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Selective initiation of replication at origin sequences of the rDNA molecule of *Physarum polycephalum* using synchronous plasmodial extracts

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ABSTRACT

A cell-free system using synchronous plasmodial extracts initiates replication selectively on the 60 kb rDNA palindrome of Physarum polycephalum. Preferential labeling of rDNA fragments by nuclear extracts, in which elongation is limited, indicates that initiation occurs at two positions corresponding to in vivo origins of replication estimated by electron microscopy. Both nuclear and whole plasmodial extracts initiate selectively within a plasmid, pPHR21, containing one of these origins. In this plasmid bubbles expand bidirectionally and generate DpnI-resistant DNA. Extracts made at prophase or early S phase, times when the nucleolus is disorganized, are most active in pPHR21 replication. Mapping positions of replication bubbles locates the initiation point in a 3.2 kb BstEII fragment at the upstream border of a series of 31 bp repeats 2.4 kb from the initiation point for ribosomal gene transcription.

INTRODUCTION

In Physarum polycephalum ribosomal RNA is encoded on multiple copies of a linear, palindromic rDNA molecule of 60 kb located in the nucleolus (1-3). In each such minichromosome two transcription units of 13 kb are positioned in inverse orientation about a central spacer of 24 kb. This rDNA molecule is one of the few eucaryotic DNA segments for which a specific origin of replication is known to function in vivo. Electron microscopic studies of rDNA molecules replicating in plasmodia have revealed that initiation occurs at either one of two origins located in each half of the central spacer (4). Origins have thus far been mapped approximately to positions about 20 and 27 kb away from each end of the molecule. Although 4 potential origins exist on each molecule, only one is observed active on a given replicating molecule (4). The rDNA replicates semi-conservatively (5,6) with forks progressing bidirectionally from an origin toward the

telomeres (4). There is no evidence for amplification of rDNA at any stage of the Physarum life cycle. Although rDNA may replicate in unscheduled fashion during proliferation (4), a stable copy number of 200-400 molecules is maintained in the nucleolus at all life stages (6,7). The timing of rDNA replication differs from that of the bulk of nuclear DNA since rDNA replicates from the latter half of S throughout G_2 phases (5,6).

There is presently no in vitro system with which to study initiation of replication at a cellular DNA origin in a synchronous eucaryotic system. Physarum rDNA offers several advantages for such in vitro studies. The high rDNA copy number assures a relatively high proportion of potential initiating factors. The rDNA with its associated proteins is located entirely within the nucleolus and can be isolated in milligram quantities (3). Since Physarum plasmodia grow naturally synchronously, extracts may be optimized for components controlling rDNA replication. In addition, it is not yet known what factors control selection of one of the four origins on the rDNA molecule at each round of replication. Here we report that an in vitro system based on Physarum plasmodial extracts can initiate selectively at one origin of the rDNA molecule cloned in a bacterial plasmid. Using this system we localize this initiation site to a position approximately 2.4 kb upstream of the ribosomal RNA transcription initiation point.

MATERIALS AND METHODS

Preparation of extracts from synchronously-dividing macroplasmodia.

Macroplasmodia were synchronized following microplasmodial fusion and outer growth regions harvested at selected cell cycle points beginning at early prophase of mitosis II (8). To prepare plasmodial cytoplasmic extracts rinsed plasmodia corresponding to 1-5 x 10^6 nuclei were lysed by 40 strokes with a Dounce homogenizer (B pestle) after swelling 10 min on ice in an equal volume of buffer: 20 mM Hepes (pH 7.5), 5.0 mM KCl, 1.5 mM $MgCl_2$ and 1.0 mM dithiothreitol. After adjustment to 0.2 M NaCl, the lysates were centrifuged at 50,000 x g for 30 min. Supernatants were dialyzed against 20 mM Hepes (pH 7.5), 1.0 mM

dithiothreitol, 0.1 mM EDTA, 10% glycerol (v/v), 50 mM NaCl and recentrifuged as above (9). Aliquots were stored at -135°C. Protein concentrations were 1.7-2.3 mg/ml. Nuclei were prepared for extraction by homogenizing as described above and centrifuging the lysates at 2000 x g for 5 min. Pellets were resuspended in 2.0 ml of 50.0 mM Hepes (pH 7.5), 10% sucrose, 0.2 M NaCl, incubated on ice and centrifuged as described (10). Supernatants were dialyzed and stored as above. Protein concentrations were 2.0-2.5 mg/ml.

Preparation of rDNA and plasmid DNA.

Physarum rDNA was purified from isolated nonsynchronous microplasmodial nuclei (3) as described by Ferris and Vogt (11). Plasmids were propagated in *E. coli* strain HB101 (*dam*$^{+}$) without chloramphenicol amplification and purified by CsCl equilibrium density gradient centrifugation, after lysis of bacteria by either rapid boiling or alkali following standard procedures. Preparations were >80% supercoiled form I DNA. Plasmid DNA preparations were tested for possible adventitious primers by assaying for DNA synthesis catalyzed by the Klenow fragment of *E. coli* DNA polymerase I according to a method previously described (12). There was virtually no incorporation of labeled dCTP into either plasmid pPHR21 or pBR322 using the Klenow enzyme regardless of whether plasmids were purified by alkaline lysis or rapid boiling. In contrast, incorporation was high in controls where plasmids were denatured and synthesis carried out with added oligonucleotide primers. These tests indicate that adventitious primers are not present in our purified plasmid DNA.

