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Replication timing of the H4 histone genes in *Physarum polycephalum*

(DNA replication/cell cycle/gene expression)

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ABSTRACT **The time of replication of the two H4 histone genes (H41 and H42) was determined during the naturally synchronous mitotic cycle of *Physarum polycephalum*. 5-Bromo-2′-deoxyuridine labeling and density gradient centrifugation was used to isolate newly synthesized DNA from defined periods of S phase. The DNA was analyzed by Southern hybridization with a cloned probe containing one of the H4 histone genes of *Physarum*. The results indicate that the two H4 histone genes are replicated in the first 30 min of S phase but not exactly at the same time. H41 is replicated during the first 10 min of S phase, when only 15% of the genome is duplicated, whereas H42 replicates between 20 and 30 min after the onset of S phase. The possible relationship between the periodic expression of the genes and the timing of their replication is discussed.**

In eukaryotes, as in prokaryotes, the replication of specific DNA sequences follows a distinct temporal order (1, 2). DNA replicated in one period of the S phase is replicated in the same temporal interval of the next S phase. In early experiments autoradiography and cytology was used to demonstrate that the chronological order of replication of specific DNA segments is invariant from one cell generation to the next (3).

In mammalian cells, multiple copy sequences have been found to replicate mainly late in S phase, whereas families of middle-repetitive sequences replicate either early or late (for references see ref. 4). "Housekeeping" or active tissue-specific genes are generally replicated early. Furst *et al.* (5) and Epner *et al.* (6) have, for example, demonstrated that the replication of globin genes in mouse erythroleukemia cells is predominantly restricted to the first third of S phase. In the same manner, studies by Braunstein *et al.* (7) have shown that the replication of the immunoglobulin heavy chain constant region gene segments C_α, C_γ, and C_μ is restricted to the first half of S and follows the linear order in which they are arranged in the genome. Recently Calza *et al.* (8) have presented evidence that certain low-copy-number genes can be replicated late and have suggested that the gene's position in the chromosome is important in determining the time during S at which it is replicated. All the results presented on the timing of gene replication support the idea that early-replicating genes are capable of expression, and Goldman *et al.* (4) suggest that the switching of specific genes from an early to a late replication region reflects the commitment of that gene to quiescence.

The sustained synchrony over several successive nuclear division cycles in the syncytial plasmodia phase of the myxomycete *Physarum polycephalum* was exploited by Braun *et al.* (9) to demonstrate that chromosomal DNA is replicated in a defined temporal sequence. In addition, Fouquet and Sauer (10) have shown that repetitive DNA of *Physarum* is synthesized during late S phase. At the level of specific genes, Pierron *et al.* (11) have recently found that the four actin loci are replicated in an invariant temporal order over a period of at least 400 generation times.

In this communication we have studied the replication timing of the H4 histone genes during the mitotic cycle of *Physarum polycephalum*. This lower eukaryote is particularly well suited for this kind of study because it is naturally synchronous throughout the entire cell cycle. Also, it is interesting to study replication of histone genes because their expression is tightly regulated by the cell cycle. It has been suggested that replication could be involved in the regulation of the transcriptional activity of histone genes (12, 13). The possible relationship between the periodic expression of the H4 histone genes and the timing of their replication is discussed.

MATERIALS AND METHODS

Strains and Cultures. The strain Tu 291 was kindly provided by G. Pierron (Villejuif, France). It is a diploid derivative of the Wis 1 natural isolate obtained by crossing RSD4 and RSD8 amoebae (14). Synchronous macroplasmodia were grown on filter paper supported by glass beads according to Daniel and Rusch (15). The cell cycle stage was determined by phase-contrast microscopy. Surface cultures were grown overnight and labeled after the second or third mitosis.

Labeling with 5-Bromo-2′-deoxyuridine (BrdUrd). Surface cultures were incubated in the presence of BrdUrd, 5-fluoro-2′-deoxyuridine (FdUrd), and uridine during the appropriate periods. The drug concentrations described by Braun *et al.* (9) were used—i.e., BrdUrd at 100 μg/ml, FdUrd at 5 μg/ml, and uridine at 100 μg/ml. FdUrd is used to inhibit thymidine synthesis (16) in order to obtain maximal BrdUrd incorporation. Uridine serves to overcome the inhibiting effects of FdUrd breakdown products on the synthesis of RNA.

