The Transition Between Growth and Sporulation in *Physarum polycephalum* CL

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To Mum and Dad

Summary of Contents

SUMMARY

The overall aim of this project was to examine the relationship between the cell cycle and sporulation in Physarum polycephalum: in particular, to determine when sporulation specific genes were transcribed.

The work fell into three sections. The first made use of the anti-fungal agent nocodazole to determine if a plasmodium competent to sporulate after 72 h starvation was in the G1 or the G2 phase of the cell cycle. Clear evidence was obtained that differentiation was initiated from the G2 phase of the cell cycle. The effect of blocking mitosis with nocodazole on the subsequent DNA replication was investigated in both growing and starving plasmodia. It had previously been reported, and was shown in this work, that P. polycephalum has no G1 phase in the cell cycle during growth. The fact that growing and starving plasmodia responded similarly to nocodazole with regard to the onset of DNA synthesis indicated that there was no prolonged G1 period during the starvation period in P. polycephalum. It is postulated that nocodazole may interfere with a temperature-sensitive pathway that controls both the increase in thymidine kinase activity and metaphase onset.

The second part of the investigation was to approach the problem of pinpointing when in the G2 phase of the cell cycle, there was sporulation specific transcription. It was assumed that this question might be answered by differential screening of a genomic library of P. polycephalum, using as probes radiolabelled copy DNA prepared from poly(A)$^+$ RNA from growing and starving plasmodia. The first requirement was a genomic library of P. polycephalum CL DNA. Of the two phage vectors, Charon 4AP and λ1059 which were compared, the latter proved to be superior as it was shown that a genomic library prepared in Charon 4AP would be diluted by the presence of a considerable number of non-recombinant phage.

To generate libraries of P. polycephalum DNA it was necessary to digest it with suitable restriction endonucleases. P. polycephalum DNA was partially digested with either Sau3A or BamHI and the 15-25 kb fragments were isolated by electroelution. These fragments were then used to generate two genomic libraries. In each case only one type of recombinant phage was created which was derived from λ1059 and contained a fragment of Physarum DNA. The DNA used to prepare these gene banks was found to be contaminated by a second type of DNA. This contaminating DNA was tentatively identified as mitochondrial in origin.

This difficulty was eliminated when Physarum DNA was isolated by the method of Hardman & Jack (1978). DNA was partially digested with Sau3A and the 15-25 kb fragments isolated. A genomic library was prepared in λ1059 and restriction analysis of a random sample of phage showed that all were derived from λ1059 and all had restriction patterns different from the parental phage. Hybridization of [^{32}P] nick-translated Physarum DNA to filter replicas of phage identified the inserts as Physarum DNA.

The third part of the work involved the isolation of RNA from P. polycephalum. A requirement for screening the library was the preparation of undegraded poly(A)$^+$ RNA from which copy DNA probes could be made. Initially an attempt was made to isolate RNA that was being actively translated on polysomes at the time of isolation. However, all attempts to prepare polysomes in sufficient quantity were unsuccessful. Cytoplasmic RNA was isolated from growing plasmodia but was highly contaminated by a polysaccharide material. This contaminant was removed by cetyltrimethylammonium bromide precipitation. Examination of the RNA,

after electrophoresis under denaturing conditions showed that the RNA was very susceptible to degradation even when prepared in the presence of two inhibitors of RNase activity, RNasin and vanadyl ribonucleoside complex.

Less degraded RNA was isolated in a buffer containing 4M guanidine thiocyanate, an inhibitor of RNase activity. This total RNA preparation was less degraded than the cytoplasmic RNA. When poly(A)$^+$ RNA was isolated by oligo (dT) cellulose chromatography it directed the synthesis of very short copy DNA.

The purest and most undegraded RNA was isolated by a modified version of the method described by Cox & Smulian (1983). After the initial isolation procedure the poly(A)$^+$ RNA was further purified by a cetyltrimethylammonium bromide precipitation and a phenol/chloroform extraction. The poly(A)$^+$ RNA was used as a template for the synthesis of cDNA *in vitro* which was found to be 200-900 nucleotides in length. This cDNA hybridized to filter replicas of recombinant phage.

The overall conclusion from this work was that the molecular genetical techniques applied in this study have a good potential for investigating the detailed sequence of events in sporulation of *Physarum polycephalum*.

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ClPC	:	isopropyl-N (3 chlorophenyl carbamate)
CTAB	:	cetyltrimethylammonium bromide
EDTA	:	ethylenediaminetetra acetic acid
GM	:	growth medium
PSB	:	plaque storage buffer
SDS	:	sodium dodecylsulphate
SpoM	:	sporulation medium
SSC	:	standard saline citrate
TCA	:	Trichloroacetic acid
TE	:	Tris/EDTA
TSM	:	Tris/sodium/EDTA
VRC	:	vanadyl ribonucleoside complex

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INTRODUCTION

Physarum polycephalum was first described by Schweinitz in 1822 (cited by Guttes et al, 1961). It is one of over 425 morphologically and physiologically linked protozoan-like species frequently referred to as the acellular or true slime moulds.

The genus Physarum belongs to the class Myxomyceteae, phylum Myxomycophyta. In these multinucleate organisms a vein-like system of channels, which supports a cyclic reciprocal flow of cytoplasm, replaces conventional cellular organisation. Syncitial organisation, synchronous mitotic division and several discrete phases of their life cycle typify many of the Myxomycetes.

The artificial culture of plasmodial myxomycetes in the laboratory began over one hundred years ago. Plasmodia collected in the field or derived from spores were grown in moist chambers on their natural substrate, eg rotting wood or tan bark (de Barry, 1864; cited by Daniel & Baldwin, 1964). Lister (1888) fed myxomycetes with several species of fungi and observed that they engulfed and digested particles as an amoeba does. Crude cultures such as these were frequently overgrown by contaminating moulds, bacteria and protozoa. Howard (1931a, 1932) was first to report 'the uncontaminated growth' of Physarum polycephalum. The most common substrates used for pure culture of the myxomycetes had been particulate sterile food placed in non-nutrient buffered agar, such as killed autolysed yeast (Cohen, 1939; Hok, 1954), killed bacteria (Hok, 1954), or rolled oats (Camp, 1937; Daniel & Rusch, 1961). While it appeared to many workers that particles were essential to the nutrition of the myxomycetes, others tested growth on a variety of soluble nutrient media (Howard, 1931b; Alexopoulos, 1959). The culture of myxomycetes on insoluble natural

media was unsuitable for detailed biochemical studies. Only when pure culture methods were developed was it shown that a myxomycete could grow on soluble substrates. Daniel & Rusch (1961) successfully grew P. polycephalum in a semi-defined soluble medium. Chick embryo extract was an absolute requirement, but later it was found that this could be replaced by haematin (Daniel et al, 1962). A complete synthetic medium for growth of P. polycephalum allowed detailed nutritional and biochemical studies to be made (Daniel & Baldwin, 1964).

These advances in culture methods have allowed detailed biochemical and morphological studies on the life cycle of Physarum to be done. In recent years this myxomycete has increased in popularity as a biochemical model in many areas of research.

