Preferential binding of S-phase proteins to temporally-characteristic units of replication in *Physarum polycephalum*

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ABSTRACT

Synchronous plasmodia of *Physarum polycephalum* were pulse-labeled with 3H-thymidine in early or late portions of the S-phase, and the binding capacity of the replicated DNA for isochronous S-phase plasmodial proteins assessed by nitrocellulose filter binding assay. Replication units replicating during the first one-third of the S-phase preferentially bind cytosol proteins present in plasmodia engaged in early S DNA replication, while late S replicating DNA exhibits a corresponding preferential binding of plasmodial proteins present only in late S plasmodia. Temporally-characteristic nascent replication units were isolated by Hydroxylapatite column chromatography and were found to contain binding sites for isochronous proteins.

INTRODUCTION

In the slime mold, *Physarum polycephalum*, nuclear DNA molecules replicating in any given one-fifth of the plasmodial S-phase are only replicated again in the same one-fifth of subsequent S-phases (Braun & Willi, 1969)². Programmed DNA synthesis during the S-phase has also been demonstrated in other eukarotic cell types (Plaut, 1969³; Mueller & Kajiwara, 1966⁴; Braun et al., 1968¹; Adegoke & Taylor, 1977)⁵. Previously, we showed that the replication order of late S replication units in *Physarum polycephalum* can be advanced by a cytoplasmic diffusible substance present only in late S plasmodia (Wille & Kauffman, 1975)⁶. Presumably, the putative phase-specific diffusible substance is a short-lived protein(s), although this has not been rigorously established. In *Physarum*, both the initiation and continuance of programmed DNA synthesis during the S-phase require short-lived proteins (Cummins & Rusch, 1966;⁷ Muldoon et al., 1971)⁸. Recently, by employing a cycloheximide-resistant strain of *Physarum polycephalum*, short-lived proteins were shown to be directly involved in three steps of DNA synthesis, the formation of primary (Okazaki size fragments) replication units, the ligation of primary units into secondary ("replicon" size) units, and the ligation

of secondary units into mature DNA. (Funderud & Haugli, 1977)⁹. However, the question of which, if any, of these proteins are control elements for the "clock" regulating sequence programming of DNA synthesis during the S-phase remains unanswered.

If specific short-lived proteins are responsible for the regulation of programmed DNA synthesis as our previous results suggest, then one possible mode of action for them is to trigger the start of specific replication units via the recognition of target replicon binding sites. We have explored this possibility by investigating the DNA-binding affinity of temporally-characteristic replication units with plasmodial S-phase proteins. In this paper we show that early S and late S replicating DNA molecules possess physically separable binding sites for isochronous S-phase proteins.

MATERIALS AND METHODS

Culture techniques: The wild type strain, M₃CIV, of the slime mold, Physarum polycephalum, was grown as microplasmodial cultures, and as surface macroplasmodia as previously described (Wille & Kauffman, 1975). The cell cycle time was 12 hours.

Isotopic labelling: Surface macroplasmodia were pulse labelled by transfer to culture medium containing 10 μ Ci ³H-thymidine (67 Ci/mM, NEN, Boston, Mass.) per ml of culture fluid for 60 minutes either at the start of the S-phase, i.e. early S, or 90 minutes after the start of synchronous S-phase, i.e., late S. The time of synchronous metaphase was chosen as the reference point for the start of the S-phase. For the nasent DNA experiments, 2 cm² pieces of surface macroplasmodia were cut from the parent plasmodia and transferred along with their underlying millipore filter paper to petri dishes containing 200 μ Ci of ³H-thymidine in 0.2 ml of sterile water for 10 minutes at room temperature. The parent plasmodium had been grown for two full cell cycles in culture medium containing 0.5 μ Ci/ml of ¹⁴C-thymidine (54.7 Ci/mM, NEM, Boston, Mass.), and placed prior to pulse-labelling during the next S-phase on culture medium for 6 hours of the last G₂-phase in order to remove excess ¹⁴C-radioactivity.

