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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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Selective Initiation of Replication at Origin Sequences of the rDNA Molecule of *Physarum polycephalum* Using Synchronous Plasmodial Extracts

by

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Abstract

Replication Initiated *in vitro* at Origin Sequences of the rDNA Molecule of *Physarum polycephalum*

by

Dianne C. Daniel

Adviser: Professor Edward M. Johnson

A cell-free system using synchronous plasmodial extracts initiates DNA replication selectively on the 60 kb rDNA palindrome of *Physarum polycephalum*. Initiation occurs at two positions corresponding to *in vivo* origins of replication estimated by electron microscopy. The system also initiates selectively within a plasmid, pPHR21, containing one of these origins. In this plasmid bubbles expand bidirectionally and generate DpnI-resistant DNA. Whole cell extracts made at prophase or early S phase, times when the nucleolus is disorganized, are most active in pPHR21 replication. An early S-phase nuclear extract accumulates replication intermediates, which are visualized at a level three fold higher than with the whole cell extract. Using the nuclear extract, selective labeling of restriction fragments locates the initiation point in a 3.2 kb BstEII fragment. Centers

of bubbles mapped by electron microscopy cluster at the upstream border of a series of 31 bp repeats 2.4 kb from the initiation point for ribosomal gene transcription. Two-dimensional gel electrophoretic fractionation and electron microscopic analysis of rDNA replication intermediates, both *in vivo* and *in vitro*, provide evidence for an unusual secondary structure in early replication bubbles. Deletion mutants have been constructed in the origin region to help identify sequence elements regulating the initiation event.

DEDICATION

This work is dedicated posthumously to my beautiful mother Grace Blanks Daniel.

ACKNOWLEDGEMENTS

in reverence for that universal cornucopia from which pours forth the fruits of all our labor and without which man would be truly forlorn

I wish to acknowledge with grateful appreciation the contributions of those who made possible the completion of this document:

my father for his joie de vivre and grand sense of humor

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my mentor Dr. Edward M. Johnson for providing the initial challenge to work on DNA replication in Physarum, for allowing independence in scientific investigation and for guidance that allowed me to grow from a student to a professional.

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LIST OF TERMS AND ABBREVIATIONS

1. in vivo: In the present context, this means in an intact organism, including those grown in culture media, either syncytial or amoebal, where nuclei have not been disrupted from their original cytoplasm.
2. in vitro: In the present context, this means in a system free of all intact organisms, either syncytial or amoebal. For this study, in vitro also implies the absence of intact nuclei or nucleoli.
3. rDNA: For this study, rDNA refers to the Physarum 60 kb DNA molecule, which includes the 19S, 5.8S and 26S ribosomal RNA coding regions and their associated transcribed and nontranscribed spacers and introns.
4. whole cell: refers to whole plasmodia in contrast to isolated nuclei.
5. cis: sequences covalently linked as part of a contiguous DNA molecule.
6. Ca-PO_4: refers to amorphous calcium phosphate precipitate.

7. BrdU: bromodeoxyuridine
8. Kb: 1,000 base pairs of DNA or 1,000 nucleotides of RNA.
9. AluI, BamHI, BclI, BstEII, DpnI, HpaI, MboI, PvuII, XbaI: restriction endonucleases.
10. HEPES: N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
11. Tris: Tris(hydroxymethyl)aminomethane
12. EDTA: ethylenediaminetetraacidic acid
13. SDS: sodium dodecyl sulfate
14. uM, ul, ug: abbreviations as typed are used for micromolar, microliter and microgram, respectively.

INTRODUCTION

Evidence for specific origin sequences in eukaryotes.

The mechanism by which DNA replication initiates at a eukaryotic chromosome origin has been approached by previous investigators in a number of ways. The search has been ongoing for specific nucleotide sequences which might comprise a functional origin. Identification of a eukaryotic replication initiation protein, which would allow characterization of controlling sequences, has remained the most elusive component. Such a protein entity is believed, by analogy to bacterial (Kornberg, 1988) and viral (Depamphilis and Bradley, 1986; Tooze, 1980) systems to bind to specific genomic sequences to begin the process leading to the first deoxynucleotide coupling. The most success in dissecting specific initiation sites has been achieved with both *in vivo* and *in vitro* studies of replication origins in the papovaviruses, SV40 and polyoma, which code for their own initiator proteins while utilizing eukaryotic cellular machinery to continue their replication (Tegtmeyer *et al.*, 1988; Depamphilis *et al.*, 1988; Kelly, 1988; Stillman *et al.*, 1985). According to present theory, eukaryotic replication machinery would be the same as

that used by viruses except for the origin-specific initiation event. Sequence homologies to the viral origins have not translated into locating a functional eukaryotic origin. There are many recent references on viral DNA origins, and since they may not all be relevant to chromosomal origins, the topic is not considered in detail here. Two recent reviews cover this subject (DePamphilis, 1988; Kelly, 1988).

Studies with the 2 um plasmid and with autonomously replicating sequence (ARS) elements that confer autonomous replication on plasmids transfected into yeast have led to a yeast ARS consensus sequence (Broach et al., 1983). This sequence correlates with known viral origins only in terms of the requirement for a flanking A-T rich region thought to function in DNA bending (Snyder et al., 1988) and\or origin-specific DNA unwinding (Umek and Kowalski, 1988). However, the lack of an initiator protein or initiation complex has thus far hindered characterization of initiation. Work by Jazwinski and Edelman (1982) detecting a putative initiation complex in vitro, as visualized by electron microscopy, has made a promising approach.

Some early interest has focused on the Alu repeat

family as potential origins of mammalian DNA replication because of homologies to sequences at DNA viral origins (Jelinek et al., 1980). In 1984, Ariga reported initiation of replication within an human Alu element contained within the construct BLUR8 in a soluble extract system previously developed for T antigen-promoted initiation of SV40 replication (Ariga and Sugano, 1983; Ariga, 1984). However, when utilizing a somewhat different system for in vitro replication, Li and Kelly (1985) were not able to reproduce replication of BLUR8. The reason for this difference has not been clarified. Support for the involvement of Alu sequences in human chromosome origins of replication has come from a trioxsalen crosslinking experiment involving extrusion of short DNA fragments from early replication bubbles generated by the diverging bidirectional replication forks between crosslinks (Anachkova et al., 1985). These nascent strands contain an abundance of BLUR8 Alu sequences represented in amounts larger than expected based on their genomic frequency. Five out of nine clones obtained by a branch migration method for nascent strand extrusion from replication loops also contain Alu sequences (Kaufman et al., 1985). Initiation within the

human Alu insert of BLUR8 has been shown to occur *in vivo* by Johnson and Jelinek (1986) by a DpnI assay for transient replication of BLUR8 in COS-7 cells at various points after transfection and through electron microscopic mapping of the initiation site. When BLUR8 is disrupted in the sequence with homology to the SV40 T antigen binding site, it does not replicate. Since BLUR8 replicates in COS-7 cells, but not in CV-1 cells, this indicates a role for T antigen participation in initiation (Johnson and Jelinek, 1986). However, T antigen dependent initiation of replication in the Alu insert represents an artificial construct relative to the usual nuclear state of the genome. More recently, Anachkova and Hamlin (1989) have reported the presence of a repetitive DNA element near one of the two initiation sites within the 28 kb locus for initiation of replication downstream from the DHFR (dihydrofolate reductase) gene in CHOC400 cells. The existence of the repetitive element had been noted earlier by Burhans *et al.*, 1986. The early replicating DNA from CHO cells is also highly enriched in the repeated DNA element, supporting previous reports regarding the presence of repetitive DNA sequences near some genomic initiation

sites (Anachkova and Hamlin, 1989). The association of Alu sequences with early replicating Reverse (R) bands, one of three structural sets of regions defining the human karyotype, has been viewed as suggestive evidence for the involvement of Alu sequences in replication (Korenberg and Rykowski, 1988).

Another plasmid bearing a human DNA insert from near the c-myc gene (upstream region) has been reported to contain a chromosome replication origin based on studies showing that it permits autonomous plasmid replication in both HeLa and HL-60 cells (Iguchi-Ariga et al., 1988; McWhinney and Leffak, 1988). Iguchi-Ariga et al., 1987b; 1988) report that c-myc protein binds a specific cellular c-myc consensus sequence, which can perform efficiently as an ARS element in cells expressing the c-myc oncogene. However, the precise location and proof of a chromosomal origin near the c-myc gene remains to be determined.

There are some conditions in which initiation of replication appears to be nonspecific. A temperature sensitive SV40 mutant initiates replication at random sites around the genome at the restrictive temperature in contrast to the sequence specific recognition,

unwinding and initiation events which lead to replication of the SV40 genome or of origin containing constructs at the permissive temperature (Martin and Setlow, 1980). Random initiation of replication occurs on a wide variety of DNA templates microinjected into Xenopus laevis eggs (Harland and Laskey, 1980; Mechali and Kearsey, 1984). Replication occurs after the DNA has acquired a nuclear membrane structure inside the egg cytoplasm or within the cell free egg extract (Blow and Laskey, 1986; Blow et al., 1988). Blumenthal et al. (1974) propose that more sequences can be recognized as origins in Drosophila cleavage nuclei than in blastoderm nuclei of the organism. In Drosophila many changes in replication occur during development of polyploid tissues; where daughter chromosomes are no longer required to segregate, origin selection appears more random in space and time (Spradling and Orr-Weaver, 1987; Spradling and Leys, 1988). These random initiation events may be explained as a response of the replication machinery to the lack of presentation by the replicative environment of conditions necessary for specificity. Such conditions may be developmentally determined as has been suggested for the Xenopus egg and

for Drosophila (see below) or brought about by the removal of a specific determinant or more energetically favorable condition such as the specific recognition sequence for the SV40 initiator protein T antigen. In contrast to the random initiation of replication of SV40 or polyoma viral DNA microinjected into Xenopus eggs (Harland and Laskey, 1980; McTiernan and Stambrook, 1984), the same viral DNA injected into nuclei of mouse embryos requires the specific viral origins in order to replicate (Wirak et al., 1985). The inherent difference in the developmental rates of Xenopus and Drosophila embryos versus mouse embryos is thought to make a critical difference (e.g., different concentrations of replication factors) (Wirak et al., 1988). However, since no replication has been detected for other plasmids lacking the SV40 or polyoma viral origins when injected into mouse embryos, there may be a requirement for unidentified cis-acting sequences in mammalian chromosomes in order for initiation of replication to occur (Depamphilis et al., 1988). Developmental variations have been observed in regard to the exact origin sequence requirements in mouse embryos. The use of different portions of the polyoma origin in one-cell mouse

embryos versus differentiated tissues has been discussed (Depamphilis et al., 1988). Two-cell embryos are already more permissive for polyoma than for SV40 (DePamphilis et al., 1988).

Yeast ARS sequences and potential mammalian ARS sequences.

What argument can be made for participation of specific sequences, or specific functional regions within a stretch of DNA, in the origin-specific recognition event? As mentioned above certain stretches of DNA have been shown to confer autonomous replication on vectors containing selectable markers when they are transfected into cultured cells (see below) or into yeast (Stinchcomb et al., 1979; Stinchcomb et al., 1980). The strength of the ARS activity in yeast has been correlated with the ability of the ARS plasmid vector to transform yeast at high frequency (Stinchcomb et al., 1980). A stable partitioning event during mitosis has been shown to depend on centromeric sequences for circular plasmids and on centromeric and telomeric sequences as well as length restrictions for linear constructs; (Murray and Szostak, 1983; Dani and Zakian, 1983; Szostak et al., 1984). The activity of ARS

elements has not been investigated in a living organism except yeast so that cells proliferating in culture must be analyzed for evidence of mammalian ARS activity. There is one exception: Iguchi-Ariga et al. (1988) report that when the c-myc plasmid, pmyc(H-P), is injected into fertilized mouse eggs, it replicates and is ultimately transmitted as an episome from F0 to F4 transgenic mice. The most likely candidates at the moment for possessing such mammalian ARS activity are a plasmid containing an Alu-family DNA sequence (Johnson and Jelinek, 1986; Ariga, 1984), plasmids containing c-myc gene sequences (McWhinney and Leffak, 1988 and Iguchi-Ariga et al., 1988) and plasmids containing mouse genomic DNA seqences (Frappier and Zannis-Hadjopoulos, 1987). The Alu-insert plasmid BLUR8 is reported to function in the presence of T antigen (Johnson and Jelinek, 1986), and the c-myc plasmid is reported to function in HL-60 cells which express high levels of c-myc protein (Iguchi-Ariga et al., 1988). Transient replication assays in cultured cells have utilized the enzymes DpnI and MboI to detect replication as a transient event in constructs which do not have centromeric sequences (Vovis and Lacks, 1977). This separates the

replication assay from a large number of mitotic partitioning events that lead to loss of input centromeric-lacking plasmid in a long-term assay. This DpnI\MboI assay (see discussion of the DpnI assay on page 66 of this document) has been used for in vivo study of BLUR8 (human Alu insert) replication (Johnson and Jelinek, 1986) and with c-myc plasmids in HL-60 cells (Iguchi-Ariga et al., 1988). Transient DpnI or MboI assays for replication have been used to identify yet other mouse and human sequences which confer autonomos replication on plasmids when they are transfected into mouse and human cells (Frappier and Zannis-Hadjopoulos, 1987; Ariga et al., 1985; Ariga et al., 1987). BrdU-labeling experiments have also been used to identify ARS activity of such sequences both in vivo (Frappier and Zannis-Hadjopoulos, 1987) and in vitro (Ariga et al., 1985; Ariga et al., 1987). In another case (Holst et al., 1988), in vitro autonomous replication has been refuted with the discovery that the plasmids were actually integrated into the genomic DNA as tandem repeats (Weidle et al., 1988; Gilbert and Cohen, 1989). In one case above involving an ARS plasmid containing a putative mouse origin of replication (Iguchi-Ariga et al.,

1987b), the results were not reproduced by another group (Gutierrez et al., 1988). There are reports of the ability of cells producing high levels of c-myc protein to replicate SV40, as well as a plasmid containing mouse sequences apparently somewhat homologous to the SV40 origin, without the need for T antigen (Iguchi-Ariga et al., 1987a; 1987b). As noted above, these reports have not been substantiated; instead a more indirect role for c-myc protein in DNA relication has been suggested (Gutierrez et al., 1988). Should the results with any of the mammalian ARS clones prove reproducible, there is still the need to map these sequences on the chromosomes and to prove their function as origins of replication within the chromosomal environment. In the case of the plasmids containing the upstream c-myc region (discussed earlier) two groups have independently reproduced the same mammalian ARS activity (McWhinney and Leffak, 1988; Iguchi-Ariga et al., 1988). In one case of ARS activity above, recombinant plasmids were obtained from the strand extrusion method (early S phase) for chromosome origin identification mentioned earlier (Kaufman et al., 1985). Interestingly, two of the four recombinant plasmids showing positive ARS activity contain Alu

family sequences, one is from alpha-satellite family DNA and the other one contains unique sequences (Frappier and Zannis-Hadjopoulos, 1987).

In the process of gene amplification, mammalian cells can apparently permit autonomous replication of closed circular molecules which contain sequence specificity and are under cell cycle control, e.g., c-myc in early passage HL-60 cells (Carroll et al., 1987; Von Hoff et al., 1988). Whether this may relate to the ability of the plasmids with c-myc inserts to replicate autonomously in HeLa and HL-60 cells (McWhinney and Leffak, 1988; Iguchi-Ariga et al., 1988) and to be retained in a long term assay in absence of selective pressure (McWhinney and Leffak, 1988) remains to be determined. There is one report that EBNA-I (Epstein-Barr virus nuclear antigen) and the family of tandem repeats from the Epstein-Barr virus oriP can under selective conditions permit long term replication and nuclear retention of plasmid constructs containing human sequences which show ARS activity in 293 cells during short term assay (Krysan et al., 1989).

The yeast ARSs present the strongest case that ARS elements are indeed specific sequences used for

chromosome initiation of replication. The yeast ARS sequence elements in the 2 um plasmid and the ARS1-containing plasmid have been shown via two-dimensional gel electrophoretic technique to be consistent with the origin of replication for these two templates (Brewer and Fangman, 1987; Huberman et al., 1987; Nawotka and Huberman, 1988). Therefore, the yeast ARSs present the strongest case that ARS elements are indeed specific sequences used for chromosome initiation of replication. The core consensus sequence for yeast ARS replicators, within its own variations, may have differing affinity for putative initiator protein as suggested by the evidence that some ARS elements are stronger replicators than others (Umek et al., 1989). At least part of the origin specificity is believed to arise from their flanking DUE (DNA unwinding element), this being explained as an energetically favored event (Umek et al., 1989). However, some ARS relicator sequences do not appear to be utilized as yeast chromosome origins of replication (Linskens and Huberman, 1988; Huberman et al., 1988; Palzkill et al., 1986; Feldman et al., 1984). Origin efficiency or the frequency of a given ARS sequence to act as an origin is therefore thought to

be determined by protein-DNA interactions, as well as template energetics and chromosome context (Umek et al., 1989). However, the cis sequences essential for replication initiation do not regulate replication to once per cell cycle. Cis control has not been demonstrated using ARS plasmids (Umek et al., 1989).

Origin localization utilizing techniques other than autonomous replication.

The two-dimensional gel electrophoretic analysis has since been extended to other stretches of DNA shown to contain ARS elements. These include yeast rDNA (Linskens and Huberman, 1988) and yeast chromosome III (Huberman et al., 1988). The neutral/neutral and neutral/alkaline two-dimensional gel techniques, together, allow identification of the regions of initiation and termination of replication on chromosomes as well as the directions of fork movement (Linskens and Huberman, 1988 and Huberman et al., 1988). The 2 um plasmid and ARS1 plasmid origin regions have been localized (within experimental error) to the ARS element, but another origin identification is more ambiguous (Linskens and Huberman, 1988). Markers were not included in the two-dimensional gel experiments with

SV40, so the specific degree of resolution obtained using of this technique cannot be compared directly to the well-characterized SV40 origin of bidirectional replication, OBR, (DePamphilis et al., 1988) based on the model described by Hay and DePamphilis (1982).

Several other approaches have been used to locate specific origins of replication in chromosomes. Regions of a gene cluster which replicate early and therefore should be located close to an origin of replication have been investigated for the mouse immunoglobulin heavy chain constant region and for the human beta-globin locus (Brown et al., 1987 and Dhar et al., 1988). One such replication timing study suggests that initiation occurs 3' of the IgC_H gene cluster in Friend virus-transformed murine erthroleukemia (MEL) cells, but it does not prove that initiation always takes place at one specific site (Brown et al., 1987).

Two different techniques have been utilized to investigate origin-derived nascent strands released from early replication loops. Strands extruded by branch migration occurring at elevated temperatures (Zannis-Hadjopoulos et al., 1981; Zannis-Hadjopoulos et al., 1985) or by alkaline denaturation after

psoralen-type cross-linking (Russev and Vassilev, 1982) can be purified and studied for origin properties. Some caveats are that ultraviolet\psoralen-type cross-linking has been shown to activate additional origins (Francis et al., 1985) and that nascent strand preparations also contain contaminating non-origin fragments at the level of individual cloned inserts (Francis et al., 1985 and Zannis-Hadjopoulos et al., 1985). However, studies have been carried out on the alpha-globin gene domain (Razin et al., 1986) and the amplified DHFR domain in CHOC400 cells (Anachkova and Hamlin, 1989) utilizing labeled nascent strands for hybridization to various restriction fragments to determine (at the level of restriction fragment resolution) where the origins might be located. Results from the hybridization of extruded oriDNA (nascent strands) to DHFR restriction fragments (Anachkova and Hamlin, 1989) are consistent with labeling of restriction fragments obtained utilizing an in-gel renaturation procedure which eliminated label contributed by single-copy sequences. Two origins regions have been identified downstream of the DHFR gene at the resolution of two restriction fragments of 1.7 and 1.8 kb, respectively, which are separated by approximately

22 kb (Leu and Hamlin, 1989). Burhans et al. (1986) utilizing benzoylated naphthylated DEAE-cellulose (BND) chromatography for purifying replication intermediates with partial single strands from synchronous CHOC400 cells, have mapped an origin zone within the amplified DHFR domain to a 4.3 kb XbaI fragment which agrees with the localization of one of the origins downstream of the DHFR gene (Leu and Hamlin, 1989). Handeli et al. (1989) have confirmed the existence of two origin regions downstream of the amplified DHFR gene in CHOC400 cells through the use of a protein synthesis inhibitor which permits preferential segregation of nucleosomes to leading strands, thereby protecting these strands from micrococcal nuclease digestion. The technique of in vitro runoff in asynchronous nuclei utilizing BrdU and restriction digestion barriers give a result (i.e. possible upstream origin) for the chicken alpha-globin domain (James and Leffak, 1986) that agreeds with the strand extrusion approach mentioned above (Razin et al., 1986). The assumption has to be made that this technique does not cause new initiation and termination sites and that replication continues normally. The approach has also been used to show the direction of

replication through transcriptionally active versus transcriptionally inactive loci for the chicken histone H5 and c-myc genes (Trempe et al., 1988; Leffak and James, 1989). Preferred polarity does not prove initiation occurred at one origin, however (Leffak and James, 1989). These studies suggest that replication can proceed with different polarity depending on cell type specificity, chromosomal location and trans-acting factors (Leffak and James, 1989 and Trempe et al., 1988).

Transcriptional activity correlates with gene replication in the direction of transcription for histone H5, alpha-globin, and c-myc genes (James and Leffak, 1986; Trempe et al., 1988; Leffak and James, 1989). Movement of replication forks in the direction of transcription has been observed to occur in housekeeping genes or genes that are heavily transcribed during S phase where there might be interferance between transcription and replication machinery (McKnight and Miller, 1977; Seidman et al., 1979; James and Leffak, 1986; Brewer, 1988; Trempe et al., 1988; Linskens and Huberman, 1988). Wolffe and Brown (1986) have shown that passage of the replication fork can erase the transcription complex in vitro. This is significant

because otherwise transcription would need to be turned off and on during S phase for replication to occur in some cases.

Controls on replication operate at different levels in eukaryotic cells. There is the point of entry into S phase of the cell cycle, the initiation of replication on subsections of the chromosome, and the initiation of replication units (reviewed by Hand, 1978). This dissertation focuses on initiation of a single replicon, and the above levels of control are beyond the scope of this treatment of initiation of replication. The reference Handeli et al. (1989), discussed above, describes an approach to investigate initiation of replication at the level of nucleosome segregation on nascent strands of individual replicons. Several investigative approaches described above show the polarity of replication fork movement in relation to the direction of transcription. These various levels of regulation and interaction are also discussed by Laskey and Harland (1981).

Distinction between those origins used for amplification and those used for the usual S phase initiation event must be made. Although implicating

the utilization of specific origin regions, it is not clear at this time that origins of amplification, such as those for the DHFR gene (Anachkova and Hamlin, 1989; Leu and Hamlin, 1989; Burhans et al., 1986), the amplified c-myc gene (Von Hoff et al., 1988) or the Drosophila chorion genes (Spradling and Leys, 1988) are also origins of replication for normal unamplified alleles. A recent report by Handeli et al. (1989) suggests that an unamplified DHFR origin in CHO cells is at the same position as the origin of amplification. However, type of integration of transfected vector -- and thus rearrangements relative to termination sites -- are not characterized (Handeli et al., 1989).

A more precise technique for localizing origins of replication is a procedure which maps small replication bubbles through size measurements made on enlarged electron micrographs of replication intermediates generated by high copy number ribosomal DNA (rDNA) genes. This technique has been used to localize the origin regions of the rDNA of Tetrahymena, Physarum and sea urchin relative to the ends or centers of the molecules, thus identifying origin zones within the non-transcribed central spacers of the rDNA of these

organisms (Vogt and Braun, 1977; Truett and Gall, 1977; Cech and Brehm, 1981; Botchan and Dayton, 1982). These more precisely located origin regions are open to further study.

Statement of the thesis problem.