Preparation and Fractionation of BrdUrd-Substituted DNA. After the pulse of BrdUrd, *Physarum* nuclei were purified as follows: Macroplasmodia were harvested by scraping the culture off the filter paper and washed once in 0.25 M sucrose/5 mM EDTA/10 mM Tris·HCl, pH 8.0. One macroplasmodium containing about 10^8 nuclei was homogenized in 100 ml of buffer containing 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, and 10 mM Tris·HCl, pH 7.6. The nuclei suspension was passed through two layers of cheesecloth and the filtrate was sedimented over 10 ml of a sucrose cushion containing 1 M sucrose, 5 mM $MgCl_2$, and 10 mM Tris·HCl, pH 7.6. The nuclei were then lysed in the presence of 20 mM EDTA, 1% sodium dodecyl sulfate, and proteinase K at 50 μg/μl for 60 min at 37°C. Purification was accomplished by extracting

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Abbreviations: kbp, kilobase pairs; HL, heavy–light; LL, light–light.

with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol) followed by one extraction with chloroform/isoamyl alcohol (24:1, vol/vol). Pancreatic ribonuclease at 100 μg/ml was used to hydrolyze RNA. Finally, DNA was extracted once more with phenol/chloroform/isoamyl alcohol and precipitated with 2 vol of ethanol. The DNA was solubilized and cleaved to completion with the restriction enzymes *Eco*RI and *Hin*dIII according to the conditions specified by the supplier (Boehringer Mannheim). The BrdUrd-substituted DNA was separated from the unsubstituted DNA by preparative ultracentrifugation in CsCl gradients: The DNA samples were diluted to 9 ml with 10 mM Tris·HCl, pH 7.6/0.1 mM EDTA (TE) and 11 g of solid CsCl was added (refractive index n = 1.40). Centrifugation was for 48 hr at 48,000 rpm in a Beckman R65 rotor. Fractions were collected and the absorbance at 260 nm of the fractions was measured. The pooled heavy–light (HL) BrdUrd-substituted and light–light (LL) unsubstituted fractions were dialyzed against TE and precipitated with 2 vol of ethanol.

Electrophoresis, Blotting, and Hybridization of DNA. Equal amounts (5 μg) of *Eco*RI/*Hin*dIII-digested DNA were fractionated on a 1% agarose gel at 4 V/cm. DNA was transferred overnight to nitrocellulose filters (BA 83, Schleicher & Schüll) as described by Southern (17), and the filters were then baked under reduced pressure for 2 hr at 80°C. Hybridization with the radioactive probe was carried out at 65–68°C for 16–18 hr in 3× SET/10× Denhardt solution/0.5 M NaCl/0.1% sodium dodecyl sulfate/100 μg of heat-denatured *Escherichia coli* DNA per ml/10^6–10^7 cpm of denatured labeled DNA per ml (20× SET = 3 M NaCl/0.6 M Tris·HCl, pH 7.6/0.04 M EDTA; 50× Denhardt solution = 1% Ficoll 400/1% polyvinylpyrrolidone 40/1% bovine serum albumin. The probe was labeled by nick-translation with [^{32}P]dCTP as described by Maniatis *et al.* (18).

Prior to autoradiography the filters were washed at 65°C for 1 hr with 250 ml each of the following: (*i*) 0.5 M NaCl/3× SET/0.1% sodium dodecyl sulfate/0.1% $NaPP_i$/5× Denhardt solution; (*ii*) 3× SET/0.1% sodium dodecyl sulfate/0.1% $NaPP_i$; (*iii*) 2× SET/0.1% sodium dodecyl sulfate/0.1% $NaPP_i$; (*iv*) 1× SET/0.1% sodium dodecyl sulfate/0.1% $NaPP_i$. Filters were exposed to x-ray films at −70°C for 16–48 hr, using intensifying screens.