In vitro replication conditions.

Reaction mixtures (50 µl) contained 250 ng of plasmid or rDNA in 30 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 µM each dATP, dGTP and dTTP, 10 µM α-^{32}P-dCTP (10-30 Ci/mmole, New England Nuclear), 200 µM each GTP, UTP, and CTP, 4.0 mM ATP, 40 mM phosphocreatine, 1.5 µg creatine kinase and 30 µl of *Physarum* plasmodial extract or nuclear extract (added last). Reactions, at 37°C, were stopped by addition of EDTA to 25 mM and SDS to 0.5%. DNA was purified from reactions by treatment with RNase A and proteinase K and extraction with phenol and chloroform. Samples were digested with restriction endonucleases

TABLE 1. Requirements for Physarum rDNA synthesis in vitro with a plasmodial prophase extract

Reaction components omitted\added*	pmol dCMP incorporated	Relative activity
System complete, 10 µM dCTP	37	100
(100 µM dCTP)	(112)	(304)
- CTP, GTP, UTP	39	106
- dATP, dGTP, dTTP	9	24
- ATP	5	13
- $MgCl_2$	7	18
- Creatine phosphate,creatine kinase	13	36
+ Aphidicolin, 10 µM	10	28
+ Camptothecin, 100 µM	14	38
+ RNases A + T_1, 1.0 µg each	30	82

*2 hour reactions were carried out using 250 ng of added purified rDNA as described in Materials and Methods. Reactions were stopped by addition of EDTA to 10 mM. Samples were placed on ice for 15 min after the addition of carrier DNA and 1.0 ml of 10% trichloroacetic acid (TCA) containing 1% sodium pyrophosphate. Each sample was filtered through a Whatman GF-C filter to collect the precipitate, which was then washed and dried. Radioactivity was assayed by liquid scintillation spectrometry.

and analyzed by autoradiography after 1.0% agarose gel electrophoresis or spread for electron microscopy, stained with uranyl acetate and rotary shadowed with Pt-Pd as described (13). Length measurements were made on enlarged electron micrographs using a Numonics digital integrator.

RESULTS

Preferential labeling of Physarum rDNA segments at potential initiation sites.

Purified, intact rDNA was used as a template for in vitro replication. The rDNA labeling was completely dependent on the presence of added purified template. The absence of labeled rDNA bands without this addition verifies that the extracts are free of endogenous rDNA. General characteristics of this system are presented in Table I. Labeling of rDNA is highly dependent on inclusion of ATP, Mg^{++} and deoxyribonucleoside triphosphates, as is generally seen with other in vitro systems. Synthesis is inhibited >70% by inclusion of aphidicolin, indicating a strong dependence on DNA polymerase alpha. Omission of ribonucleotides has little effect on incorporation indicating either that new RNA

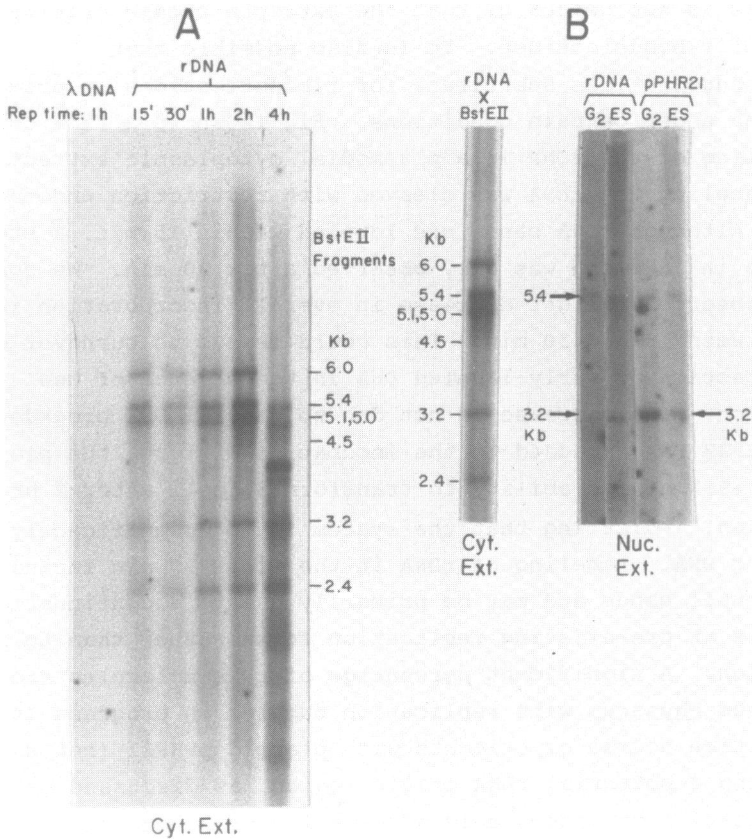

Figure 1. Labeling of rDNA in vitro: selective cleavage of a replicated region and preferential labeling of a specific cloned segment. A. Time course of labeling rDNA using a cytoplasmic extract and cleavage of a 6.0 kb Bst EII segment. The rDNA (250 ng) was subjected to in vitro replication using an extract from prophase, digested with Bst EII and subjected to agarose gel electrophoresis and autoradiography as described in Materials and Methods. As a control for selectivity, lambda phage DNA (250 ng) was also subjected to the system and digested with Bst EII prior to electrophoresis (left lane). B. Selective labeling of fragments containing potential origins of replication in rDNA and in plasmid pPHR21 using nuclear extracts. Nuclear extracts were prepared from early S (ES) and late G_2 (G_2) as described and used to label either rDNA or plasmid pPHR21 (containing the rDNA 3.2 kb Bst EII segment) for 3 hrs. The purified, labeled DNA was then digested with Bst EII and autoradiographed after gel electrophoresis as in A. A lane showing labeling of all the rDNA fragments by a cytoplasmic prophase extract is provided at left for comparison. The four lanes at right show selective labeling of the 3.2 and 5.4 kb Bst EII bands of rDNA (arrows at left) and of the 3.2 kb Bst EII band of pPHR21 (arrow at right).