1. The Life Cycle of Physarum polycephalum

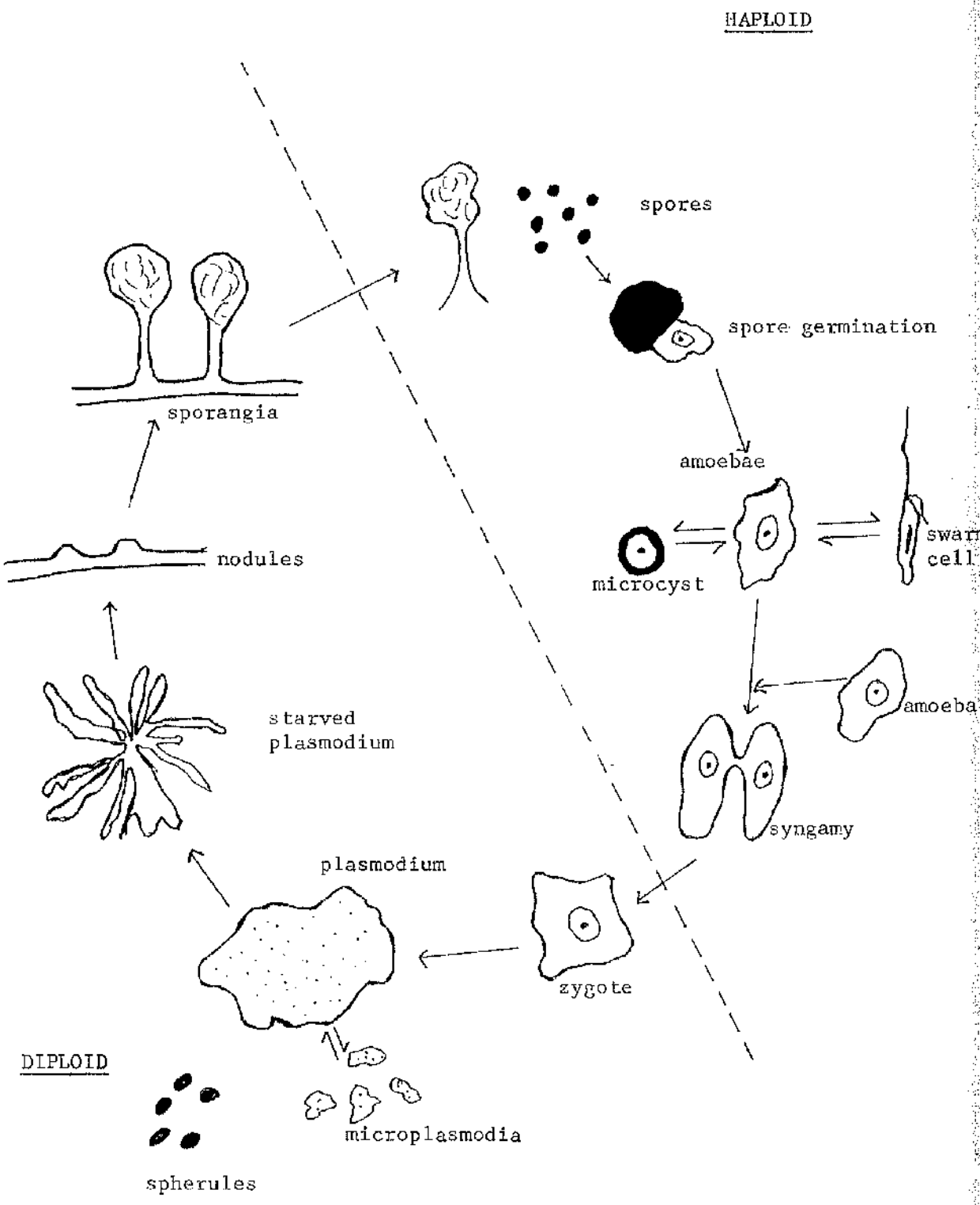
The life cycle of Physarum polycephalum, first described by Howard (1931a), can be described as an alternation between two very different cellular forms; the microscopic uninucleate amoebal cell and the macroscopic plasmodium (Fig 1). These two cell types differentiate into each other by characteristic pathways.

a) Amoeba (Myxamoebae)

The gamete stage of the life cycle is initiated with the germination of myxospores and the release of amoebae.

Myxamoebae range in size from 14-18 μm and contain numerous mitochondria and a single nucleus; both structures appear morphologically similar to homologous structures found in the plasmodium (Aldrich & Blackwell, 1976; Turner & Johnson, 1975). Amoebae undergo a typical

Figure 1. The life cycle of Physarum polycephalum


HAPLOID
spores
spore germination
sporangia
amoebae
nodules
microcyst
swarm cell
amoeba
starved plasmodium
syngamy
plasmodium
zygote
DIPLOID
microplasmodia
spherules

cell division involving open mitosis (complete dissolution of the nuclear envelope by metaphase), the participation of centrioles, and cleavage of the cytoplasm following karyokinesis.

Workers have grown amoebae successfully on lawns of bacteria. Goodman (1972) first grew amoebae axenically in liquid culture. Under these conditions growth is about four times slower compared with the four to eight hours generation time in two-membered cultures (McCullough & Dee, 1976). However, few strains have adapted to axenic culture and two unlinked genes (axe-1 and axe-2) have been implicated in controlling the amoebal growth (McCullough et al, 1978).

When on a solid surface, amoebae move by pseudopodial extensions. However, when placed in a liquid environment the amoebae rapidly undergo a complex structural change that allows a different motile behaviour. These motile cells, called 'swarm cells' neither divide nor feed and rapidly revert to the amoebal form in the absence of free water. The swarm cells are biflagellate, possessing one large active flagellum directed forward and one short flagellum directed backwards; the latter often so tightly pressed against the cell that its appearance is not apparent in the light microscope (Gray & Alexopoulos, 1968). The flagella arise from basal bodies and show a nine plus two set of axonemal microtubules (Aldrich, 1968; Jacobson & Adelman, 1975). The flagella, the basal bodies and the nucleus are all firmly interconnected as a complex, which has been isolated and carefully analysed by serial sectioning and examination in the electron microscope (Wright et al, 1979). The ability to undergo this transformation is facultative and after extended periods of axenic growth the capacity is lost (Goodman, 1972).

When amoebae are subjected to adverse conditions such as starvation, osmotic shock, accumulation of metabolic products or drought they can round up, become immobile and produce a wall. These resistant structures are called microcysts (Goodman, 1972; Turner & Johnson, 1975; Aldrich & Blackwell, 1976). The microcysts are stable structures provided that conditions that induce the transition continue; if the conditions become more favourable they rapidly excyst and resume normal growth (Gorman & Wilkins, 1980).

b) The plasmodium

The plasmodial stage is established by the differentiation of cells within the amoebal population. Two sexual types of mating systems have been described in the Physarales; homothallism and heterothallism. Homothallic strains are characterized by the capacity to form plasmodia in pure amoebal clones: they are self-fertile. These plasmodia are haploid. One such strain of P. polycephalum is the Colonia isolate. Wheals (1970) was the first to show that the amoebae of the Colonia isolate produced plasmodia within individual clones. Genetic analysis of the progeny of crosses between the Colonia strain C50 and heterothallic amoebae showed that the ability of C50 to produce plasmodia within clones was due to a single allele at the mating type (mt) locus which was called mt_h (Wheals, 1970). It was originally thought that two amoebae of like mating type fused and underwent karyogamy to produce a diploid plasmodium; however DNA analysis by Cooke & Dee (1974), using the methods of Mohberg et al (1971, 1973), demonstrated that plasmodia formed in these amoebal clones were haploid. These results were confirmed by Mohberg (1977). A cinematographic analysis of these amoebae by Anderson et al (1976) revealed that the plasmodial formation

could occur without the fusion of amoebae. Clarification of these 'apogamic' amoebae has allowed genetic studies to progress as it eliminates the need to outcross and backcross when selecting mutants. After mutagenesis it is necessary to screen large numbers of clonally derived plasmodia in order to isolate plasmodially expressed mutants. Thus mutagenised mt_h amoebae must be plated on a bacterial lawn so that they grow and form well separated plaques. C50 amoebae however only produce plasmodia when grown on a dense bacterial streak. A derivative of the C50 strain, C5-1, was isolated. This strain could form plasmodia in plaques with 100% efficiency; the plasmodia however were unable to sporulate (Wheals, 1973). It was therefore necessary to isolate a strain which could complete the life cycle. Cooke & Dee (1975), isolated the CL (Colonia Leicester) strain which produces plasmodia in clones with high efficiency and completes the life cycle repeatedly in single clones. Since then the strain has been used in numerous genetic studies.

Sexual formation of a plasmodium occurs with the fusion of haploid amoebae carrying different alleles. Dee (1962), while working on the amoebal-plasmodial transition, noticed that amoebae were of different mating types. These were called + and - and only amoebae which were of different mating types could fuse and form plasmodia. Later the number of mating types rose to four (Dee, 1966) and then to 12 or 14 (Collins, 1975). However, Dee (1978) reported that a gene locus (rac) affects plasmodial formation; the new gene is not linked to the mt alleles. The presence of a second mating type locus was confirmed by Anderson (1979), Pallotta et al (1979) and Youngman et al (1979). This locus affects the mating of amoebae in such a way that strains

differing in their mt alleles cross more readily if they also differ in their rac alleles. All evidence to date indicates that the rac locus and that of Anderson, Pallotta and Youngman are the same. Youngman et al (1979) named the new compatibility locus matB and renamed the original mt locus matA. The evidence available suggests that the matB locus controls amoebal cell fusion (Youngman et al, 1979) and that the matA locus, in P. polycephalum, controls the differentiation of amoebae into plasmodia (Adler & Holt, 1975). Both the matA and matB loci are multiallelic. To date 13 matB alleles are known (Kirovac-Brunet et al, 1981) and there is a minimum of 13 alleles at the matA locus (Collins & Tang, 1977).