RESULTS

Analysis of Histone H4 Genomic Organization of *Physarum* Strain Tu 291. To determine the temporal sequence of replication of the histone H4 genes of *Physarum* we have chosen strain Tu 291, which has recently been used successfully to study the replication of the four actin gene loci (11) and is well characterized with respect to the mechanism of DNA synthesis (19). The organization of histone H4 gene sequences was examined by hybridization of blotted restriction endonuclease fragments of *Physarum* DNA with a ^{32}P-labeled histone H4 probe. The probe used was a 0.6-kilobase-pair (kbp) fragment containing the coding sequence of one of the H4 genes of *Physarum* strain M3CVIII and about 0.2 kbp of noncoding sequence 5′ of the gene (20). *Physarum* DNA was digested with restriction endonuclease *Hin*dIII, *Eco*RI, or both. The pattern of bands obtained with strain Tu 291 is similar to that observed with strain M3CVIII and is shown in Fig. 1: Two bands are observed in the *Eco*RI and *Hin*dIII digests, suggesting that *Physarum polycephalum* contains two types of H4 histone genes (H41 and H42). When the DNA is digested with both *Eco*RI and *Hin*dIII, three fragments, 0.6, 1.2, and 1.4 kbp, hybridize to the probe. The smallest fragment (0.6 kbp), which is also obtained when *Physarum* DNA is digested with *Hin*dIII alone, contains the entire coding sequence of one of the genes (H41). The 1.4-kbp fragment contains the second gene. The 1.2-kbp fragment

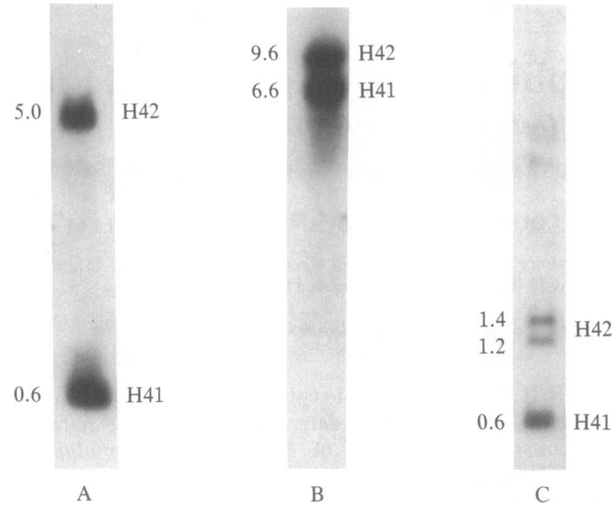

FIG. 1. Autoradiography of Southern blots of *Physarum polycephalum* DNA hybridized to the H4 histone probe labeled by nick-translation. Five micrograms of DNA was digested with *Hin*dIII (*A*), *Eco*RI (*B*), or *Hin*dIII and *Eco*RI (*C*), electrophoresed on a 1% agarose gel, blotted to nitrocellulose paper, and hybridized to the probe. Between 10^6 and 10^7 cpm/ml of probe at approximately 10^8 cpm/μg was used. The lengths of the histone gene restriction fragments were calculated by using fragments of λ DNA digested with *Hin*dIII as molecular weight markers. The lengths of the fragments are given in kbp.

cross-hybridizes to the probe because it contains 0.2 kbp of noncoding sequence and is a *Hin*dIII/*Eco*RI fragment located 2 kbp downstream from the H41 gene. The copy number of H4 genes was determined for strain Tu 291 as previously described (21). The results (data not shown) suggest that, as in strain M3CVIII, there are only one or two copies of each H4 gene in the haploid genome of this strain.

Fractionation of Newly Replicated Nuclear DNA. BrdUrd labeling and density gradient centrifugation was used to isolate newly synthesized DNA. Earlier reports have shown that BrdUrd allows quantitatively normal DNA synthesis during S phase (9). The onset of DNA synthesis occurs during a period immediately following the completion of mitosis, and S phase occupies about one third of a cell cycle of 8–10 hr. The rate of DNA synthesis increases sharply at the beginning of S phase. After 20–30 min, DNA synthesis reaches its full rate. It is maintained at a high level during about 1 hr and then decreases to reach its premitotic rate at the end of the S phase.