synthesis is not needed or that the extracts retain critical levels of ribonucleotides. It is also possible that deoxyribonucleotides substitute for ribonucleotides in priming reactions under certain conditions. Fig 1A shows a time course of labeling of the rDNA in a plasmodial cytoplasmic extract. After labeling the rDNA was cleaved with restriction endonuclease BstEII. Although rDNA bands are labeled within 15 min, a steady increase in labeling was only observed after 30 min. We consistently observe a slight decrease in overall incorporation into rDNA between 15 and 30 min. This could be due to turnover of a small fraction of early-labeled DNA in the absence of new initiation. The decrease is not due to general DNA breakdown. When pBR322 DNA is added to the incubation mixture, the plasmid retains >95% of its ability to transform E. coli after 2 hrs incubation, indicating that the system is not significantly degrading DNA. Labeling of rDNA in the first 30 min includes all major BstEII bands and may be primarily due to a continuation of synthesis at pre-existing replication forks rather than to new initiation. A significant percentage of rDNA molecules are isolated from Physarum with replication bubbles in progress (4). In similar time course experiments with plasmid pPHR21 (not shown), containing a potential rDNA origin region, as discussed below, new initiation commences only after a lag of 30 min and no labeling is seen before this time. A lag in new initiation of 15 min has previously been reported for a mammalian in vitro replication system (9). Repair synthesis contributes little to the early rDNA labeling, as shown by lack of labeling of a lambda phage control. The phage DNA was incubated with the system for 1 hr and would be subject to the same nicking and repairing as the rDNA. At 4 hrs the system generates specific cleavage within the labeled rDNA band at 6.0 kb to yield discrete bands of smaller size. Thus far such cleavage has not been observed in the bulk of nonreplicated rDNA subjected to the system, but any relation between this cleavage and replication has yet to be established.

Experiments shown in Figs 1B and 2 were performed to distinguish rDNA labeling at new initiation sites from labeling at previously-existing replication forks. We examined labeling using extracts of isolated nuclei, taken at either late G_2 or

early S-phase of the cell cycle. When rDNA is incubated for times up to 3 hrs with a nuclear extract, differential labeling of the BstEII fragments is observed (Fig 1B). The bands most intensely labeled are the 3.2 kb and 5.4 kb fragments from the central spacer. These fragments are in the vicinity of origins of replication observed _in vivo_ (4). We examined as a template with nuclear extracts plasmid pPHR21, a pBR322 clone with a 3.9 kb BclI insert including the 3.2 kb BstEII fragment (11,14). Fig 1B shows that in this plasmid, as well as in rDNA, preferential labeling occurs within the 3.2 kb BstEII fragment. Labeling of this fragment is more pronounced in the plasmid than it is in the rDNA when utilizing the late G_2 nuclear extract. The 5 kb BstEII fragment of pPHR21 containing primarily the pBR322 vector is not significantly labeled (Fig 1B). Electron microscopy, described below, reveals that nuclear extracts facilitate accumulation of a high percentage of early replicating plasmid forms. However, overall labeling with the nuclear extract is lower than with cytoplasmic extracts. Together with the selective labeling, these results suggest that nuclear extracts initiate selectively but elongate more slowly than do cytoplasmic extracts.

Fig 2 shows a densitometric comparison of rDNA labeling by cytoplasmic and nuclear extracts. The nuclear extract, center panel, shows a clear preference for labeling of the 3.2 kb and 5.4 kb fragments. The agent aphidicolin selectively inhibits elongation due to DNA polymerase alpha while having no effect on repair polymerases (15). At concentrations >10 µM, aphidicolin abolishes detectable labeling of rDNA. However, at 10 µM aphidicolin the inhibition is not complete and one rDNA band remains clearly labeled, the 3.2 kb BstEII fragment (Fig 2, bottom panel). Similar observations on inhibition of labeling of SV40 DNA _in vitro_ by aphidicolin revealed that the least inhibited fragment contained the replication initiation site (16). It was noted that since aphidicolin inhibits elongation while having little effect on initiation, this type of experiment could allow identification of _ori_ sequences in a variety of systems if conditions are available for accumulation of early-replicating intermediates (16). Such conditions are obtained _in vitro_ with the _Physarum_ nuclear extract. If elongation following initation