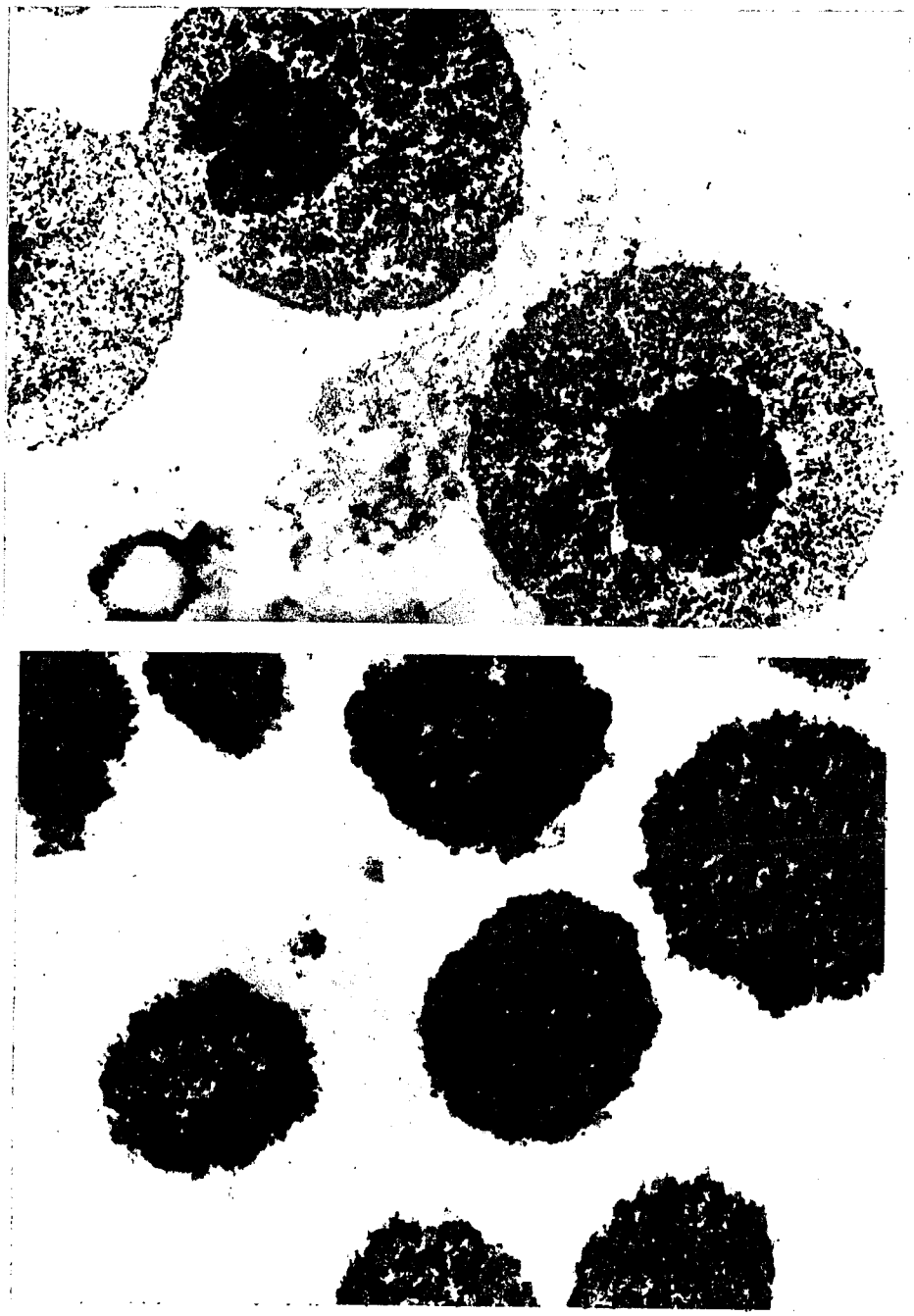
The overall question addressed in this research is: How does replication initiate at a natural chromosome origin? To focus on this question, examination of Physarum presents an advantage because there is natural synchrony of the macroplasmodial life form, and it contains approximately 200-400 copies of a ribosomal DNA (rDNA) minichromosome separated from major nuclear DNA within the nucleolus (Guttes and Guttes, 1969; Braun and Evans, 1969; Zellweger et al., 1972; Newlon et al., 1973; Molgaard et al., 1976; Vogt and Braun, 1976; Hall and Turnock, 1976; Hall and Braun, 1977; Grainger and Ogle, 1978). The rDNA minichromosome is also amenable to in vivo replication studies. This broad question is narrowed down to the examination of initiation of replication in vitro on the rDNA palindrome of Physarum polycephalum. Based on previous electron microscopic studies by Vogt and Braun (1977), the hypothesis is that initiation can be localized to

specific rDNA sequences, that these sequences will be the same both *in vivo* and *in vitro*, and that a dissection of controlling sequences will be accessible *in vitro*.

Physarum rDNA can be purified by centrifugation through equilibrium sedimentation in a CsCl gradient since it has G + C content higher than the bands of nuclear and of mitochondrial DNA (Zellweger *et al.*, 1972; Bradbury *et al.*, 1973). Ferris and Vogt (1982) have utilized isopycnic centrifugation, but have included the bouyant dye Bisbenzimid H to increase the separation of rDNA from chromosomal DNA. Since *Physarum* rDNA makes up the nucleolar DNA, milligram quantities can also be obtained from purified nucleoli (Mohberg and Rusch, 1971; Bradbury *et al.*, 1973; Johnson *et al.*, 1978). Procedures for obtaining purified nucleoli (see Figure 1) also make possible the efficient isolation of associated proteins (Seebeck *et al.*, 1979; Cheung *et al.*, 1981). If the DNA origins of replication and the proteins making up the replication machinery were to be identified, they could likely be isolated from nucleoli. The high rDNA copy number assures a relatively high

Figure 1. Electron micrographs of isolated Physarum nuclei and nucleoli. Nuclei were isolated by the procedure of Mohberg and Rusch (1971), and nucleoli were isolated by the procedure of Bradbury et al. (1973) after disruption of nuclei in a French pressure cell. Preparations were fixed, stained with osmium tetroxide, embedded and sectioned. Top: nuclei x 25,000. Bottom: nucleoli x 32,500. (Courtesy of Dr. Edward M. Johnson and Dr. V. C. Littau.)

Figure 1



proportion of potential initiating factors. Since Physarum plasmodia grow in synchrony, 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.

The Physarum ribosomal RNA is encoded on the 60 kb linear rDNA molecule which is palindromic around a central spacer region (Molgaard et al., 1976; Vogt and Braun, 1976; Campbell et al., 1979). In each minichromosome, two transcription units of 13 kb are positioned in inverse orientation about a central spacer of 24 kb. 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 (Vogt and Braun, 1977). Origins have thus far been mapped to approximate positions about 20 and 27 kb away from each end of the molecule. Although four potential origins exist on each molecule, only one has been observed to be active on a given replicating molecule (Vogt and Braun, 1977).

In vivo replication studies can be done on Physarum by growing macroplasmodia for a desired time period on

medium containing radioactive deoxynucleoside triphosphates or other chemicals to be employed in the studies. Similar studies can be carried out with microplasmodia by addition of the required chemical or chemicals to the liquid medium of the shaker culture. Studies with macroplasmodia have the advantage that rDNA can be isolated during G_2 phase when chromosomal DNA does not replicate. Such *in vivo* studies involving density-shift experiments indicate that rDNA replicates semiconservatively (Zellweger *et al.*, 1972; Newlon *et al.*, 1973). One such study also shows via electron microscopy that replication forks on rDNA molecules are bidirectional (Vogt and Braun, 1977).

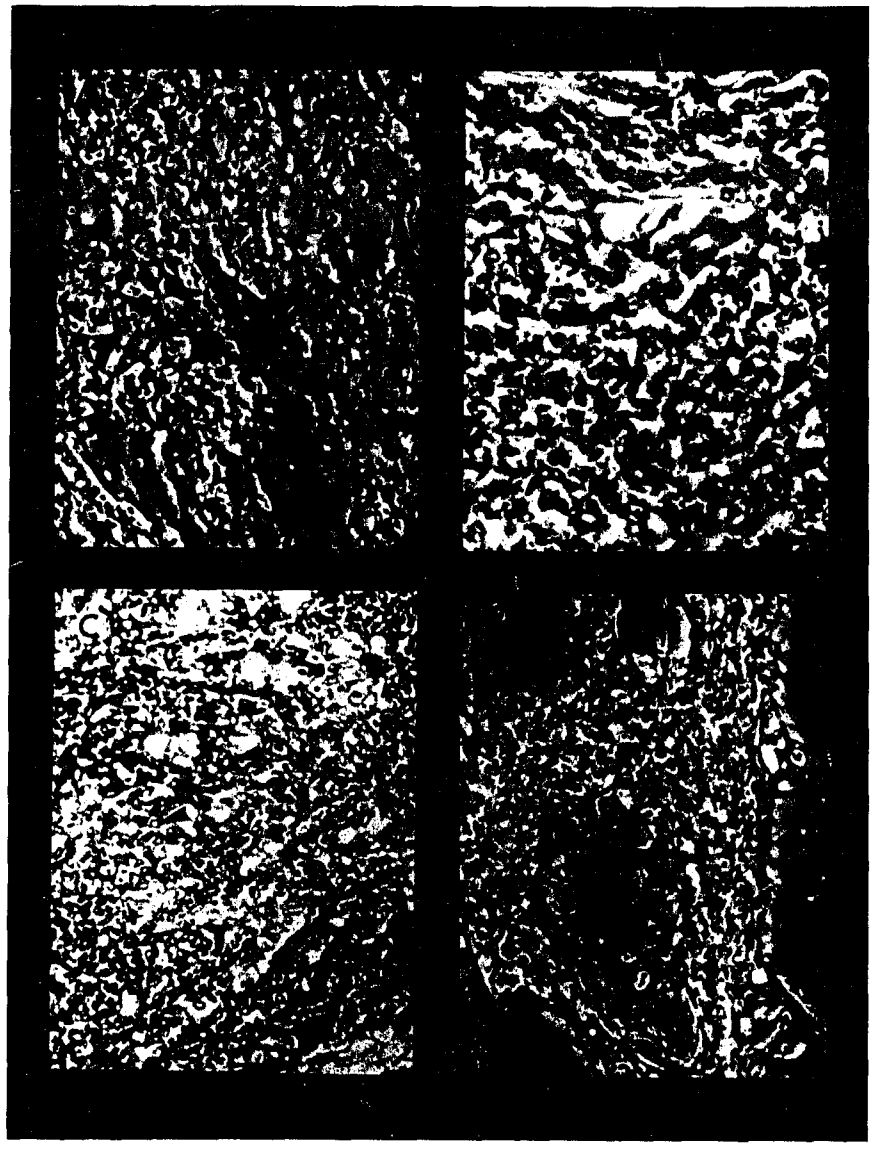
Replication of nuclear DNA in *Physarum* has been shown (by double-labeling density-shift experiments) to be ordered in a temporal sequence with an S period of approximately 3 hours and a G_2 period of approximately 5 hours. There is no G_1 period in the interphase since synthesis of nuclear DNA begins immediately after synchronous mitosis in macroplasmodia (Nygaard *et al.*, 1960; Braun *et al.*, 1965; Braun and Wili, 1969). Further *in vivo* studies have shown rDNA replication to be unscheduled and to occur both in the S and G_2 periods of

the cell cycle (Zellweger et al., 1972; Newlon et al., 1973; Vogt and Braun, 1977). Although rDNA may replicate in unscheduled fashion during proliferation (Vogt and Braun, 1977), a stable copy number of molecules is maintained in the nucleolus at all life stages (Newlon et al., 1973; Affolter and Braun, 1978). There is only one hour in very early S period while the nucleolus is reforming that incorporation of isotope into rDNA does not occur (Zellweger et al., 1972). Although the Physarum rDNA molecule is present in multiple copies within the nucleolus, there is no evidence for an integrated copy at any life cycle stage. In contrast to extrachromosomal Tetrahymena rDNA (Yao and Gorovsky, 1974), evidence has accumulated that Physarum rDNA is not only extrachromosomal, but that it replicates through duplication and not by amplification at all life stages (Braun and Evans, 1969; Newlon et al., 1973; Vogt and Braun, 1976; Vogt and Braun, 1977).

The synchronous division cycle in Physarum can be followed by phase microscopy. The stages of the Physarum cell cycle have been well characterized (Guttes et al., 1961). Figure 2 illustrates such a characterization of cell cycle stages. The natural

Figure 2. Phase microscopy photographs of synchronous Physarum plasmodia taken at different stages of the nuclear division cycle. A. Late interphase -- Chromosomes moving away from the periphery of the nucleus. B. Early prophase -- nucleolus has moved to the periphery of the nucleus and has taken on a crescent shape. C. Late Prophase -- nucleolus has disappeared. D. Metaphase -- side and polar views.

Figure 2



synchrony allows cell cycle extract preparation, and at mitosis II, where such extracts were prepared for this project, the synchrony is such that 99% of the nuclei divide within five minutes of each other (reviewed by Rusch, 1970).

Although cell extract systems for *in vitro* replication have been developed for viruses which make use of the eukaryote host replication machinery (Challberg and Kelly, 1979; Ariga and Sugano, 1983; Ariga, 1984; Li and Kelly, 1984; Wobbe *et al.*, 1985; Stillman and Gluzman, 1985; Murakami *et al.*, 1986), no such *in vitro* system exists for looking at initiation of replication at a cellular DNA origin in a synchronous eukaryotic system. Although several *in vitro* cell extract systems have been developed for yeast plasmid replication (2 um circle and ARS-containing chimeric plasmid), these systems do not initiate efficiently and present a large background of repair synthesis (reviewed by Campbell, 1986).

Overview of approach to thesis problem.

This work involves the development of an *in vitro* system utilizing naturally synchronous extract preparations for studying initiation of replication in the *Physarum* rDNA palindrome and in an rDNA origin zone

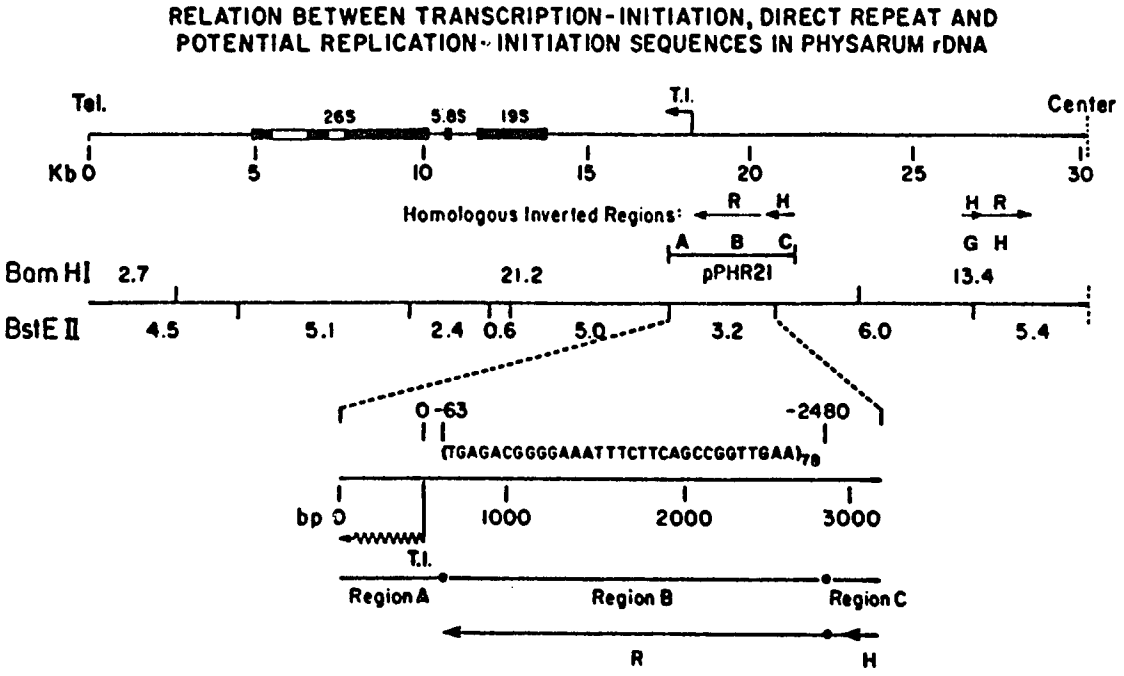
contained within a circular plasmid. Nuclear extracts are shown to differentially label restriction fragments. Such differential labeling allows localization of origin regions at the resolution of restriction fragments. Electron microscopy studies utilizing such nuclear extracts allow more precise localization at the level of structural zones on the template. Cytoplasmic whole cell extracts make possible the production of full-length product as assayed by DpnI resistance. Since extracts can be made at synchronous points in the cell cycle (see Figure 2), the cell cycle points which most efficiently support the production of full-length product can be determined, as well as generating data on replication control. Physarum has never been successfully transfected with bacterial plasmids, but the synchronously growing macroplasmodial state permits a unique way to approach in vitro studies of initiation of replication and cell cycle controls. The Physarum polymerases, both alpha and beta-types, have been purified (Baer and Schiebel, 1978; Weber et al., 1988). A cell cycle study where portions of the replication machinery are found missing helps identify where in the cell cycle vital proteins are most available for

extraction.

Figure 3 shows the structure of the rDNA palindrome of Physarum. Several regions having particular structural features were identified and later sequenced (Ferris, 1985) following characterization of the non-transcribed central spacer by Vogt and Braun (1976) and Ferris and Vogt (1982). Region A designates the zone on the rDNA palindrome containing the transcription initiation site. Within each origin zone is a 31 bp consensus element repeated approximately 78 times. This repeat region is designated as Region B for the transcription-proximal origin and as Region H for a similar structure in inverted orientation in the more central origin. The repeat regions of the two origins consist of similar sequences, but the transcription-proximal origin has about twice the total number of repeats. A unique Region C lies adjacent to and 5′ of Region B. This same unique sequence, denoted Region G, exists in inverted orientation at the site of the more central origin in the same orientation relative to the repeats as it is in the transcription-proximal origin zone. However, the transcription-proximal origin contains a sequence element of approximately 65 bp at the beginning of Region C

Figure 3. Map of Physarum polycephalum rDNA. One half of the 60 kb palindrome is shown. Two regions to which origins have been approximately localized by Vogt and Braun (1977) are indicated as arrows in inverted orientation under the map. The notation HR from Daniel and Johnson (1989, in press) indicates sequence similarities in the two regions. The notations A,B,C,G and H refer to regions sequenced by Ferris (1985). R (repeats) represents Region B and Region H of the origin zones. H (homology) designates Region C and Region G, the unique region in the two origin zones. Region B consists of 78 repeats of the 31 bp element shown in the expanded map of the 3.2 kb BstEII fragment at the bottom (consensus sequence of Ferris, 1985).

Figure 3


RELATION BETWEEN TRANSCRIPTION-INITIATION, DIRECT REPEAT AND POTENTIAL REPLICATION-INITIATION SEQUENCES IN PHYSARUM rDNA
Tel.
26S
5.8S
18S
T.I.
Center
Kb 0
5
10
15
20
25
30
Homologous Inverted Regions:
R H
H R
A B C
G H
pPHR21
BamHI
2.7
21.2
13.4
BstE II
4.5
5.1
2.4
0.6
5.0
3.2
6.0
5.4
0-63
-2480
(TGAGACGGGGAAATTTCTTCAGCCGGTTGAA)78
bp 0
1000
2000
3000
T.I.
Region A
Region B
Region C
R
H

that is not seen in the corresponding position in the more central origin. For this thesis project, origin zones are designated RH (repeat and homology). Both terminologies are used in further discussion: Regions A,B,C,G and H as described by Ferris and Vogt (1982) and designations R and H, Daniel and Johnson (1989, in press). Restriction endonucleases BstEII and HpaI delete nucleotide sequences within specific structural zones of the rDNA insert contained within a circular pBR322 plasmid vector. These restriction endonuclease deletions are utilized to generate mutants, which along with other available plasmid vectors containing rDNA inserts, set the stage for analysis of structural zones of the origin region.

MATERIALS AND METHODS

Preparation of rDNA and plasmid DNA.

Physarum rDNA was purified from isolated non-synchronous microplasmodial nuclei (Campbell *et al.*, 1979) as described by Ferris and Vogt (1982). 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 (Maniatis *et al.*, 1982). 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 (Jong and Scott, 1985).

Preparation of macroplasmodial extracts.

Protocols were modifications of procedures used by Challberg and Kelly (1979) for preparation of nuclear extracts and by Wobbe *et al.* (1985) for 0.2 M NaCl extraction of cells.

Preparation of nuclear extracts from macroplasmodia.

Microplasmodia were harvested and placed on number 40 Whatman filters according to the procedure described

by Guttes et al. (1961). Fusion was for approximately 1 hour and 45 minutes after which the filters with the plasmodia were transferred to bacterial plates containing medium and hemitin in 1.1% bactoagar. Cell cycle points in the synchronous macroplasmodia were then identified beginning at early prophase of mitosis II utilizing phase microscopy. Control plates were used as indicators after the method of Guttes et al. (1961). Preparation of extracts was carried out in the cold and where possible on ice. Filters holding macroplasmodia were washed once by dipping them into an excess of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCL, 10.6 mM Na_2HPO_4, 1.4 mM NaH_2PO_4); a second wash followed using an excess of hypotonic buffer [20 mM HEPES (pH7.5), 5.0 mM KCL, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol]. Macroplasmodial new growth, approximately 2.5 ml and without innoculum, was scraped into one tube and resuspended in an equal volume of hypotonic buffer. After swelling for 10 minutes on ice, it was disrupted by 40 strokes of a Dounce homogenizer (B pestle). Nuclei were identified by phase microscopy of an aliquot. Lysate centrifugation was at 2000 x g for 5 minutes. After resuspension in 2 ml of buffer [50

mm Hepes (pH7.5), 10% sucrose], the nuclear suspension was extracted with 0.2 M NaCl for 1 hour. After centrifugation of the nuclei at 10,000 rpm for 20 minutes, the clear supernatant was dialyzed for 3 hours in 20 mM HEPES (pH7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 10% vol\vol glycerol, 50 mM NaCl with one change of buffer. The extract was then centrifuged at 25,000 rpm for 30 minutes and stored at -135^{o}C. Proteins concentrations were 2.0-2.5 mg/ml as determined by the method of Lowry et al. (1951).

Preparation of 0.2 M NaCl cytoplasmic extracts from macroplasmodia.

Preparation of the 0.2 M NaCl extracts from macroplasmodia followed the same procedure as for preparation of nuclei above until after the dounce homogenization step. Hypotonic buffer modifications were: $MgCl_2$ was present at 1.5 mM and dithiothreitol was present at 1 mM. At this point the entire lysate was brought to 0.2 M NaCl and centrifuged immediately at 25,000 rpm for 30 minutes. Dialysis, centrifugation and storage of the supernatant followed the same procedure as for the nuclear extract. Protein concentrations were 1.7-2.3 mg/ml as determined by the procedure of Lowry et al.

(1951).

In vitro replication conditions.