Generally, shortly after fusion of the amoebae the nuclei fuse in interphase, forming a zygote nucleus which has two nucleoli. After about two hours a closed mitosis occurs, yielding two diploid nuclei, in which a single nucleolus becomes reconstructed. This binucleate cell develops into a multinucleate plasmodium by synchronous nuclear division without further cell division.

The plasmodial stage represents the macroscopic vegetative phase of Physarum. The plasmodium of P. polycephalum has a bright yellow colour, which is caused by numerous yellow pigment granules (Guttes et al, 1961). On surfaces the plasmodium of Physarum is disc-like and appears as a naked, reticulate mass of protoplasm surrounded by a protective glycocalyx that is primarily composed of a sulphated galactose polymer containing trace amounts of rhamnose (McCormick et al, 1970). The strands of protoplasm exhibit a rapid to and fro streaming. With adequate nutrition, at a temperature optimum of between 21°C and 24°C, mitosis occurs every eight hours and is synchronous in all nuclei (Guttes

et al, 1961). Thus the plasmodium can be regarded as one giant cell where the events of the cell cycle occur in a synchronous manner. In shaken liquid medium the plasmodium is fragmented and these small pieces of vegetative slime mould are called microplasmodia (Daniel & Baldwin, 1964). Each microplasmodium contains about 100 nuclei which exhibit synchronous mitosis, but there is no synchrony among the various microplasmodia.

Plasmodia have two mechanisms for dealing with adverse environmental conditions. The first is encystment, an asexual process that allows a reasonably rapid response (36-48 hours for completion) to an unfavourable environment. If plasmodia are in liquid culture (as microplasmodia) the process is called spherulation; if cultures are on a solid or semi-solid surface (macroplasmodia) the process is called sclerotization.

Differentiation begins at the tail end of the plasmodium where, after the protoplasmic streaming has stopped, portions of the protoplasm round up into little balls. This process was first described as spherulation by Brandza (1926). Most work has been done on microplasmodia grown in liquid culture. Microplasmodia are routinely induced to differentiate by placing them either into a balanced salts solution (Daniel & Baldwin, 1964; Goodman et al, 1969) or by osmotic shock (Jump, 1954; Chet & Rusch, 1969). The spherules each contain several cell nuclei. Walls are established between the spherules and the whole mass appears cellular. The spherulation is accompanied by very complex cytological and metabolic changes in an ordered time sequence (Goodman & Rusch, 1970; Zaar & Kleing, 1975; Schreckenbach & Verfuerth, 1982). The process of spherulation is mainly characterised

by the induction of numerous new translatable mRNA species. Blue light has been shown to inhibit this differentiation by suppressing, at the mRNA level, all changes in protein synthesis characteristic of the plasmodium - spherule transition (Putzer et al, 1983). Germination follows the return to normal growth conditions.

The second response to an adverse environment is sporulation, a slower response (5 to 8 days) with more stringent requirements for induction. When surface plasmodia are starved by removal of nutrients, they begin to move about and form a branched network of streaming protoplasm. If the starvation is continued in the dark for three to four days and the plasmodium is then exposed to light for a minimum of 30 minutes, sporulation is induced (Daniel & Rusch, 1962a; Sauer et al, 1969a; Chapman & Coote, 1982). If nutrients are returned to the starving plasmodium at any time during the first three hours following the end of illumination, growth resumes. After three hours, the process leading to sporulation becomes irreversible.

Sporulation consists of the formation of sporangial strands and the development of many pillars by the pulsating protoplasm. Spores are formed in sporangial caps after a meiotic division. The completion of meiosis is closely coupled with spore viability (Laffler & Dove, 1977). The spores germinate after a maturation period and release amoebae thus completing the complex life cycle of P. polycephalum. A more detailed analysis of the sporulation process in Physarum is given below (Section 4).

2. The Cell Cycle of Physarum

a) The eukaryotic cell cycle

The process by which one cell becomes two is essentially the

same in most eukaryotic organisms. It involves the replication of the DNA and its accurate distribution between the two daughter cells by means of a mitosis.

Howard & Pelc (1953) presented terminology for the phases of the life cycle of eukaryotic cells. The DNA synthetic period, termed S, and mitosis called M, are two clearly defined stages in the cell cycle. The period after M but before S is called G1 and the one following S but prior to M is called G2. These gaps in the cell cycle (hence the term G) are not devoid of cellular activity as their name suggests.
P. polycephalum, because of the synchronous manner of mitosis and because growth and differentiation occur as separate and distinct phases, is a useful organism in which to study various aspects of the cell cycle.

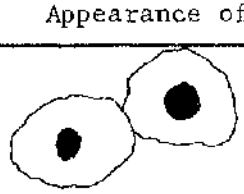
b(i) The mitotic cycle of Physarum

Details of the nuclear division cycle in the growing plasmodium have been provided by both light and electron microscopy (Guttes et al, 1961; Mohberg & Rusch, 1969; Laane & Haugli, 1974).

Figure 2 illustrates the appearance of the nuclei and the times occupied by specific stages of the mitotic cycle. A single large nucleolus is the dominant cytological feature of the interphase nucleus, and it is the characteristic changes undergone by this organelle that identify in particular the early stages of mitosis. The onset of anaphase is the most abrupt change that has been detected in a film analysis (Wolf et al, 1979). This change serves as the most stringent marker of mitotic synchrony. In a plasmodium of about 5 cm diameter containing 10^7 nuclei, over 98% are in metaphase within 5 minutes. In a larger plasmodium, containing 10^8-10^9 nuclei, mitosis in different areas can vary by up to 20-40 minutes (Mohberg & Rusch, 1969).

Figure 2. The Mitotic Cycle of Physarum

(from Mohberg & Rusch, 1969)

Stage of Mitosis	Duration in minutes	Appearance of nuclei
Interphase	480	
Early Prophase	15-20	
Prophase	5	
Metaphase	7	
Anaphase	3	
Telophase	5	
Reconstruction	75	

The nuclear division in the plasmodium is not typical for higher cells, in that an intranuclear spindle exists. As the amoebal stage of Physarum has a typical open mitosis one explanation for the closed mitosis in the plasmodial form is that it avoids the mixing of the chromosomes of adjacent nuclei after membrane breakdown.

A better understanding of the mechanisms controlling the alteration between interphase and mitosis depends on the availability of cellular material. As single cells are too small for this purpose, methods were designed which synchronised large numbers of cells (Maruyama & Yanagita, 1956). Critics of these methods said that they interfered with the natural cycling of the cells. Others looked for material which exhibited natural synchrony such as lily anthers (Erikson, 1947), giant amoebae, and the myxomycetes (Lister, 1893; Howard, 1932). The myxomycetes, and particularly P. polycephalum, are attractive because of their natural synchrony, giant size and ease of cultivation (Daniel & Rusch, 1961; Daniel & Baldwin, 1964). The growing plasmodium of Physarum has been used in many studies which have looked at the mechanisms which may control mitosis.