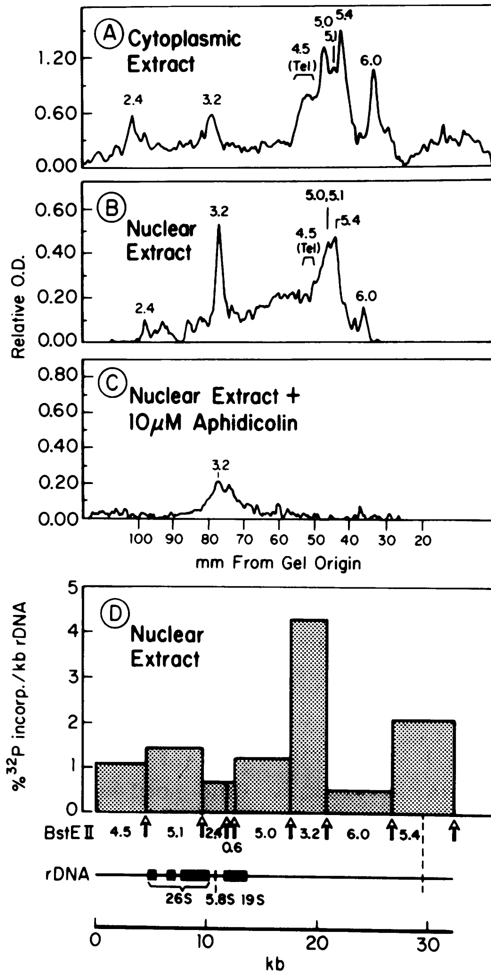

Figure 2. Preferential labeling by a nuclear extract of rDNA regions containing origins of replication. Intact rDNA was subjected to in vitro replication with either cytoplasmic extract or nuclear extract, from early S-phase plasmodia, as described for Fig 1B. After labeling, the rDNA was purified, digested with Bst EII and subjected to agarose gel electrophoresis and autoradiography as described in Materials and Methods. Gels were scanned using a Bio-Rad 620 video densitometer. Panel A: cytoplasmic extract; panel B: nuclear extract; panel C: nuclear extract with 10 μM aphidicolin. Tel refers to the diffuse telomeric fragment. Numbers at peaks are fragment sizes in kb.
Panel D: Relative incorporation, using a nuclear extract, corrected for size of the rDNA Bst EII fragments. Per cent of incorporation, calculated from peak areas in panel B, was divided by the rDNA fragment size and plotted as a histogram above an rDNA Bst EII map.

proceeds only slightly or not at all, then the labeled fragment would remain at its original double-stranded position as seen for the 3.2 kb fragment in Fig 2. Labeling seen between the 3.2 and 4.5 kb bands could represent elongation at bubbles in the 3.2 kb fragment. (Two dimensional gel analyses of labeled rDNA intermediates yielded complex results, possibly due to internal sequence repetition or secondary structure formation. Hybridization analysis of intermediates on 2-D gels, used to map yeast origins of replication in vivo [17], is not feasible here due to extensive sequence repetition in the rDNA.) It is possible that the 5.4 kb BstEII fragment is utilized less effectively for initiation by our system than is the 3.2 kb fragment, explaining why the 5.4 kb fragment labels less and is not seen after treatment with aphidicolin. Based on electron microscopic mapping, it is likely that BstEII cleaves at or near the central rDNA origin, thus also possibly contributing to the lower intensity of label seen in the 5.4 kb band. Results of Figs 1 and 2 implicate the 3.2 kb BstEII fragment as a potential replication initiation site both in rDNA and in a plasmid clone.

Selective initiation and replication in vitro of plasmid pPHR21.

We sought to determine whether plasmid pPHR21, containing a potential replication origin as indicated in Fig 1, initiates selectively and replicates fully in vitro. DpnI digestion is a demonstrated means of assaying plasmid replication in eucaryotic systems (9,13,18). Plasmids propagated in dam^+ strains of E. coli are methylated at adenosine residues and are sensitive to DpnI (19). They become resistant after replication in eucaryotic systems, which do not methylate adenosines. Plasmid pBR322 has 22 DpnI sites, so that complete DpnI resistance of pBR322 or of pPHR21 implies complete replication. For gel electrophoretic visualization of labeled bands, shown in Fig 3, each plasmid was linearized after replication and before DpnI treatment to consolidate replicated circular forms into a single band. Full-length pPHR21 DNA resistant to DpnI is clearly detected after 2 hrs of replication with cytoplasmic extracts from early S, G_2 or prophase of the cell cycle (Fig 3 left). Plasmid pBR322, subjected to the in vitro system, is completely digested by DpnI indicating that it does not replicate (Fig 3 left, last lane).