Reaction mixtures (50 ul) contained 250 ng of plasmid or rDNA in 30 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 uM each dATP, dGTP and dTTP, 10 uM alpha-^{32}P-dCTP (10-30 Ci/mmole, New England Nuclear), 200 uM each GTP, UTP, and CTP, 4.0 mM ATP, 40 mM phosphocreatine, 1.5 ug creatine kinase and 30 ul of Physarum plasmodial extract or nuclear extract (added last). Where applicable, aphidicolin was added to 10 um. Aphidicolin solution was prepared by dissolving 1 mg (Sigma) in 500 ul of dimethyl sulfoxide and diluting with dimethyl sulfoxide prior to addition to the in vitro replication mixture. Reactions, at 37°C, were stopped by addition of EDTA to 25 mM and SDS to 0.5%. Samples were treated with 40 ug proteinase K (Boehringer) for 1 hour at 37°C. In some cases samples were treated with RNAase A for 1 hour prior to the step above. After phenol (twice), one chloroform\isoamyl alcohol and one ether extraction, unincorporated nucleoside triphosphates were removed by two sequential isopropanol extractions. The pellets were resuspended in buffer, digested with the appropriate restriction

enzyme(s), and run on high-resolution neutral (0.8-1.0%) agarose gels based on modification of the procedure of Sundin and Varshavsky (1980). Conditions for running the gels were: 50 volts, 200 milliamps, 17-20 hours in an electrophoresis apparatus of 25.5 cm x 19.5 cm diameter with recirculation of buffer at room temperature. Gels and gel buffers were prepared according to Sundin and Varshavsky (1980). Gels were dried by standard procedure (Maniatis et al., 1982) and analyzed by autoradiography. After treatment with the appropriate restriction enzymes, template products of some of the in vitro reactions were again purified. These samples were spread for electron microscopy, stained with uranyl acetate and rotary shadowed with Pt-Pd as described (Johnson and Jelinek, 1986). Length measurements were made on enlarged electron micrographs using a Numonics digital integrator.

Preparation of high-salt nuclear extract replication mixtures.

High salt nuclear extracts were prepared according to the procedure of Decker et al. (1986) with slight modification. After harvesting plasmodia at known cell cycle points, the homogenization step was as described

for the 0.2 M NaCl nuclear extract preparation. Hypotonic buffer was prepared according to Decker et al. (1986) and included 20 mM HEPES (pH 7.8), 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol. Centrifugation of the lysate was at 1,200 x g for 5 minutes. Supernatant was then centrifuged at 100,000 x g for 1 hour. This supernatant (cytosol) was aliquoted and stored at -135°C. Nuclear pellet (from lysate centrifugation above) was suspended in 1.3 ml of hypotonic buffer with high salt (20 mM HEPES, pH 7.8; 500 mM potassium acetate; 0.5 mM $MgCl_2$ and 0.5 mM dithiothreitol) and extracted for 90 minutes on ice with occasional gentle vortexing. Centrifugation of nuclei was at 8,000 x g for 10 minutes. Supernatant (high salt nuclear extract) was stored at -135°C.

The in vitro replication mixture was prepared according to Decker et al. (1986) and included polyethylene glycol and aphidicolin as indicated in the **RESULTS**. Phosphocreatine and creatine kinase were substituted for phosphoenolpyruvate and pyruvate kinase. 0.25 ug of rDNA was used per 50 ul reaction.

Preparation of deletion mutants.

Plasmid pPHR21 was digested with restriction

endonuclease BstEII (68 units/ug) or HpaI (30 units/ug) for 2 hours at 37°C, and reactions were stopped by the addition of EDTA to 20 mM. In order to separate HpaI restriction fragments, which usually migrated with a smear from cleavage within the repeats (Region B and H, see Figure 3), high resolution electrophoretic fractionation was utilized (Sundin and Varshavsky, 1980). The appropriate restriction fragment generated by the restriction endonuclease treatment was cut out of a 1.0% low melt BRL (Bethesda Research Laboratory) agarose gel by longwave ultraviolet light and purified by GENECLEAN procedure (BIO 101, Inc.) following the manufacturer's instructions. Fragments were ligated to yield the deletion construct by use of T4 ligase, following BRL protocol. HpaI blunt end ligation was at 26°C for 4 hours; BstEII protruding-end ligation was at 14°C for 4 hours. 2 ul of each ligation reaction mix was diluted to 10 ul with 10 mM Tris, 1 mM EDTA, and 1 ul of the diluted ligation reaction mix was used to transform E coli HB101 competent cells.

Preparation and transformation of E. coli HB101 competent cells.

Preparation of E. coli HB101 cells competent for

transformation was carried out according to the procedure of Mandel and Higa (1970). Transformation procedure followed the protocol of Hanahan (1985). 1 ul of ligation mixture was added to 200 ul of competent HB101 cells and incubated on ice for 60 minutes. Cells were heat-shocked at 42°C for 90 seconds and returned immediately to ice. 800 ul of SOB (plus 20 mM glucose) medium was added with incubation for 60 minutes at 37°C. 4 ml of top agar plus antibiotic was added to the tube, mixed briefly and poured on LB plates containing the antibiotic. LB and SOB medium were prepared according to Maniatis et al. (1982). LB (Luria-Bertani) medium included per liter: bacto-tryptone, 10 g; bacto-yeast extract, 5 g; NaCl 10 g (pH 7.5). SOB medium contained: bacto-tryptone, 2%; bacto-yeast extract, 0.5%; NaCl, 10 mM; KCl, 2.5 mM; $MgCl_2$, 10 mM; $MgSO_4$, 10 mM (pH 7.0).

Labeling of plasmid DNA probes by the random-primer method.

The following solutions were used for labeling (Feinberg and Vogelstein, 1984). Solution O: 1.25 M Tris-HCl and 0.125 M $MgCl_2$ (pH 8.0). Solution A: 1.0 ml of solution O plus 0.08 ml of 2-mercaptoethanol plus 5 ul each of dATP, dGTP and TTP (each dissolved in 3.0

mM Tris-HCl and 0.2 mM EDTA, pH 7.0, at 0.1 M). Solution B: 2.0 M HEPES (pH 6.6) with 4 M NH_4OH. Solution C: random hexadeoxyribonucleotides (P.-L. Laboratories) evenly suspended in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) at 90 absorbance units per ml. Solution OLB: a mixture of solutions A:B:C at a ratio of 100:250:150. Labeling was performed by mixing in the following order: 0.6 ul of DNA (30-50 ng, heat-denatured at 100°C for 10 minutes and chilled on ice), 31 ul of deionized H_2O, 10 ul of solution OLB, 2 ul of bovine serum albumin (10.0 mg/ml), 5 ul of alpha-^{32}P-dCTP (NEN, at least 3000 Ci/mmole; 50 uCi) and 1 ul containing 2 units of the Klenow fragment of E. coli DNA polymerase I (Boehringer) in distilled water. The enzyme was added last to begin the labeling reaction, which was carried out for 2.5 hours at room temperature. Reactions (50 ul) were stopped by addition of 200 ul of a solution containing 20 mM NaCl, 2 mM EDTA, 0.25% SDS and 20 mM Tris-HCl (pH 7.5), plus 1.0 uM dCTP. Labeled probes were used directly for hybridization with no further purification. Incorporation, monitored by assaying radioactivity of samples precipitated 3 times with alcohol, ranged from 0.5 to 2.0 x 10^9 cpm/ug of DNA.

Blotting of membrane filters.

Agarose gels with DNA to be blotted were treated first with 0.25 M HCl for 30 mintes to partially depurinate, then in 0.4 M NaOH, 0.6 M NaCl for 30 minutes to denature the DNA. Gels were then neutralized by immersion in 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) for 30 minutes. A Gene-Screen Plus membrane filter, cut to the exact size of the gel to be blotted, was wet in deionized water and soaked for 10 minutes in 10 x SSC (SSC = 0.15 M NaCl-0.015 M Na-citrate, pH 7.0). Each neutralized gel was placed on a piece of porous polyethylene (Bel-Art Products) rested, but not immersed, in 10 x SSC in a standard cafeteria tray. The Gene-Screen-Plus filter was placed on the gel with convex side down. All bubbles were carefully pressed out from between the gel and filter with a clean, gloved hand. Six pieces of Whatman 3 MM paper, cut to the exact size of the gel, were then placed on the filter and on top of this 4 inches of cut paper towels. Care was taken not to let any of the paper touch the 10 x SSC solution surrounding the porous plastic or the gel. Transfer by this method was complete in 2 hours, but gels were usually left overnight. The membrane was then

taken from the gel and treated with 0.4 M NaOH for 1 minute followed by 0.1 M ammonium acetate, pH 7.0, for 10 minutes. Membranes were blotted dry and stored at room temperature between sheets of 3 MM paper.

Hybridization and washing of blotted membrane filters.

The following hybridization buffers were utilized. Prehybridization buffer contained: 1.0 M NaCl, 1.0% SDS, 10% dextran sulfate (Pharmacia) and 50 mM Tris-HCl (pH 7.5) plus 0.1 mg/ml of boiled, sonicated herring sperm DNA. Hybridization buffer contained: 0.3 M NaCl; 1.0% SDS; 10% dextran sulfate; 50 mM Tris-HCl (pH 7.5); 0.1 mg/ml of boiled, sonicated herring sperm DNA and labeled probe (10^6 cpm/ml of hybridization buffer, 0.5 -2.0 x 10^9 cpm/ug of probe DNA). Membrane filters were placed in a Scotch Seal-a-Meal plastic bag and incubated with prehybridization buffer (30 ml per one 15 cm x 15 cm membrane) for 4 hours at 68°C. During this time, hybridization buffer (30 ml without the herring sperm and probe DNAs) was warmed to 68°C. Herring sperm DNA (0.3 ml of a 10 mg/ml solution in H_2O) and labeled probe DNA (3 x 10^7 cpm, usually 1/3 of a random-primer labeling reaction as described, approximately 80 ul) were added together, heated to 100°C in a heating block for

10 minutes and added to 30 ml of the pre-warmed hybridization buffer. A corner was then cut from the bag containing the membrane, and as much prehybridization buffer as possible expressed using a glass rod or pipet. The complete, warmed hybridization buffer (30 ml) was then added through a glass funnel, the bag resealed, and hybridization commenced. For both prehybridization and hybridization, the filled bags were attached to a Nutator rotator with metal clamps (at edge of bag) and rotated at 1 cycle per 2 seconds throughout the incubation. Hybridization was for 16 hours at 68^{o}C (Longer times increased background). Membranes were removed from the plastic bags after cutting the bags open and draining (and carefully disposing of) the labeling buffer. Washes were performed with the membrane in plastic boxes. The washes, in order, were: 5 minutes in 2 x SSC, 1.0% SDS at room temperature; 1 hour in 2 x SSC, 1.0% SDS at 68^{o}C; 2 washes of 30 minutes in 2 x SSC, 1.0% SDS at room temperature; 1 hour in 0.1 x SSC, 1.0% SDS at room temperature. Membranes were blotted dry with Whatman 3 MM paper, wrapped in Saran wrap and autoradiographed for 24 hours to 2 weeks at -70^{o}C using Kodak X-omat AR X-ray film and one Dupont

Cronex Hi-Plus intensifying screen.

RESULTS

Purification of supercoiled plasmids.

Since it has been shown for viral systems that supercoiled plasmids are preferential templates for *in vitro* replication, it is desirable to utilize a fully-supercoiled, form I, plasmid template (Li and Kelly, 1984; Li and Kelly, 1985; Stillman and Gluzman, 1985). The plasmids used in this work were not amplified because of what appeared to be endogenous primers in the yeast *in vitro* systems due to amplification of plasmids in bacteria. Additional treatment with proteinase K, RNAase A and T1, and RNAase H was utilized. This further diminished the plasmid yield of the unamplified pBR322 vectors prepared for this *in vitro* replication system. Although supercoiled plasmid can be purified by two bandings in a cesium chloride\ethidium bromide gradient, quick methods were sought to purify small quantities of supercoiled plasmid. Procedures were utilized to prepare both pBR322 and pPHR21. Two methods were utilized: GENECLEAN (BIO 101 Inc) and acid-phenol extraction (Zasloff *et al.*, 1978).

GENECLEAN (BIO 101 Inc) purification relaxes and linearizes supercoiled plasmid.

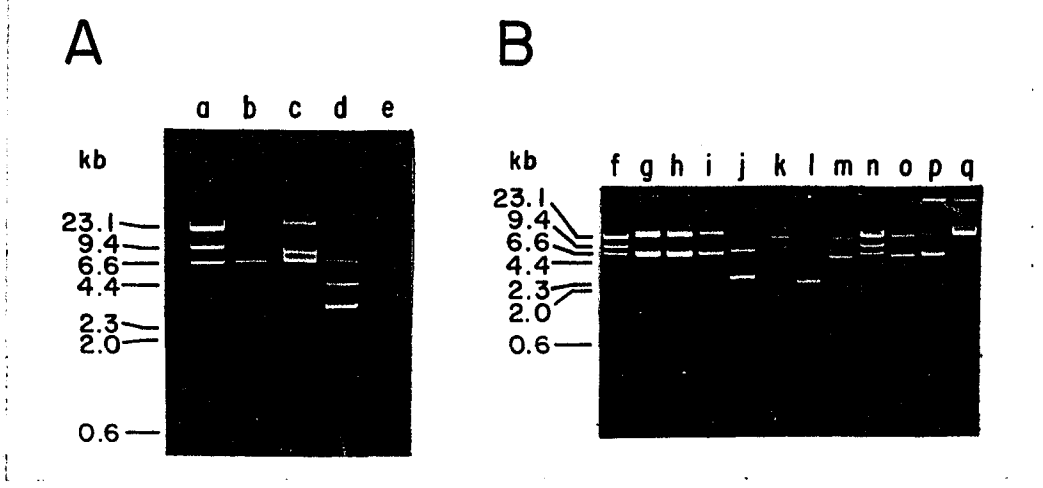
Plasmid material from one banding in a cesium chloride\ethidium bromide gradient was placed in slots across a 1.0% neutral agarose gel. After the run, one marker lane was stained with ethidium bromide. The bands representing supercoiled (form I) plasmid were removed by reference to the marker and subjected to GENECLEAN (BIO 101 Inc.) according to the manufacturer's instructions. As can be seen in Figure 4A, the supercoiled material (pPHR21 and pBR322) was partially relaxed and linearized by the procedure such that there were approximately equal amounts of each form of the plasmid.

Acid-phenol extraction for purification of supercoiled plasmid.

Acid-phenol extraction is a technique which can provide quick preparation of supercoiled material, and has been especially useful when there are small quantities of material that would not be efficiently banded in a second cesium chloride\ethidium bromide gradient. The key step in this purification is a phenol extraction performed at pH 4.0. Figure 4B shows the purification of supercoiled material by the acid-phenol treatment exactly as described by Zasloff et al. (1978).

Figure 4. Gel electrophoresis showing different percentages of form I, supercoiled plasmid DNA obtained by different purification methods. 1.0% agarose minigels were run at 100 volts for approximately 40 minutes. Buffer were prepared as described in **Materials and Methods**. Ethidium bromide was used for staining. Lanes are: Figure 4A: lambda treated with HindIII (lane a)\ markers (pPHR21, lane b; pBR322, lane e)\ GENECLEAN preparations (pPHR21, lane c; pPBR322, lane d). Figure 4B: lambda treated with HindIII (lanes f, k, n)\ marker lanes (pPHR21, lanes i and o; lambda uncut, lane q; pBR322 dimer, lane m)\ CsCl gradient preparation -- after one gradient: (pPHR21, lanes g, h; pBR322, lane j)\ acid-phenol preparations (pPHR21, lane p; pBR322, lane l).

FIGURE 4


A
a b c d e
kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6
B
kb
f g h i j k l m n o p q
23.1
9.4
6.6
4.4
2.3
2.0
0.6

These data are included although the macroplasmodial *in vitro* system developed in this research project did not produce random initiation. Plasmids used were not amplified (Jong and Scott, 1985), but additional treatment with RNAase H as was utilized in the *Xenopus* system proved unnecessary (Blow and Laskey, 1986). 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 this purified plasmid DNA.

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 is 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 1. Labeling of rDNA is highly dependent on inclusion of ATP, Mg^{++} and deoxyribonucleoside triphosphates, as expected. 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 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. Figure 5A shows a time course of labeling of the rDNA in the in vitro mixture utilizing a plasmodial cytoplasmic extract from prophase. After labeling, the rDNA was cleaved with restriction endonuclease BstEII. Although rDNA bands are labeled within 15 minutes, a steady increase in labeling is only observed after 30 minutes. When pBR322 DNA is added to the incubation mixture, the plasmid retains >95% of its ability to transform E.coli after 2 hours incubation, indicating that the system is not degrading DNA. The small decrease in incorporation between 15 and 30 minutes could be due to turnover of a small fraction of early-labeled DNA in the absence of new initiation. The

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

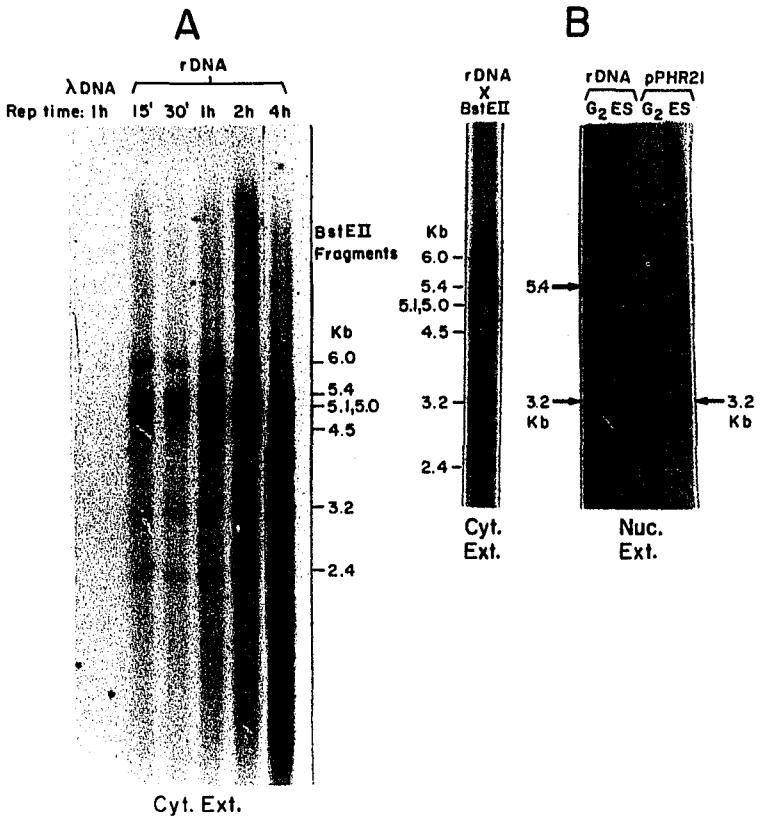
Reaction components omitted\added*	pmol dCMP incorporated	Relative activity
System complete, 10 μM dCTP	37	100
(100 μM dCTP)	(112)	(304)
- CTP, GTP, UTP	39	106
- dATP, dGTP, dTTP	9	24
- ATP	5	13
- $MgCl_2$	7	18
- Creatine phosphate, creatine kinase	13	36
+ Aphidicolin, 10 μM	10	28
+ Camptothecin, 100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

Figures 5. 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 BstEII segment. The rDNA (250 ng) was subjected to in vitro replication using an extract from prophase, digested with BstEII 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 BstEII 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 BstEII segment) for 3 hours. The purified, labeled DNA was then digested with

BstEII 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 BstEII bands of rDNA (arrows at left) and of the 3.2 kb BstEII band of pPHR21 (arrow at right).

Figure 5


A
rDNA
λDNA
Rep time: 1h 15' 30' 1h 2h 4h
BstEII Fragments
Kb
6.0
5.4
5.1,5.0
4.5
3.2
2.4
Cyt. Ext.
B
rDNA X BstEII
Kb
6.0
5.4
5.1,5.0
4.5
3.2
2.4
Cyt. Ext.
rDNA pPHR21
G2 ES G2 ES
5.4
3.2 Kb
3.2 Kb
Nuc. Ext.

decrease is not due to DNA breakdown. Labeling of rDNA in the first 30 minutes includes all major BstEII bands and could 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 (Vogt and Braun, 1977). In similar time course experiments with plasmid pPHR21 (not shown), containing a potential rDNA origin region, new initiation commences only after a lag of 30 minutes, and no labeling is seen before this time. A lag in new initiation of 15 minutes has previously been reported for a mammalian in vitro replication system (Wobbe et al., 1985). Repair synthesis contributes little to the early rDNA labeling, as shown by lack of labeling of a lambda phage control (Figure 5A, lane 1). The phage DNA was incubated with the in vitro mixture containing a prophase whole cell extract for 1 hour and would be subject to the same nicking and repairing as the rDNA. At 4 hours, incubation of rDNA template in the in vitro mixture utilizing the whole cell extract generates specific cleavage within the labeled rDNA band at 6.0 kb to yield discrete bands of smaller size (Figure 5A, lane

6). Thus far, such cleavage has not been observed in the bulk of nonreplicated rDNA subjected to the system, but any relation between the cleavage of the labeled 6.0 kb restriction fragment and replication has yet to be established.

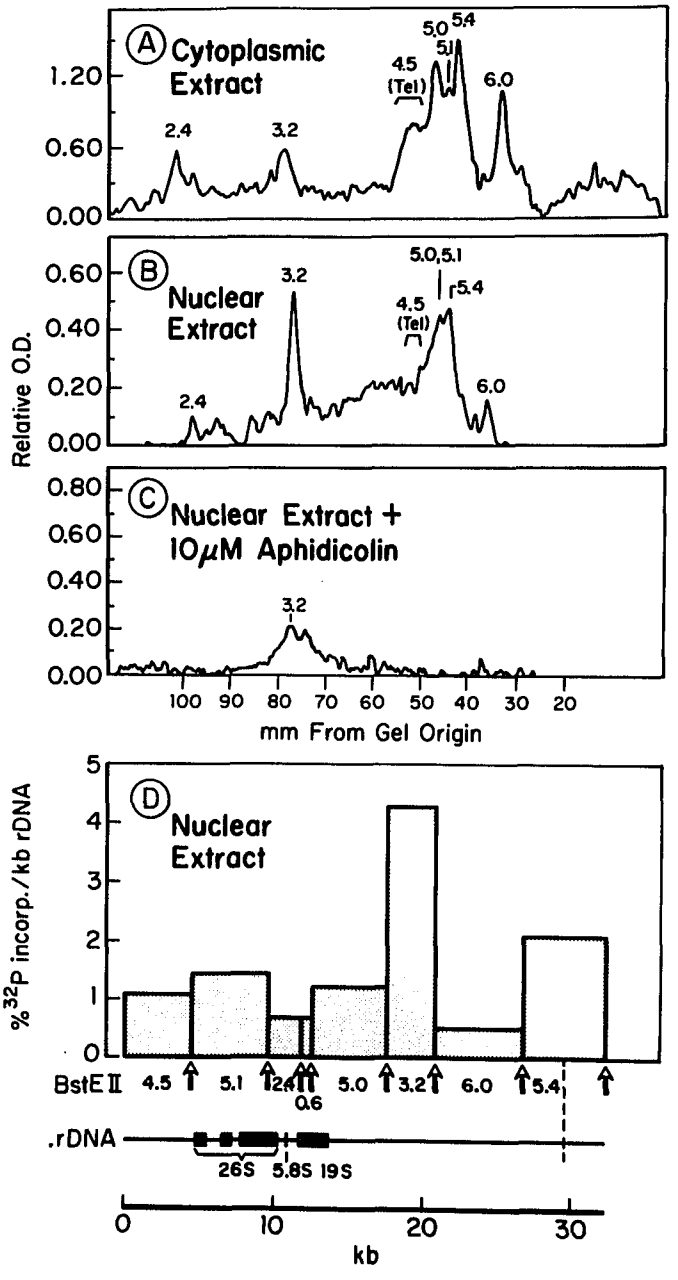
Experiments were performed to distinguish rDNA labeling at new initiation sites from labeling at previously-existing replication forks (Figures 5B and 6). Labeling was examined using extracts of isolated nuclei taken at either late G_2 or early S phase of the cell cycle. When rDNA is incubated for times up to 3 hours with such nuclear extracts, differential labeling of the BstEII fragments is observed (Figure 5B). 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 (Vogt and Braun, 1977). The template, pPHR21, is a pBR322 plasmid with an rDNA BclI restriction fragment insert of 3.9 kb. The BclI insert contains the 3.2 kb BstEII restriction fragment (Ferris and Vogt, 1982; Ferris, 1985). Figure 5B shows that in this recombinant plasmid, as well as in rDNA, preferential labeling occurs within an rDNA BstEII fragment of 3.2 kb. 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 (Figure 5B). 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 replication selectively but elongate nascent strands more slowly than do cytoplasmic extracts.