ii) Control mechanisms of mitosis

As post-fusion mitoses are so highly synchronous in the plasmodium of P. polycephalum, the controlling mechanism has attracted much attention. There are two possibilities, either each nucleus within the cytoplasm has a biological clock or a freely diffusible cytoplasmic factor is produced, the concentration of which determines when mitosis occurs in all nuclei. A large number of experiments suggest that the latter is the case. Pairwise fusion of macroplasmodia were first studied by Rusch et al (1966). Fused plasmodia were prepared from two

plasmodia two or three hours apart from one another in the cell cycle and late in the G2 period. The fused plasmodia underwent nuclear division at a time about halfway between the division times of control pieces of the two parent plasmodia. Pieces of plasmodia which differed not only in their cell cycle but also in size were then fused. Mitosis in the fused piece was again intermediate between controls, but closer to the time of mitosis in the control plasmodium supplying the larger piece (Rusch et al, 1966; Sachsenmaier et al, 1972). It was therefore assumed that the controlling factor was synthesized and increased in concentration in G2 phase until a crucial level was reached and mitosis occurred. Heat shock delays mitosis (Nygaard & Guttes, 1962). Maximum sensitivity was observed two hours before mitosis. High temperature applied at 15 minutes before mitosis for 10 minutes, blocked mitosis at metaphase or anaphase. Thereafter the plasmodium returned to interphase (Brewer & Rusch, 1968). This result indicates a very late event in the cell cycle that marks the transition point beyond which the nuclei are committed to divide. This was confirmed by experiments which asked how close to mitosis a nucleus could be and still be stopped from completing the mitosis by fusion with a plasmodium in S phase. It was shown that nuclei become irreversibly committed at prophase, at 15-20 minutes before metaphase (Loidl, 1979, cited by Sauer, 1982). This transition point coincides with two other observations: the execution point of a temperature-sensitive mutant, which at the restrictive temperature, becomes blocked at metaphase (Laffler et al, 1979); the high phosphorous content of histone H1 which has been suspected of causing the condensation of chromatin into chromosomes (Fischer & Laemmli, 1980). Bradbury et al (1974) advanced mitosis by

treatment with plasmodial extracts displaying histone H1 phosphokinase activity. Oppenheim & Katzir (1971) found that they could advance mitosis by one hour in macroplasmodia that were treated with plasmodial extracts placed on their surface. The extracts were most active from G2 phase plasmodia and they were shown to be heat-labile and non-dialysable. Immunological methods have also been used. An antiserum was prepared against S phase plasmodial extracts. This was used to precipitate S phase components from a G2 phase extract. This enriched material was then shown to advance mitosis (Blessing & Lempp, 1978). Irradiation of plasmodia with ultra-violet light alters the timing of one or more subsequent mitoses (Sachsenmaier, 1966; Devi et al, 1968; Sudbery & Grant, 1975). X-ray treatment also delays mitosis (Nygaard & Guttes, 1962). Addition of cycloheximide, a drug which inhibits protein synthesis, before metaphase or earlier in the cell cycle prevents the onset of mitosis (Cummins et al, 1965; Cummins & Rusch, 1966). These various results suggest that the factor controlling the synchronous mitosis is found in the cytoplasm and is probably a heat-labile protein.

A variety of models to explain the plasmodial mitosis have been proposed.

The concentration model; the simplest theory is that the cytoplasmic concentration of some substance changes linearly during the cell cycle, and when it reaches a critical value, the substance triggers mitosis (Rusch et al, 1966). Sachsenmaier et al (1972) suggested that the concentration of a mitotic activator is maintained at a constant value. As the plasmodium grows the total number of activator molecules increases. These molecules enter the nuclei and bind to nuclear sites. When all the sites are occupied by activator molecules mitosis occurs.

By doubling the number of nuclei the number of nuclear sites doubles producing unoccupied sites which must be filled during the next cell cycle.

The unstable-inhibitor model was proposed by Sudbery & Grant (1975). In this model an inhibitor is synthesised proportionally to the amount of DNA and it breaks down spontaneously. As the plasmodium grows, inhibitor concentration decreases because of dilution. When the concentration is halved from its post-mitotic value, mitosis is triggered. The total amount of inhibitor doubles during the next S phase.

Tyson et al (1979) suggested that the synchronous mitoses were controlled by the accumulation of an unstable activator. The activator was thought to be a protein or to be dependent on the synthesis of an unstable protein. No single explanation has been undoubtedly confirmed to date.

c) DNA synthesis

The duration of the DNA synthetic period was originally delineated by Nygaard et al (1960). They found that nuclear DNA was replicated immediately after mitosis. Braun et al (1965) observed that the maximum rate of DNA synthesis was reached within 5 minutes of the uncoiling of the telophase chromosomes. The maximum rate of synthesis was maintained for around 1½ hours and subsequently decreased reaching the low premitotic value around 4 hours post-mitosis. The same authors showed that DNA synthesis starts simultaneously in 99% of plasmodial nuclei, but the duration of synthesis varied between nuclei of a plasmodium. One strain of P. polycephalum used recently in flow-cytometry studies is M_3cIV. This strain contains two types of nuclei

within its macroplasmodial structure. The two types of nuclei contain different amounts of DNA but a constant duration of the S phase is maintained via differential slowdown of replication rates during the second and third hours of replication (Kubbies & Pierron, 1983).

Isotope dilution methods also showed rapid DNA synthesis during the first two hours of the S phase so that by the end of this period approximately 80% of the DNA had been replicated. Subsequently there is a large decrease in the rate of DNA synthesis, which continues until the end of S phase (Fink & Nygaard, 1978; Hall & Turnock, 1976). Nuclei which were found to incorporate [^{3}H] thymidine during G2 were shown to be exceptionally large and frequently to contain several small nucleoli rather than the one nucleolus characteristic of normal nuclei (Guttes & Guttes, 1969). Vogt & Braun (1977) found that chromosomal DNA replicated during G2 did not replicate again during the next cell cycle. These results seem to indicate that G2 synthesis of DNA occurs in abnormal nuclei which cease to synthesise DNA. G2 synthesis of DNA has also been shown to be partly due to the replication of mitochondrial DNA (Evans, 1966) and the replication of nucleolar DNA (Braun & Evans, 1969).

Braun et al (1965) and Braun & Wilt (1969) demonstrated that DNA replication is synchronous at the molecular level. Using a combination of radioactive and density labelling of DNA they showed that a DNA sequence replicated in a given subinterval of an S phase is replicated in the corresponding subinterval of the next S phase. Thus DNA molecules do not replicate randomly but in a fixed temporal sequence. DNA replication has also been shown to be discontinuous and bidirectional (Funderud & Haugli, 1977b; Funderud et al, 1978a,b,c; Gillespie & Hardman, 1979). Okazaki type fragments of around 10^5 daltons were

detected by short labelling pulses throughout the S phase. These short pieces increased in weight in a discontinuous fashion. Full-size single strand pieces of more than 10^8 were apparently formed by the fusion of relatively long-lived intermediates of molecular weight 2×10^7. The 2×10^7 intermediate is thought to represent the replicon size in P. polycephalum.

The role of protein synthesis in the synthesis of DNA in P. polycephalum was first investigated by Cummins & Rusch (1966). The drug cycloheximide, an inhibitor of protein synthesis, was used in this study. When the inhibitor is added just before prophase, neither mitosis nor DNA synthesis occurs; addition during mitosis or at different times during S phase allows only a small amount of DNA synthesis. By adding the drug at different times during S phase, the effect of cycloheximide led to the claim that the genome of P. polycephalum consists of at least 10 replication units controlled by proteins synthesized at defined times during the S period (Muldoon et al, 1971). It was found that cycloheximide blocks the molecular weight increase of replicating DNA molecules (Evans et al, 1976). Funderud & Haugli (1977a) also found that cycloheximide blocks elongation of progeny strands in vivo. The drug has also been found to inhibit the expansion of the nucleotide triphosphate pools in P. polycephalum (Bersier & Braun, 1974; Evans et al, 1976). In a later study, Evans & Evans (1980) determined that the effect of cycloheximide on DNA replication is at least as rapid as the effect on protein synthesis and that the level of inhibition obtained is quite similar. Thus cycloheximide has several effects in P. polycephalum in addition to inhibiting protein synthesis. As yet these effects have not been separated and the relationships between them remain unclear. However, a

cycloheximide resistant strain has been isolated and segregation data indicated that the resistance was due to a single mutation (Evans & Evans, 1980). They found that the effect of cycloheximide on DNA synthesis, the level of thymidine triphosphate and the incorporation of [^{3}H]thymidine into thymidine triphosphate paralleled the effect of cycloheximide on radioactive leucine incorporation into protein in the homozygote, the heterozygote and the heterokaryons. They suggested that the correlation could be the result of a relationship between the effects. If the effects were independent, the induction of cycloheximide resistance with respect to growth would necessarily involve two or more mutations. It was known that the resistance was due to a single mutation, therefore it appeared that the effects of cycloheximide on DNA synthesis and nucleotide metabolism are secondary to the effect of the drug on protein synthesis in P. polycephalum.