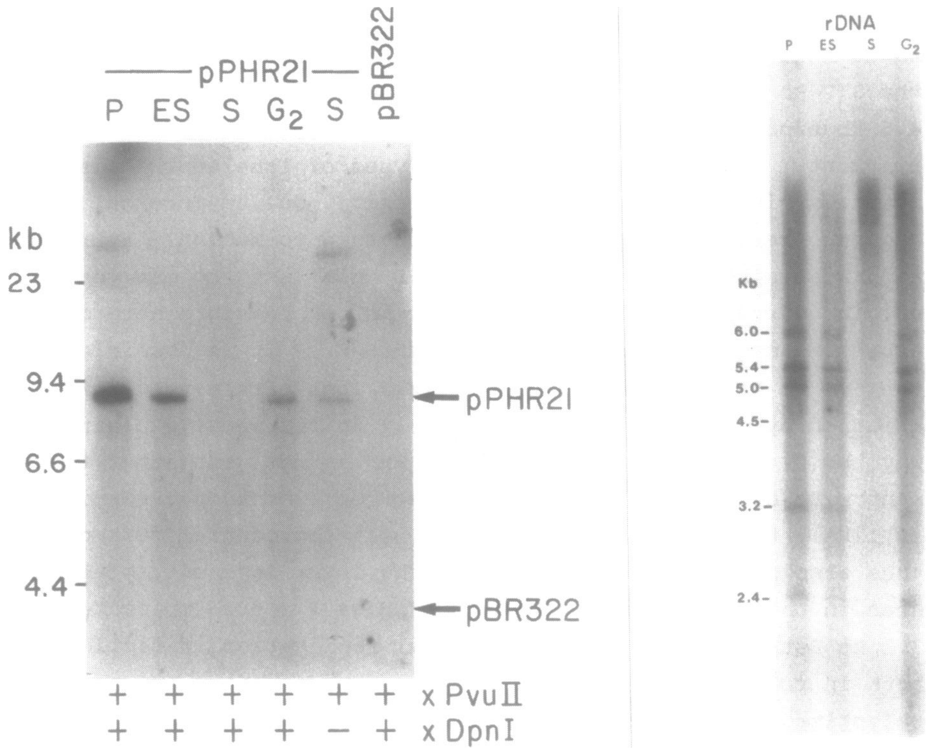

Figure 3. DpnI-resistance of plasmid pPHR21 DNA after replication using cytoplasmic extracts from different points in the synchronous division cycle. Left: Plasmid pPHR21 DNA (250 ng) was subjected to the *in vitro* replication system for 2 hrs using cytoplasmic extracts from synchronous plasmodia. Points taken were: Prophase (P) = 30 min prior to metaphase II; Early S (ES) = immediately after telophase II; S = 1.5 hrs after telophase II; G_2 = 4 hrs after telophase II. Purified, labeled DNA was then linearized with PvuII (60 units/µg DNA) and treated with DpnI (35 units/µg of DNA) for 1 hr at 37°C and analyzed as in Fig 1A. Control plasmid pBR322 DNA was also subjected to the system and treated with the same restriction enzymes prior to electrophoresis (right lane). In indicated lanes DNA labeled with an S-phase extract was analyzed before and after treatment with DpnI. Right: Purified *Physarum* rDNA was subjected to *in vitro* replication as described for pPHR21 DNA at left using extracts from different points of the cell cycle. The DNA was then repurified as described in Materials and Methods, treated with Bst EII and analyzed as in Fig 1A.

Plasmid pPHR21, subjected to the S-phase extract, is detected slightly labeled after PvuII linearization, but this labeled band is digested by DpnI indicating that the S extract labeling is not

TABLE 2. Replication of plasmid pPHR21 *in vitro* relative to control pBR322 as measured by DpnI resistance and transformation of *E. coli*

Plasmids incubated[a]	DpnI	Colonies counted[b] (Amp+)	(Tc+)
pPHR21 (Amp+)	-	5724	0
pBR322 (Amp+, Tc+)	-	5508	6012
pPHR21 + pBR322	+	648	18
pPHR21 + 2 ng pBR322[c]	+	315	0

[a]Plasmids (300 ng each) were subjected to *in vitro* replication separately for 2 hours using a synchronous early S-phase extract as described in Materials and Methods.
[b]Following incubation DNA was purified from the reaction mixtures as described, equal aliquots of different plasmids mixed, and samples either treated or not treated with 15U of DpnI for 2 hours at 37°C. DNA was precipitated with isopropanol-ammonium acetate and equal aliquots used to transform *E. coli* strain HB101. Equal aliquots of tranformed bacteria were then spread on plates containing either ampicillin (50 μg/ml) or tetracycline (12.5 μg/ml) for analysis of DpnI resistance internally controlled as previously described (18). Colonies per μg of input DNA are presented.
[c]Purified pBR322 DNA (2.0 ng), not subjected to the *in vitro* replication reaction, was added to pPHR21 DNA subjected to the replication mixture as described above.

due to complete replication. We performed experiments in which the negative replication control, pBR322, was included with pPHR21 in the DpnI digestion reaction. For these experiments we did not linearize the plasmid DNA with a second enzyme since it has been reported that linearization can affect the reliability of DpnI digestion (20), thus complicating an internally-controlled experiment. Rather than analyze the mixed plasmids by gel electrophoresis, which could lead to ambiguities due to multiple bands, we quantitatively determined the extent of DpnI digestion and levels of DpnI resistance using a recently-described bacterial transformation assay (18). This assay exploits the differential sensitivities of *E. coli*, transformed with pPHR21 or pBR322, to ampicillin and tetracycline. The results, shown in Table II, clearly show DpnI resistance of replicated pPHR21 relative to pBR322. It can be seen in the Tc^+ column that 99.7% to 100% of pBR322 molecules are digested by the DpnI. In the same

reactions 6 to 11% of recovered, circular pPHR21 molecules are DpnI resistant, as seen by comparing the Amp^+ and Tc^+ columns. (One effect of mixing the two plasmids from the replication reaction is that slightly more molecules of pPHR21 survive DpnI digestion than when 2 ng of unreacted pBR322, instead of reacted pBR322, are mixed. This could be due to an overall difference in DNA concentration during the digestion and suggests that over-digestion could cleave some replicated pPHR21 molecules. In all cases the replicated pPHR21 was preferentially resistant to the DpnI vs the pBR322.) The results of Fig 3 and Table II indicate that replication initiates in the Physarum rDNA insert of pPHR21 and yields full-length plasmid. They further show that replication is highly dependent on the cell cycle phase at which extracts are taken.

Replication activity of extracts at different points in the synchronous division cycle.