Figure 6 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 by DNA DNA polymerase alpha while having no effect on repair polymerases (Huberman, 1981). At concentrations >10 uM, aphidicolin abolishes detectable labeling of rDNA. However, at 10 uM, the inhibition is not complete and one rDNA band remains clearly labeled, the 3.2 kb BstEII

Figure 6. 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 Figure 5B. After labeling, the rDNA was purified, digested with BstEII 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 uM 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 BstEII 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 BstEII map.

Figure 6


A Cytoplasmic Extract
1.20
0.60
0.00
2.4
3.2
4.5 (Tel)
5.0
5.1
5.4
6.0
B Nuclear Extract
0.60
0.40
0.20
0.00
2.4
3.2
4.5 (Tel)
5.0,5.1
5.4
6.0
Relative O.D.
C Nuclear Extract + 10μM Aphidicolin
0.80
0.60
0.40
0.20
0.00
3.2
100 90 80 70 60 50 40 30 20
mm From Gel Origin
D Nuclear Extract
%32P incorp./kb rDNA
5
4
3
2
1
0
BstEII 4.5 5.1 2.4 0.6 5.0 3.2 6.0 5.4
.rDNA
26S 5.8S 19S
0 10 20 30
kb

fragment (Figure 6, panel C). Similar observations on inhibition of labeling of SV40 DNA *in vitro* by aphidicolin reveal that the least inhibited fragment contains the replication initiation site (Decker *et al.*, 1986). 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 were available for accumulation of early-replicating intermediates (Decker *et al.*, 1986). Such conditions are obtained *in vitro* with the *Physarum* nuclear extract. If elongation following initiation were to proceed 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 Figure 6. Labeling seen between the 3.2 and 4.5 kb bands could represent elongation at bubbles in the 3.2 kb fragment. 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. Due to some difference in nucleotide sequence between the two origin regions, 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. The small amount of label left at the ends of such cleaved origin fragments when aphidicolin is included in the replication mixture might be lost during purification of the replication products. Results in Figures 5 and 6 implicate the 3.2 kb BstEII fragment as a potential replication initiation site both in rDNA and in a plasmid containing this rDNA restriction 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 two-dimensional gels, used to map yeast origins of replication *in vivo* (Huberman *et al.*, 1987), is not feasible here due to extensive sequence repetition in the rDNA.

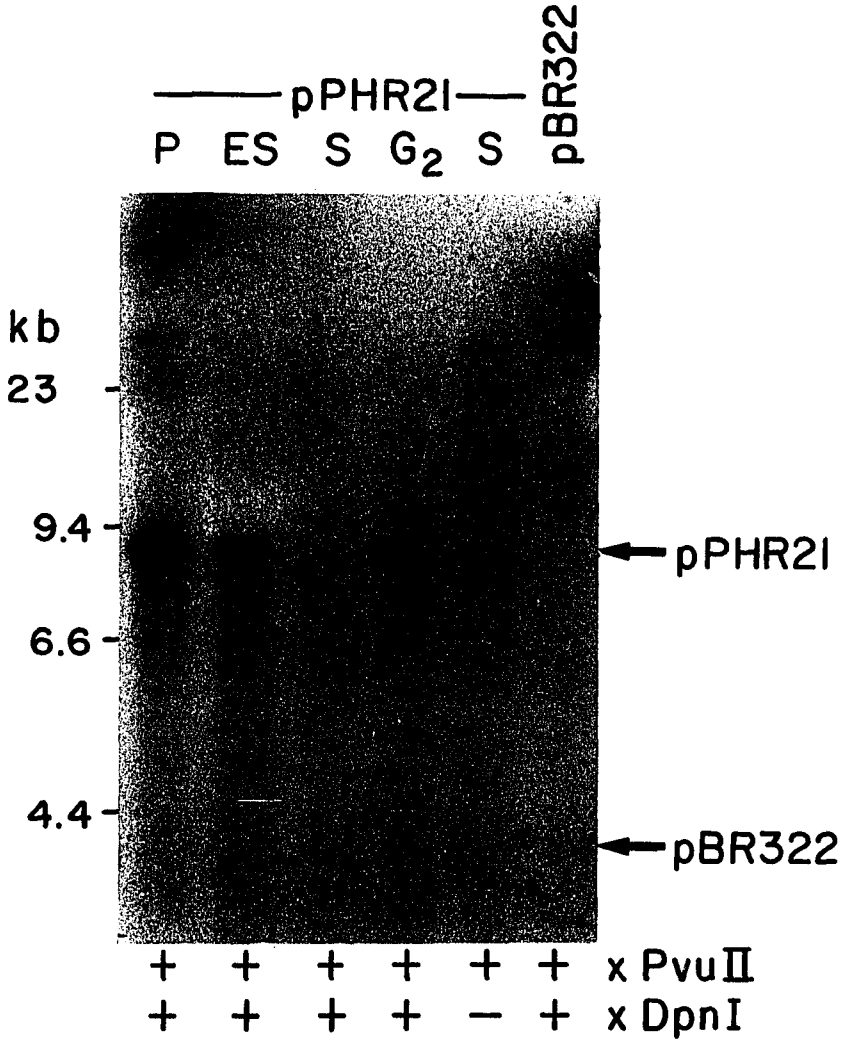
Selective initiation and replication in vitro of plasmid pPHR21.

Experiments were done to determine whether plasmid pPHR21, containing a potential replication origin (Figures 5 and 6), initiates selectively and replicates fully *in vitro*. DpnI digestion is a demonstrated means

of assaying plasmid replication in eucaryotic systems (Wobbe et al., 1985; Johnson and Jelinek, 1986; Vassilev and Johnson, 1988). Plasmids propagated in dam^+ strains of E. coli are methylated at adenosine residues and are sensitive to DpnI (Lacks and Greenberg, 1977). 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, (Figure 7), 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 hours of replication with cytoplasmic extracts from early S, G_2 or prophase of the cell cycle (Figure 7). When plasmid pBR322 is subjected to the in vitro system, it is completely digested by DpnI, indicating that it does not replicate (Figure 7, last lane). 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 due to complete replication.

Figure 7. DpnI-resistance of plasmid pPHR21 DNA after replication using cytoplasmic extracts from different points in the synchronous division cycle. Plasmid pPHR21 DNA (250 ng) was subjected to the *in vitro* replication system for 2 hours using cytoplasmic extracts from synchronous plasmodia. Points taken were: Prophase (P) = 30 minutes prior to metaphase II; Early S (ES) = immediately after telophase II; S = 1.5 hours after telophase II; G_2 = 4 hours after telophase II. Purified, labeled DNA was then linearized with PvuII (60 units/ug DNA) and treated with DpnI (35 units/ug of DNA) for 1 hour at 37°C and analyzed as in Figure 5A. Control plasmid pBR322 DNA was also subjected to the system and treated with the same restriction enzymes prior to electrophoresis (right lane). DNA labeled with an S-phase extract was analyzed before (lane 5) and after (lane 3) treatment with DpnI.

Figure 7


pPHR21
pBR322
P ES S G_2 S
kb
23
9.4
6.6
4.4
pPHR21
pBR322
+ + + + + + x PvuII
+ + + + − + x DpnI

Experiments were performed in which the negative replication control, pBR322, was included with pPHR21 in the DpnI digestion reaction. In these experiments, the plasmid DNA was not linearized with a second enzyme since it has been reported that linearization can affect the reliability of DpnI digestion (Rao and Martin, 1988), thus complicating an internally-controlled experiment. Rather than analyze the mixed plasmids by gel electrophoresis, which could lead to ambiguities due to multiple bands, the extent of DpnI digestion and levels of DpnI resistance were quantitatively determined using a recently-described bacterial transformation assay (Vassilev and Johnson, 1988). This assay serves as an internal control for DpnI cleavage by exploiting the differential sensitivities of E. coli, transformed with pPHR21 or pBR322, to ampicillin (Amp) and tetracycline (Tc). The results, shown in TABLE 2, 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

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.

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 overdigestion could cleave some replicated pPHR21 molecules. In all cases the replicated pPHR21 is preferentially resistant to the DpnI versus the pBR322. The results of Figure 7 and TABLE 2 indicate that replication initiation is specified by sequences in the Physarum rDNA insert of pPHR21. These results also indicate that the replication mixture yields the most full-length plasmid product when 0.2 M NaCl cytoplasmic extracts from either prophase and early S phase are utilized in the mixture. 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. Experiments were done to examine the ability of extracts taken at different points

in the cell cycle to support replication. The cell cycle for strain a x i consists of a 3 hour S phase, a 5 hour G_2 phase and mitosis, which is prefaced by a relatively long prophase. Prophase is easily identified microscopically beginning about 50 minutes prior to metaphase, when the nucleolus starts to move to the nuclear membrane and begins to disorganize (Guttes et al., 1961). 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 hours (Zellweger et al., 1972). The nucleolus reorganizes in about the first 1.5 hours of S phase (Guttes et al., 1961). Endogenous rDNA replicates during the latter part of S and throughout G_2 (Zellweger et al., 1972; Newlon et al., 1973).

Extracts vary greatly in their ability to replicate plasmid pPHR21. The most effective cytoplasmic extracts are 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 cause plasmid pPHR21 to be labeled only slightly, as seen in Figure 7. Extracts also differ in ability to label exogenous rDNA

Figures 8 and 9. Labeling of rDNA bands using cytoplasmic extracts taken at different points of the nuclear division cycle. The rDNA was subjected to *in vitro* replication using whole cell extracts from different points in the cell cycle in the presence of alpha-^{32}P-dCTP. It was then cleaved with restriction endonuclease BstEII and subjected to electrophoresis as described for Figure 6. The numbered peaks are fragments of size: 1 = 2.4 kb; 2 = 3.2 kb; 3 = 4.5 kb (telomere); 4 = 5.0 kb; 5 = 5.1 kb; 6 = 5.4 kb; 7 = 6.0 kb.

Figure 8

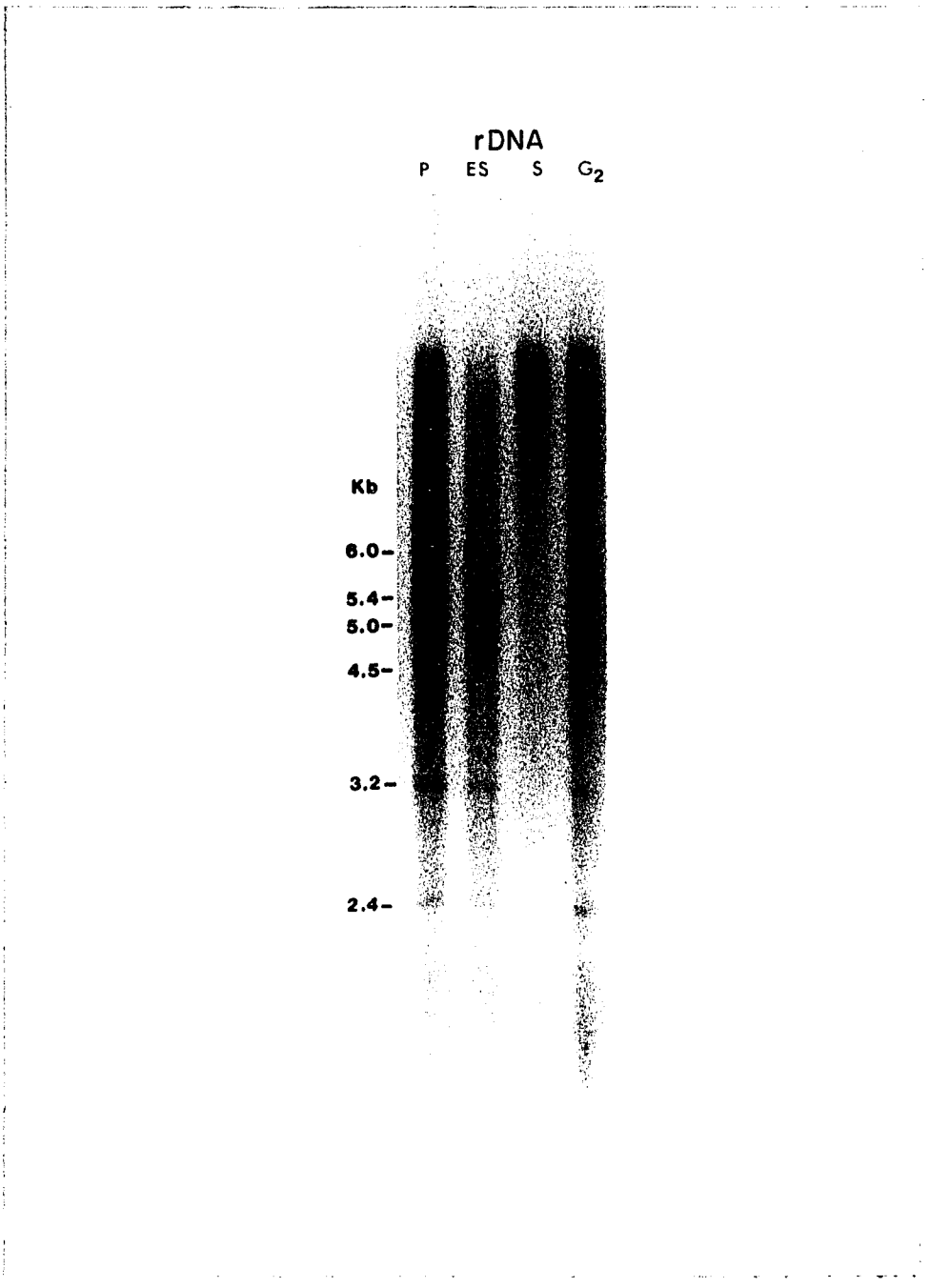
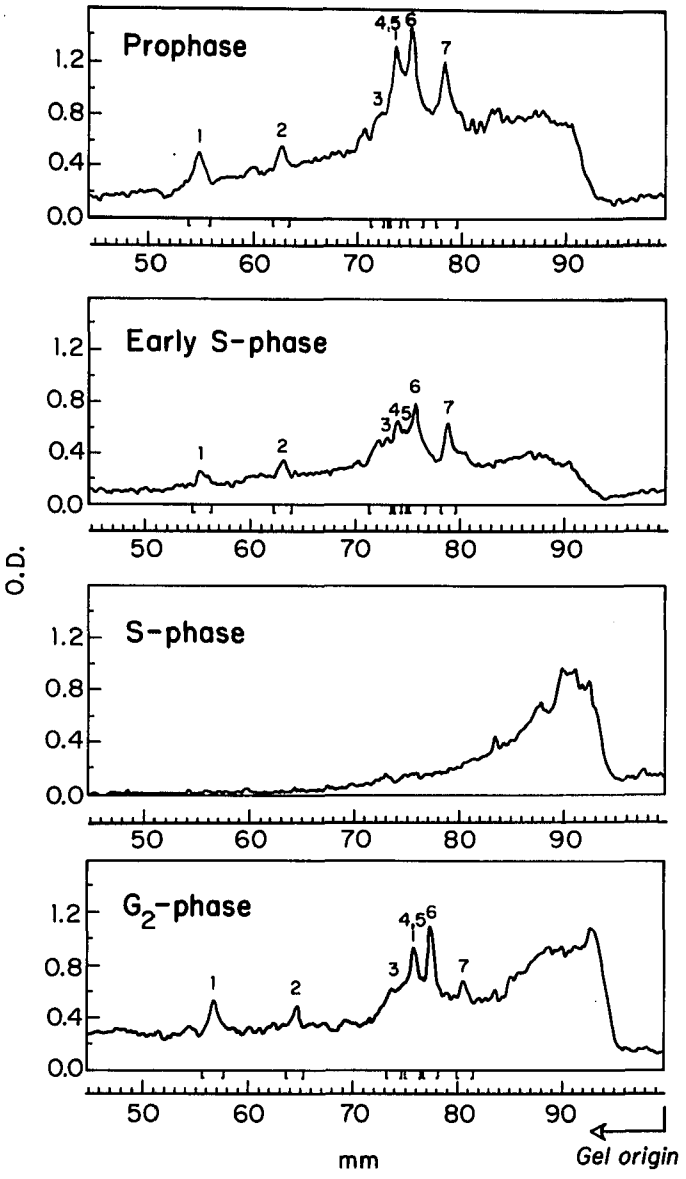


Figure 9


Prophase
Early S-phase
S-phase
G2-phase
O.D.
1.2
0.8
0.4
0.0
50
60
70
80
90
mm
Gel origin

template, with prophase extracts being most active and S-phase extracts least active (Figures 8 and 9). Nuclear extracts taken at early S phase (as described earlier) were also examined. These extracts initiate selectively, as determined by electron microscopy (next section), although elongation is limited. Others have reported that isolated nuclei from S phase retain ability to initiate replication with temporal specificity and to elongate (Sauer et al., 1987). 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.

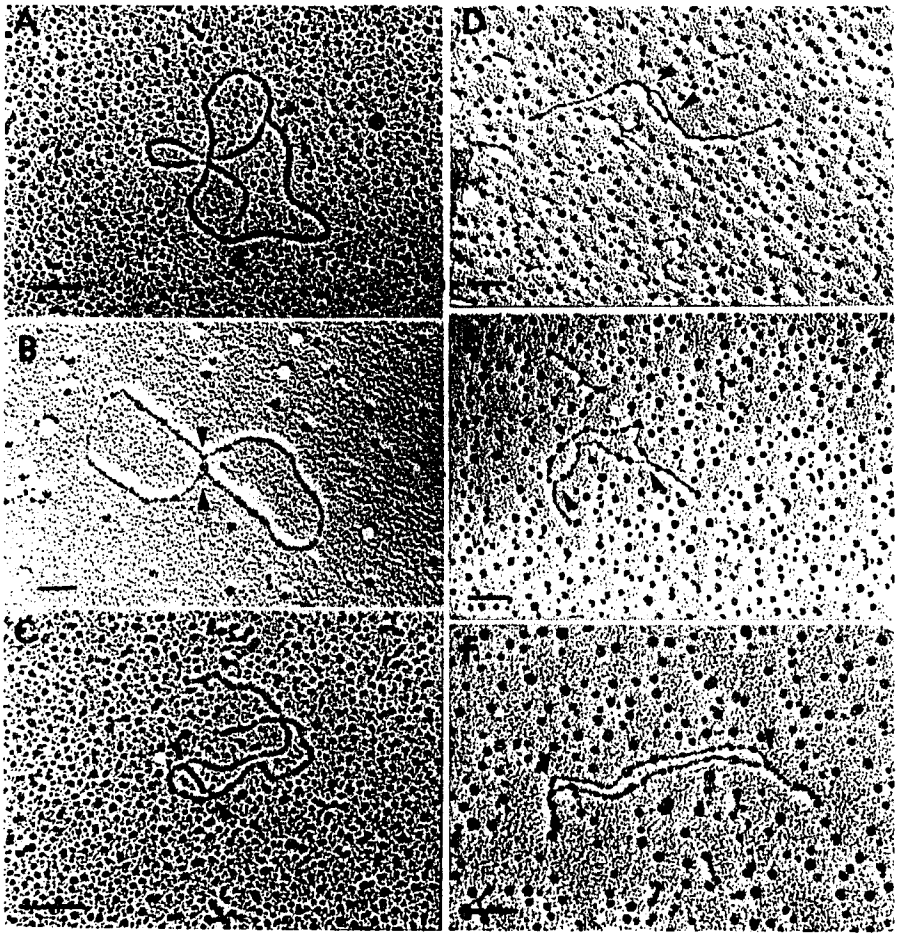
Electron microscopic mapping of an rDNA initiation site for bidirectional replication in plasmid pPHR21.

Electron microscopy was used in conjunction with in vitro replication to localize the origin of replication within known sequence regions of the rDNA template as well as of pPHR21. This is the method of choice here due to limitations on two-dimensional gel techniques with Physarum rDNA, as discussed earlier. For some experiments early-S nuclear extracts (50 minutes post-telophase) were used since these accumulated three-fold

higher levels of Cairns structures and early replication bubbles than did whole plasmodial extracts. Following incubation for 2 hours, pPHR21 DNA was purified from the replication mixture, treated with PvuII and spread for electron microscopy as described in **Materials and Methods**. Figures 10A and 10B show typical pPHR21 molecules as late Cairns structures. 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, 2.4% of all full-length molecules scored were seen as linear DNA with a replication bubble after PvuII cleavage. Circular uncut molecules with a replication bubble constituted 0.8% of full-length molecules measured with a Numonics digital integrator, as did circular uncut molecules with no bubble. These molecules probably represent pPHR21 plasmids that escaped cleavage by PvuII. 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

Figure 10. Electron microscopic visualization of plasmid pPHR21 replication intermediates generated in vitro. Purified plasmid DNA was subjected to *in vitro* replication for 2 hours 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 (no restriction endonuclease treatment). C-F: replication intermediates treated with PvuII, which cleaves approximately opposite the rDNA insert. Bars=1 kb.

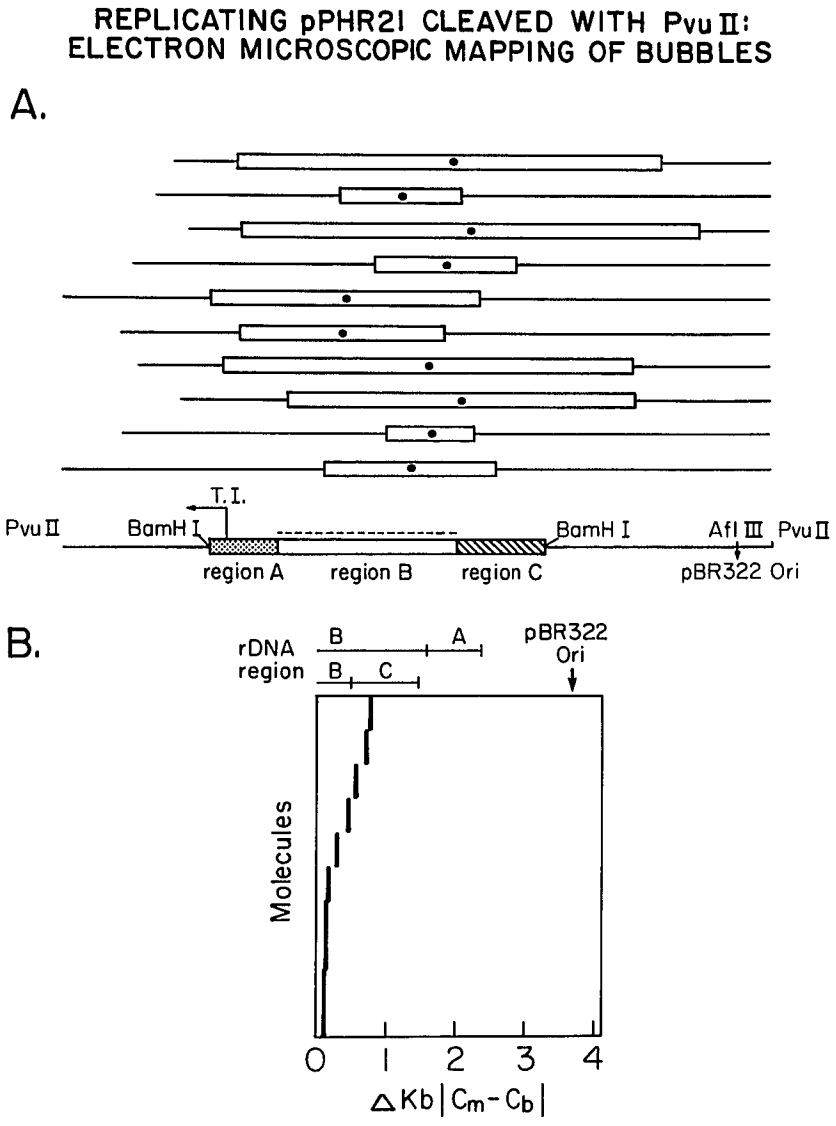
Figure 10



seen with a bubble. More than 10,000 pPHR21 molecles were scored. Several linearized pPHR21 molecules with replication bubbles are shown in Figure 10, C-F. Ten representative molecules with different-sized bubbles and no branch migration were mapped as shown in Figure 11. Here the position of the center of each replication bubble is mapped 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 replication is indicated by the configurations of bubbles shown in Figure 10, C-F and mapped in Figure 11: 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 (Figure 10F). The centers of bubbles map to a position within the rDNA insert as shown for 10 molecules in Figure 11A. No bubbles were seen that correspond to the position of the pBR322 origin of 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 (Ferris and Vogt,

Figure 11. 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 Figure 10. 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 Figure 10, D-F, measured and positioned with long arms at the right above a map of PvuII-linearized pPHR21. Dots mark the centers of bubbles. Regions A-C are as previously described (Figure 3). 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.