Sachsenmaier & Rusch (1964) used flurodeoxyuridine as a tool to study the role of DNA synthesis in mitosis. 5-Flurodeoxyuridine (FUdR) inhibits the enzyme thymidylate synthetase creating a thymidine deficiency which results in the inhibition of DNA synthesis. They found that mitosis cannot occur, in Physarum, unless DNA replication is completed. Is mitosis then necessary for initiation of DNA replication? When mitosis was prevented, by protein inhibition, in early prophase, DNA replication was also prevented. When protein synthesis was inhibited in late prophase both mitosis and limited DNA replication took place (Cummins & Rusch, 1966). These results suggest that mitosis normally plays some role in the initiation of DNA synthesis. Brewer & Rusch (1968) showed that heat shock caused a pseudomitosis. The mitotic spindles were formed, but mitosis did not take place. Nuclei then

returned to their intermitotic state and DNA synthesis continued. When G2 nuclei were placed in S phase cytoplasm they did not undergo another round of DNA replication (Guttes & Guttes, 1968). The results suggest that initiation of mitosis, but not necessarily its completion, is necessary for DNA synthesis and that some cytoplasmic factor related to mitosis is needed to initiate DNA replication.

d) DNA transcription

RNA synthesis was first investigated by Nygaard et al (1960). When radioactive precursors were used to measure RNA synthesis during the cell cycle of Physarum it was found to be biphasic, with peaks of isotope incorporation at S phase and G2 phase (Mittermayer et al, 1964; Braun et al, 1966 ; Sauer et al, 1969c). It has been shown that RNA synthesis is continuous through the cycle, except during mitosis, when it has been shown by autoradiography that precursor incorporation decreases to an undetectable level as the system passes from prophase to metaphase through anaphase, then returning during telophase (Kessler, 1967). This arrest of transcription only lasts about 5 minutes. Ribosomal RNA (rRNA) synthesis increases five to six-fold during the mitotic cycle (Hall & Turnock, 1976). The isotope dilution method was used in these experiments in order to minimise errors due to precursor pool fluctuations. The accumulation of rRNA was also measured and it was found to double during the cycle in a non-exponential way (Hall & Turnock, 1976). Fink & Turnock (1977) looked at the synthesis of transfer RNA (tRNA) and found it increased during the cell cycle. The accumulation of tRNA was found to closely follow that of rRNA, since the ratio of tRNA:rRNA measured at hourly intervals during the cell cycle did not vary significantly (Melera & Rusch, 1973).

The synthesis of poly(A)$^+$ RNA is detectable throughout the intermitotic period (Fouquet et al, 1974), although a disproportionate amount of RNA synthesized in early S phase appears to contain poly(A)$^+$ RNA.

Fouquet et al (1975a) used hydroxyurea to block incorporation of thymidine into DNA. Under these conditions uridine incorporation into TCA-precipitable material was significantly (up to 70%) inhibited in early S phase of the cell cycle. In addition to this, the extent of uridine incorporation into poly(A)$^+$ RNA was considerably reduced as determined by gel electrophoresis and DNA-RNA hybridization reactions. These results suggest that poly(A)$^+$ RNA synthesis during S phase is in some way coupled to DNA replication.

There appears to be a close connection between certain functions of RNA and the replication of DNA. The DNA of Physarum is replicated in Okazaki fragments about 200 nucleotides long (Funderud & Haugli, 1977a). These Okazaki fragments have a short polyribonucleotide sequence at the 5' end (Waqar & Huberman, 1975). By inhibiting DNA replication, incorporation of radioactive nucleotides into RNA is greatly reduced (Rao & Gontcharoff, 1969; Fouquet et al, 1975b; Pierron & Sauer, 1980).

Work with actinomycin D demonstrated that RNA synthesis was necessary for mitosis to occur (Mittermayer et al, 1965). Using actinomycin C, the time of synthesis of the last messenger RNA (mRNA) necessary for mitosis was estimated as being 1 hour before the beginning of telophase (Sachsenmaier et al, 1967). Cummins and Rusch (1967) found that synthesis of the last mRNA took place around 14 minutes before metaphase.

e) Protein synthesis during the cell cycle

Protein synthesis has been shown to be necessary for mitosis in the plasmodium of Physarum, but addition of cycloheximide just before prophase allows mitosis to occur, therefore all necessary structural proteins were synthesized before this time (Cummins et al, 1965). Protein synthesis was originally shown to be biphasic and to follow the pattern of RNA synthesis (Mittermayer et al, 1966a). Such experiments however did not take into account the free unlabelled amino acids in the cellular pools. Isotope dilution methods showed protein synthesis to be continuous with an increase in rate in G2 phase (Birch & Turnock, 1977). Protein synthesis has been shown to continue during mitosis (Turnock et al, 1981). This study also looked at individual proteins during the cell cycle. They labelled proteins with [^{3}H] lysine for 1 hour and then fractionated extracts of plasmodia by two dimensional electrophoresis. Results indicated that most if not all proteins are synthesized throughout the cell cycle. Two metabolically stable polypeptides were detected whose synthesis occurs mainly in G2 phase (Turnock et al, 1981). In a similar study Laffler et al (1981) found two polypeptides that were preferentially labelled during G2 phase. The proteins were found to have molecular weights similar to those of tubulins from lower eukaryotes including Physarum and were capable of coassembly with porcine microtubular proteins. The identity of these polypeptides was confirmed by Chang et al (1983). They compared the proteins preferentially labelled during G2 with tubulins enriched from Physarum myxamoebae by electrophoresis and peptide mapping. One major and one minor species of protein matched the myxamoebal α- and β-chains respectively.

Four tubulin isotypes have now been identified in the plasmodium of P. polycephalum, two α-species and two β-species. All four tubulin isotypes are present in the mitotic nucleus (Paul et al, 1984; in the 6th European Physarum Workshop).

Pahlic & Tyson (1983a) found one protein of molecular weight 32,000 and isoelectric point 4.9, that showed a ten-fold increase in total amount between early G2 phase and metaphase. This protein appears to be cytoplasmic and its identity and function are unknown.

Most enzymes that have been studied in Physarum follow a continuous pattern. This is true for the polymerases of DNA and RNA. There are, however, around ten examples of enzyme fluctuations in the mitotic cycle of Physarum. Among those are peak enzymes whose activity goes up and down again, such as thymidine kinase, adenylate cyclase and histone kinase (Sauer, 1982).

Thus it seems that the synthesis of all macromolecules is closely interrelated during the cell cycle and it may be this relationship that is important in driving the plasmodia through their cell cycles.

3. Eukaryotic Differentiation

a) Cellular differentiation and the cell cycle

In a bacterial cell cycle the S and G2 periods are 40 and 20 minutes long respectively. That is, it takes 40 minutes to replicate the DNA of the cell and there is a period of 20 minutes between termination of replication and cell division (Cooper, 1979). For cells growing with a doubling time greater than 60 minutes there is a G1 period as well as the S and G2 periods. When bacteria have doubling times less

than 60 minutes there is no G1 and instead DNA synthesis occurs during cell division, having started in the previous division cycle. Where the doubling time is less than 40 minutes DNA synthesis will commence before the previous rounds of DNA replication have been completed (Cooper, 1979).

This cannot happen in eukaryotic cells since cell division is preceded, during either meiosis or mitosis, by the separation of two sets of daughter chromosomes during which DNA replication cannot occur, therefore the cell cycle of eukaryotes must be precisely regulated, and this led to the question: Do the controls which regulate the cell cycle also control the cells potential to differentiate?

It was once thought that cell differentiation and cell division were antagonistic processes, but in a number of systems it has been shown that the induction of development is coupled to certain aspects of the cell cycle (Stockdale & Topper, 1966; Shininger, 1975). It is of interest to determine whether cells in the interphase of their cell cycle can become committed to differentiate or whether they require to continue through the cell cycle and mitosis before making the choice. This question has been addressed to varying degrees in a number of experimental systems which favour an analysis of differentiation at the molecular level.

b) Eukaryotic model systems

i) Dictyostelium discoideum

The life cycle of Dictyostelium discoideum consists of two distinct phases. During vegetative growth amoebae feed, grow and divide as mononucleate unicellular organisms. When the food supply is exhausted

the cells enter the developmental phase and begin multicellular morphogenesis. Differentiation of amoebae within newly formed aggregates generates the three distinct cell types found in the mature fruiting body; spore cells, stalk cells and basal discs (Loomis, 1975). Raper (1941) assumed that, since development occurred in response to starvation, cell division was absent during the developmental phase of D. discoideum. Subsequent studies have shown that this is not true. One of the initial consequences of starvation in D. discoideum is the cessation of cell division. However, by the time the fruiting body is formed there has been a substantial increase in cell numbers (Bonner & Frascella, 1952; Wilson, 1953; Katz & Bourguignon, 1974; Atryzek, 1976), but it has been concluded that differentiation in D. discoideum is not dependent on cell division (Sussman & Sussman, 1960).