Preparation of Nuclear DNA: At the completion of the labelling period, plasmodial tissue was washed briefly by submersion in ice-cold distilled water and nuclei isolated by the methods of Mohberg and Rusch (1971).¹⁰ Purified nuclei were incubated for 30 minutes with 10 μ g/ml RNAase, lysed by addition of 0.2% sarkosyl, and the lysate incubated at 37° for 16 hours with 2mg/ml pronase. Following pronase digestion, the lysate was extracted

with a equal volume of phenol (saturated with 10 mM Tris-HCl, pH=7.2 containing 1 mM Na₂EDTA) and the DNA fibers collected from the aqueous phase by addition of 2 volumes of 95% ethanol. The DNA fibers were washed by ethanol rinses and resuspended in 10 mM Tris-HCl, pH=7.2, 1mM Na₂EDTA solution. Purified DNA as prepared above contained negligible amount of protein as determined by the Lowry test for protein, less than 5% RNA by the orcinol test for RNA.

Preparation of nascent replicating DNA: After the completion of the 10-minute ³H-thymidine pulse labelling period, the 2 cm² pieces of plasmodia were washed by dipping in ice-cold distilled water, and frozen by submersion in liquid nitrogen. Nuclei were isolated by the following modification of the standard procedure: To ensure high recovery of the labelled nuclei, excess "carrier" unlabelled isochronous S-phase plasmodial nuclei were added to the labelled nuclear pellet, and the combined nuclei carried through the isolation procedure. Purified nuclei were lysed as described above, and spun for 15 minutes at 20,000 RPM in a refrigerated centrifuge to remove debris. The clarified supernatant was dialysed overnight against several changes of 0.01 M phosphate buffer, pH=6.8. The dialysate was passed thru a 25G hypodermic needle 5 times to shear the nuclear DNA, and loaded on 1 g of hydroxylapatite column (20 cm x 0.6 cm) which had been pre-equilibrated by washing with 0.01 M phosphate buffer, pH=6.8. Figure 1 shows the result of step-wise elution of the HA column for one such nuclear lysate with increasing phosphate molarities. The bulk of the ¹⁴C-radioactivity eluted with material found under peak II, which eluted with 0.4 M phosphate buffer, while substantial amounts of the ³H-radioactivity eluted under both peak II and peak I, which was eluted with 0.2 M phosphate buffer. The individual fractions collected under peaks I and peaks II were separately pooled, and their ³H/¹⁴C ratios determined by scintillation counting of 20 µl aliquots dried on to millipore filters. Peak II contains only double-stranded DNA as determined by comparison of the nitrocellulose filter retention of radioactivity of heat denatured (100°C, 5 minutes) versus unheated peak II material, i.e., no counts were retained in the unheated, and all of the input count were retained in the heated samples. In contrast, material eluting under peak I possessed partial single-stranded DNA, i.e., approximately 50% of the input count were bound on nitrocellulose filters in unheated samples, while all of the input counts were bound in heated samples. Enrichment for nascent replicating DNA molecules is evident from the two-

1. Separation of nascent DNA by hydroxylapatite column chromatography. After 2 generations of growth in medium containing 2- ^{14}C -thymidine, plasmodia were pulse-labeled ^3H -thymidine for the first 10 minutes of the next S-phase, and a nuclear lysate prepared as described in Methods. The material eluted off at 0.2 M phosphate buffer, pH=6.8 is shown as peak I, and is enriched in pulse-labeled DNA by 4-5 fold over the ^3H -labeled DNA molecules eluting off the column at 0.4 M phosphate buffer, (peak II). ●—●, ^3H -cpm; ○—○, ^{14}C -cpm.

five-fold higher $^3\text{H}/^{14}\text{C}$ ratios routinely obtained in the pooled material eluted in peak I, as compared to peak II.

Preparation of cytosol protein extracts from timed-plasmodia: Well-timed synchronous macroplasmodia were collected at different times in the S-phase and G2-phase by scraping plasmodial tissue off their underlying millipore filter, and immediately freezing by submersion in liquid nitrogen. Hereafter, all extraction procedures were performed at 4°C . To the thawed plasmodial tissue was added 4.0 ml of ice cold buffer A (0.5 M NaCl, 0.01 M Tris-HCl, pH=8.1, 0.01 M MgSO_4 , 1 mM EDTA, 0.014 M mercaptoethanol) and homogenized by blending 30 sec. in a microblender, "low" speed. The homogenate was centrifuged at 20,000 g for 10 minutes to remove most particulate matter, and polyethylene glycol 6000 added to a final concentration of 10%. After 30 minutes, the solution was centrifuged at 10,000x g to remove the precipitate. The supernatant was dialyzed for 24 hours against several changes of 50 volumes of buffer A which had 0.05 M NaCl and 10% glycerol. The dialysate was centrifuged for 15 minutes at 20,000 g to remove the precipitate, and the supernatant was used in all

further DNA-protein binding assays as crude "cytosol" plasmodial protein.