Figure 11


REPLICATING pPHR21 CLEAVED WITH PvuII:
ELECTRON MICROSCOPIC MAPPING OF BUBBLES
A.
T.I.
PvuII
BamH I
BamH I
AflIII
PvuII
region A
region B
region C
pBR322 Ori
B.
rDNA region
B
A
B
C
pBR322 Ori
Molecules
0
1
2
3
4
ΔKb|Cm-Cb|

1982; Ferris, 1985), are positioned in the bars above Figure 11B. 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 Figure 11B, both regions 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 7 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. There is a bias inherent in aligning all the plasmid molecules with long arms at the right, as shown in Figure 11A. This is due to the fact that there is no intrinsic reason for all length variability to occur at the left end of these molecules. Mapping this way tends to place centers of bubbles nearer Region C than Region A. This is reasonable in the present case since it most closely agrees with

previous in vivo mapping of rDNA. The bias of Figure 11A is eliminated by mapping centers of bubbles relative to centers of molecules, as performed for Figure 11B, since in this case length variability is not arbitrarily localized to one end of the molecule. It can be seen that no matter how bubbles are mapped, a clustering of centers is localized over Region B near its border with Region C. Figure 12 summarizes the relationship of this site to the transcription initiation point and to repeats near the more central origin of replication.

Figure 13 shows the 21.2 kb BamHI restriction fragment containing the transcription-proximal origin zone as shown in Figure 12. These BamHI restriction fragments were derived from rDNA molecules isolated from in vivo (top figure) and from purified rDNA template which had been incubated in the replication mixture containing the early S extract (bottom figure). The replication bubbles seen in the two rDNA molecules map in the same cluster as those localized for the plasmid template, pPHR21.

Figure 12. 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, 1985). H = a region repeated once with inverted homology. R and H are in approximate locations of origins of replication in vivo (Vogt and Braun, 1977). The dotted line in the drawn bubble at the bottom of Figure 12 indicates the average position of the 10 bubbles shown in Figure 11A and mapped relative to the centers of molecules as in Figure 11B. The lines at either side of the drawn bubble indicate the range of the centers of the molecules positioned as in Figure 11A with the two least conforming values omitted.

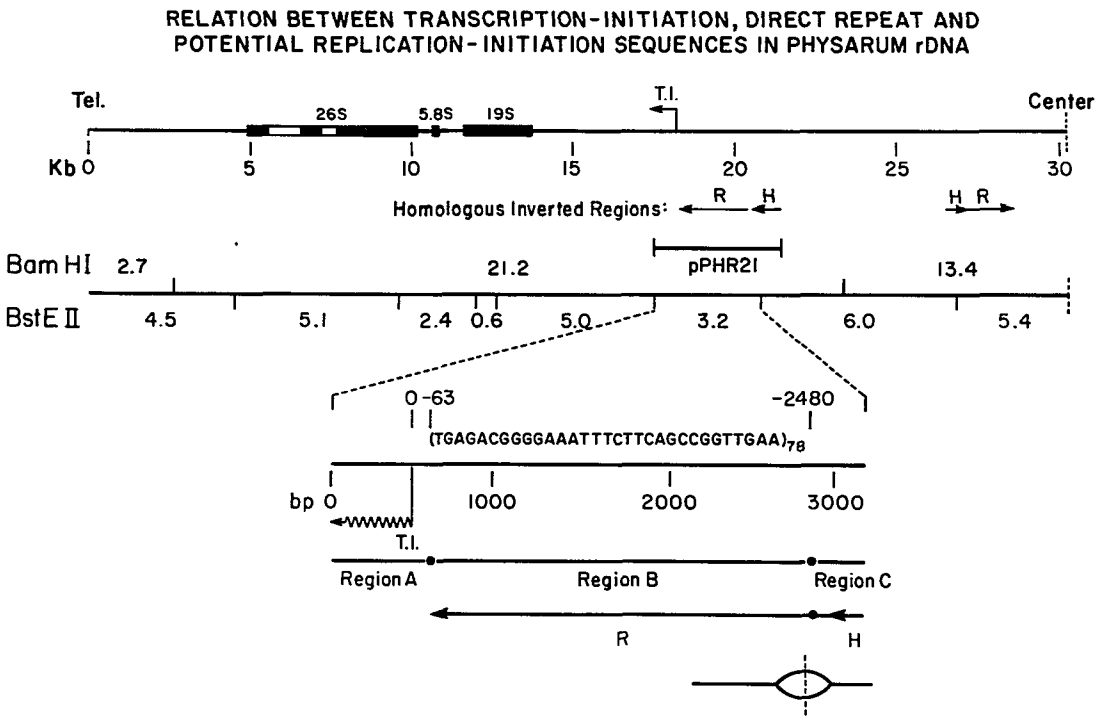
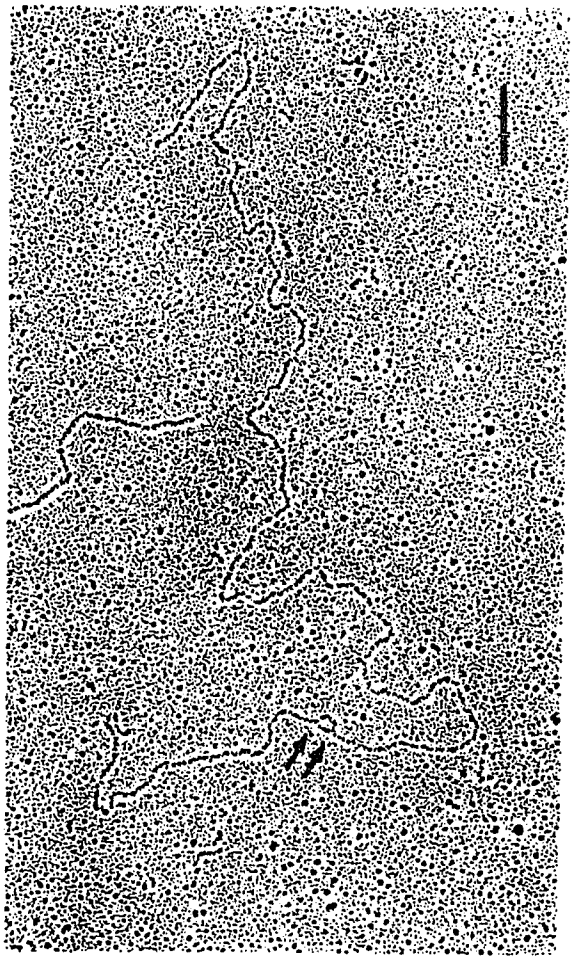
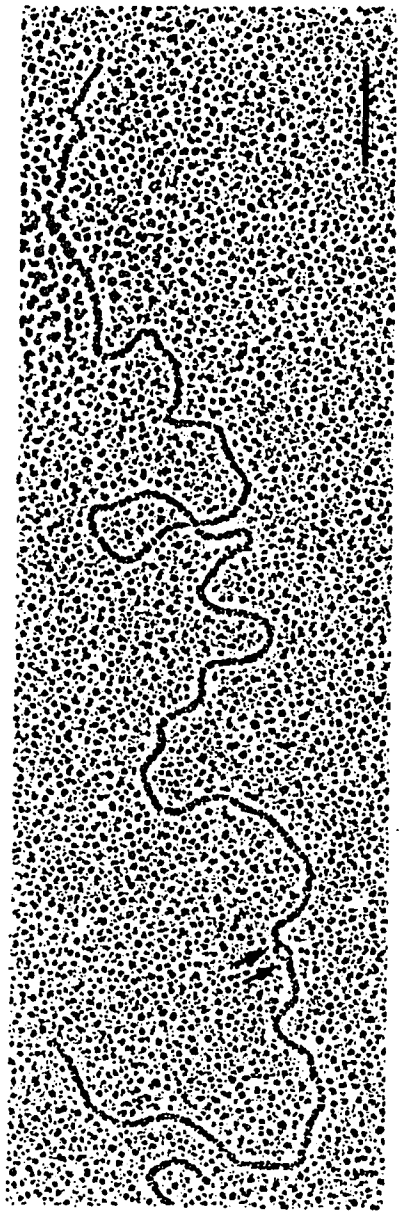

RELATION BETWEEN TRANSCRIPTION-INITIATION, DIRECT REPEAT AND
POTENTIAL REPLICATION-INITIATION SEQUENCES IN PHYSARUM rDNA
Tel.
26S
5.8S
19S
T.I.
Center
Kb 0
5
10
15
20
25
30
Homologous Inverted Regions:
R H
H R
Bam HI
2.7
21.2
pPHR21
13.4
BstE II
4.5
5.1
2.4
0.6
5.0
3.2
6.0
5.4
0
-63
-2480
(TGAGACGGGGAAATTTCTTCAGCCGGTTGAA)78
bp 0
1000
2000
3000
T.I.
Region A
Region B
Region C
R
H

Figure 12

Figure 13. Electron micrographs of restriction fragments of Physarum rDNA showing similar location of replication bubbles generated in vivo and in vitro. Top: the 21.2 kb rDNA BamHI fragment (see rDNA map, Figure 12) spread for electron microscopy after subjecting intact rDNA to in vitro replication for 2 hour as described in **Materials and Methods** and treating it for 15 minutes with BamHI (68 units/ug DNA). Bottom: the 21.2 kb BamHI fragment of rDNA isolated from microplasmodia and treated with restriction endonuclease as above. The rDNA was complexed with cytochrome c, spread from a mixture containing 50% formamide onto a hypophase of 20% formamide, stained with uranyl acetate, and rotary shadowed with Pt-Pd as previously described (Campbell et al., 1979). Bars = 1 kb. Arrows point to replication forks.

Figure 13



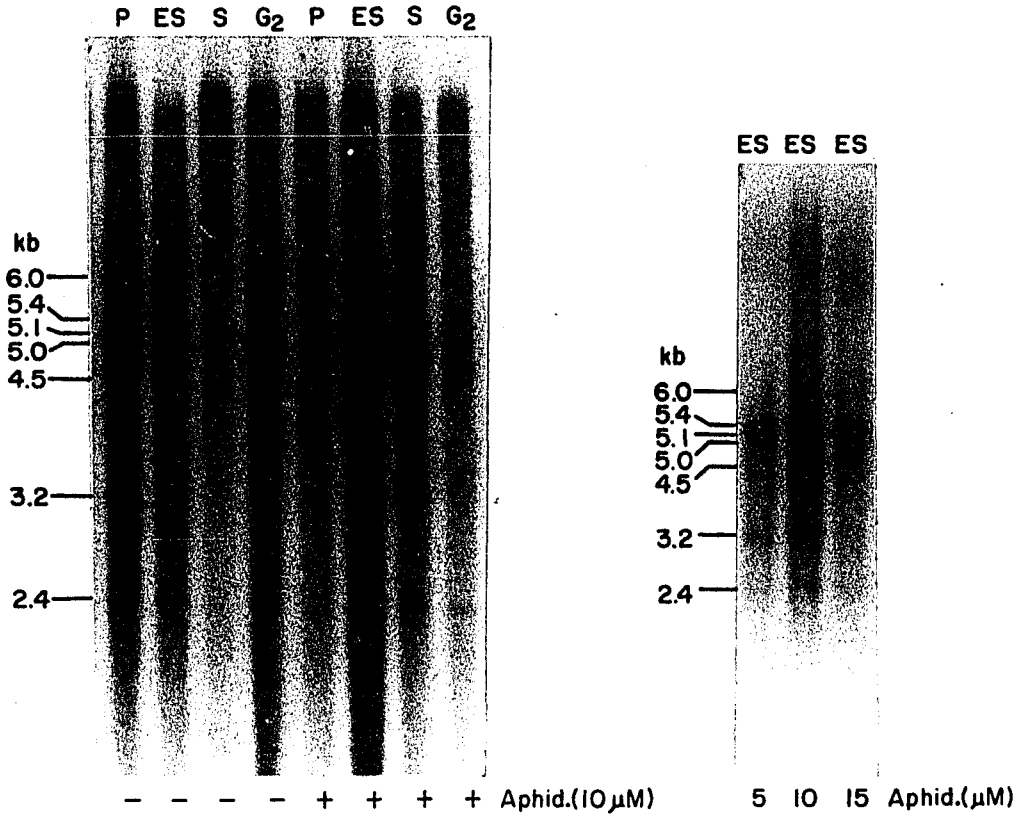


Aphidicolin effect on labeling of rDNA in various cell cycle extracts.

Although aphidicolin proves inhibitory to the labeling of rDNA by the replication mixture when nuclear extracts are included in the mixture (e.g., see Figure 6), its effect on the same replication mixture when cytoplasmic extracts are utilized depends on the cell cycle point at which the extract was taken. As seen in Figure 14 (lane 1 versus lane 5 and lane 4 versus lane 8), aphidicolin at 10 uM diminishes most of the label generated by use of the 0.2 M cytoplasmic whole cell extracts from the prophase and G_2 cell cycle points, indicating involvement of polymerase alpha in the reactions. Surprisingly, there is an increase in label when extracts from ES or S phase are utilized in the replication mixture containing 10 uM aphidicolin as seen in Figure 14 (compare lane 6 with lane 2 and compare lane 7 with lane 3). In Figure 14 (lane 3 versus lane 7) there is a shift in the S-phase label when aphidicolin is present in the replication mixture. Label migrating where higher molecular weight replication intermediates would be expected to migrate in the gel (i.e., above the 6 kb restriction fragment, lane 3)

Figure 14. Effects of aphidicolin on labeling of rDNA bands in vitro using cytoplasmic extracts taken at different points in the nuclear division cycle. Reactions were carried out for 2 hours in the presence or absence of aphidicolin as described for Figure 5. P = prophase. ES = early S phase. S = S phase. G_2 = G_2 phase.

Figure 14


P ES S G2 P ES S G2
kb
6.0
5.4
5.1
5.0
4.5
3.2
2.4
- - - - + + + + Aphid.(10 μM)
ES ES ES
kb
6.0
5.4
5.1
5.0
4.5
3.2
2.4
5 10 15 Aphid.(μM)

shifts to labeled full-length restriction fragments, as well as to smears between restriction fragments (lane 7) in the presence of aphidicolin. Such smear of product above full length restriction fragments has been shown to correspond to the position in the gel where replication intermediates would migrate (Buckler-White and Pigiet, 1982 and Nawotka and Huberman, 1988).

Evidence for secondary structure at an origin of replication.

Two-dimensional (neutral/alkaline) gel electrophoresis of in vitro labeled rDNA:

0.2 M NaCl cytoplasmic extract prepared from macroplasmodia during prophase was utilized in the replication mixture containing rDNA template, and samples were incubated for various time periods to generate a time course as described for Figure 5A. The rDNA was treated and run on a neutral gel in one dimension as described in **Materials and Methods**. The lane was then cut out and run in the second dimension under denaturing conditions in the manner described in **Materials and Methods**. Reactions in Figure 15 were performed at 37^{o}C, and those in Figure 16 at 26^{o}C. The running time varied for the two gels from 17 to 26 hours, respectively. Although

separation was not as good during the longer run due to skewing of the curves, certain aspects can be observed for each of the two time courses.

When replication reactions are carried out at 37°C (Figure 15C, dark arrow), the 3.2 kb BstEII restriction fragment migrates more slowly in the second alkaline direction at 2 hours than it does at earlier time points. High molecular weight material above the 6.0 kb restriction fragment also migrates more slowly and generates a curve during the alkaline run, which becomes very obvious by 1 hour (Figure 15B,Δ) and which becomes very strongly labeled by 2 hours (Figure 15C,Δ). With the 26°C incubations, the effect shows up more slowly, but by 3 hours the high molecular weight material above the 6 kb restriction fragment is beginning to accumulate as it did at 37°C (Figure 16C,Δ). However, it never accumulates to the same extent as it does at the 2 hour point shown for the 37°C incubations. In fact by 4 hours, it is degraded in this sample incubated at 26°C (Figure 16D). One way that labeled intermediates can be generated that run higher than the 6 kb BstEII fragment in the alkaline dimension is if there is some covalent joining of free ends of intermediate strands,

Figure 15. Two-dimensional agarose gel electrophoretic fractionation of rDNA products of in vitro replication taken at different time points. In vitro replication reactions, DNA purification and enzyme digestion were performed as in Figure 5A. Time points were: 15 minutes (A), one hour (B). two hours (C). The first dimension gel was of 0.8% agarose subjected to electrophoresis for 20 hours at 50 volts in first dimension neutral buffer prepared according to Sundin and Varshavsky (1980). The second dimension gel was of 1.0% agarose. After incubation of each first-dimension lane in alkaline buffer (0.03 N NaCl) according to Sundin and Varshavsky (1980), gels were subjected to electrophoresis for 17 hours at 50 volts. Gels were then dried by blotting between Whatman 3 MM paper as described in Maniatis et al., (1982).

Figure 15

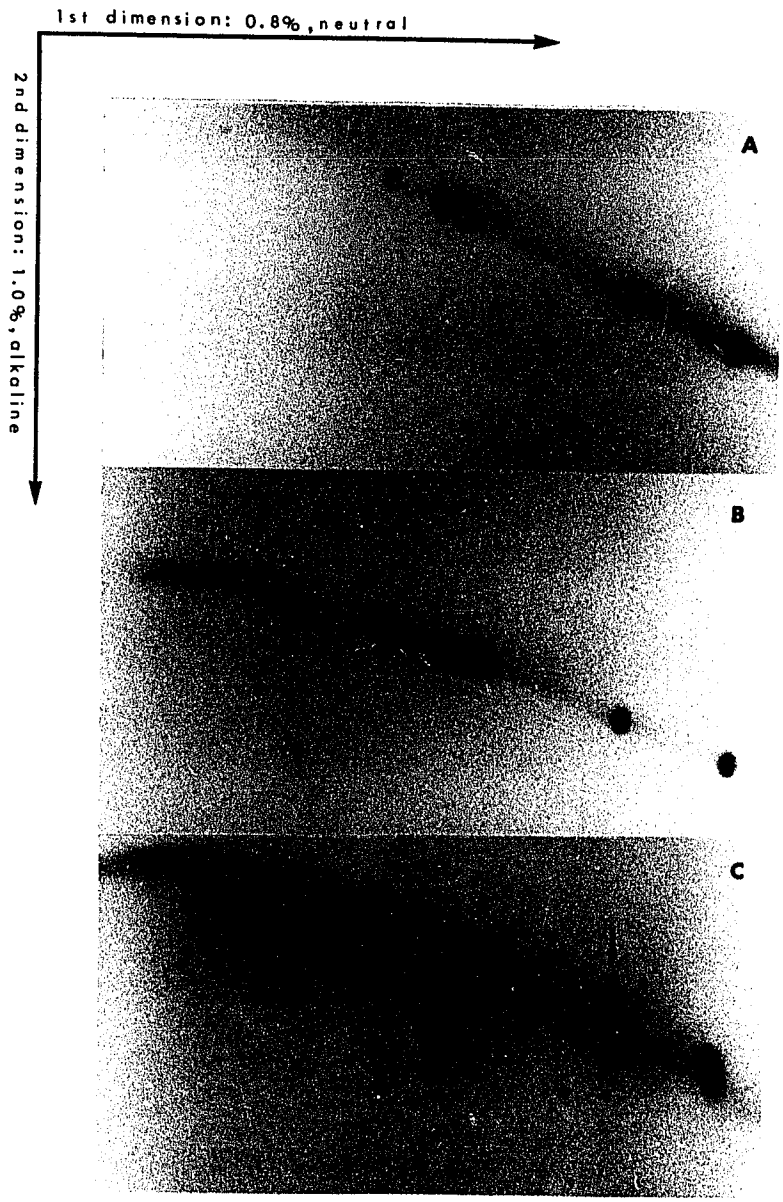
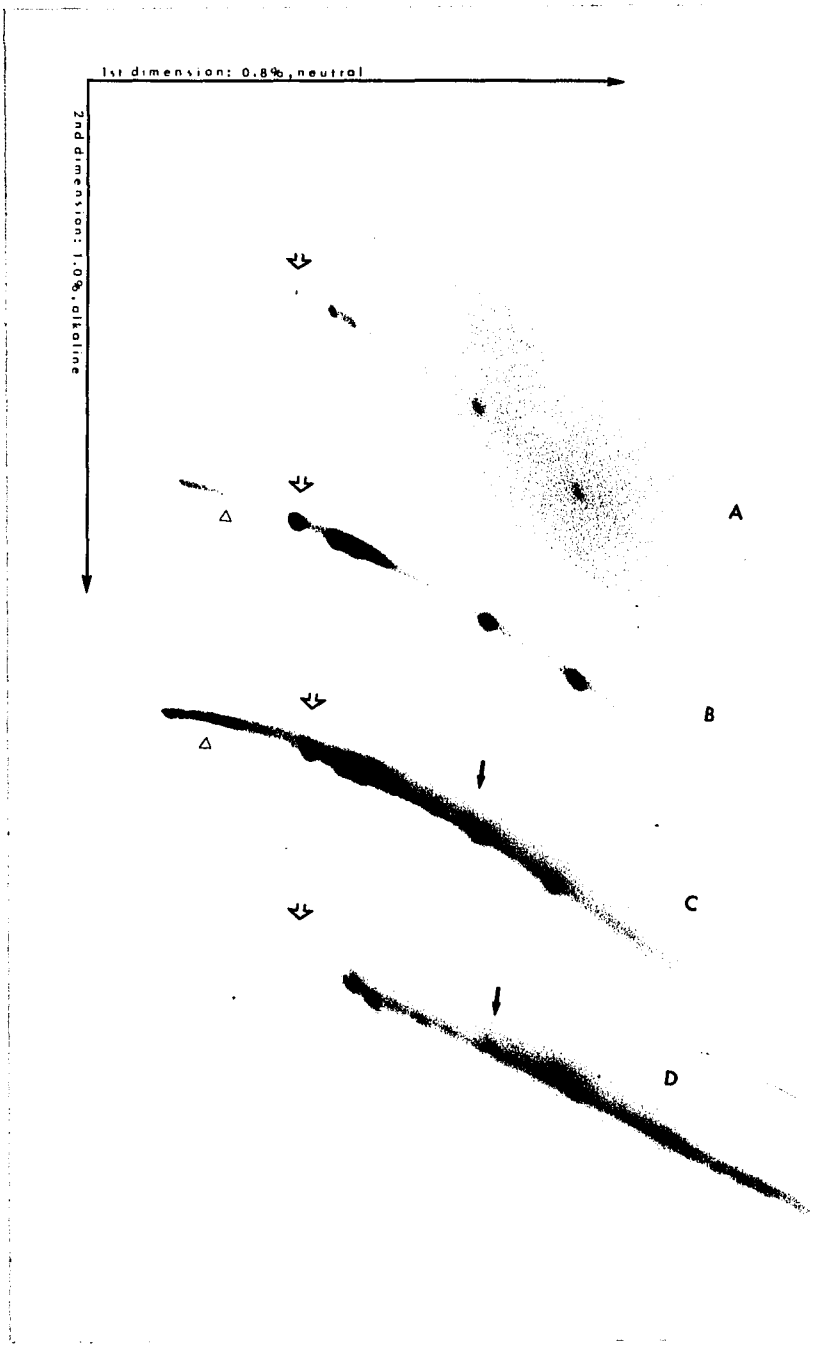


Figure 16. Two-dimensional agarose gel electrophoretic fractionation of rDNA products of in vitro replication with a prophase cytoplasmic extract at 26°C. Reactions, as in Figure 5A, were for 30 minutes (A), one hour (B), three hours (C), four hours (D). Second-dimension gels were run for 26 hours, but otherwise the procedure was the same as described in Figure 15.

