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Invariant temporal order of replication of the four actin gene loci during the naturally synchronous mitotic cycles of *Physarum polycephalum*

(sequential DNA replication/cell cycle)

GERARD PIERRON*†, DAVID S. DURICA‡, AND HELMUT W. SAUER*

*Department of Biology and Institute of Developmental Biology and ‡Department of Medical Biochemistry, Texas A&M University, College Station, TX 77843

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ABSTRACT **The chronological sequence of replication for the four unlinked actin gene loci of *Physarum* has been established. Southern hybridization analysis of density-labeled, bromodeoxyuridine-substituted DNA isolated from defined periods of S phase demonstrates that three actin loci (*ardB*, *ardC*, *ardD*) are duplicated early, corresponding to the first 10% of the genome. The fourth locus (*ardA*) replicates later, between 80 and 100 min into S phase and after 75% of DNA synthesis is completed. Gene-dosage determinations, based on the quantitation of hybridization signals from DNAs isolated from various times during S phase, confirm the results obtained with bromodeoxyuridine-substituted DNA and increase the temporal resolution. The chronological order of replication in the macroplasmodium appears constant through two consecutive cell cycles and after prolonged growth in suspension culture. The precise chronology of DNA synthesis at the gene level extends to the coordinate replication of allele pairs.**

It has been clearly demonstrated, both by cytological methods and density-labeling, that certain segments of the eukaryotic genome replicate at defined time intervals in S phase of the cell cycle (1, 2) in lower eukaryotes like *Physarum* (3) as well as in human cells (4). Heterochromatin is generally found to replicate late in S phase (5–7), whereas certain potentially active genes (8, 9) replicate early. Employing electron microscopic spreads of chromatin, we have recently visualized actively transcribing genes in early-replicated DNA of *Physarum*. Typically, both coding strands of a newly replicated locus are transcribed in early S phase (10). A relationship between the onset of DNA replication in S phase and transcription has been documented for yeast (11), *Drosophila* embryos (12), and tissue culture cells (13, 14) as well as for *Physarum* (15), although the significance of this correlation is not understood. A detailed analysis of the temporal sequence of replication and expression of defined genes should further our understanding of the mechanisms underlying cell proliferation and cell differentiation (16). *Physarum* is aptly suited for such an analysis, due to the unparalleled mitotic synchrony of a macroplasmodium, in which 10^8 nuclei divide and initiate DNA replication within <5 min every 10 hr.

Taking advantage of restriction fragment length polymorphisms and *Physarum*'s amenability for genetic analysis, it is possible to analyze the Mendelian segregation of specific multigene family members, as was recently done for the actin genes of *Physarum* (17). This knowledge about the segregation pattern of the actin gene family (17), coupled with the natural synchrony of the nuclear replication cycle of *Physarum* (16, 18), the evidence for the temporal order of DNA synthesis (3), and the preferential transcription of newly replicated DNA in early S phase (10, 15), prompted us to determine the chronology of replication of the four actin loci.

In this communication we demonstrate that three of the four actin loci are replicated at the time when only 5–10% of the genome has replicated. The synchrony of DNA replication at the gene level is such that two allelic DNA sequences of one actin locus are coordinately replicated. The fourth actin locus is replicated when DNA synthesis is 75% completed. Moreover, this temporal order of replication remains invariant, when monitored either through two consecutive S phases in a single macroplasmodium or over a period of at least 400 generation times in suspension culture. The significance of this fixed order of replication is discussed in light of the replication–transcription–coupling hypothesis.

MATERIALS AND METHODS

Cultures. The strain Tu 291, provided by F. Haugli (Tromsø University, Norway), was used throughout this investigation. Obtained by crossing stains RSD_4 by RSD_8 amoebae, it is a diploid derivative of the Wis 1 natural isolate (19). The synchronous cultures (macroplasmodia) were made according to published procedures on Whatman filter paper no. 4. (20). Surface cultures (6–7 cm in diameter) were harvested after mitosis II or III (MII or MIII).