Since Physarum plasmodia grow naturally synchronously, each extract is from a highly specific point in the cell cycle. We examined the ability of extracts taken at different points in the cell cycle to support replication. The cycle for strain a x i consists of a 3 hr S phase, a 5 hr G_2 phase and mitosis, which is prefaced by a relatively long prophase. Prophase is easily identified microscopically beginning about 50 min prior to metaphase, when the nucleolus starts to move to the nuclear membrane and begins to disorganize (8). Plasmodia have no G_1 period in their cell cycle. DNA synthesis begins immediately after mitosis, and the bulk of chromosomal DNA replicates in the first 2 hrs (5). The nucleolus reorganizes in about the first 1.5 hrs of S-phase (8). Endogenous rDNA replicates during the latter part of S and throughout G_2 (5,6). Extracts varied greatly in their ability to replicate plasmid pPHR21. The most effective cytoplasmic extracts were prepared from very early S (immediately after telophase) and prophase, two times in the cell cycle, bracketing mitosis, when the nucleolus is disorganized. Later S-phase cytoplasmic extracts labeled plasmid pPHR21 only slightly, as seen in Fig 3, left. Extracts also differed in ability to label exogenous rDNA template, with prophase extracts being most active and S-phase extracts least active (Fig 3, right). We also ex-

amined nuclear extracts taken at early S-phase. These extracts initiated selectively, as determined by electron microscopy, although elongation was limited. Others have reported that isolated nuclei from S-phase retain ability to initiate replication with temporal specificity and to elongate (21). It is thus likely that certain components necessary for replication during S-phase resist extraction from nuclei by our procedure, but other possibilities remain to be examined.

Electron microscopic mapping of an rDNA initiation site for bidirectional replication in plasmid pPHR21.

We used electron microscopy in conjunction with *in vitro* replication to localize the origin of replication within known sequence regions of pPHR21. This is the method of choice here due to limitations on 2-D gel techniques with *Physarum* rDNA, as discussed earlier. For some experiments early-S nuclear extracts (50 min post-telophase) were used since these accumulated 3-fold higher levels of Cairns structures and early replication bubbles than did whole plasmodial extracts. Fig 4A shows a typical pPHR21 molecule as a late Cairns structure. Following incubation for 2 hrs, pPHR21 DNA was purified from the replication mixture, treated with PvuII and spread for electron microscopy as described in Materials and Methods. The plasmid is cleaved once by PvuII approximately opposite the rDNA insert so that any early replication bubbles initiating within the insert will appear intact, and their centers can be mapped relative to the PvuII site. Using an early S-phase nuclear extract, after PvuII cleavage 2.4% of all full-length molecules scored were seen as linear DNA with a replication bubble. Circular uncut molecules with a replication bubble constituted 0.8% as did circular uncut molecules with no bubble. Linear, full-length plasmid molecules with no bubble constituted 96%. With control plasmid pBR322, either subjected or not subjected to the replication reaction, or with pPHR21 not subjected to the reaction, <0.01% of molecules were seen with a bubble. More than 10,000 pPHR21 molecules were scored. Several linearized pPHR21 molecules with replication bubbles are shown in Fig 4, D-F. Ten representative molecules with different-sized bubbles and no branch migration were mapped as shown in Fig 5. We mapped the position of the center of each