Figure 16



e.g., recombination. There is as yet no further evidence for this. Another more likely possibility is that some secondary structure or modification in the replicating DNA prevents normal cleavage by BstEII. Figures 16C and 16D (dark arrows) show the position that the 3.2 kb restriction fragment migrates in the gel during the second dimensional run when derived from the 26°C in vitro replication incubations. Either this fragment does not migrate more slowly at the three hour time point (Figure 16C, dark arrow), or separation is not sufficient due to the skewed nature of the curves (when gels are run for 26 hours) to allow one to clearly visualize the migration. At 26°C there is extensive degradation at the 4 hour time point, and in particular, there is cleavage of the 6 kb restriction fragment (Figure 16D, open arrow). Compare with Figure 5A where this cleavage is also seen. The slower migration (Figure 15C, dark arrow) of the 3.2 kb restriction fragment at 2 hours observed in the second dimension alkaline run may indicate that there is a secondary structure within that fragment. Were such a structure to be resistant to restriction cleavage at some point in its formation, there could be an accumulation of

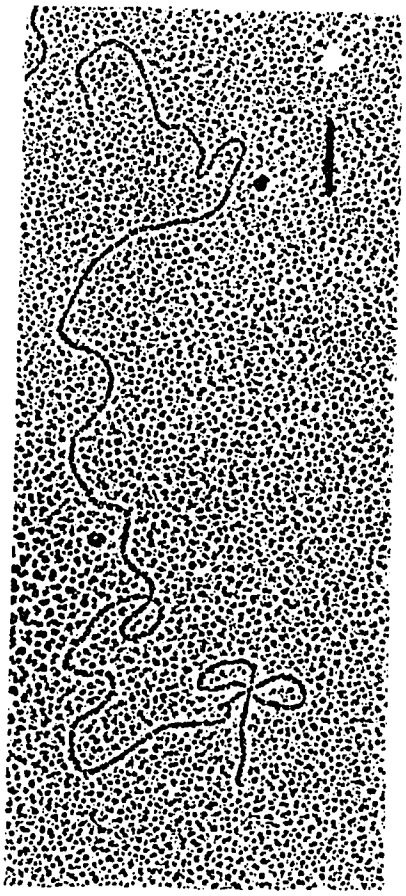
aberrantly-migrating high molecular weight material. Since BstEII cuts within the more central origin, a corresponding higher migration of those origin fragments such as is seen for the 3.2 kb restriction fragment may not be observed. This in vitro two-dimensional gel technique proves useful to display gross changes such as the slower migration of the 3.2 kb fragment and high molecular weight material, especially were these particular areas of the curve to be electroeluted at appropriate time points and products viewed by electron microscopy.

Electron microscopic evidence for secondary structure at the origin zones:

Structures observed in electron micrographs provide supportive evidence that secondary structure may be present at the origin regions. Figures 17 and 18 show electron micrographs of rDNA molecules isolated from in vivo which possess secondary structure at the origin region. Mapping through measurements made on electron micrographs with a Numonics digital integrator places the structures within the repeat regions of the origin zones. Similar structures are seen on in vitro -incubated rDNA templates, but further study is needed

Figure 17. Possible secondary structures in replication initiation zones of Physarum rDNA. The rDNA was isolated from proliferating microplasmodia as described in **Materials and Methods** and treated for 15 minutes at 37°C with BamHI (68 units/ug DNA). Top: the 21.2 kb fragment containing the potential origin nearest the rRNA transcription unit. Bottom: the 13.4 kb fragment containing both potential origins nearest the center of the rDNA palindrome.

Figure 17



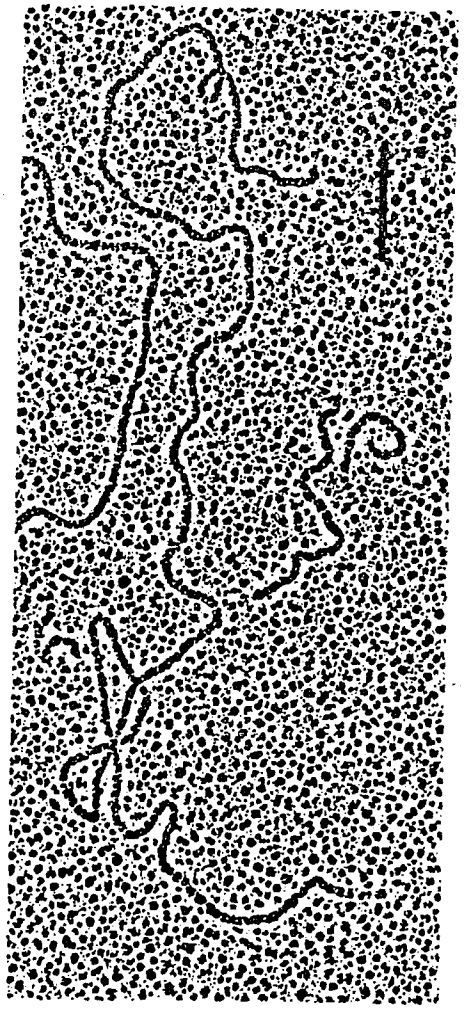
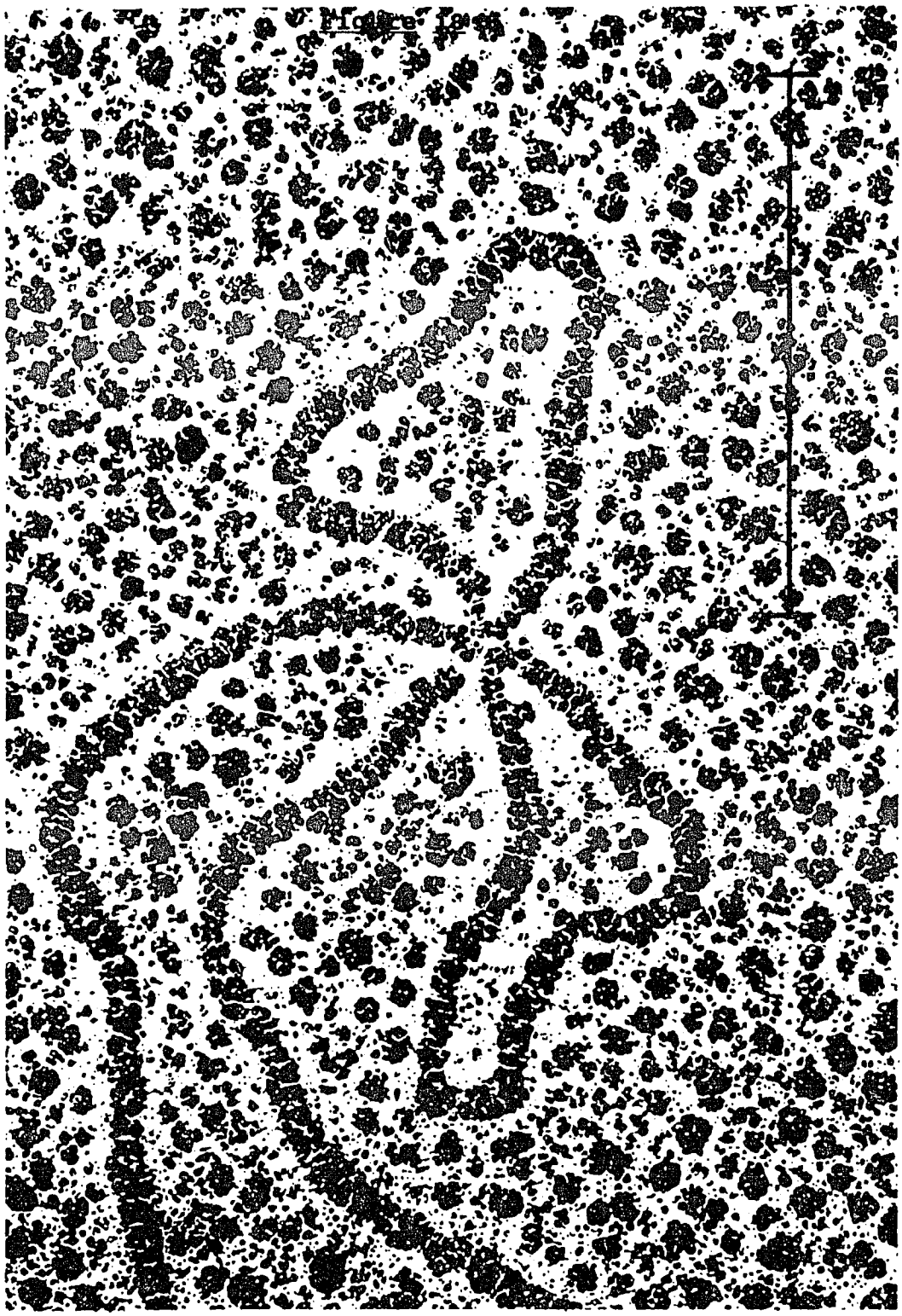


Figure 18. Secondary structure in a replication initiation zone of Physarum rDNA. The rDNA was prepared and treated with BamHI as described for Figure 17. Shown is a detail of the molecular junction of a secondary structure in the potential origin region of the central, 13.4 kb restriction fragment.



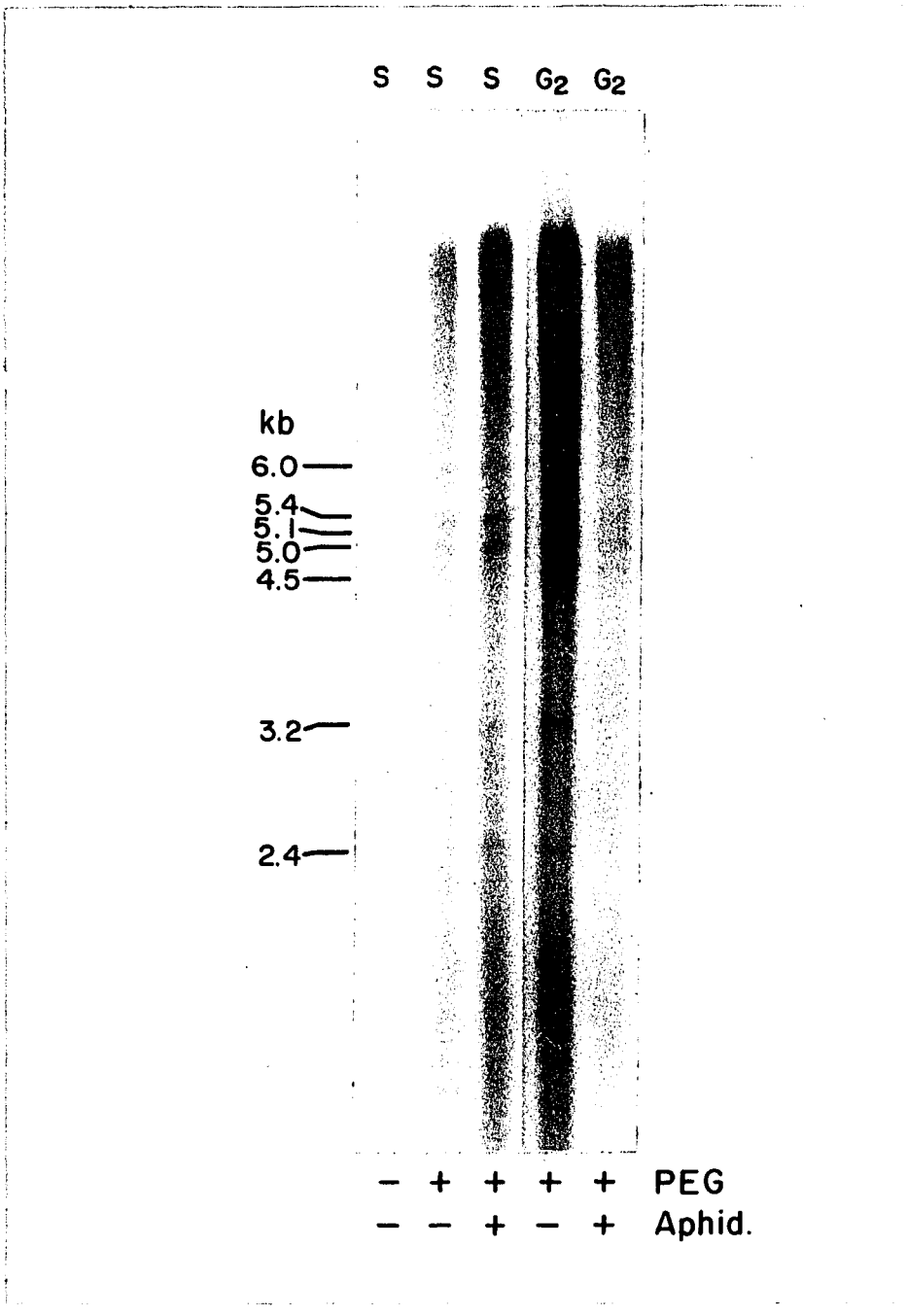
to determine if they actually form or resolve in vitro. Similar studies utilizing in vitro-incubated plasmids have not been carried out at this time.

Utilization of combined high-salt nuclear and cytoplasmic extracts.

In order to determine if high salt could extract out additional proteins which would help generate DpnI resistant replication product when the extract was prepared during S phase and utilized in a replication mixture, a high-salt nuclear and a cytoplasmic extract were prepared as described in **MATERIALS and METHODS**. The earlier replication mixture was also altered; preparation was according to Decker et al. (1986) as described in **Materials and Methods**. Combining a high-salt nuclear extract with cytoplasmic extract does not improve the efficiency of replication relative to the 0.2 M whole cell cytoplasmic extract when either is prepared during the S phase and utilized in a replication mixture. Neither of the high-salt extracts generates DpnI resistant product when plasmid pPHR21 is utilized as a template (data not shown). As can be seen in Figure 19 (lane 4 versus lane 5), aphidicolin at 10 um greatly diminishes label when an extract prepared

Figure 19. Differential effects of aphidicolin on labeling of rDNA using high salt nuclear replication mixture taken during either S or G_2 phases of the nuclear division cycle. The rDNA was subjected to *in vitro* replication for 2 hours as described in **Materials and Methods** using high-salt nuclear extracts prepared essentially as described by Decker *et al*. (1986). Reactions were performed in the presence or absence of polyethylene glycol-6000 according to Decker *et al*., (1986) and in the presence or absence of 10 um aphidicolin as described in **Materials and Methods**. The labeled rDNA was subjected to 1.0% agarose gel electrophoresis, and the gel was dried and autoradiographed.

Figure 19



during G_2 is utilized. Aphidicolin has a slight effect on rDNA labeling of the S-phase sample utilizing this extract system (Figure 19, lane 2 versus lane 3). This label may not be due to polymerase alpha activity. An alternative interpretation is that aphidicolin acts to slightly stimulate label in a similar fashion to that seen when utilizing the 0.2 M cytoplasmic extract prepared during S phase (compare to Figure 14, lanes 3 and 7).

Construction of deletion mutants from pPHR21 and preparation of pPHR20 and pPHR11.3.

Plasmids pPHR21, pPHR20 and pPHR11.3 were obtained from Dr. Volker M. Vogt. Figure 20 shows two deletion mutants constructed from pPHR21 for this thesis project, through the use of two restriction endonucleases, HpaI and BstEII. These two restriction endonucleases delete certain structural features of the origin zone located within the rDNA insert. The use of the two different restriction enzymes was followed in each case by religation of the plasmid: HpaI--The deleted fragments in this case contain most of the large number of direct repeats (Region B) upstream from the transcription initiation site, which have been sequenced by Ferris

Figure 20. Map of plasmid pPHR21.

KEY

Circle: thin line = pBR322, thick line = rDNA insert

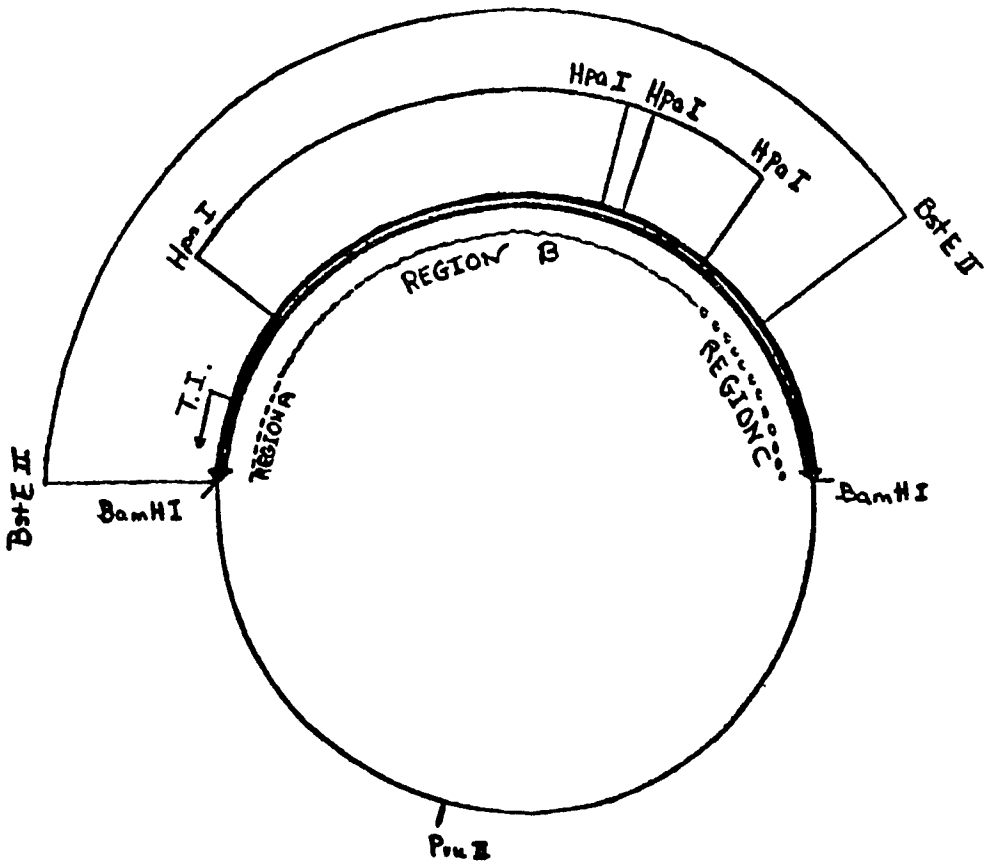
Region C = *unique sequences

Region B = 78 direct repeats of a 31 base pair unit

Region A = *unique sequences and transcription initiation site (T.I.)

[*unique sequence region = region does not contain extensive internal repeats]

Figure 20


HpaI
HpaI
HpaI
HpaI
HpaI
BstEII
BstEII
REGION B
REGION A
REGION C
T.I.
BamHI
BamHI
PvuII

(1985). Although initial in vivo mapping by electron microscopy places the putative origin in the part of Region B lying near to Region C, Ferris and Vogt (1982) have speculated that the origin actually lies in the unique sequences of Region C that are nearest to the direct repeats of Region B. At the time of their research, in vivo mapping of replication bubbles was not very precise. This deletion plasmid would not replicate if the essential in vitro origin region were in the deleted direct repeats of Region B. BstEII--The part of Region C lying near the direct repeats of Region B is contained within this deleted fragment. The sequences bear homology as compared to origin regions in other organisms (see Figure 24, designations A., B.,C.). Regions B and A are also deleted. Therefore, this deletion plasmid would replicate only if the functional in vitro origin were in the part of Region C remaining (i.e., the part further from Region B). Thus, through the elimination process one might determine which regions of the rDNA insert might be essential for in vitro initiation of replication on the plasmid.

Figure 21 shows linearized pPHR21 as well as the two deletion mutants constructed from it through use of

Figure 21. Plasmid constructs and deletion mutants for analysis of the rDNA region spanning the potential transcription initiation and origin-of-replication sites. Plasmids pPHR21, pPHR20 and pPHR11.3 were obtained from Dr. Volker M. Vogt. Plasmids pPHR21ΔB and pPHR21ΔH were constructed as part of this thesis project by deleting between BstEII and HpaI restriction sites, respectively, as described in **Materials and Methods**. (Note: There is a missprint on the photograph -- 64 bp should be 65 bp).

PLASMID CONSTRUCTS AND DELETION MUTANTS:
PHYSARUM rDNA REPLICATION AND TRANSCRIPTION INITIATION REGIONS

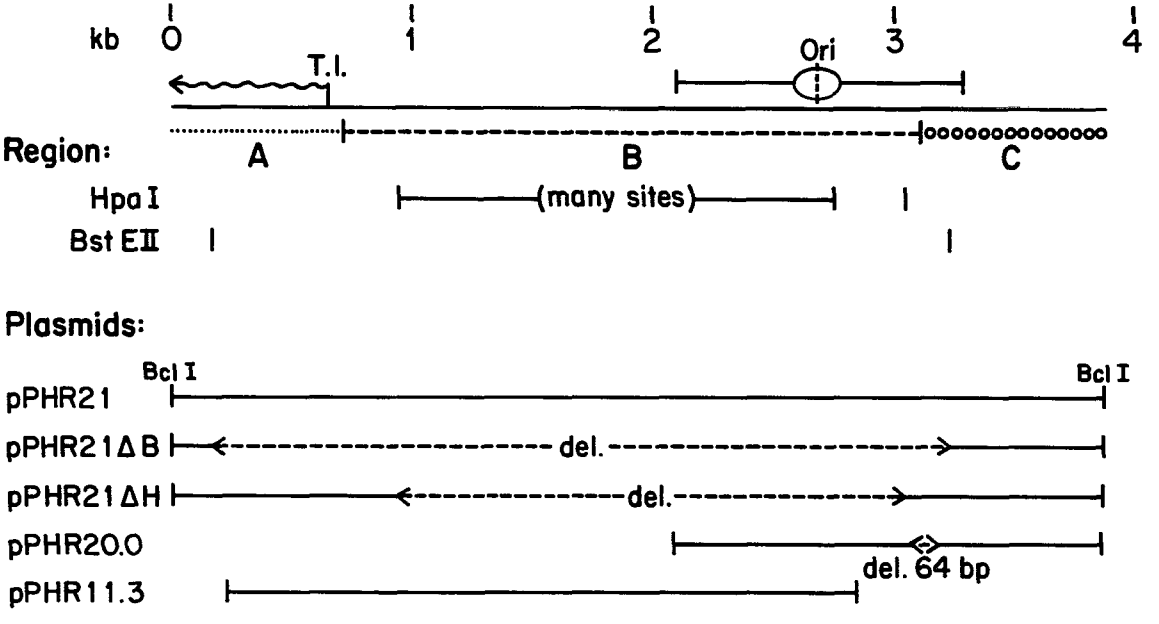


Figure 21

the restriction endonucleases described above. Figure 21 also shows linearized pPHR20 and pPHR11.3. Plasmid pPHR20 contains the more central origin region, which consists of two regions homologous to the origin zone contained within pPHR21 (see also Figures 3 and 12). However, the origin zone (rDNA insert within pPHR20) is missing the 65 bp contained within the transcription-proximal origin. The origin zone can also be seen to contain half as many repeats as are present in pPHR21 (transcription-proximal origin zone). pPHR11.3 contains the portion of pPHR21 shown in Figure 21; this plasmid rDNA insert omits Region C and the repeats immediately bordering Region C.