DNA-protein binding assay: The specificity of *Physarum* DNA binding proteins was studied by the use of the nitrocellulose filter assay system for detection of protein-DNA complexes (Riggs et al., 1968). Each filter binding assay consisted of two consecutive 10 minute incubations at 23°C. First 0.1 ml of calf thymus DNA (50 µg in 50 mM NaCl buffer A containing 15% dimethylsulfoxide) was incubated with 0.2 ml of *Physarum* cytosol protein at various concentrations, then 20 µl of ³H-labelled early S or late S *Physarum* DNA was added, and the entire mixture filtered through a nitrocellulose filter (S & S, type B-6, 25 mm, Sartorius), which had previously been washed in buffer A. The filters were washed three times with 0.33 ml of ice cold buffer A, dried, and counted in a liquid scintillation counter. To control for non-specific protein binding to *Physarum* DNA, parallel binding assays were carried out with bovine serum albumin instead of the cytosol proteins, and the amount of non-specific binding to BSA controls subtracted as background from the cytosol protein binding to *Physarum* DNA. The first incubation step with calf thymus DNA was employed to control for non-specific binding of *Physarum* cytosol proteins with any DNA. The ratio of thymus DNA added was 50 times that of the added ³H-labelled *Physarum* DNA.

Colorimetric determinations: DNA was determined by the diphenylamine method of Burton (Burton, 1956).¹³ Protein was determined according to Lowry et al., (1951)¹⁴ using bovine serum albumin as standard.

RESULTS AND CONCLUSIONS

1. Specificity of S-phase cytosol proteins for early S and late S DNA.

The technique of nitrocellulose filter binding of DNA-protein complexes has been employed to demonstrate the specific interaction of *Lac* repressor protein with the operator region of the *Lac* gene in *Escherichia coli* (Riggs and Bourgeois, 1968)¹² and for detecting species-specific DNA-binding proteins in many eukaryotic DNAs (Sevall et al., 1975).¹⁶ In our studies the technique of DNA-protein filter binding was modified to permit detection of temporally-specific protein interaction with replicating *Physarum* nuclear DNAs. Control experiments established: (1) that amount of radioactive DNA bound per filter was linear with increasing amounts of DNA at a constant sub-saturating level of plasmodial protein, (2) that pancreatic DNASE I (10 µg/assay) reduced the amount of DNA bound to background levels, while RNASE (10 µg/assay) has no effect on the amount of DNA bound, and (3) the binding of *Physarum* DNA to plasmodial proteins was optimal at 50

mM sodium chloride in the binding medium. The latter concentration of salt was used in all of the reported DNA-protein binding experiments.

Figure 2 (A panel) shows a typical result when increasing amounts of either early S cytosol protein, prepared 42 minutes after mitosis,

2. Binding characteristics of *Physarum* cytosol proteins to early S and late S replicating *Physarum* DNA. A. Different cytosol proteins were bound in the nitrocellulose filter assay to 1 μg of ³H-labeled early S replicating DNA, pulse-labeled for the first 60 minutes of the S-phase (specific activity = 6.5×10^3 cpm/μg). B. Different cytosol protein preparations were bound in the nitrocellulose filter assay to 1 μg of ³H-labeled late S replicating DNA, pulse-labeled for 60 minutes 90 minutes after the start of the S-phase. (Specific activity = 9.5×10^3 cpm/μg). Cytosol proteins were prepared from plasmodia at 42 minutes after synchronous mitosis (○—○), or 125 minutes after mitosis (●—●), prophase cytosol proteins were prepared from plasmodia 15 minutes before synchronous metaphase. (▲—▲). Each point represents the mean of triplicate determinations, minus the background counts for bovine serum albumin binding to *Physarum* labeled DNAs, which was always less than 10% of the input counts.