DNA Extraction. About 10^8 nuclei were isolated from a macroplasmodium according to ref. 21 and immediately resuspended in 50 mM Tris·HCl, pH 8.0/50 mM NaCl/10 mM EDTA. DNA was extracted and purified as described (22). As monitored by gel electrophoresis, the DNA was consistently 50 kilobases (kb) or greater in size.

BrdUrd Incorporation. Surface cultures were treated at 10 min before mitosis (prophase) until harvested in S phase. Drug concentrations in normal growth medium (20) were 100, 5, and 100 μg/ml for BrdUrd, fluorodeoxyuridine, and uridine, respectively (3). Under these conditions, at least two mitotic cycles take place on 15 ml of medium.

Isolation of BrdUrd-Substituted DNA. After a BrdUrd pulse, the DNA was isolated and purified on CsCl gradients. To recover the newly replicated heavy-light (HL) DNA from unreplicated adjacent sequences, we digested the isolated DNA with *Hin*dIII. Typically 100–150 μg of DNA was digested overnight in 100–150 units of the enzyme. An aliquot of 10–20 μg of restricted DNA was removed for Southern hybridizations (23) and the rest of the restricted DNA samples was diluted to 9 ml in 50 mM Tris·HCl, pH 8.0/50 mM NaCl/10 mM EDTA/0.1% Sarkosyl. After addition of 11 g of solid CsCl, the restricted DNA was centrifuged for 60–72 hr at 36,000 rpm in a Beckman 50 Ti rotor. Pooled fractions

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Abbreviations: kb, kilobase(s); HL, heavy-light; LL, light-light; MII and MIII, second and third mitoses.

†Present address: Laboratoire de Microscopie Electronique, Institut de Recherches Scientifiques sur le Cancer, Centre National de la Recherche Scientifique, 94802 VilleJuif Cedex, France.

of light-light (LL) and HL DNA were dialyzed against 10 mM Tris·HCl, pH 8.0/1 mM EDTA and precipitated with ethanol.

Hybridization Analysis. Restricted DNA was analyzed by the protocol of Southern (23) under standard conditions (0.45 M NaCl/0.045 M sodium citrate, pH 7, 68°C) for 20 hr in the presence of 10% dextran sulfate. The actin gene probe was a 3.6-kb *Hin*dIII fragment isolated by gel electrophoresis and electroelution (24) from plasmid pSpG17 (25). It contains the complete coding sequence of a sea urchin cytoskeletal actin gene (25, 26). The fragment was labeled by nick-translation (27) to a specific activity of 5–20 × 10^7 cpm/μg of DNA. Autoradiographs were scanned in a Beckman DU8 spectrophotometer equipped with a gel scan compu-set. Main parameters were 500 nm; slit, 0.2 nm; gel slit, 0.05 mm; gel and chart speed, 0.4 cm/min; integration of selected areas without background subtraction.

RESULTS

Analysis of the Actin Gene Loci of *Physarum* Strain Tu 291. By hybridization of nick-translated yeast and *Drosophila* actin probes to restricted DNA from the diploid strain Wis 1, and its haploid progeny, four unlinked actin loci have been detected in *Physarum* (17). For each of three of these loci, two alleles were defined as single *Hin*dIII (or single *Eco*RI) restriction fragments of different length. The fourth locus is complex because two *Hin*dIII (or three *Eco*RI) restriction fragments cosegregate upon meiotic assortment.

To determine the temporal sequence of replication of the actin genes in *Physarum*, we first utilized a combination of the classic density-shift experiment (3) with the recent successful molecular approach (17). We chose strain Tu 291 for several reasons: (*i*) it is the best-characterized strain of *Physarum* with respect to the mechanisms of DNA replication (28, 29); (*ii*) its precise synchrony of replication throughout S phase has recently been proven and the kinetics of DNA synthesis has been accurately established by high-resolution flow cytometry (30); (*iii*) it is known to be fully sensitive to the thymidylate synthesis inhibitor 5′-fluorodeoxyuridine (31), which is advantageous for maximal BrdUrd incorporation into DNA. Since strain Tu 291 is a derivative of the strain Wis 1 (19), we reasoned that the *Hin*dIII fragments containing actin-like sequences should be identifiable from the hybridization pattern of the parent strain (17).