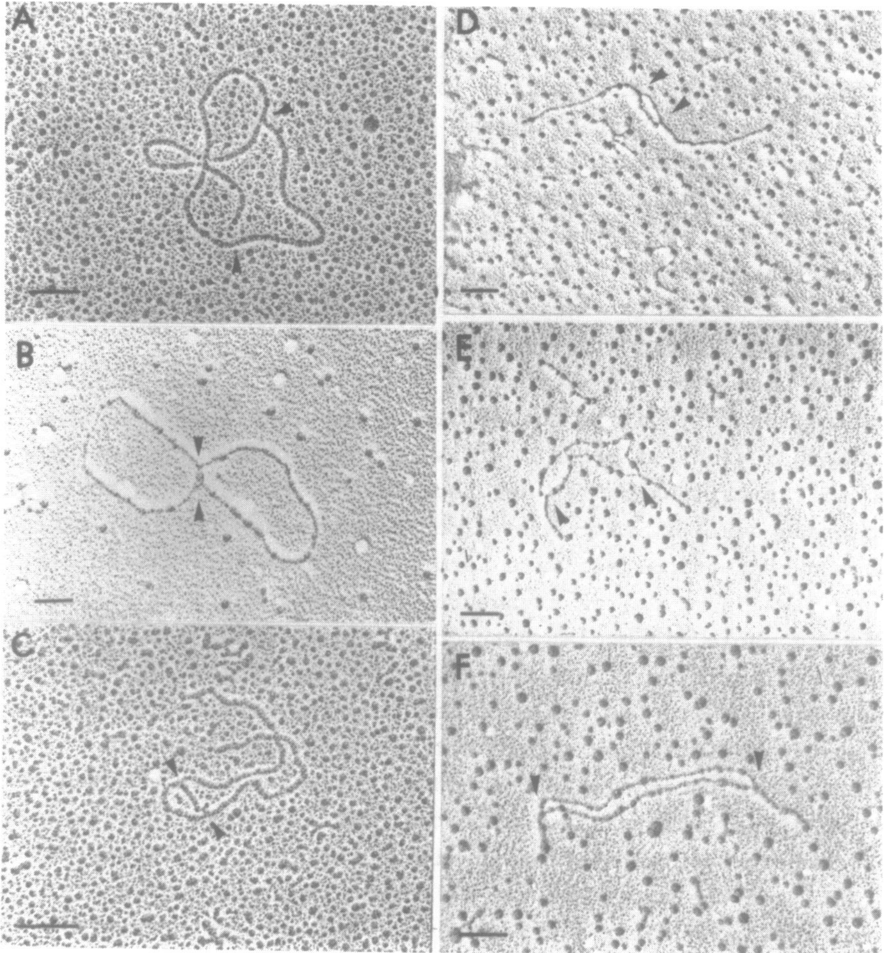

Figure 4. Electron microscopic visualization of plasmid pPHR21 replication intermediates generated in vitro. Purified plasmid DNA was subjected to in vitro replication for 2 hrs and prepared for electron microscopy as described in Materials and Methods. Samples A-E were generated with an early-S nuclear extract; sample F with a prophase cytoplasmic extract. A,B: replication intermediates uncleaved with restriction enzyme. C-F: replication intermediates treated with Pvu II, which cleaves approximately opposite the rDNA insert. Bars=1 kb.

replication bubble relative to the center of each linear molecule. If replication proceeds bidirectionally, and assuming approximately equal rates of fork progression, the center of each bubble will map closely to the initiation site. Bidirectional

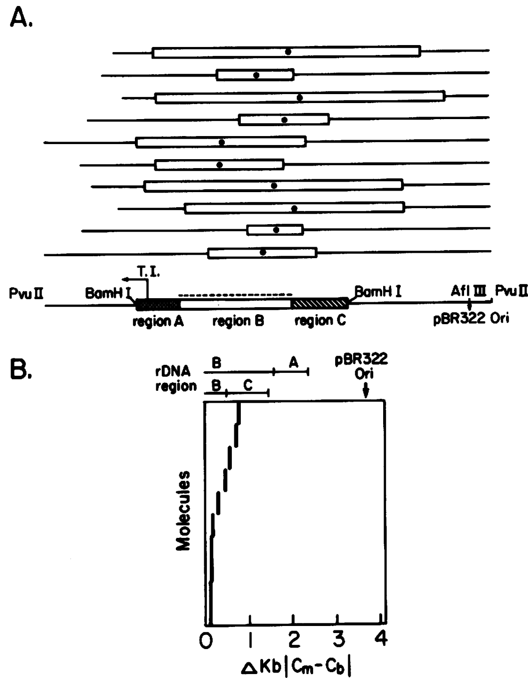

Figure 5. Electron microscopic mapping of replication bubbles in linearized pPHR21 plasmid DNA. Plasmid DNA was subjected to *in vitro* replication, linearized with Pvu II and spread for electron microscopy as described for Fig 4. Linear DNA molecules (8.2 kb ±15%) with replication bubbles were recorded. No molecules were seen that had more than one replication bubble. A: Depiction of 10 molecules, as in Fig 4, D-F, measured and positioned with long arms at the right above a map of Pvu II-linearized pPHR21. Dots mark the centers of bubbles. Regions A-C are as previously described (11). The dotted line marks the region of repeated 31 bp elements. B: Mapping positions of replication bubbles. The absolute value of the difference between the positions of the center of each bubble (C_b) and the center of its molecule (C_m) was determined. Positions of *Physarum* rDNA regions and the pBR322 origin of replication are shown at top.

replication is indicated by the configurations of bubbles shown in Fig 4, D-F and mapped in Fig 5: while the centers of bubbles are localized narrowly relative to the PvuII site, the distal forks range in position outward to near both ends of the molecule (Fig 4F). The centers of bubbles map to a position within the rDNA insert as shown for 10 molecules in Fig 5A. No bubbles were seen that correspond to the position of the pBR322 origin of

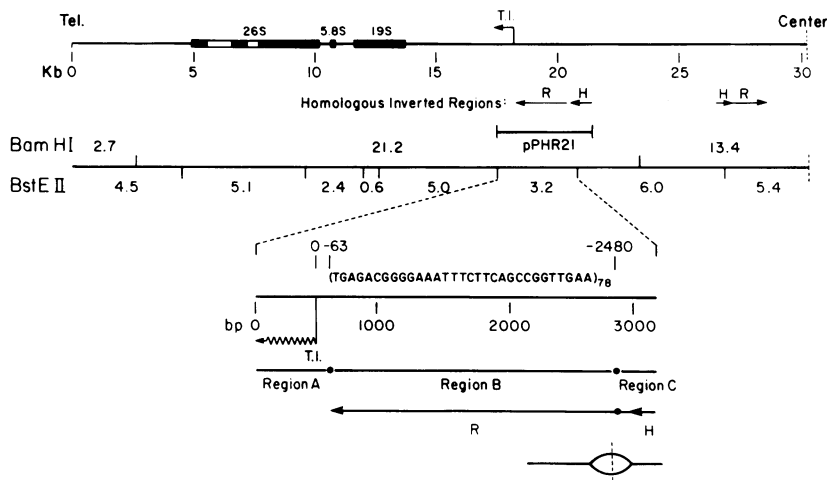

Figure 6. Replication initiation sites in the Physarum rDNA molecule. A restriction map of half of the 60 kb rDNA palindrome is shown with the 3.2 kb Bst EII fragment of pPHR21 expanded at bottom. T.I. = the initiation site of ribosomal RNA transcription. R = a region of 31 bp repeat elements (the consensus sequence is that of Ferris [14]). H = a region repeated once with inverted homology. R and H are in approximate locations of origins of replication in vivo (4). The bubble at bottom indicates the average position of initiation sites mapped by electron microscopy in plasmid pPHR21.