These early studies used bacterially grown cells which were known to retain a significant number of bacteria when transferred to the developmental system. It was thought that this carryover may have accounted for the continued cell division during development. This doubt was eliminated when Zada-Hames & Ashworth (1978) carried out experiments using axenically grown amoebae. They found there were two distinct periods when mitotic cell division occurred. To demonstrate conclusively that the increase in cell numbers that they saw were the result of mitotic nuclear division the presence of mitotic figures was verified. They found that the first phase begins at the onset of development and that the second reaches a maximum of activity during the grex stage.

In order to determine whether mitosis and DNA synthesis were indispensable for cell division, chemical inhibitors of these processes

were used. Flurodeoxyuridine was used to inhibit DNA synthesis and colchicine was used to prevent mitosis. It seemed that differentiation was dependent on these events since development was inhibited in the presence of those drugs (Cappucinelli & Ashworth, 1976).

Cappucinelli et al (1979) used nocodazole to obtain large numbers of mitotically inhibited amoebae. They found that nocodazole treated amoebae did differentiate but with some delay when compared with controls. They concluded that mitosis, which is naturally present during development of D. discoideum, was dispensable for development.

White et al (1981), using another mitotic inhibitor, isopropyl-N(3 chlorophenylcarbamate) (CIPC), claimed that mitosis was not required for development of D. discoideum. In addition, these authors also isolated mutants that were resistant to the drug during growth but were sensitive to its action during development. They suggested that this indicated that CIPC did not inhibit development by blocking mitosis. They suggested that cytoplasmic microtubules may play a role during development and it was this function that was being affected since CIPC had its effect on the function of microtubules.

It therefore remains unclear as to whether it is a mitosis that is a strict requirement for development or some cytoplasmic microtubule-requiring event that is necessary for the successful development of D. discoideum.

It has been suggested that D. discoideum amoebae became blocked mid-way through the G2 phase of their cell cycles during aggregation. Katz & Bourguignon (1974) postulated that starvation triggers some signal that programmes the cells to aggregate at a specific time. This signal may allow the cells to continue through the

cell cycle until they reach the block in G2. The block is later released. When cells are grown in suspension culture to a high density they stop dividing and are called stationary cells. They appear to be blocked in the G2 phase of the cell cycle (Soll et al, 1976). When these cells develop they do not undergo any preaggregation division (Zada-Hames & Ashworth, 1978).

A recent study suggested that cells that were in S phase and very early G2 at the onset of starvation demonstrate a strong tendency to sort to the tip of the resulting slugs (McDonald & Durston, 1984). They suggest that the cells may sort out due to cell cycle based adhesion differences but there is no direct evidence for this. The mechanism of cell sorting and the basis of its relation to the cell cycle remains to be clarified.

It appears that the cell cycle and its relationship with the differentiation of D. discoideum amoebae is complex and many questions still need to be answered before it is completely understood.

ii) Saccharomyces cerevisiae

The yeast Saccharomyces cerevisiae is a single-celled organism capable of rapid division on defined media. Each haploid cell reproduces by budding and the bud grows in size throughout the cell cycle. The sexual stage of the life cycle is initiated by fusion of haploid cells of the opposite mating type. Meiosis then occurs and sporulation results in 4 spores contained in a single ascus. Occasionally 8 ascospores may be found in a single ascus if they have undergone a post-meiotic mitosis. Meiosis and ascospore formation is normally induced in laboratory strains by transferring vegetative cells to a nitrogen free medium (Esposito et al, 1969; Fowell, 1969; Pinon, 1977).

One of the main advantages of S. cerevisiae as an experimental organism is its genetic system (Mortimer & Hawthorn, 1969). This characteristic has been exploited for cell cycle analysis by the isolation of cell division cycle (cdc) mutants (Hartwell et al, 1970). Examination of a great many temperature sensitive clones revealed the presence of 150 cdc mutant clones. Complementation studies showed that those mutants belonged to 32 complementation groups (Hartwell et al, 1973). These mutations define 32 genes, each of whose products plays an essential role in the successful completion of the cell cycle. Hartwell et al (1974) studied twenty cdc mutants in detail. They looked at the points of the cell cycle where the mutants became blocked and tried to develop a model of the cell cycle. Hirschberg & Simchen (1977) tried to determine whether only cells at a particular stage of the cell cycle could differentiate. They found that only cells blocked in early G1 could go directly into differentiation. Mutants that were blocked later in the cell cycle had to first complete the cycle and go through mitosis before being able to differentiate.

It was also known that as yeast cells exhaust their nutrients they finish cell cycles already initiated and become arrested in early G1 (Hartwell et al, 1974). This led to the concept of 'start' in the cell cycle of S. cerecisiae (Hartwell et al, 1974; Nurse, 1981). This is the earliest known gene controlled event in the cell cycle and occurs in G1. Once past 'start' cells are committed to the mitotic cycle. Only cells that reach a critical size are able to complete 'start' (Nurse, 1981) and so cells deprived of nutrients accumulate at 'start' before differentiation occurs.

A similar approach was used to investigate the cell cycle of

the fission yeast Schizosaccharomyces pombe and a similar 'start' point in the cell cycle has also been found (Nurse & Bisset, 1981). One mutant, cdc-10 which is a known 'start' mutant, was used in another study on the dependency relations between events of the cell cycle. It was found that completion of the cdc-10 function was dependent on the occurrence of mitosis in the previous cycle (Fantes, 1982).

Using new in vitro genetic techniques it has been shown that the cdc-28 cell cycle start gene of S. cerevisiae can complement the cdc-2 mutant of Schizosaccharomyces pombe. It was concluded therefore that the cdc-2 and cdc-28 genes perform homologous cell cycle control functions (Beach et al, 1982).

Therefore it has been possible to determine an exact point in the cell cycle of S. cerevisiae when cells make the decision to continue through the cell cycle or to differentiate. This 'start' point occurs early in G1 and once past this point the yeast cells must continue through S, G2 and complete mitosis before differentiation can occur.

iii) Other model systems

In more complex eukaryotic organisms it has been shown that certain types of development are linked to some aspects of the cell cycle.

Terminal differentiation of the cells of the mammary gland in mice only occurs after a hormone-induced cell division (Stockdale & Topper, 1966; Vanderhaar & Topper, 1974); continued cell cycling is required for the formation of tracheary elements in higher plants (Torrey & Fosket, 1970; Shininger, 1975), and cell fusion during myogenesis can only occur during the G1 period of the cell cycle (Okazaki & Holtzer, 1966

A point analogous to the 'start' point in the cell cycle of the budding and fission yeasts has been reported to occur in mammalian

cells. Cells accumulate at this point in the cell cycle in conditions of minimal metabolism. Again this point is early in G1 and Pardee (1974) called it the 'restriction point'. It is thought that cells which are arrested at this point may be resting in the specialized G1 state called Go (Pragnell et al, 1980). Friend erythroleukemia cells temperature-sensitive for growth have been isolated. Using those cells it was found that they also have a requirement for a cell cycle dependent event which occurs in G1. This event must occur before the cells can undergo differentiation and become capable of synthesizing haemoglobin (Conkie et al, 1981).

Thus it seems that controls which govern the progress through the cell cycle may play some part in controlling differentiation at least in some systems.