Table 1 summarizes the analysis of the actin loci of strain Tu 291 (see also Figs. 3 and 4). Total purified DNA was restricted with *Hin*dIII and hybridized with a specific sea urchin actin probe (see *Materials and Methods*). The six hybridization bands are very similar in molecular weight to the *Hin*dIII restriction fragments of the parental strain Wis 1, detected with yeast and *Drosophila* probes (17). By comparing the pattern of alleles for the actin gene family of *Physarum* we conclude that strain Tu 291 is homozygous for three of the four *ard* (actin restriction-defined fragments, ref. 17 and Table 1): *ardA* (allele A_2, 17.4 kb and 7.2 kb), *ardB* (allele B_1, 13.6 kb), and *ardD* (allele D_1, 4.5 kb) and heterozygous for *ardC* (allele C_1, 5.6 kb, and C_2, 5 kb).

Table 1. Comparative patterns of *Hin*dIII restriction fragments of *Physarum* hybridizing with actin gene probes in strains Wis 1 and Tu 291

Locus*	Allele*	*Hin*dIII fragment, kb		Designation of hybridization bands
		Wis 1*	Tu 291†	
ardA	A_1	36.2	—	—
		7.8	—	—
	A_2	17.8	17.4 ± 0.9	A_{17}
		7.0	7.2 ± 0.5	A_7
ardB	B_1	13.5 ± 0.6	13.6 ± 0.8	B
	B_2	12.5 ± 0.6	—	—
ardC	C_1	5.3 ± 0.1	5.6 ± 0.35	C_1
	C_2	4.8 ± 0.1	5.0 ± 0.3	C_2
ardD	D_1	4.2 ± 0.1	4.5 ± 0.25	D
	D_2	4.0 ± 0.1	—	—

*From ref. 17.
†Values are expressed as mean ± SD based on five independent determinations.

The genotype of strain Tu 291 allows us to assay the order of replication of the four actin linkage groups as well as the replication of one pair of actin alleles. For convenience, we have labeled the restriction bands (in order of decreasing size): A_{17}, B, A_7, C_1, C_2, and D, where the letter designates the locus, numbers 1 and 2 designate the alleles, and numbers 17 and 7 designate the two fragments derived from the single complex locus (Table 1 and Figs. 2–4).

Separation of Early- and Late-Replicated Nuclear DNA Fractions. To probe for the sequence of actin gene replication, we first increased the resolution of preparative separation of unifiliary BrdUrd-substituted HL from nonreplicated LL fractions of the nuclear DNA.

Partially BrdUrd-substituted DNA was obtained from cultures treated from 10 min before telophase until distinct time points in S phase (see *Materials and Methods*). Labeling periods were based on our knowledge of the kinetics of DNA replication (30), the lack of G_1 phase (3, 18), and the high degree of mitotic synchrony in *Physarum*. Purified DNA was centrifuged on CsCl gradients and monitored by UV absorbance during fractionation. As seen in Fig. 1*d*, after incorporation of BrdUrd for a sufficient time (60–90 min of S phase, when 50–75% of the genome is replicated) we detect a bimodal distribution of nonreplicated LL DNA (ρ = 1.70 g/ml) and newly replicated HL DNA (ρ = 1.73 g/ml). This has been shown previously for *Physarum* (see refs. 3 and 18 for review) and mammalian cells (8, 32). For shorter incuba-