To separate newly replicated DNA during specific periods of S phase, plasmodia were grown in the presence of BrdUrd so that DNA replicated during those periods would be labeled with bromouracil. In one experiment the DNA was labeled for 45 min between the end of telophase (beginning of S phase) and 45 min after mitosis or for 45 min between 45 min and 90 min after mitosis. In a second set of experiments BrdUrd-substituted DNA was obtained from cultures labeled between 10 min before mitosis and 10, 20, or 30 min after it. After the labeling period the DNA was purified and doubly digested with *Eco*RI and *Hin*dIII. The digested samples were run on 1% agarose gels and transferred to nitrocellulose paper, and the blots were hybridized with the H4 probe to ensure complete digestion of the DNA. The digested BrdUrd-substituted DNA was separated from unsubstituted DNA by CsCl density gradient centrifugation and the absorbance at 260 nm of the fractions was monitored. In Fig. 2 we show the profiles obtained when the DNA was labeled for 10, 20, or 30 min after mitosis; a bimodal distribution of nonreplicated light DNA (LL) and newly replicated heavy DNA (HL) is observed. For the shortest BrdUrd incorporation period the

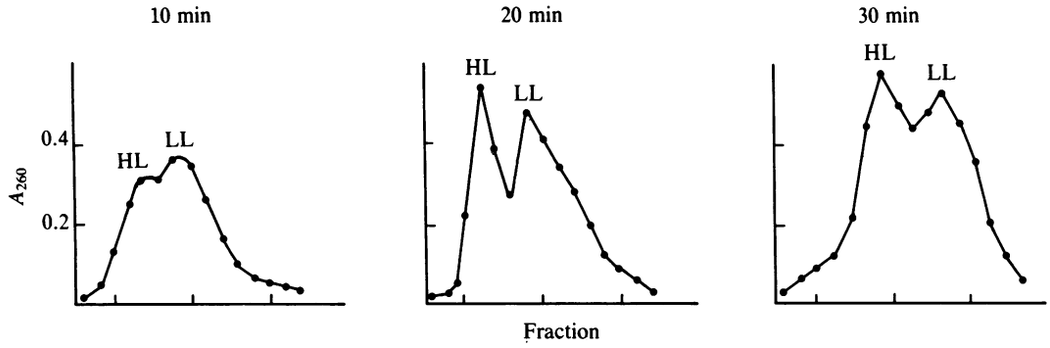

FIG. 2. Purification of early- and late-replicating *Physarum polycephalum* DNA. *Physarum* plasmodia were grown in the presence of BrdUrd between 10 min before mitosis and 10, 20, or 30 min after it. Nuclei were isolated, and the DNA was purified and digested to completion with *Hin*dIII and *Eco*RI. BrdUrd-substituted DNA was separated from the unsubstituted DNA by fractionation on a preparative CsCl density gradient. The absorbance at 260 nm of the fractions was monitored. HL (BrdUrd-substituted DNA) and LL (nonreplicated DNA) peak fractions were collected and used in subsequent experiments.

light and heavy peak are only partially separated. To improve the separation, the pooled LL and HL fractions were sedimented in a second CsCl gradient.

Replication Timing: The H4 Histone Genes Replicate Early in S Phrase. To determine the time of replication of the two H4 histone genes the amount of histone genes in the replicating and nonreplicating DNA during a given period of S phase was quantitated. The pooled HL and LL DNA fractions were analyzed on 1% agarose gels. The DNA was then denatured, transferred to nitrocellulose paper, and hybridized to the H4 probe. If a histone gene is replicated during the pulse of BrdUrd it will be present in high concentration in the HL BrdUrd-substituted DNA and absent or present in very low concentrations in the LL DNA fraction. The result of the first experiment, in which the cultures were labeled for 45 min between 0 min and 45 min or 45 min and 90 min after mitosis, is shown in Fig. 3. Clearly, the two histone genes replicate before 45 min, since the newly replicated DNA labeled in the first 45 min of S (HL fraction) hybridized preferentially to the H4 probe, whereas the H4 restriction fragments were over-represented in the LL nonreplicating DNA when the cultures were labeled between 45 min and 90 min after mitosis. The fact that the histone genes are found first in the HL fraction and then in the LL fraction shows that the separation on the CsCl gradient is not caused by an abnormal G+C content of the histone genes but that the fractionation is due to substitution of thymine by bromouracil. Hybridization is not completely absent from lanes b and c in Fig. 3, but we believe that this reflects imperfect separation of the half-substituted from the unsubstituted DNA strands on CsCl gradients because the spill-over is much less pronounced when the pooled HL and LL fractions are sedimented on a second CsCl gradient (see Fig. 4).