(M+42 protein), or late S cytosol protein, prepared 125 minutes after mitosis, (M+125 protein), were incubated in the binding assay with early S replicating DNA, prepared by pulse-labeling plasmodia for the first 60 minutes after mitosis with ^3H -thymidine followed by isolation and purification of nuclear DNA. Early S protein saturates early S DNA at 18% of the input Physarum DNA, while late S protein saturates early S DNA at roughly one-half the early S protein level of saturation. Hence, early S replicating DNA preferentially binds early S plasmodial proteins. Conversely, it can be seen (Figure 2, B panel) that M+125 protein saturates late S replicating DNA, pulse-labeled for 60 minutes with ^3H -thymidine 90 minutes after mitosis, at 22% of the input Physarum DNA, while M+42 protein now only reaches saturation on late S DNA at 8% of the total input DNA.

Additional experiments (not shown) revealed that plasmodial protein preparations, M+30 protein and M+110 protein, had the same binding properties on early S and Late S replicating DNAs as M+42 protein and M+125 protein, respectively. Likewise, different preparations of ^3H -labeled early S and Late S DNAs displayed preferential binding to their corresponding isochronal S-phase cytosol proteins.

Figure 2 also shows the result of comparing the binding of prophase-proteins with early S and late S replicating Physarum DNAs. Prophase-proteins bind linearly with increasing amount to early S DNA over the range of concentrations tested, while they saturate on late S DNA roughly (7%) to the same extent as M+42 protein. This result implies that just after nucleolar breakdown and prior to the initiation of DNA synthesis a substantial amount of early S phase DNA-binding proteins are present in pre-metaphase plasmodia. Figure 3 shows that cytosol proteins prepared from plasmodia in early G-2, (5 hours after synchronous mitosis), bind to late S DNA at the same level as M+42 protein (12%) and bind early S DNA about as well as M+125 protein. The latter result suggests that late S protein binding on early S DNA and early S protein binding on late S DNA is a general non-specific DNA-protein binding of Physarum proteins on Physarum double-stranded DNA, unrelated to S-phase specific protein binding to replicating DNA. As a further comparison of specificity of Physarum cytosol S-phase proteins binding to Physarum DNA, the relative binding capacity of early S and late S replicating Physarum DNA to purified total calf thymus is shown in figure 4. Both early S and late S Physarum DNAs were bound linearly with increasing amounts of calf thymus histone with

3. Comparison of the binding characteristics of S-phase and G2-phase cytosol proteins to early S and late S replicating *Physarum* DNA. Binding of late S cytosol protein, prepared from a plasmodium 125 minutes after mitosis, to late S DNA (●—●), or to early S DNA (○—○); binding of Mid-G2 cytosol proteins to either late S DNA (▲—▲), or to early S DNA (△—△). Conditions for binding assay were the same as in legend of figure 2.

nearly identical efficiency over the range of 3 to 30 ug histone. Figure 5 compares the specificity of cytosol proteins for a heterologous ³H-labeled DNA, *Tetrahymena pyriformis* (W). Surprisingly, steady state labeled *T. pyriformis* binds both early S cytosol and late S cytosol proteins about equally well (saturated at 20% of input DNA).

Taken as a whole, the above results clearly establish that synchronous plasmodia at many different times in the S-phase contain short-lived proteins which bind to temporally-characteristic regions of DNA molecules currently being replicated.

2. Cytosol protein binding to nascent DNA. We have investigated the binding affinities of *Physarum* cytosol proteins with rapidly-synthesized or nascent, DNA molecules isolated from plasmodia which had been pulse-labeled for 10-minute intervals at different times in the S-phase. In *Physarum*, as in other eukaryotic organisms, DNA synthesis proceeds through the formation of short DNA chain intermediates. (Tseng and Goulian, 1975)¹⁶ and advantage has been taken of their single-stranded properties to separate them from the bulk nuclear DNA by hydroxylapatite column fraction-

4. Nitrocellulose binding of total calf thymus histone with $1\mu\text{g}$ of ^3H -labeled early S Physarum DNA (○—○), or to $1\mu\text{g}$ of ^3H -labeled late S Physarum DNA (●—●). Binding assay modified by omission of the preincubation step with calf thymus DNA. Otherwise identical with conditions given in legend of figure 2.