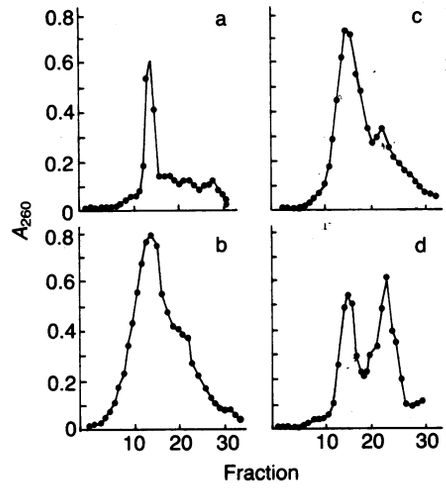

FIG. 1. Separation of early- and late-replicating DNA by preparative CsCl gradient centrifugation after BrdUrd incorporation *in vivo*. Nuclei were isolated and DNA was purified and centrifuged on CsCl gradients and fractionated. LL nonreplicated DNA, ρ = 1.70 g/ml; HL BrdUrd-substituted DNA, ρ = 1.73 g/ml. (*a*) Control, no BrdUrd incorporation, one CsCl gradient centrifugation. Fractions 12–16 were pooled for total DNA preparations. (*b*) Twenty minutes of BrdUrd incorporation, digested with *Hin*dIII following first CsCl gradient. Fractions 5–16 and 19–28 were pooled for LL and HL DNA preparations, respectively. (*c*) Forty minutes of BrdUrd incorporation, digested with *Hin*dIII following first CsCl gradient. Fractions 6–18 and 19–30 were pooled for LL and HL DNA preparations, respectively. (*d*) Ninety minutes of BrdUrd incorporation. Fractions 11–17 and 20–28 were pooled for LL and HL DNA preparations; no *Hin*dIII digestion.

tion in BrdUrd we utilized *Hin*dIII digestion before density gradient centrifugation to enrich for HL and LL DNA in the shorter DNA molecules. The restriction fragments of LL and HL DNA can be partially separated after a labeling period with BrdUrd for 20 or 40 min of S phase (Fig. 1 *b* and *c*).

Chronology of Actin Gene Replication in *Physarum*. On ethidium bromide staining, analysis by gel electrophoresis of *Hin*dIII-restricted DNA yields a broad smear ranging from 3 to 45 kb, with two prominent bands of 3.4- and 5-kb fragments that are derived from the nuclear ribosomal DNA (18). DNA gels were blotted on nitrocellulose and hybridized to a nick-translated sea urchin actin gene probe. The distribution of the specific restriction fragments (see Table 1) in the newly replicated HL DNA and late-replicated LL DNA at different time points in S phase is shown in Fig. 2. The *Hin*dIII digestion pattern of Fig. 2 *Left* is representative of 20-min S phase after 15% of the DNA has replicated. As compared to the total DNA (lane a), two of the actin restriction fragments (A_{17}, A_7) are clearly over-represented in the LL nonreplicated DNA (lane b), whereas the four others (B, C_1, C_2, D) are enriched in the newly replicated HL DNA (lane c). Fig. 2 *Right* represents the pattern of hybridization for 40 min of BrdUrd incorporation. In this case, there is a better fractionation as the HL DNA (35% of genome) is not as much overlapped by the unsubstituted DNA after the CsCl gradient centrifugation (see Fig. 1*c*). Only the two linked restriction fragments (A_{17}, A_7) of locus *ardA* are present in the LL DNA, whereas about 95% of the four other fragments are found in the newly replicated HL DNA. These two independent experiments clearly indicate the early replication of three of the four loci (*ardB*, *ardC*, *ardD*) and suggest a later activation of the replicon(s) of the fourth locus (*ardA*).

Estimation of the Relative "Gene Dosage" of the *ard* Loci During the Mitotic Cycle of *Physarum*. From the density-shift experiments and preparative separation of replicated from nonreplicated DNA, the data suggest that by 20–40 min of S phase, four of the six actin-related restriction fragments have replicated and two have not. As all six fragments are displayed in a single DNA preparation, the relative concentration should increase transiently for each gene as the putative actin locus becomes replicated in S phase. Therefore, we determined the *relative* intensity of the six hybridization bands (i.e., the gene dosage) at different times in S and G_2 phase.