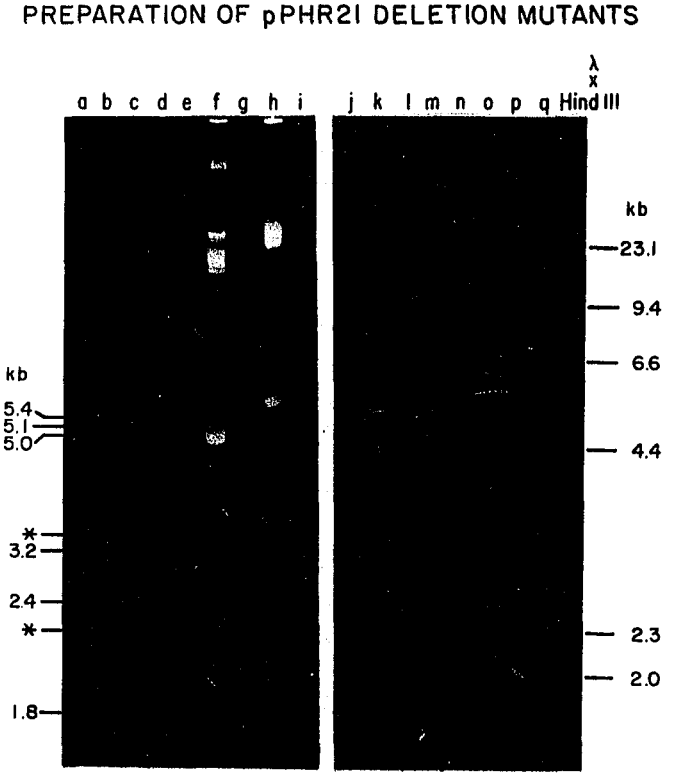
Figure 22 (lanes b, d, f) shows plasmid preparations obtained from the laboratory of Dr. Volker M. Vogt. [An additional plasmid preparation (Figure 22, lanes h and i) was not useful since it contained material that did not generate monomer plasmid of the appropriate size after transformation.] Figure 22 (lanes c, e, g) shows alkaline-lysis plasmid preparations (following transformation of HB101 bacteria with the DNA from Dr. Vogt) after one CsCl gradient purification. Lanes j-p show plasmid preparations of

the two deletion mutants constructed from pPHR21 as part of this thesis project. Although the desired HpaI fragment was obtained in the HpaI deletion mutant (Figure 22, lanes n, o, p), there is extra material in the vector as seen by linearization with PvuII (Figure 22, lane q) and by comparison of the uncut mutant with the original pPHR21 (Figure 22, lane b). This mutant requires further characterization, but may prove interesting since a preliminary result indicates that it is no longer able to be labeled very well in the in vitro replication system.

Figure 22. Gel electrophoresis of deletion mutants constructed from plasmid pPHR21. Deletions were performed as described in the legend to Figure 21. A 1.0% agarose gel was run as described in **Materials and Methods.** The gel lanes b, d, f and h show plasmid preparations (0.5 ug) obtained from Dr. Volker M. Vogt. Gel lanes c, e, g and i show alkaline-lysis plasmid preparations (0.5 ug) made after tranformation of HB101 bacteria using the plasmid DNA obtained as a gift. (Plasmid preparations are shown after one CsCl gradient preparation.) Gel lanes j-q show plasmid preparations (1 ug for lanes j, k, l, n, o, p; 0.5 ug for lanes m and q) of two deletion mutants prepared as part of this thesis project. The samples shown are: pPHR21ΔB not treated with restriction endonuclease (lane j); pPHR21ΔB treated with BstEII (lane k); pPHR21 marker treated with BstEII (lane l) and not treated with restriction enzyme (lanes b and c); pPHR21ΔB treated with PvuII (lane m); pPHR21ΔH not

treated with restriction endonuclease (lane n); pPHR21ΔH treated with HpaI (lane o); pPHR21 marker treated with HpaI (lane p) and compare again to untreated marker (lanes b and c); pPHR21ΔH treated with PvuII (lane q). BstEII linearizes pPHR21ΔB implying that the restriction site was restored by ligation. Plasmid pPHR21ΔH is discussed further in the text. PvuII should cleave only within the pBR322 vector portion of pPHR21. (See Figures 3, 12, 20 and 21 for restriction endonclease cleavage sites).

Figure 22


PREPARATION OF pPHR21 DELETION MUTANTS
a b c d e f g h i
j k l m n o p q
λ x Hind III
kb
5.4
5.1
5.0
3.2
2.4
1.8
kb
23.1
9.4
6.6
4.4
2.3
2.0

DISCUSSION

Physarum offers two great advantages for studies of initiation of replication in eucaryotes. The *Physarum* rDNA molecule is highly repeated and constitutes approximately 2% of the total DNA in plasmodial nuclei (Zellweger *et al.*, 1972). Thus any initiator proteins required for replication may be present at high concentrations in extracts. Plasmodia normally grow in synchrony, and one can make extracts at cell cycle points where components of the rDNA replication apparatus are maximally accessible.

Replication *in vitro* has been previously reported for yeast (Kojo *et al.*, 1981; Jazwinski *et al.*, 1983; Celniker and Campbell, 1982). Although selective initiation is observed, the usefulness of these systems in dissecting the replication machinery has been hampered by low efficiencies, possibly due to limiting quantities of initiator proteins for yeast replication (Campbell, 1986). Careful controls were utilized here to avoid 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 (Jong and Scott, 1985). None of our plasmid preparations show

any template activity with the Klenow fragment of E. coli DNA polymerase I, whereas plasmids containing adventitious primers do show such activity (Jong and Scott, 1985). 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 (Campbell et al., 1979).

A cell-free replication system utilizing synchronous Physarum macroplasmodial extracts selectively labels Physarum rDNA sequences both in the rDNA palindrome as well as in a specific rDNA sequence within the plasmid pPHR21. A 3.2 kb BstEII restriction fragment from the rDNA insert within pPHR21 preferentially labels in a synchronous early S phase nuclear extract that initiates but does not elongate efficiently. Two BstEII restriction fragments from the linear rDNA molecule also preferentially label in this extract, one of these also being the 3.2 fragment. The clonal and rDNA labeled fragments correspond to positions of in vivo origins located via electron microscopy relative to the ends of the linear molecule (Vogt and Braun, 1977). Plasmid pPHR21 was utilized for selective labeling studies since the 3.2 kb BstEII restriction fragment

from the rDNA molecule was the most resistant to 10uM aphidicolin. Resistance to inhibition by aphidicolin indicates that the rDNA origin insert might contain more accumulated early intermediates, as suggested by the findings of Decker et al. (1986). Results from the electron microscopy experiments show that bidirectional replication bubbles initiate on pPHR21 when it is incubated in this in vitro system utilizing synchronous macroplasmodial extracts. Bidirectional intermediates accumulate relative to the elongated product when the nuclear ES extract is utilized. A mapping of the centers of the replication bubbles on molecules showing various degrees of replication elongation places the origin in or near a series of 31-base pair repeats approximately 2.4 kb upstream from the initiation point for ribosomal gene transcription. Taken together, these two studies map the initiation sequences to a more narrowly defined region within that identified by in vivo electron microscopy (Vogt and Braun, 1977). Similar localization is obtained when the rDNA molecule is utilized, rather than pPHR21. The rDNA template was isolated from microplasmodia, which are not synchronous among themselves, although nuclei within the common

cytoplasm of one microplasmodial fragment divide in synchrony. Whereas replication bubbles on the exogenously added rDNA palindrome could conceivably have been present from in vivo isolation, these have previously been observed at about 1:1000 molecules when replication intermediates were isolated without enrichment from synchronous macroplasmodia in the G_2 phase; rDNA has been shown to replicate as extensively throughout G_2 as it does in S phase (Vogt and Braun, 1977 and Newlon et al., 1973). The replication bubbles on pPHR21 would, however, represent origins that are selectively initiated in vitro utilizing plasmodial extracts since extracts give no labeled or ethidium bromide-stained bands when incubated in the same manner but without added pPHR21 or rDNA. Also, plasmids pPHR21 or pBR322 do not show any theta structures when visualized by electron microscopy without incubation in extract (i.e., plasmids isolated from stationary phase HB101, purified and spread directly for electron microscopy). These bubble intermediates are seen approximately three fold enriched relative to whole cell extract when the nuclear extract from ES phase is utilized. Deletion mutants should help to determine

which sequences in the rDNA insert of pPHR21 are critical for efficient initiation.

Structually one might visualize a model for initiation of replication corresponding to that of Umek and Kowalski for Saccharomyces cerevisiae (Umek et al., 1989) based on DNA unwinding experiments with both bacterial oriC and the yeast H4 ARS (Umek et al., 1988; Umek and Kowalski, 1988). Borrowing from both bacterial structural models as well as the SV40 model, the authors propose that the essential core consensus sequence binds to the putative initiator protein, and as a consequence there is local unidirectional unwinding of a DNA unwinding element (DUE). The DUE is an additional sequence flanking the core consensus sequence that is thought to be a DNA element with a low unwinding energy. Such a flanking sequence 3' to the T-rich core consensus strand has been shown necessary for efficient initiation. Such a locally unwound region would provide for the entry of a helicase and of the machinery for bidirectional replication.

A similar scenario might be envisioned for the Physarum rDNA origin where a specific sequence in Region C (Figures 3 and 12) might bind an initiator protein.

As a consequence of specific binding by an initiator protein or complex within the adjacent Region C, Region B (31 bp repeat units) might be unwound unidirectionally allowing entry of helicase activity. Perhaps with the help of topoisomerase I, these activities would open the duplex for entry of the polymerase machinery, generating bidirectional replication. The polymerase machinery might bind at specific sequences or in a sequence-blind or stochastic manner within a narrow zone localized by contact with the initiator protein. If such a functional structure exists, it represents evolutionary conservation of structure from prokaryotes (discussed by Umek et al., 1989).

Whether such a model holds can be explored with the mutants described earlier in which specific structural features of the rDNA origin region contained within pPHR21 are deleted. Gel retardation assays along with DNAaseI footprinting might give information on protein binding as would fractionation of the nuclear extract (which is specific for accumulating replication intermediates) combined with electron microscopy of any bound protein complexes as described by Jazwinski and Edelman (1982) for the 2 um plasmid. In chromatin

studies of rDNA palindromes from microplasmodia, 25% of the molecules show single-stranded bubbles larger than the nucleosomal-size bubble (protein-protected bubbles) in Regions C and G of the two origins, respectively, after psoralen cross-linking performed at low salt under saturation conditions (Lucchini et al., 1987). These bubbles may correlate with inaccessible cleavage sites seen for restriction endonucleases (MboI and HinfI) which also map to the Region C or G of the two origins, respectively (Kunzler et al., 1984). These data support a model for binding of an initiator protein or complex to the C and G unique regions of the two origins.

The studies of chromatin structure on rDNA, along with studies on naked plasmid DNA containing an rDNA insert spanning Region B direct repeats (as seen in Figure 3 and Figure 12), suggests that Region B is probably not packaged in nucleosomes, but is probably covered by non-nucleosomal components (Lucchini et al., 1987). Based on the chromatin studies, in combination with a circular dichroism study, an unusual conformation--perhaps an atypical B-DNA--is suggested (Lucchini et al., 1987). Similar structure is observed for the repeat region of the more central origin which also contains similar

(although fewer) repeats. The secondary structures reported in this thesis research also map to the direct repeats of the two origin regions (Figures 17 and 18). Two-dimensional gel analysis combined with electron microscopic analysis of DNA products eluted from portions of such a curve (Figures 15 and 16) should indicate the types of rDNA intermediates generated at various time points, whether such secondary-structure intermediates or other complex events might be transient in the replication intermediate material accumulating over time within the whole cell extracts and whether such structures change or resolve within the extract. Aanalysis of electroluted material larger that full-length restriction fragments should provide information on the presence of replication intermediates, their migration position in the gel and the effect on migration caused by the presence of any unusual structure within the origin zones. Such analysis should give additional indication of how well initiation is occurring on the rDNA template compared to the plasmid template <u>in vitro</u> when utilizing a specific extract. One might question the role of the additional repeats and of an extra cassette of approximately 65 bp located in the unique

Region C of the origin zone proximal to the transcription initiation site but not in the origin zone closer to the center of the molecule. The role of the direct repeats and the proximity of one origin versus another to the rRNA transcription unit adds another dimension of complexity to initiation of rDNA replication.

0.2 M NaCl cytoplasmic extracts prepared during prophase and early S phase show the most replication active when plasmid pPHR21 is utilized as template (Figure 7). It is known that Physarum thymidine kinase activity increases at about 1.25 hrs prior to metaphase and is maximal just after telophase (Sachsenmaier et al., 1967). Nuclear DNA polymerase levels increase sharply 1 hr prior to metaphase, corresponding closely to thymidine kinase levels (Brewer and Rusch, 1966). Studies with a cycloheximide block have shown that initiator proteins, required during early S-phase, are synthesized during the early prophase immediately preceding (Cummins and Rusch, 1966). The nucleolus disintegrates during prophase and reforms during the first 1.5 hrs after mitosis (Guttes et al., 1961). 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. Such sequestering of replication components by endogenous episomes through *cis*-acting elements has been reported for the viral bovine papilloma-SV40 chimeric plasmid when an SV40 origin-containing plasmid (pSV-ori) is supertransfected into these cells under conditions where both vectors share the same endogenous initiator protein (Roberts and Weintraub, 1988b). There is also the possibility, as discussed by Roberts and Weintraub (1988b) that sequestering might occur as a result of binding of initiator elements into an inactive (stable) form of replicative complexes on endogenous templates. Since rDNA is under copy number control, perhaps a variation of that theme might be causing such an effect after nucleolar reorganization (i.e., during rDNA replication). Interestingly, Kunzler *et al*. (1984) interpret the disappearance of their endonuclease insensitivity sites on rDNA chromatin through the use of high salt on mitotic nucleoli to mean that complexes binding rDNA to the nucleolar matrix during S and G_2 cell cycle

phases are released from the matrix during mitosis, but remain bound to the rDNA template at the C and G regions of the two origins. Whether such putative complexes would be released in prophase extracts at the 0.2 M salt concentration used in preparation of the extracts for this *in vitro* assay was not examined in that work. Initiator protein or complex might be less extractable in 0.2 M salt if rDNA origin regions were bound to the matrix in S and G_2 as has been proposed for *Physarum* origins in general (Aelen *et al.*, 1983). More severe extraction, sonication and extract fractionation would be another approach, although problems with polymerase stability are reported as purification increases (Weber *et al.*, 1988). Isolated nucleoli (Bradbury *et al.*, 1973) might be used as a source of extract preparation for a comparison of activity with nuclear extracts which function best during nucleolar disorganization. However, it must be noted that since macroplasmodia have no G_1 cell cycle stage, but begin DNA synthesis immediately after telophase, accumulation of positive factors necessary for DNA replication (e.g. DNA polymerase and thymidine kinase activities mentioned earlier) must be available at the time of mitosis relative to other

cell types having a G_1 phase where positive factors for DNA replication begin to accumulate in preparation for S phase.

Efficiency of generating full-length DpnI resistant material through use of whole cell extracts appears also to be related to the growth cycle stage of microplasmodia at the time of harvest for fusion into macroplasmodia. Although the time remains to be precisely quantitated, Weber et al. (1988) have described a destabilization of polymerase alpha activity after exponential growth, 48 hours in shaker culture representing maximum polymerase alpha activity under standard growth conditions. A similar destabilization of initiator complex has been reported when yeast cell fractionation occurred in late logarithmic phase versus early logarithmic phase (Jazwinski and Edelman, 1982). A cytoplasmic inhibitor of DNA polymerase activity has been isolated from Physarum (Murakami-Murofushi et al., 1976). Although the activity has not been precisely characterized, excess DNA eliminates the inhibitory effect. Weber et al. (1988) find an intrinsic polymerase alpha inhibitor can be removed at the beginning of protein purification through the use of

poly(ethyleneimine) precipitation. A nuclear-localized polymerase alpha inhibitory activity has also been described (Holler, 1989). Whether this inhibitor relates to the truncation of elongation activity seen in nuclear extracts or there are other controls operating or whether there is simply missing cytoplasmic component remains to be determined. The role of such inhibitors in the extract assays can be examined in the future, but could implicate the presence of negative regulatory controls in the process of DNA replication as a whole in nonsynchronous Physarum microplasmodia. Mixing experiments with extracts from various cell cycle points should provide evidence for or against the different levels of inhibitory activity over the cell cycle. Through the use of both types of extracts (nuclear and whole cell) taken at the various cell cycle points both positive and negative regulatory controls may be investigated.

Results with aphidicolin differ when utilizing 0.2 M NaCl cytoplasmic ES and S extracts from results obtained when using 0.2 M NaCl cytoplasmic P and G_2 extracts. The difference indicates an alteration in mechanism of action of aphidicolin at these S-phase

times. Since the differential effect is not seen in ES nuclear extracts, generation of this effect apparently involves cytoplasmic participation when utilizing whole cell extracts from S phase. The existance of different mechanisms of action of aphidicolin are discussed by Huberman (1981). Some differences relate to whether the inhibitor is added in vivo or to isolated nuclei, and different templates also modify aphidicolin effect. In experiments for induction of methotrexate resistance, Hoy et al. (1987) have reported a different effect of aphidicolin on cells in S phase at the time of inhibition of DNA synthesis. Overreplication of DNA through misfiring of initiation sites has been suggested, along with dissociation of cell cycle progression which misaligns S\M phases. Gene amplification and methotrexate resistance are thought to be related to the overreplication event. The type of product represented by the increase in label seen in Figure 14 (lanes 6 versus 7) must first be investigated. Preliminary observation would suggest an increase in replication product due to increase in material migrating more slowly than normal full length fragments. Further work with aphidicolin may give information on control of S phase

replicons through mechanisms which involve DNA-polymerase complexes.

Use of nuclear extracts prepared during ES phase and 0.2 M NaCl cytoplasmic extracts may provide information on controls over separate phases of replication such as distinguishing controls for initiation from those for elongation. An example from this research would be the preferential labeling by the G_2 nuclear extract of the origin-containing restriction fragment in pPHR21 versus the same restriction fragment cleaved from the rDNA template. However, when the cytoplasmic component is present in the G_2 sample, there is paradoxically better labeling of rDNA versus generation of full-length DpnI resistant material from pPHR21, which contains this origin region (see Figures 5B, 7 and 8). The difference can be compared with the ES nuclear extract sample where the rDNA and the plasmid clone pPHR21 label similarly in the 3.2 kb origin-containing restriction fragment common to both, and also label in a similar fashion when the cytoplasmic extract is utilized (see Figures 5B, 7 and 8). Nuclear transplantation experiments performed by Guttes and Guttes (1968) have shown that synchronous plasmodia whose nuclei are in ES phase

continue replication when allowed to fuse with synchronous plasmodia whose nuclei are in late interphase\prophase of the cell cycle. In contrast late interphase\prophase nuclei do not begin DNA synthesis when sharing a common cytoplasm with nuclei in ES phase (Guttes and Guttes, 1968). Similar experiments have been discussed by Roberts and Weintraub (1987) in interpreting cis-acting effects of the viral bovine papilloma\SV40 chimeric plasmid during S phase. However, since rDNA replication occurs during both S and G_2 cell cycle phases, any rDNA cis-acting effects may relate to its copy number control and may function at times other than S phase.

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 (Cooney et al., 1988). Data presented in this thesis indicate the existence of secondary structure at both origin regions (see Figures

18 and 19), but the role of such structures in replication, if any, is presently unknown. Further analysis through neutral/alkaline two-dimensional gel electrophoresis of *in vitro* labeled rDNA using various enzymes to distinguish the two origin regions while leaving each origin intact would help clarify whether both are functional *in vitro* as they have been shown to be *in vivo*, and the use of plasmid pPHR20 (Figure 21) of the more central origin region should provide information on whether the structural features differ functionally for the two origin regions on the rDNA template.

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 (Fouquet *et al.*, 1975). At this point no origin of replication near a protein-coding gene has been definitively identified in *Physarum*. Availability of such cloned chromosomal origin regions would allow a comparison of *in vitro*

function and distinguish any differences between labeling of extrachromosomal rDNA and a chromosomal replicon. Such differences viewed across the cell cycle might give clues to copy number control of rDNA. The differential effect of aphidicolin could further be explored using both types of replicons. Use of a chromosomal repliconn might provide further information on the extent of the role of endogenous DNA in sequestering up needed replication machinery in comparison to rDNA which replicates at a different time.

Characteristics of Physarum rDNA differ from those recently reported for ribosomal genes of yeast. In yeast most rDNA is replicated unidirectionally by forks moving in the direction of transcription (Linskens and Huberman, 1988; Brewer and Fangman, 1988). 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 (Figures 3 and 12). Thus bidirectional replication of Physarum rDNA does result in forks progressing in the direction of transcription. It remains to be determined whether or not the repeat

sequences bridging the transcription and replication initiation sites play any role in regulation of either transcription or replication. Multiple enhancer-like repeats bridging the analogous sites in Tetrahymena rDNA are believed to function as binding sites for positive regulators of rDNA replication (Larson et al., 1986). However, the number of enhancerlike repeats contained in the Drosophila rDNA genes (insert$^+$ and insert$^-$) do not appear to determine replication dominance (Spradling and Orr-Weaver, 1987).

The system described here offers a unique opportunity to dissect control of replication of a linear replicon with multiple, distinct origins. Possibility exists for further expansion of in vitro studies to look at choice of one rDNA origin region over another utilizing various cell cycle preparations, controls of replication of various types of replicons over the cell cycle (e.g., comparison of in vitro activity of rDNA origins to chromosomal origins), methylation effect if any, and in vitro chromatin assembly. If controls were to be identified, in vitro studies might localize these to nuclear or whole cell preparations. There is also the need to identify any cis-acting sequences and

related cell cycle effect, copy number control(s) and its position in cell cycle, as well as to explore the mechanism of action of aphidicolin.

Since the Physarum rDNA origin regions have been sequenced (Ferris, 1985), several types of specific sequence homologies to other origin types are presented (Figure 23 and accompanying figure legend). Three characteristics of the replication mechanism at known prokaryotic and eukaryotic origins of replication include an AT-rich region(s), helicase activity and topoisomerase activity. AT-domains have been found in some instances to allow helical bending for proper protein fit (Deb et al., 1986b; Zahn and Blattner, 1987; Kelly, 1988) or to perhaps contribute along with, or as part of, other flanking sequences to form more easily unwound regions on the duplex as shown for oriC and yeast ARS sequences (Dean et al., 1987; Kornberg, 1988; Umek et al., 1989). The yeast ARS core consensus sequence (Broach et al., 1983), which is expected to bind a putative initiator protein or complex, is also AT-rich (reviewed by Umek et al., 1989). A helicase activity has been associated with initition mechanism at origins in prokaryotes and with T antigen-specific

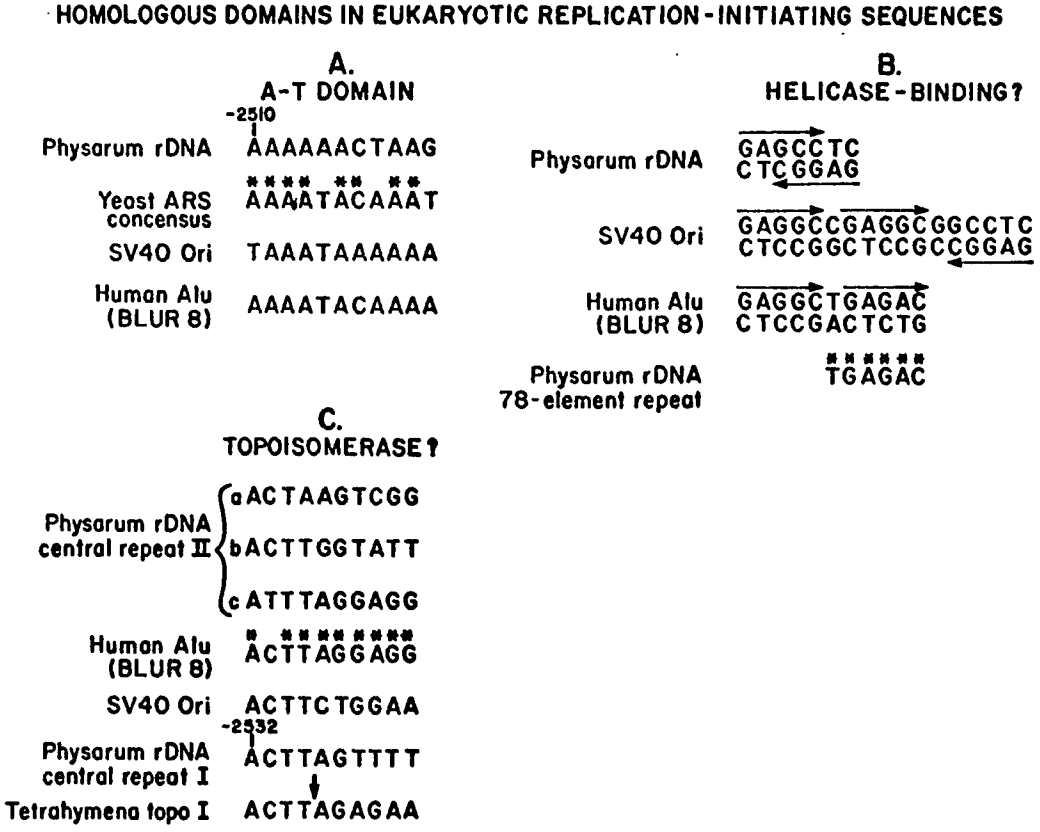
unwinding at the core origin, as well as at replication forks (Kornberg, 1988; Kelly 1988). Helicase activity has also been isolated from mammalian extracts (Hubscher and Stalder, 1985; Seki et al., 1987). Such unwinding activity is necessary ahead of the elongation fork, but creates tortional stress (positive supercoiling) in the constrained template (Kornberg, 1988). Both in vitro and/or in vivo studies with oriC, SV40, as well as yeast, have shown the need for either topoisomerase (type I or type II) for providing swival action in the removal of energy barriers on the template (e.g., caused by local unwinding through helicase activity ahead of replication forks.)(Cozzarelli, 1980; Zeng et al., 1985; Snapka, 1986; Yang et al., 1987; Brill et al., 1987; Avemann et al., 1988; Champoux, 1988).

