore, we displayed HindIII digested S- and G_2 -phase DNA samples of the M3CIV strain on a 0.7% agarose gel. In order to estimate experimental variations, duplicated samples from 2 G_2-phase DNA preparations (G2a and G2b isolated respectively 7 and 8 hours after mitosis) were loaded on the same gel. After electrophoresis and blotting, these samples were hybridized with a mixture of the LAV1-1 and LAV3-2 P^{32} -labelled cDNAs, generating the hybridization signals of the 25kb LAV3-2 HindIII fragment and of the 21 and 10kb LAV1-1 fragments. We restricted the quantitative analysis by densitometry to the 25kb LAV3-2 and 21kb LAV1-1 fragments (Figure 4). In G_2 phase, we found that the relative intensities of the LAV3-2 to the LAV1-1 hybridization signals were almost identical in the duplicated samples from the 2 DNA preparations (106 and 102% for the G2a samples and 112 and 101% for the G2b samples, Figure 4). This defines the ratio of the hybridisation signals at a cell-cycle stage where there are 2 copies of each gene. In contrast, in the DNA extracted 60min after the onset of S-phase,at a stage where we knew that LAV1-1 is replicated and LAV3-2 is not, the relative intensity decreased to 64%. Moreover, this low ratio was also observed at 80 and 100 min in S-phase, 42 and 62% respectively, suggesting that this unequal gene copy number is maintained. However, the relative intensity of the two hybridization signals changed again between 100 and 120 min in S-phase and returned to a G_2 like value (113%).We conclude that the LAV3-2 gene replicates during this interval of late S-phase,at a stage where 80-85% of the genome has already replicated (16).

These results are in perfect agreement with the data obtained with our BrdUrd incorporation studies which had shown that by 90 min in S-phase the LAV1-1 gene was replicated whereas the LAV3-2 gene was not (Fig.2c).

Timing of replication of four inactive LAV genes

As mentioned above, 4 of the cDNAs used in this study are specific of genes highly expressed at distinct stages of the Physarum life cycle but apparently not active in the plasmodium because they do not hybridize to plasmodial RNA on Northern Blot (26,27,28).

By BrdUrd incorporation studies, we found that these 4 inactive genes could not be assigned to a single compartment in S-phase (Fig.5). One gene, LAV2-1, which is highly expressed during spherulation, was not replicated by 90min in S-phase. This is shown in Fig.5a where after 40 or 60 min of BrdUrd incorporation the two LAV2-1 Eco R1 fragments were exclusively found in the LL fraction. We obtained a similar result for 90 min of BrdUrd treatment (data not shown)

In contrast, the two cDNAs LAV3-1 and LAV3-3, specific to the amoebal stage, recognized genes replicated in mid S-phase (Fig. 5). Both were found in the LL fraction after 40min of

BrdUrd incorporation but were density-shifted in the DNA preparations obtained after 60 min of BrdUrd incorporation.This is shown in Fig.5b for the LAV3-1 gene and in Fig.5c for the LAV3-3 gene. We conclude that they replicate between 40 and 60 min in S-phase, in the interval of 33-50% of genome replication.

The last presumably inactive gene that we tested was found to replicate early in S-phase. This gene, LAV5-1, is highly expressed during sporulation (28). We analysed its timing of replication by a double-hybridization experiment. The LAV5-1 probe was mixed with the LAV2-1 cDNA and hybridized to the LL and HL fractions of a 40 min BrdUrd incorporation DNA preparation (Fig.5d). The 13 kb Eco R_1 fragment of the LAV5-1 gene was present in the HL fraction whereas the 9 and 6.6kb fragments of the late replicating LAV2-1 gene were, as expected, found in the LL lane. This demonstrate the replication of LAV5-1 by 40min in S-phase.

DISCUSSION

The inactive X chromosome in mammals has long been known to be replicated later in S-phase than its active counterpart (33). More recent results obtained at the gene level have strengthened this apparent relation between early replication and gene activity since the active genes analysed so far have been found to replicate in the first half of S-phase (3-11).Moreover, some genes - either rearranged or not - are replicated early in cell-lines in which they are expressed and at later times in cell-lines in which they are not expressed (7,11,34).This demonstrates that the fixed temporal order of replication within one cell-type can vary during differentiation. Hypotheses have been proposed in which late replication in S-phase would preclude gene activity (5,6,14). Therefore, a late replicating and consequently inactive gene would have first to be recruited in an early replicating compartment of the genome before it could be activated and transcribed. This is clearly not always the case in Physarum where we show that 2 active genes are replicated very late in S-phase, at a stage where over 80% of the genome has duplicated.