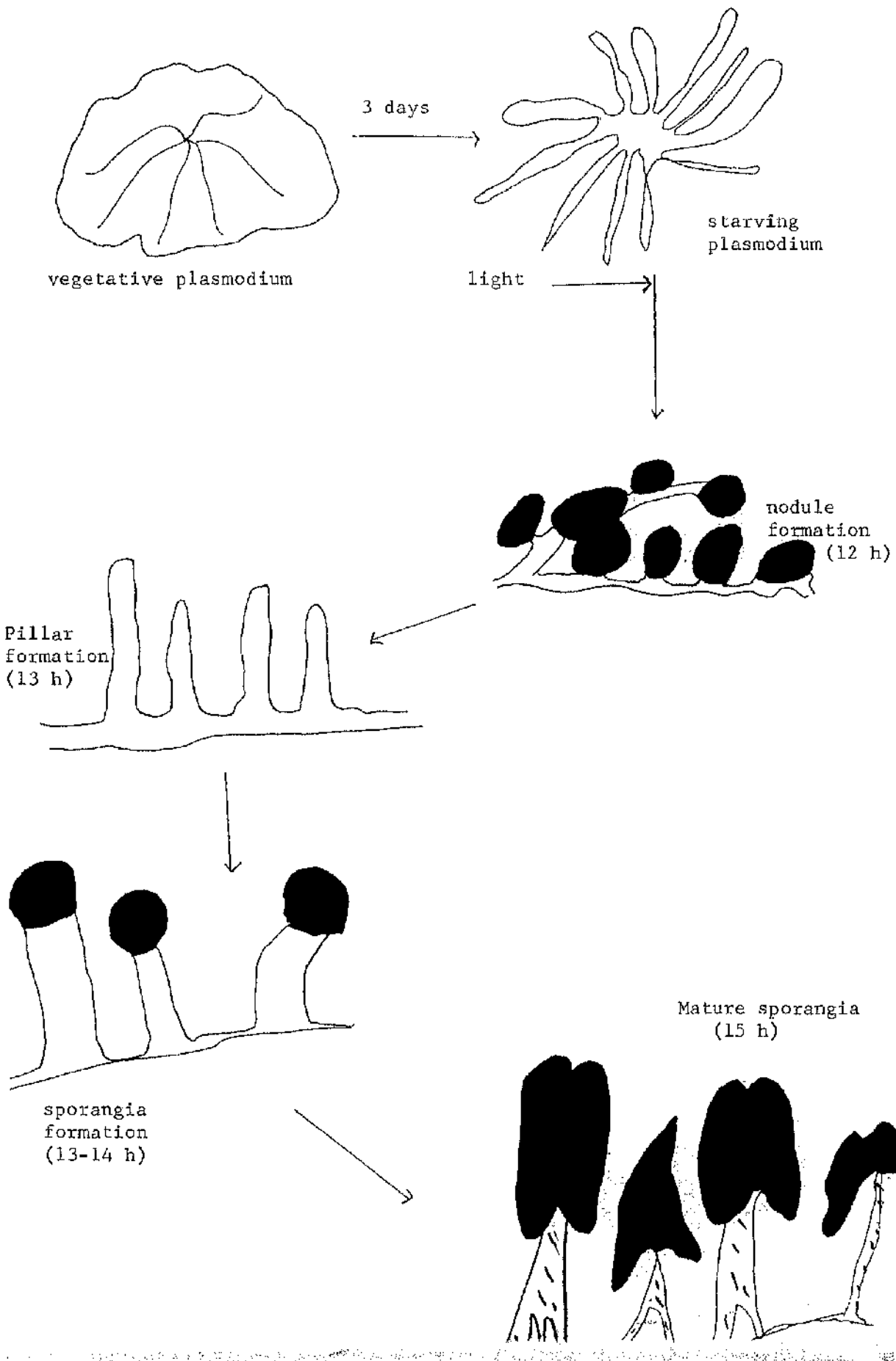
4. Cellular Differentiation in Physarum polycephalum

a) Sporulation

Sporulation is a phenomenon of surface macroplasmodia and when carried out on a defined salts medium consists of 3 stages (Guttes et al, 1961; Daniel & Rusch, 1962a; 1962b) (Fig 3).

The first stage is an initial starvation period. During this time the plasmodium changes from a compact, almost solid appearing mass to readily distinguishable, individual veins or strands of protoplasm showing extensive mobility. This starvation period is necessary to induce a state of competence to sporulate in response to light; the most effective light being between 360 μm and 500 μm (Gorman & Wilkins, 1980). The illumination period necessary to induce sporulation has been

Figure 3. Sporulation in Physarum polycephalum


3 days
starving plasmodium
vegetative plasmodium
light
nodule formation (12 h)
Pillar formation (13 h)
sporangia formation (13-14 h)
Mature sporangia (15 h)

found to vary from 4 hours to 30 minutes (Daniel & Rusch, 1962a; Chapman & Coote, 1982). After illumination the strands become thinner and become wavy. Commitment to sporulate follows within 3 to 5 hours after exposure to light. After this point a return to normal growth conditions no longer prevents sporulation (Daniel & Rusch, 1962b; Sauer et al, 1969a; Chapman & Coote, 1982).

Between 8 and 12 hours postillumination the morphogenic phase begins. The protoplasmic strands break up into nodules at regular intervals along a vein, into which the protoplasm collects. All the beads remain connected by a thin thread of plasmodial material covered by a layer of slime. These nodules soon elongate into pillars, 1-2 mm high. Within 1 hour of formation a constriction near the apical end divides each pillar into a stalk, in which the yellow pigment granules are concentrated, and a terminal sporangium which acquires a disc-like or lobular shape (Guttes et al, 1961). Partitioning of the sporangial mass takes place in 2 stages. First, the capillitium, which is a criss cross network of channels, is formed. Several hours later the cleavage divisions take place and divide the sporangium into mono- and oligo-nucleate units. Following cleavage the bilaminate spore wall forms and melanization takes place (Randall & Lynch, 1974).

b) The metabolism and biochemistry of sporulating plasmodia

In the laboratory, the metabolic changes which occur during sporulation have been studied under a rigid set of conditions. Most results have been obtained with the Wisconsin strain using the following conditions. Microplasmodia must be harvested at the end of the exponential growth phase. Only at this time will sporulation occur in macroplasmodia

prepared from these microplasmodia (Daniel & Rusch, 1962a). Recent work using the CL strain has shown that microplasmodia can be harvested during the exponential growth phase and sporulation will occur if the macroplasmodium is kept on growth medium for one day before transfer to sporulation medium (Chapman & Coote, 1979).

The sporulation medium is a buffered balanced salts-solution which must contain niacin or one of its derivatives (Daniel & Rusch, 1962a; 1962b). It was concluded that niacin is required to generate NAD^+ or one of its derivatives (Daniel & Rusch, 1962b). Three alternative effects have been postulated: i) NAD^+ could act as a cofactor for enzymes in the cytosol; ii) in its reduced form, NADH could regulate the flow of electrons and thus influence the ATP level; and iii) NAD^+ could be used to synthesise poly(ADP)ribose. It is not clear however why niacin is needed to make the plasmodium competent to sporulate. During sporulation there is a switch from glycogen synthesis to utilization of glycogen accompanied by proteolysis and degradation of RNA (Daniel, 1966). At the end of the starvation period glycogen has disappeared, protein has decreased by 50%, RNA by 70% and DNA by around 80%.

Illumination is also a strict requirement for sporulation. The identification of the photoreceptor has been attempted. Sporulation can be induced by microinjection of cytoplasm from illuminated but not unilluminated plasmodia (Wormington & Weaver, 1976). Purification of the active factor shows it to have a molecular weight of around 500D (Wormington & Weaver, 1976). The mechanism by which light energy is converted to a chemical signal is unknown. The signal has been cautiously identified as cAMP (Daniel, 1974). Exogenously added dibutyryl cyclic AMP permits sporulation at reduced illumination levels and theophylline,

an inhibitor of cyclic AMP phosphodiesterase will mimic the action of light. Although illumination has been regarded as an absolute requirement for sporulation alternative means have been found as described below. Injection of ammonium chloride and sodium chloride produces sporulation (Wormington et al, 1975). This has proved difficult to explain but it may be linked to the fact that illumination induces release of K^+ while promoting proton uptake (Daniel & Eustance, 1972). It has been found that during differentiation the intracellular pH drops to very low values (pH 6.6). Additionally, artificially lowering the intracellular pH will give rise to morphology which resembles the first stages of starvation induced differentiation (Morisawa & Steinhardt, 1982). The time of commitment to sporulate, the point of no return (PNR), coincides with the cessation of glucose transport into the plasmodium and it has been claimed that glucose is actively secreted from the committed plasmodium (Sauer, 1982). One major consequence of illumination is a change in the pattern of calcium metabolism. Calcium is required in high concentrations for sporulation and is deposited in large amounts in developing sporangia in the capillitium and on individual spores (Daniel & Jahlfors, 1972; Chet & Kislev, 1973). Sporulation requires continued RNA and protein synthesis until late in the developmental sequence. Changes in protein synthesis during sporulation have been observed. Jockusch et al (1970) found that there is a shift towards synthesis of relatively large polypeptide chains during differentiation.