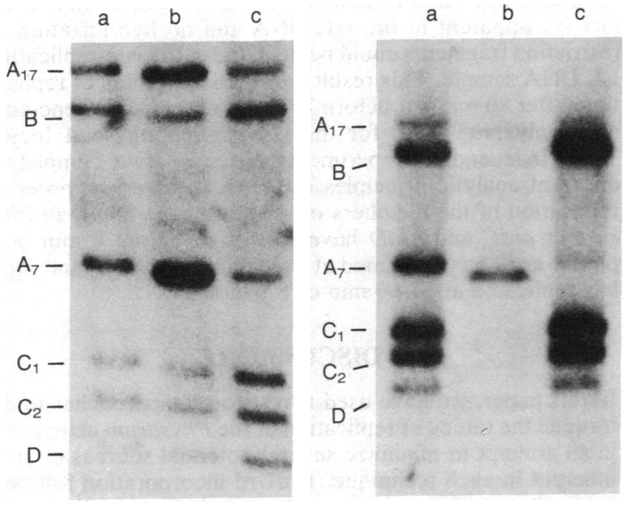

FIG. 2. Actin gene replication: Hybridization patterns of nick-translated probe to *Hin*dIII-restricted LL DNA and HL DNA of *Physarum*. After incorporation in BrdUrd for segments of S phase, preparative separation of LL and HL DNA on CsCl gradients, agarose gel electrophoresis, and Southern blotting, hybridization and autoradiography were performed (designation of the restriction fragments of the four *ard* loci *A–D* are defined in Table 1). (*Left*) BrdUrd incorporation for 0–20 min of S phase. Lanes: a, total DNA; b, LL nonreplicated DNA; c, HL replicated DNA. (*Right*) BrdUrd incorporation from 0–40 min of S phase. Lanes: a, total DNA; b, LL late-replicating DNA; c, early-replicating DNA.

A typical gene-dosage experiment is illustrated in Fig. 3 *Left*, quantitated in Fig. 3 *Right a–d*, and analyzed in Table 2. To minimize nonspecific variations, we flanked the three S-phase samples with four G_2-phase DNA samples from two independent DNA extractions prepared at an interval of 6 months (Fig. 3 *Left*). Scanning of the autoradiographs and integration of the peak areas establish that the relative intensities of the hybridization bands in G_2 phase are constant; the ratios (×100) of the peak areas are $A_{17}/B = 86 \pm 6$, $A_7/(C_1 + C_2 + D) = 65 \pm 2$, and $A_{17}/A_7 = 108 \pm 14$. These ratios can serve as an internal standard. Densitometer tracings were made from autoradiographs representing longer and shorter exposure times of the blot shown in Fig. 3. These results show ratios similar to the data presented in Table 2. Control blots yielded a linear densitometric response over an 8-fold range of DNA concentration. The BrdUrd experiment (Fig. 2) indicated that band B was replicated at 20 min, whereas band A_{17} was unreplicated at 40 min of S phase. Therefore, the G_2 ratio of the intensities of band A_{17} to band B should decrease in S phase. The following ratios (×100) were obtained (Table 2): 94 at 8 min, 58 at 30 min, and 52 at 45 min of S phase. The A_{17}/B ratios at G_2 phase and 8 min of S phase are significantly different from the ratios at 30 and 45 min of S phase ($P < 0.001$). This result indicates that locus *B* has not replicated during the first 5% of genome replication yet has effectively replicated between 8 and 30 min of S phase. In contrast, locus *A* has not replicated prior to 45 min but has done so by the following G_2 phase.

The density-shift experiment also predicted that actin loci *C* and *D* replicated before locus *A*. Consequently, their hybridization intensities (grouped together because the relative ratios of bands C_1, C_2, and D, as determined by densitometry, do not vary measurably in Fig. 3 *Right a*) should increase relative to band A_7, thus yielding a smaller ratio of $A_7/(C_1 + C_2 + D)$ at the time of effective replication. As indicated in Table 2, a decrease in the ratios (×100) was detected: 42, 43, and 30 at 8, 30, and 45 min of S phase, respectively, as compared to the G_2 ratio (×100) of 65 ($P < 0.01$). This result indicates that the homozygous actin locus *D* and the two alleles C_1 and C_2 of the heterozygous actin locus *C* replicate within the first 8 min of S phase, which is again concordant with the BrdUrd data.