This timing of replication has been obtained through two independent experimental approaches. One, the classical BrdUrd-dependent density labelling of the newly replicated DNA, indicated that the LAV1-4 and LAV3-2 genes are not replicated at a time where 75% of the genome has duplicated. This contrasts with the early replication of four other active genes (LAV1-1; 1-2; 1-3; 1-5),that we eventually used as internal controls in double-hybridization experiments (See Fig.2c and3c). These results were confirmed and extended by gene dosage analysis.In this case,the replication of a gene is deduced from the comparison of the hybridization signals throughout S-phase of two restriction fragments which are not replicated in the same interval of S-phase (18).In total agreement with the density-shift

experiments, the gene dosage analysis pinpoints the replication of the LAV3-2 gene between 100 and 120 min in S-phase i.e about 80 to 85% of genome replication (Fig.4). Moreover, this experiment confirmed the early replication of the LAV1-1 gene in the absence of any treatment of the cells, since they were neither synchronized nor treated with a thymidine analog. Based on these evidence, we firmly conclude that in Physarum at least two actively transcribed genes are duplicated late in S-phase.

At first sight,the chronology of gene replication appears to be different in Physarum and in the mammalian cell-lines where a high number of active genes were found to be replicated early (3,11). However, there are recent indications that both the dihydrofolate reductase and an active H2A gene might be replicated late in the S-phase of one out of 9 different murine cell lines (11). Furthermore, in Physarum too , most of the active genes are duplicated in the first 40 min of a 3 hours S-phase.This is true for 2 actin genes (18) , 2 histones H4 genes (21) and 4 LAV genes studied in this com- munication (Fig.1). Finally, of the 5 genes potentially inactive within the plasmodium which have been identified so far, only one ,the sporulation specific LAV5-1 gene is replicated during the first third of S-phase (Fig.5d). Two others genes (LAV3-1 and 3-3), which are highly expressed only in amoeba, were found to replicate in mid S-phase (33-50% genome replication; Figs 5b and 5c). Lastly, the encystment specific LAV2-1 gene is duplicated late in S-phase (Fig.5a) ,as is the inactive actin ardA gene (18). Therefore, the relation between early replication and gene activity is often seen in Physarum too, yet there are exceptions as demonstrated in this communication. From the late replication of the two active LAV3-2 and LAV1-4 genes of Physarum, we conclude that late replication does not prevent gene activity.

In turn, our results do not support a model in which the activity of a gene would determine its timing of replication. Indeed, one could have hypothesized a greater accessibility of the replication factors to the active genes known to be in an "open" chromatin structure (35). This would result in a precocious replication of the genes transcribed within one cell. Yet, the LAV1-4 and LAV3-2 genes which are expressed and consequently are in an active chromatin conformation within the plasmodium were invariably found to replicate late in S-phase (Fig.2and3). Clearly, what dictates the chronology of gene replication is unknown.

It has been postulated that the timing of replication could provide a mechanism of coarse control for the expression of the genes in the complex genome of eukaryotic organisms. In a particularly attractive hypothesis, early replication would provide an advantage for gaining access to limiting amount of transcriptional factors (36). A possible example for such a mechanism is the selective expression of the early replicating somatic 5S rRNA genes in Xenopus at low level of the transcription TFIIIA during embryogenesis (8), and the expression of some oocyte specific 5S rRNA in a cell line where some of the respective genes

have been rearranged into an early replicating chromosomal region (9). This model, based on competition for trans-acting factors, can accomodate our results if it is assumed that the concentration of the trans-acting factors fluctuates during development (as is the case for TFIIIA which is abundant in the oocyte). In limiting the concentration of a specific factor, only the corresponding early replicating genes would have access to the factor whereas under non-limiting conditions, the factor would bind to, and activate both early and late replicating genes. This would provide a general advantage to the early replicated genes and would not preclude, in some instances, the expression of late replicating genes.

Obviously, the understanding of the coordination of the replication and transcription activities in S-phase or during the development of an organism will require a better knowledge of specific chromosomal replicons.The characterization of chromosomal origins of replication and their position relative to the transcription units on the genome would be of particular interest (37-40). In this context, we are studying more closely some of the active and early replicating genes characterized in this study.For 3 of them,we have established that they are replicated in the first 10 min in S-phase,suggesting that they are flanked by functional origins of replication (M.B andG.P; manuscript in preparation).They might correspond ,therefore, to some of the genes we had observed on chromatin spreads as active transcription units located at the center of nascent replicons (17).Whether the origins of replication of these 3 replicons are contained within these genes, as tentatively concluded from our electron microscope observations, is currently under investigation.

ACKNOWLEDGMENTS

We thank Jacqueline Pedron for expert technical assistance. M.B. is a fellow of the "Institut de Formation Supérieure Bio-Médicale" (Villejuif). G.P. is supported by grant n°1301 from the "Association pour la Recherche sur le Cancer" (Villejuif). H.W.S. is supported by grant DCB-860817 from NSF.

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