Even though sequence homology to other origins can be located in the rDNA origin zones (Figure 23), there are many ambiguities lacking correlation to known functions which remain to be investigated. For example, the sequence GAGGC is found as four pentanucleotide inverted repeats for binding of T-antigen in the SV40 core origin (Tegtmeyer et al., 1983; Tegtmeyer et al., 1988; Deb et al., 1986a). A different number and arrangement of this

sequence exists in flanking region I of the SV40 core origin (Ryder et al., 1985; DeLucia et al., 1986). However, in Physarum rDNA a somewhat similar sequence GAGAC is present both in the unique region as well as in each of the flanking 78 repeat units, but without the same arrangement pattern. Such a sequence homology has been indicated by I.P. in the Region C of Figure 24. The AT-domain flanks the binding site for T-antigen in the SV40 core origin (Deb et al., 1986a,b; Dean and Hurwitz, 1987; Wold et al., 1987), but an AT-rich core consensus is thought to bind an initiator protein in yeast, followed by an AT rich flanking sequence (Umek et al., 1989). Likewise, Physarum origin regions also show such AT-rich structures both in Region C as well as in the Region B repeat unit. Whether there is actually any sequence conservation between prokaryote and eukaryote origin regions seems less likely than conservation of structural features.

Figure 23. Homologous domains in eukaryotic replication initiating sequences. Three elements found in Region C of Physarum rDNA, approximately 60 kb from the border of the B-region (see Figure 24) and in the zone of initiation mapped by electron microscopy are shown. Other global homologies from Region C and Region B are included. Comparisons are made to similar sequences found at various other reported initiation sites. References: yeast ARS (Campbell, 1986); SV40 core origin (Deb et al., 1986a; DeLucia et al. 1986; Tegtmeyer et al., 1988); human Alu (Jelinek et al., 1980); SV40 and tetrahymena topoisomerase I cleavage site (Porter and Champoux, 1989, Anderson et al., 1985).

Figure 23

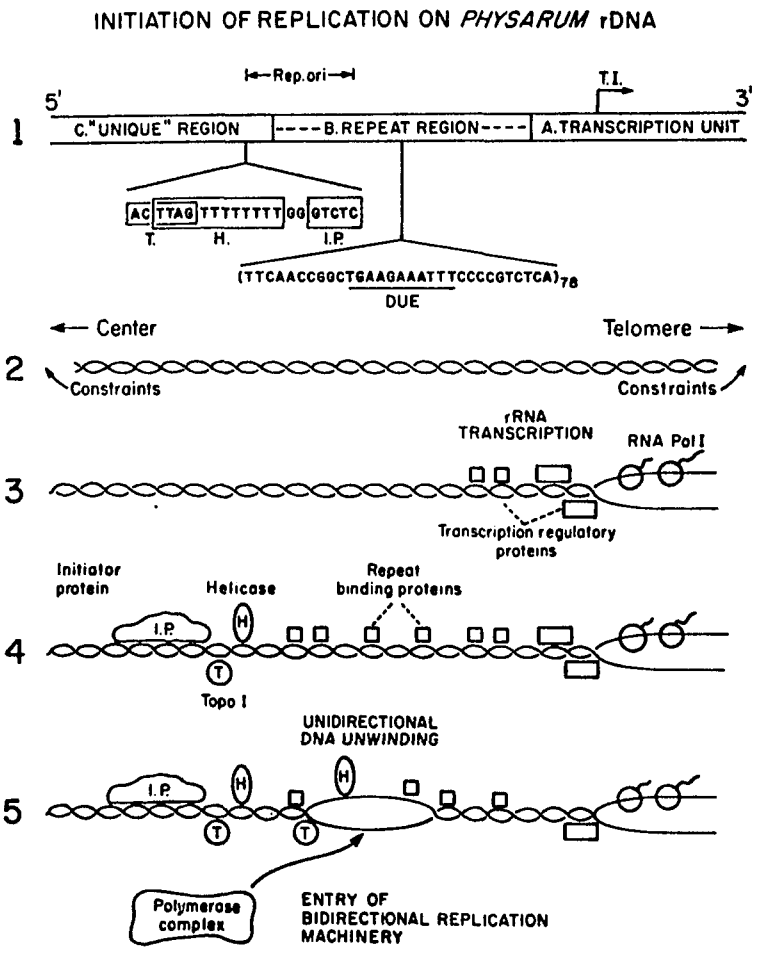

HOMOLOGOUS DOMAINS IN EUKARYOTIC REPLICATION-INITIATING SEQUENCES
A.
A-T DOMAIN
-2510
Physarum rDNA AAAAAACTAAG
Yeast ARS concensus AAAATACAAAT
SV40 Ori TAAATAAAAAA
Human Alu (BLUR 8) AAAATACAAAA
B.
HELICASE-BINDING?
Physarum rDNA GAGCCTC CTCGGAG
SV40 Ori GAGGCCGAGGCGGCCTC CTCCGGCTCCGCCGGAG
Human Alu (BLUR 8) GAGGCTGAGAC CTCCGACTCTG
Physarum rDNA 78-element repeat TGAGAC
C.
TOPOISOMERASE?
Physarum rDNA central repeat II
a ACTAAGTCGG
b ACTTGGTATT
c ATTTAGGAGG
Human Alu (BLUR 8) ACTTAGGAGG
SV40 Ori ACTTCTGGAA
-2532
Physarum rDNA central repeat I ACTTAGTTTT
Tetrahymena topo I ACTTAGAGAA

Based on information available for known origin structure and on the known sequence structure of the Physarum origin regions (Ferris, 1985), the following model in Figure 24 for the transcription-proximal origin may be invoked; (other than the well-studied prokaryote and viral origins, only yeast ARS elements are available for model conjecture at this level of resolution):

Part 1 shows sequence homologies to other known origin regions which are contained within an extra 65 bp cassette found only in the transcription-proximal origin in Region C: I.P. (homology to SV40 initiator protein binding site), H (AT-rich region which might participate in site-specific helicase activity; also shows homology to the yeast core consensus sequence (Figure 23), T (Topoisomerase I binding site homology (Figure 23), which overlaps the A-T motif). Repeat Region B (containing 78 repeats of a 31 base pair consensus unit): within each repeat unit, there is found an found an AT-rich element (labeled DUE) which might contribute in some fashion, or along with other specific sequences, to ease of unwinding of a larger area of the duplex. Part 2 shows the rDNA molecule subject to constraints found in vivo. These could be attached to the nucleolar

Figure 24. A model for initiation of replication of Physarum rDNA. A map showing sequence elements present in the B and C regions is shown: (1) with orientation of the telomere and center indicated as potential sites of chromosomal constraints (Bergold et al., 1983; Kunzler et al., 1984). (2) Proteins binding to the rRNA transcription control regions are indicated as rectangles, and proteins binding to the 78 bp repeats are indicated as squares. Initiator, topoisomerase I and helicase proteins (I.P., T and H, respectively) are depicted binding to the C region initially since this region contains elements homologous to binding sites for such proteins in other systems (see Figure 23). The actual unwinding event is drawn over the B region where electron microscopic data places it, and where an A-T rich sequence may serve as a DNA-unwinding element (DUE).

Figure 24


INITIATION OF REPLICATION ON *PHYSARUM* rDNA
Rep.ori
T.I.
5'
3'
1
C."UNIQUE" REGION
B.REPEAT REGION
A.TRANSCRIPTION UNIT
AC TTAG TTTTTTTT GG GTCTC
T.
H.
I.P.
(TTCAACCGGCTGAAGAAATTTCCCCGTCTCA)78
DUE
Center
Telomere
2
Constraints
Constraints
rRNA TRANSCRIPTION
RNA Pol I
3
Transcription regulatory proteins
Initiator protein
Helicase
Repeat binding proteins
I.P.
H
4
T
Topo I
UNIDIRECTIONAL DNA UNWINDING
I.P.
H
H
5
T
T
Polymerase complex
ENTRY OF BIDIRECTIONAL REPLICATION MACHINERY

matrix as suggested by Aelen et al. (1983) or to other nuclear features. A-T rich regions are a prominent characteristic of matrix-attachment-sites (MARS) (Cockerill and Garrard, 1986). Part 3 shows the location of the transcription initiation site along with polymerase I and RNA transcripts in an unwound area. Transcriptional factors binding here might influence both transcription and replication or in some way contribute to an open chromatin structure in the area as might proteins binding to the repeat units. Transcriptional enhancers are now known to directly influence origin activity in a number of viral systems (DePamphilis, 1988). One can visualize the need for a larger area of duplex, free for unwinding (represented by Region B consisting of the many repeats), whereby polymerase machineries for transcription and for replication could gain entry at this transcription-proximal origin. Local unwinding might allow entry of helicase activity as part of another recognition complex or as an individual activity, part 4. Topoisomerase I might bind to DNA to relieve torsional stress or other constraining energy barriers. Once the duplex is open DNA primase\DNA polymerase alpha as part of a polymerase

complex or as one of separate polymerase(s) alone or in synenergy could give rise to bidirectional forks through leading and lagging strand synthesis (part 4). Such a model, though speculative, raises questions and points to directions that could be next explored with mutant clones. As more proteins are isolated and characterized, the system can be further extended in a very specific way.

APPENDIX

Several experiments were performed in search of an alternative approach to that utilizing the macroplasmodial life form. For example, an extract might have been prepared from microplasmodia where one extract could have been used rather than extracts prepared from several cell cycle points. Also, if a successful transfection method were to have been developed, the experiments could have been carried out with whole plasmodia rather than with extracts. However, these approaches did not give encouraging results. They have been included as an appendix since they are not part of the macroplasmodial *in vitro* system which was developed and utilized for the thesis project. Although not part of the macroplasmodial extract approach, the results are informative.

I. Utilization of microplasmodial extract for labeling of rDNA in vitro.

An extract from microplasmodia was prepared in the same fashion as the 0.2 M whole cell cytoplasmic extract from macroplasmodia (**Materials and Methods**). Microplasmodia are not synchronous among themselves; only the nuclei within the cytoplasm of a single microplasmodial fragment are synchronous. A single microplasmodial nucleus would have about a 55% probability of being in G_2 at any given time since 5 hours of the 9 hour cell cycle is G_2.

Figure 25 (left) shows incubations of rDNA template in the replication mixture (see **Materials and Methods**) containing microplasmodial extract and utilizing various amounts of cold dCTP as described in the accompanying figure legend. The labeling with this extract was erratic even at the same time point or with the same concentration of cold dCTP when running duplicate samples. This erratic labeling may have been due to excess insoluble polysaccharide (from the slime) which may have diluted part of the 30 ul of extract added to the replication mixture for each sample. Whereas in macroplasmodia lightly pigmented new growth only can be

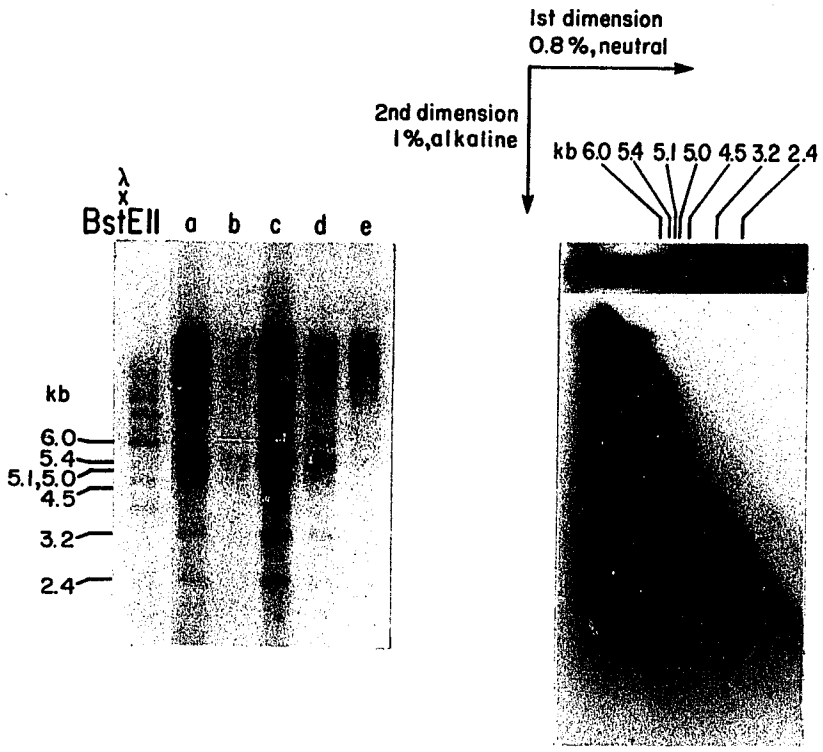
harvested, both new and old growth would be harvested in microplasmodial cultures. This might produce more slime in the extract since slime and pigment increase with the age of plasmodia. Also, macroplasmodia are grown on filter papers, separated from excess medium; the medium in shaker cultures of microplasmdia would be more difficult to remove, and contains slime, as well as inhibitors of polymerase alpha activity (Weber et al., 1988; Murakami-Murofushi et al., 1976). As seen in Figure 25 (left) lane 1, there is also a lack of specificity of labeling with this extract since the lambda DNA is also labeled.

Figure 25 (right) shows a 1 hour time point run on a neutral gel aligned over a 1 hour time point run in the second dimension on an alkaline gel. There is a large background of short product at 1 hour when utilizing this extract. This type of product can be seen as a smear of increasingly shorter product extending vertically below the restriction fragments which represent full length material on the linear curve. This vertical smear probably represents repair label or nicking which has occurred as a result of the incubation. The regions of the linear curve extending above full-length

restriction fragments would represent the positions on the curve where intermediate products of replication would migrate in the first direction (Buckler-White and Pigiet, 1982; Nawotka and Huberman, 1988). The short product extending down beneath these regions is apparently unligated material that is not covalently attached to the parental template. Once it had been shown that macroplasmodial extracts were less erratic and initiated with specificity, work was focused on macroplasmodia.

Figure 25. Labeling of rDNA in vitro with microplasmodial extracts. The rDNA was incubated for one hour in the in vitro replication mixture utilizing a microplasmodial extract described in the accompanying text. The rDNA was then purified, digested with BstEII and run on a 0.8% agarose gel. The gel was blotted dry and autoradiographed. Procedures were the same as those described in **Materials and Methods**. Left: 0.8% agarose gel showing lambda phage DNA (lane a) digested with BstEII after incubation in the in vitro replication mix as described above for rDNA. Lanes a-e are five incubations utilizing rDNA as template in which the replication mixture contained various amounts of dCTP. Lane a, no dCTP; lane b, 5 uM; lane c, 10 uM,; lane d, 25 uM; lane e, 50 uM. Right: A 0.8% agarose gel lane from a first dimensional run (described above) utilizing 2.5 uM dCTP was cut out and soaked in alkaline buffer. It was then placed horizontally, cast in an alkaline gel and run in the second dimension for 12 hours as described in Figure 15. A first demensional marker gel lane run concurrently in the first dimension was aligned above the second dimension gel to mark the restriction fragments.

Figure 25


λ
x
BstEII a b c d e
kb
6.0
5.4
5.1,5.0
4.5
3.2
2.4
1st dimension
0.8 %, neutral
2nd dimension
1%, alkaline
kb 6.0 5.4 5.1 5.0 4.5 3.2 2.4

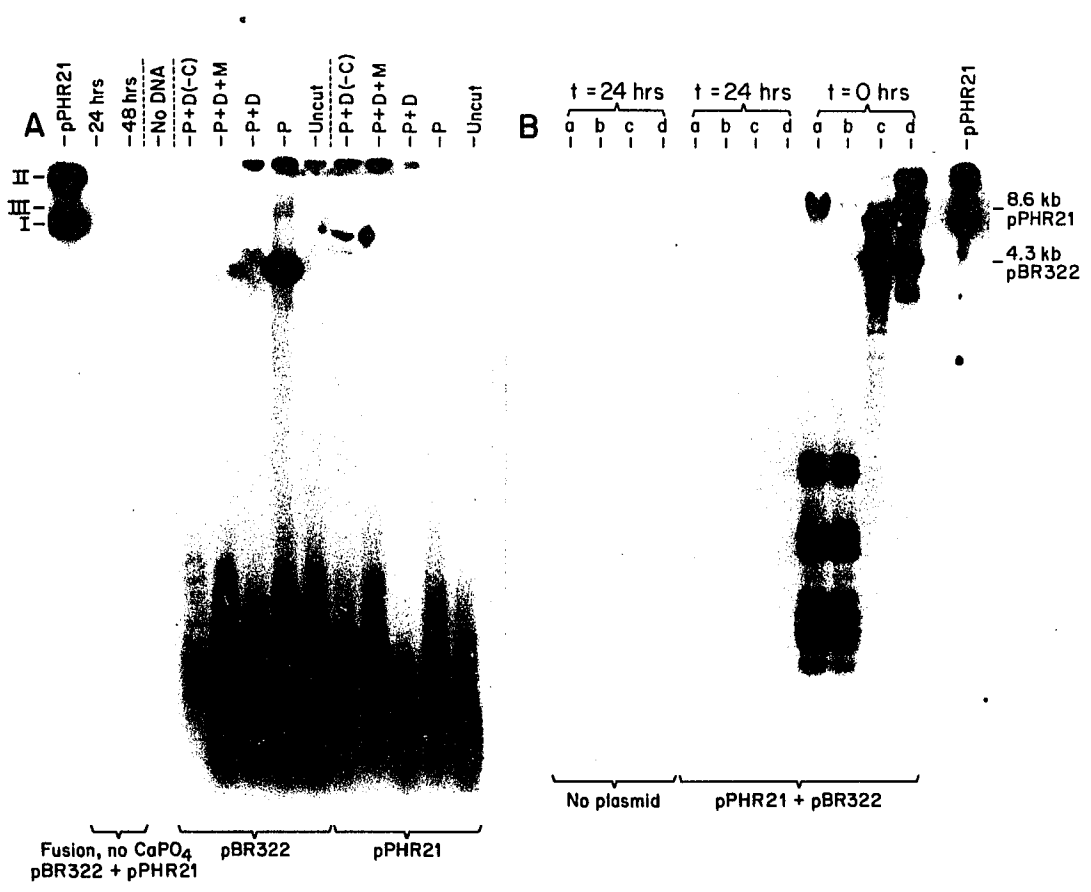
II. Transfection attempts utilizing fusing microplasmodia

Several types of procedures were attempted in trying to transfect microplasmodia at the point just prior to when they are pipetted onto filter paper for fusion into macroplasmodia (Figure 26 and accompanying figure legend). Results indicate that even though the input DNA may get into the cytoplasm, it is apparently degraded before it reaches the nucleus. Input DNA does not survive the 24 hour time point, and no DpnI resistant material is generated. A comparison of transfections performed with or without Ca-PO_4 is informative. Without Ca-PO_4, no plasmid DNA enters the plasmodia, and none is detected on the gel, even in degraded form. With Ca-PO_4, plasmid DNA is detected by hybridization to a pBR322 probe. This apparently-entering plasmid DNA is only seen intact at 0 time. However, at 24 hours it is entirely degraded, even prior to cleavage with PvuII, DpnI or MboI.

Figure 26. Attempts to transfect fusing Physarum plasmodia with pPHR21 and pBR322. Microplasmodia (at 48 hours growth) were washed with water and with phosphate-buffered saline and allowed to settle. To 0.3 ml of packed microplasmodia were added 0.5 ml of a mixture containing plasmid DNA (5 ug of pPHR21 and pBR322 together or each separately), 31 ul of 2 M $CaCl_2$, 0.25 ml of 2 x HBSP and water (2X HBSP is 1.5 mM dibasic sodium phosphate, 10 mM KCL, 280 mM NaCl, 12 mM d-glucose and 50 mM HEPES, pH 7.0). The Ca-PO_4 precipitate was allowed to form in the presence of the microplasmodia for 30 minutes before plating and fusion. At 48 hours post-transfection, low-molecular-weight DNA was extracted from the fused plasmodia essentially by the method of Hirt (1967). DNA was purified by successive extractions with phenol, chloroform and diethyl ether. Aliquots containing approximately 1 ug of DNA were treated or not treated with restriction enzymes PvuII (40 units to linearize), DpnI (20 units) and MboI (20 units), and subjected to 1.4% agarose gel electrophoresis. After blotting to Gene-Screen Plus (NEN), the DNA was hybridized to a pBR322 DNA probe labeled with alpha-^{32}P-dCTP by a random primer method

(0.5-2.0 x 10^9 cpm/ug DNA) (Feinberg and Vogelstein (1984). The filter was hybridized, washed and autoradiographed essentially as described by Johnson and Jelinek, (1986). A. Transfection with either pPBR322 or pPHR21 separately and comparison with transfection in the absence of Ca-PO_4. Lanes are: uncut = no restriction cleavage; P = PvuII; P + D = PvuII + DpnI; P + D + M = PvuII + DpnI + MboI; P + D (-C) = PvuII + DpnI treatment of DNA from a transfection with no Ca-PO_4. The two lanes at left marked 24 and 48 hours are fusion carried out with plasmid DNA added to plated fusing microplasmodia in the absence of Ca-PO_4. The lane pPHR21 is an uncut marker lane with 100 ng of plasmid. B. Time course after simultaneous transfection with pPHR21 and pBR322. Lanes are: a = PvuII + DpnI + MboI; b = PvuII + DpnI; c = PvuII; d = uncut. pPHR21 is an uncut marker lane with 100 ng of plasmid.

Figure 26


A
pPHR21
24 hrs
48 hrs
No DNA
P+D(-C)
P+D+M
P+D
P
Uncut
P+D(-C)
P+D+M
P+D
P
Uncut
II
III
I
Fusion, no CaPO4
pBR322 + pPHR21
pBR322
pPHR21
B
t = 24 hrs
t = 24 hrs
t = 0 hrs
a b c d
a b c d
a b c d
pPHR21
8.6 kb
pPHR21
4.3 kb
pBR322
No plasmid
pPHR21 + pBR322

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