To detect how late in S phase locus *ardA* is replicated, a gene-dosage analysis was made at 8 time points throughout S phase and interspersed with two G_2 phase controls (Fig. 4). For clarity, we present only the two adjacent bands, A_{17} and B. As in Fig. 3 and Table 2, the ratio of the intensities of band A_{17} to band B is close to 1 in G_2 phase (Fig. 4, lanes a and g). A coherent change occurs at 10 min of S phase, when the intensity of band B has become stronger than that of band A_{17}, thus indicating replication of band B (lane c). This relationship remains constant from 20 to 80 min of S phase (lanes d–f and h), until about 75% of the genome is replicated (30). However, at 100 and 120 min of S phase the relative intensities of bands A_{17} and B are once again similar (lanes i and j), as they are in G_2 phase. Densitometry of the bands yielded the following ratios (×100) for A_{17}/B: 96 for lane a, 89 for lane g (G_2 phase), and 61, 71, 48, 52, 64, 127, and 116 for lanes c, d, e, f, h, i, and j, respectively (S phase). A significant shift in the relative intensity of the hybridization signal of bands A_{17} and B was observed at 10 min of S phase for band B and between 80 and 100 min of S phase for band A_{17} in two further experiments (not shown), suggesting replication of actin locus *ardB* early and locus *ardA* late in S phase. This experiment confirms early replication of the *ardB* locus and indicates the replication of the *ardA* locus (both the A_{17}