replication. The precision of mapping is sufficient to allow us to identify rDNA sequence regions that could contain the initiation site. Regions A, B and C, previously characterized and sequenced by Ferris and Vogt (11, 14), are positioned in the bars above Fig 5B. The center of the pPHR21 molecule is represented by point 0 on the abcissa, and regions of the rDNA insert in both directions from the center are represented by the bars above the plot of Fig 5B, both to the right of point 0 since absolute values of C_m - C_b are plotted. Region B contains 78 tandem repeats of an element with a 31 bp consensus sequence. Region C contains a more complex sequence which is repeated on the rDNA molecule in inverse orientation approximately 6 kb nearer the center of the molecule, adjacent to another series of B repeats extending toward the center. No bubbles seen map to the A region. Approximately 80% of bubbles seen map to the B region, forming a cluster centered within 200 bp of the border between

the B and C regions. Fig 6 summarizes the relationship of this site to the transcription initiation point and to repeats near the more central origin of replication.

DISCUSSION

Physarum offers two great advantages for studies of initiation of replication in eucaryotes. Firstly, The Physarum rDNA molecule is highly repeated and constitutes approximately 2% of the total DNA in plasmodial nuclei (5). Thus any initiator proteins required for replication may be present at high concentrations in extracts. Secondly, plasmodia grow naturally synchronously, and one can make extracts at cell cycle points when components of the rDNA replication apparatus are maximally accessible. This may have worked in our favor with the extract from prophase (Fig 3), as discussed below. In vitro replication of pPHR21 provides an assay for potential initiator proteins. At certain cell cycle points these should be concentrated in nucleoli, which contain only rDNA and associated proteins (1).

Replication in vitro has previously been reported for yeast (22-24). Although selective initiation was observed, the usefulness of these systems in dissecting the initiation machinery has been hampered by low efficiencies, possibly due to limiting quantities of initiator proteins for yeast replication (25). We have carefully controlled here for a pitfall noted with some yeast in vitro systems, i.e. the presence of adventitious primers on plasmid DNA, amplified in E. coli, that initiate artefactually (12). None of our plasmid preparations showed any template activity with the Klenow fragment of E. coli DNA polymerase I whereas plasmids containing adventitious primers do show such activity (12). Initiation on plasmid pPHR21 occurs at the same site as on rDNA prepared from Physarum, and no RNA in Physarum is homologous to this region (3).

Extracts prepared during prophase and early S are the most active studied with plasmid pPHR21 (Fig 3). It is known that Physarum thymidine kinase activity increases at about 1.25 hrs prior to metaphase and is maximal just after telophase (26). Nuclear DNA polymerase levels increase sharply 1 hr prior to metaphase, corresponding closely to thymidine kinase levels (27).

Studies with a cycloheximide block have shown that initiator proteins, required during early S-phase, are synthesized during the early prophase immediately preceding (28). The nucleolus disintegrates during prophase and reforms during the first 1.5 hrs after mitosis (8). In addition to availability of various enzymes discussed above, it is conceivable that high activity of extracts from the period bracketing mitosis results from the ready extractability of essential components that are during S-phase more tightly associated with chromatin.

Origin exclusion in *Physarum* rDNA presents an intriguing problem. How is it that only one of the four potential origins--and only one of two identical versions--is used to initiate on a given molecule? It has recently been reported that most rDNA molecules are hypomethylated in the region of one of the four potential origins, raising the possibility that methylation could play a role in exclusion (29). Our data suggest that the potential role of methylation may now be accessible to analysis *in vitro*. Since *Physarum* rDNA is an extrachromosomal replicon, it may not be subject to the same constraints, regarding cell cycle timing, that govern origins of replication oriented along a chromosome. For example, there is evidence for a difference in replication-transcription coupling for rRNA genes vs. protein-coding genes in the *Physarum* cell cycle (30). At this point no origin of replication near a protein-coding gene has been definitively identified in Physarum.

Characteristics of *Physarum* rDNA replication differ from those recently reported for ribosomal genes of yeast. In that organism most rDNA is replicated unidirectionally by forks moving in the direction of transcription (31, 32). In *Physarum* the rDNA is replicated bidirectionally both *in vivo* and *in vitro*. It should be noted that the ribosomal genes of yeast are tandemly reiterated whereas these genes in *Physarum* are located on each rDNA molecule as pairs with inverse orientation. Thus bidirectional replication of *Physarum* rDNA does result in forks progressing in the direction of transcription.

Initiation sites for replication of *Physarum* rDNA *in vitro* correspond to two regions previously mapped approximately *in vivo* (4). Here we show that a plasmid clone containing one of these

regions also initiates selectively in plasmodial extracts. We localize this initiation site, which is the more distal one on the rDNA molecule, to a position approximately 2.4 kb upstream of the point of initiation of ribosomal RNA transcription (Fig 6). Deletion mutant plasmids have now been constructed to determine which sequences in this region are critical for initiation. It remains to be determined whether the repeat sequences bridging the transcription and replication initiation sites play any role in regulation of either. Multiple enhancer-like repeats bridging the analogous sites in Tetrahymena rDNA are believed to function as binding sites for positive regulators of rDNA replication (33). The system described here offers a unique opportunity to dissect control of replication of a linear replicon with multiple, distinct origins.

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