5. Binding characteristics of Physarum cytosol proteins with ^3H -labeled Tetrahymena pyriformis (w) DNA. Nitrocellulose binding assay contained $1\mu\text{g}$ of steady-state labeled T. pyriformis DNA and increasing concentrations of cytosol proteins extracted from plasmodia 30 minutes after mitosis (○—○), or extracted from plasmodia 110 minutes after mitosis (●—●).

ation after very short labeling times (Sato, et. al., 1972)¹⁷. Indeed, when *P. polycephalum* is pulse-labeled for up to 20 minutes with ³H-thymidine, the shortest labeled DNA strands contain RNA (Wagar et al., 1975)¹⁸. The aim of our experiments was to ascertain if isolated nascent DNA of *P. polycephalum* also contain the binding sites for S-phase cytosol proteins. Plasmodia pulse-labeled for 10 minutes over the first one-third of the S-phase had from 1.5 to 5 times higher ³H/¹⁴C in the single-stranded DNA than the double-stranded DNA (see figure 1). The single-stranded DNAs pooled from Peak 1 of column eluates were used in the DNA-protein binding assay to compare their respective binding capacity to S-phase cytosol proteins. Although the pulse-labeled DNAs in Peak 1 are enriched in single-stranded DNA molecules and have a high background level of binding to nitrocellulose filters, independent measurements of their binding before and after heat denaturation revealed that they had considerable secondary structure, as might be expected for putative replication intermediates. Figure 6 shows the result of comparing the binding capacity of nascent early S DNA, pulse-labeled for the first 10 minutes of the S phase, with M+1 cytosol proteins,

6. Binding characteristics of *Physarum* S-phase proteins to nascent replicating *Physarum* DNA. Plasmodial cytosol proteins were prepared from plasmodia at either 1 minute after synchronous metaphase, M+1 protein (●—●), or from plasmodia at 40 minutes after synchronous mitosis, M+40 protein (○—○), and incubated with 0.01 μg of early S replicating DNA (specific activity + 1.32×10^4 cpm/μg), prepared from plasmodia which were pulse-labeled for 10 minutes at the start of the S-phase, and enriched for nascent replicating intermediates as described in Methods. Each point represents the mean of triplicate determinations and plots only the tritium radioactivity of the DNA.

and M+40 cytosol proteins prepared from plasmodia either 1 or 40 minutes after mitosis, respectively. The preference of early S nascent DNA replicated in the first 10 minutes of S phase for cytosol proteins present in plasmodia immediately after metaphase is clearly established with M+1 protein saturating at 28% of the available input DNA. On the other hand, Figure 7 shows that early S nascent DNA, pulse-labeled for 10 minutes 30 minutes into S-phase is preferentially bound by M+40 protein, reaching saturation at 38% of the input DNA, while M+1 protein saturates at 19% of the input DNA. Figure 7 also shows that plasmodial protein prepared 67 minutes in S-phase only does as well as M+1 protein on early S DNA, replicating at 40 minutes into the S-phase. The results with nascent pulse-labeled early S DNA firmly establish that the binding sites for diverse S-phase plasmodial proteins are both temporally and physically separable and are related to the scheduled time of replication of the units of replication themselves.

In conclusion, it seems likely that the eukaryotic genome is organized into units of replication which contain specific binding sites for short-lived proteins, which appear serially during S in conjunction with their scheduled time of replication. In Physarum, the phase-specific DNA-binding

7. Binding characteristics of Physarum S-phase proteins to nascent replicating Physarum DNA. Cytosol Physarum protein extracts were prepared from plasmodia at either 1 minute after synchronous metaphase, M+1 protein (●—●), or from plasmodia at 40 minutes after synchronous mitosis, M+40 protein (○—○), or 67 minutes after mitosis (▲—▲), and incubated with 0.01 µg of early S nascent replicating Physarum DNA (specific activity = 1.6×10^4 cpm/µg), which was prepared from plasmodia pulse-labeled for 10 minutes 30 minutes after the start of the S-phase, and enriched for nascent replicating DNA as indicated above. The binding assay was the same as that described in the legend of Figure 6.

proteins decay with half-lives on the order of 20 minutes and are not synthesized earlier than 30 minutes prior to the time their target binding sites undergo replication. It would thus be of great interest to examine the non-histone chromosomal proteins synthesized during the S-phase for their binding capacity to "chromotypic" nuclear DNA as others have found them to contain specific DNA-affinity proteins only present in the S-phase of the mammalian cell cycle (Salas & Green, 1971¹⁹; Fox and Pardee, 1971)²⁰.

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