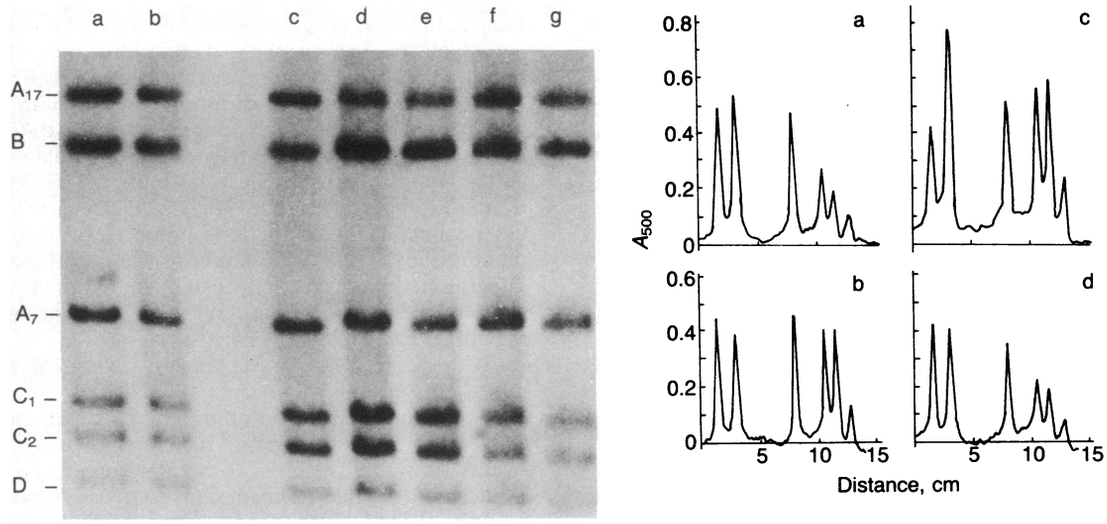

FIG. 3. Gene-dosage determination of the actin gene containing *Hin*dIII fragments of *Physarum*. Total DNA from two independent G_2-phase preparations and from three time points in S phase was purified, digested with *Hin*dIII, electrophoresed, and hybridized as described in the legend to Fig. 2. (*Left*) Hybridization spectra of the actin gene restriction fragments (defined as four *ard* loci in Table 1). Lanes: a, b, f, and g, G_2 phase; c, 8 min of S phase; d, 30 min of S phase; e, 45 min of S phase. (*Right*) Scans of the fluorograph in *Left*. (*Right a*) Representative hybridization pattern of G_2-phase DNA, before MII (lane b). (Scans of lanes a and g are very similar; not shown.) (*Right b*) Hybridization pattern of total DNA at 8 min of S phase; note relative increase in intensity of bands C_1, C_2, and D. (*Right c*) Hybridization pattern of total DNA at 30 min of S phase; note relative increase in intensity of band B. (*Right d*) Hybridization pattern of G_2-phase DNA after completion of the S phase following MII (8 hr after MII); note great similarity of the pattern with that in *Right a* and also note relative stability of the intensities of bands A_{17} and A_7 in *a–d*.

and A_7 fragments, the latter not shown) between 80 and 100 min after the onset of S phase.

Invariant Order of Actin Loci Replication in Two Consecutive Mitotic Cycles in *Physarum*. In the classical density-shift experiment with *Physarum* (3), after double labeling portions of the DNA with a pulse of [^{3}H]thymidine and [^{14}C]BrdUrd in two consecutive S phases and analyzing the DNA profiles of analytical CsCl gradients, a distinct temporal order of replication was implied.

The results of the above experiments strongly suggest that the actin gene loci should replicate in the same sequence and at the same time in the S phase following each mitotic cycle. Since all of our previous experiments were done during S phase following MII, we have repeated the analysis of the distribution of actin-related restriction fragments in replicated HL and nonreplicated LL DNA at 3 critical time points following the third synchronous mitosis (MIII). For 20 and 40 min of incorporation of BrdUrd during the S phase following MIII, the distribution of the actin restriction fragments within newly replicated HL DNA and nonreplicated LL DNA is very similar to that in the preceding S phase (see Fig. 2). The only two late-replicating fragments (enriched in LL DNA) are the two linked fragments of the *ardA* locus. Densitometry of the hybridization patterns (not shown) and determination of the relative proportions of locus *ardA* (bands A_{17} + A_7) in total HL and LL DNA preparations, we obtained values of 39% ± 4% for G_2 phase and 32% ± 4% in S phase in total DNA. At 20 min of S phase, hybridization to *ardA* represented 14% in HL DNA and 74% in LL DNA (as compared to 26% in HL DNA and 86% in LL DNA following MII, Fig. 2) and after 40 min of S phase, *ardA* amounts to 9% in HL DNA and 92% in LL DNA (as compared to 12% in HL DNA and 90% in LL DNA after MII, Fig. 3).

Following 90 min of BrdUrd incorporation, all four actin loci are apparent in the HL DNA and no hybridization to restriction fragments could be detected in the late-replicating LL DNA sample. This result confirms the timing of replication (after 80 min but before 100 min) obtained by gene-dosage analysis (Fig. 4) for the late-replicating *ardA* locus. Thus, independent experiments based on two completely different analytic principles establish an invariant order of replication of the members of the actin gene family in *Physarum*: *ardC* and *ardD* have replicated before 8 min of S phase, *ardB* has replicated at 8–10 min of S phase, and *ardA* has replicated at 80–90 min of S phase.

Table 2. Relative intensities of hybridization bands for actin-related *Hin*dIII fragments throughout the mitotic cycle of *Physarum*

Stage	Lane in Fig. 3	A_{17}/B	$A_7/(C_1 + C_2 + D)$
G_2	a	80	68
G_2	b	92	65
S, +8*	c	94	42
S, +30*	d	58	43
S, +45*	e	52	30
G_2	f	84	64
G_2	g	81	63

The ratios (×100) of intensities of hybridization bands were determined from the peak areas after scanning autoradiographs.
*Minutes of S phase; 0 min = telophase of mitosis.