We wanted then to detect how early in S phase the two H4 genes are replicated. The *Physarum* cultures were therefore labeled with BrdUrd for shorter periods (10 min, 20 min, and 30 min of S phase) and the DNA was analyzed as indicated above. The distribution of the H4 restriction fragments in the newly replicated HL DNA and in the nonreplicated LL DNA at the three time points is shown in Fig. 4. In the first 10 min of S phase one of the histone H4 genes (H41, which corresponds to the 0.6-kbp fragment) was mainly found in the newly replicated HL DNA, whereas the second gene (H42) was still present in the nonreplicated LL DNA. Thirty minutes after the beginning of S phase H42 had also disappeared from the LL fraction and was almost entirely represented in the newly replicated HL DNA. These two experiments indicate that the histone H4 genes are replicated early

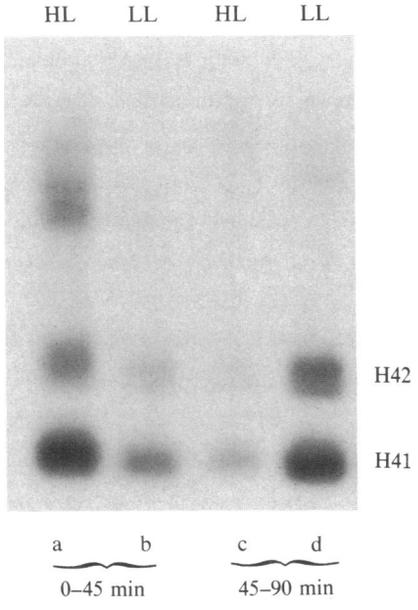

FIG. 3. Replication timing of the H4 histone genes. After incorporation of BrdUrd for 45 min between 0 and 45 min after mitosis (lanes a and b) or for 45 min between 45 and 90 min after mitosis (lanes c and d), HL and LL DNA was analyzed by Southern hybridization.

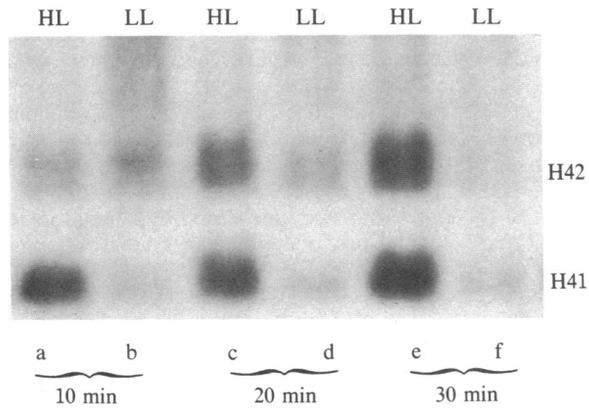

FIG. 4. H4 histone gene replication in early S phase. After incorporation of BrdUrd between 10 min before mitosis and 10 min (lanes a and b), 20 min (lanes c and d), or 30 min (lanes e and f) after it, HL and LL DNAs were separated on a CsCl density gradient (Fig. 1) and analyzed by Southern hybridization.

in S phase. The two genes are not replicated at the same time. H41 replicates within the first 10 min of S phase, whereas H42 replicates between 20 and 30 min after the onset of S phase.