DISCUSSION

In this paper, we have used two separate approaches to determine the timing of replication of the *Physarum* actin genes in an attempt to minimize several potential sources of error inherent in each technique. BrdUrd incorporation followed by CsCl centrifugation does not unambiguously distinguish between the replication of a gene or of its flanking sequences. The assumption must also be made that the sequences being compared do not differ radically in their G·C content. Estimations of gene dosage by blot hybridization analysis are complicated by the possibility of differential degradation of the large restriction fragments and transfer artifacts. In spite of these potential problems, the results obtained through both methodologies were reproducible and equivalent. As monitored either by the distribution of the respective restriction fragments after BrdUrd incorporation

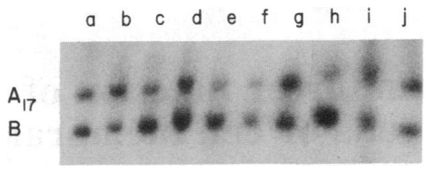

FIG. 4. High-resolution gene-dosage determination of an early- and late-replicating actin gene locus in *Physarum*. DNA from 10 accurately determined time points of S phase after MII was analyzed, as in Fig. 3, with two G_2-phase samples as internal controls (lanes a and g). Lanes: b, 5 min; c, 10 min; d, 20 min; e, 30 min; f, 40 min; h, 80 min; i, 100 min; j, 120 min of S phase. A relative increase in intensity of band B occurs by 10 min of DNA replication and regains the typical G_2-phase ratio between 80 and 100 min of S phase.

or by the variations in hybridization signal from DNA isolated from progressively later times in S phase, the four unlinked actin loci show a distinctly bimodal pattern of replication. Three of the four genes replicate during the first 6% of genome replication, corresponding to the first 10 min of S phase. The two allelic variants (*ard* C_1 and C_2) of one gene are observed to replicate at the same time. The fourth locus does not replicate until after 80 min, representing a period when DNA synthesis is ≈70% complete. These observations establish the precise order of replication of actin genes during vegetative growth of *Physarum*.

Physarum DNA (0.6 pg per diploid cell) is replicated in 180 min. The replication rate is rather constant for the first 90 min, at about 5000 kb/min, and then decreases to 1500 kb/min in late S phase (30). The elongation rate of an individual replicon as measured in four different investigations (28, 29, 33, 34) is about 1.2 kb/min. Taking into account the size of the genome, the mean replicon size of 30 kb (29), and the kinetics of replication of the strain Tu 291 (30) it can be estimated that among the 18,000 replicons of the *Physarum* genome, about 4000 are active in early S phase. The four actin gene loci, as measured by hybridization to genomic restriction fragments, are considerably smaller than the mean replicon size. Thus, it seems likely that the *Physarum* actin genes are included within a few replicons, perhaps only 4. From the rate of DNA synthesis (0.6 kb/min per fork), the time needed for the replication of the smallest and largest early-replicating fragments of *ardB*, *ardC*, and *ardD* (4.5 and 13.6 kb) can be estimated at 8 min and 22 min, respectively. We are confident that the replication of the longest fragment (*ardB* = 13.6 kb) has occurred by about 10 min after the onset of S phase. This would require the respective replication fork to move twice as fast as average or that two replication forks participate in the replication of the 13.6-kb fragment.

We have recently shown evidence in *Physarum* for the presence of an origin of replication inside the transcription unit of early-replicated and immediately activated genes (10). Based on this and other evidence for replication–transcription coupling in *Physarum* (15), we have suggested that the sequential replication of the genome of this organism directs the order of transcription during S phase, which, in turn, may contribute to the control of the proliferative mitotic cycle (16). Similar events may be required for erythrocyte differentiation (9, 14, 32, 35) and early gene expression in the ascidian embryo (36). It has been demonstrated in other organisms (37–39) that actin mRNA levels corresponding to specific family members vary during ontogenesis and are not coordinately transcribed. In these organisms, the timing of actin gene replication is not known and any relationships between replication and transcription cannot be assessed. However, with the synchrony of the mitotic cycle and a fair synchrony of the sporulation process, which includes a presporangial mitosis, this question is addressable in *Physarum*.

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