DISCUSSION

In most eukaryotic cells the biosynthesis of histone proteins occurs periodically in the cell cycle and appears to be coupled to the synthesis of DNA during the S phase (for review see ref. 22). Control at the level of transcription of the histone genes has been found to be important in maintaining the coordination of histone and DNA synthesis (12, 23–27). The close relationship between the transcription of histone genes and DNA replication has led Hereford *et al.* (12, 27) to suggest that termination of transcription is a result of the replication of histone genes. To test this suggestion we have taken advantage of the natural mitotic synchrony of *Physarum* macroplasmodia to determine the timing of replication of the genes coding for core histone H4. The temporal order of replication of the two H4 genes was established by Southern analysis of newly replicated DNA from different periods of S phase. Our results show that the two H4 histone genes are replicated in the first 30 min of S phase and that duplication of one of the genes (H41) occurs during the first 10 min, when only 15% of the genome is replicated. This finding is consistent with the observation that active genes are generally replicated early in the cell cycle (4). Of importance for the replication of H41 gene is the identification of an autonomously replicating sequence ("*ars*"), as assayed by yeast transformation, near the 5′ end of the gene. This *ars* element is located about 1.5 kbp upstream of the 5′ end of the gene, within a 2.5-kbp *Eco*RI/*Bam*HI fragment (see figure 1 in ref. 20) adjacent to the H4 gene and was identified because it promotes high-frequency yeast transformation when present on an integrative plasmid (unpublished data). The ability of *ars* sequences to allow autonomous replieation of an integrative plasmid suggests that they represent specialized replication origins. This view is supported by several observations. First, *ars* elements in yeast occur with approximately the same frequency as the number of replication units seen by electron microscopy (28). Second, *in vitro* replication systems are able to initiate DNA synthesis at *ars* sequences (29). Finally, Broach *et al.* (30) have mapped the position of the single *ars* segment of the 2-μm-circle yeast plasmid and found that it corresponds with one origin of replication defined by electron microscopy. Similar elements able to support autonomous replication in yeast have been found at the 3′ end of the two yeast histone H2B genes and to the 3′ side of the H4 gene of copy I yeast H3-H4 genes (31). The *ars* element near a yeast H2B gene coincides with a *cis*-acting element required for regulated expression of yeast histone H2A (13). This coincidence could explain the close connection between DNA replication and histone gene transcription. The presence of an *ars* element near the H41 gene of *Physarum* suggests that the same mechanism may be used to regulate the expression of histone genes in this organism. The rate of elongation of an individual replicon in *Physarum* has been measured by several investigators (19, 32, 33) and was found to be 0.6 kilobases per min per fork in the first 90 min of S phase. Since the histone H41 gene is replicated in the first 10 min of S phase it can be estimated that the distance between the replication origin and the gene must be less than 6 kbp if the origin is activated at the very beginning of S. It is therefore not unreasonable to believe that the *ars* element found near the H41 gene is a chromosomal origin of replication. Whether this segment coincides with a regulatory sequence necessary for periodic transcription of the gene remains to be determined. While this paper was in preparation Iqbal *et al.* (34) reported that the multigene family of core and H1 human histone genes replicates during the first half of the S phase and claimed that replication origins are not a requirement for the cell cycle appearance of histones. This does not preclude the possibility that a regulatory sequence that does not coincide with a replication origin exists near the human histone genes.

We have recently monitored the level of histone H4 mRNA during the cell cycle of *Physarum* (21). Our data indicate that the level of mRNA is maximal at 30 min and then decreases. At 90 min it is only 10% of that observed at 30 min. This observation would be consistent with a coincidence between the cessation of histone gene transcription and replication. The same mechanism may be used to regulate the expression of other genes in *Physarum*, but it is certainly not general, since, on the basis of electron microscopy observations, Pierron *et al.* (35) have suggested that DNA replication is a prerequisite for the transcription of certain genes. In contrast, the initiation of transcription does not seem to be correlated to the activation of a replication origin close to the gene. Indeed we have found that initiation of histone H4 transcription occurs in G_2 phase. In the second half of G_2 (4 hr before mitosis) histone H4 mRNA begins to accumulate, and at the end of G_2 phase the abundance of H4 mRNA is about the same as in S phase, when the level is maximum. Therefore it is clear that initiation of histone H4 RNA synthesis in G_2 cannot be attributed to the activation of an origin of replication and thus is not coupled to DNA replication. Other factors such as changes in chromatin structure or the presence of a histone-gene-specific transcription factor (36) must therefore be involved in this process. It seems likely that in *Physarum* initiation and termination of histone H4 gene transcription are controlled by two different mechanisms. Replication would be involved only in the cessation of transcription, but both mechanisms could mediate a change in the chromatin structure of the gene and its controlling sequences.

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