Using Actinomycin D, an inhibitor of RNA synthesis, it was estimated that the last essential RNA for sporulation was transcribed around three hours after illumination (Sauer et al, 1969b). A recent study has found that the first point of new mRNA species was nine hours

after light induction (T.H. Schrechenbach, 1984; In the 6th European Physarum Workshop). They isolated messenger RNA from induced plasmodia at different times and translated the mRNA in vitro. They found 60 induced mRNA species, some of which persisted through to the spores, others were transient. They also found very little change in the mRNA population during starvation. These results indicate that sporulation is a process that is controlled at the transcriptional level.

Differences in the above results may have been due to the impermeability of the plasmodium to actinomycin D after several hours post-illumination.

c) Nuclear changes during sporulation

It was reported that cycles of nuclear division continue, though at lengthened intervals and show prolonged nuclear reconstruction periods (Guttes et al, 1961). However, continued mitoses fail to produce a net increase in nuclear number since nuclear degeneration occurs and produces a net decline in nuclear and DNA content (Rusch, 1969). A presporangial mitosis, 12-13 h post-illumination has been reported (Guttes et al, 1961). Sauer et al (1969a) claimed that at least one mitosis and DNA replication must occur in the starved plasmodium before illumination could induce sporulation. These authors also claimed that a mitosis immediately followed by DNA replication occurred around 4 h before starved plasmodia were illuminated after 92 h starvation. More recent work using the CL strain has shown that three rounds of DNA replication take place, all within the first 24 h of starvation (Chapman & Coote, 1982). In order to determine the importance of DNA synthesis and mitosis during starvation for the CL strain they used two

inhibitory drugs, nocodazole, a benzimidazole derivative, which interferes with mitosis by preventing the assembly of the microtubule protein (Quinlan et al, 1981), and hydroxyurea which is an inhibitor of DNA replication. When the effect of these drugs on sporulation was studied it was found that sporulation began to occur normally if hydroxyurea was added later than 24 h into the starvation period. This finding was in agreement with the timing of the last S phase. Escape from nocodazole inhibition of sporulation was more delayed and was not complete until 55-60 h into starvation.

These results suggested that there may be a final mitosis, necessary for sporulation, which differed from other mitoses in that it was not followed by a round of DNA replication. It may be that this microtubule-requiring event was not a mitosis but some other event necessary for morphogenesis. If, however, the event was a mitosis it implied that the starving plasmodium entered a G1 phase from which sporulation competence developed.

In other eukaryotic model systems the events of the cell cycle, and in particular the G1 phase, have been found to be important during the differentiation process. It is therefore necessary to determine whether the microtubule-requiring event that occurs around 60 h of starvation in P. polycephalum is a mitosis and whether differentiation is initiated from a G1 phase of the cell cycle.

The most significant change during sporulation is the occurrence of meiosis. The meiotic division takes place during the final phase of morphogenesis, ie the cleavage divisions, but the timing is not rigidly controlled (Laane & Haugli, 1976). Generally, meiosis takes place one day after individual spores have been formed. The

onset of the second meiotic division has yet to be definitely defined (Mohberg et al, 1973). Nuclear degeneration may follow either the first or second meiotic division resulting in uninucleate spores (Laane & Haugli, 1976). Occasionally, multinucleate swarm cells are released upon germination, and the release of two swarm cells from a single spore is not an uncommon event.

It is generally believed that every non-mutational permanent change in cell structure or behaviour is a result of a differentiative process. Clearly sporulation in Physarum is an example of cellular differentiation and the study of the changes involved could contribute to our knowledge of eukaryotic cellular differentiation. Other studies of eukaryotic differentiation are often hampered by the fact that in many of the organisms available for study the cells are in various stages of the cell cycle. The synchrony of cell cycle events in the large plasmodium should allow a more convenient study of the relationship of cell cycle events to the onset of differentiation.

In recent years questions have been asked about the genetic control of differentiation. These include the regulation of genomic expression and the time at which differentiation specific genes are "switched on". New techniques which have been developed now allow those questions to be answered. The techniques are those involved in in vitro genetic manipulation. Although a number of eukaryotic models have been used in these studies, Physarum has a great deal to offer in this area of research. Again one of the most attractive features of this organism is the synchrony of its cell cycle. As a large plasmodium can contain as many as 10^{11} nuclei all within a common cytoplasm it can be regarded as one giant cell. Using Physarum as an experimental organism the worker has

a large amount of material which not only progresses through the cell cycle with perfect synchrony but also undergoes differentiation in synchrony. This synchrony offers a unique opportunity to study the time of expression of differentiation-specific genes.

5. In Vitro Genetic Manipulation

a) Introduction

In the past, understanding the molecular biology of prokaryotic gene expression has relied mainly on studies involving bacteriophage and bacterial plasmids. The *E. coli* phage lambda (λ) is probably the best characterized. The interaction of λ with the host cell is particularly interesting as it consists of a set of genes which can either direct cell lysis or allow λ to become associated with the host chromosome in lysogeny (Lewin, 1974). In the production of infectious phage from lysogens the excision can be imperfect. This results in a phage transductant which carries the segment of the bacterial chromosome which was next to the phage attachment site. Such phage allowed a map of the part of the bacterial chromosome next to the attachment site to be constructed (Franklin, 1971).

Research on bacterial plasmids has followed the same path. The discovery and rationalisation of the mechanism whereby F factors promote bacterial conjugation was crucial to the development of *E. coli* genetics. Just as the imperfect excision of λ from its lysogenic state can result in a phage genome carrying a segment of *E. coli* chromosome, so the imperfect excision of an F plasmid from an Hfr strain results in an F' (prime) plasmid which also carries a segment of bacterial DNA.

These F' plasmids have been valuable in working out the control mechanisms of several bacterial operons (Smith-Keary, 1974; Broda, 1979).

The principles of genetic engineering are similar to these *in vitro* events, but they overcome the limitation of a dependence upon the *in vivo* recombinational mechanisms of the *E. coli* cell. The techniques for recombining DNA *in vitro* make it possible to insert DNA from any organism into a plasmid or viral replicon to form a hybrid molecule which can replicate in a host organism (Sinsheimer, 1977; Morrow, 1979). The plasmids and phage used for constructing recombinant DNA molecules are called vectors. Plasmids and phage have their own advantages and disadvantages which make them attractive to the worker.

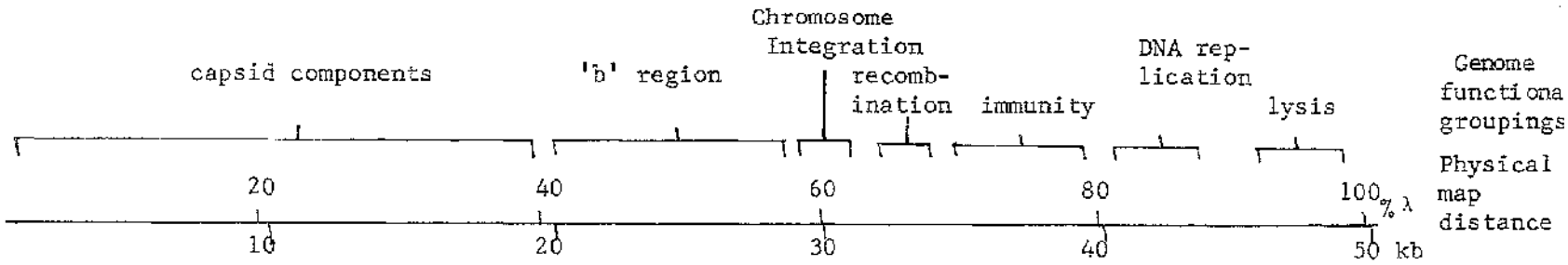
Phage vectors allow the insertion of pieces of foreign DNA up to 25 kb in length. This is much longer than the piece of DNA which can be cloned when using a plasmid vector. When plasmid and foreign DNAs have been ligated they must be transformed into *E. coli* cells. This method usually gives rise to around 10^5-10^6 clones per μg of DNA. Recombinant phage molecules no longer have to be transfected into *E. coli* cells to recover phage particles. They can be *in vitro* packaged, a process that yields 10^7-10^8 recombinant phage per μg of DNA (Hohn & Murray, 1977) (see Section C below). Thus the use of phage vectors and *in vitro* packaging is a much more efficient process in yielding recombinant molecules. In general plasmid libraries are more difficult to work with at all subsequent stages of cloning and DNA analysis. When screening a library by hybridization, a phage library can be screened using 10^4 cpm ml^{-1} for phage plaque filters. A cosmid library requires much more probe, around 1-5 x 10^6 cpm ml^{-1} for cosmid colony filters (Hadfield, 1983).

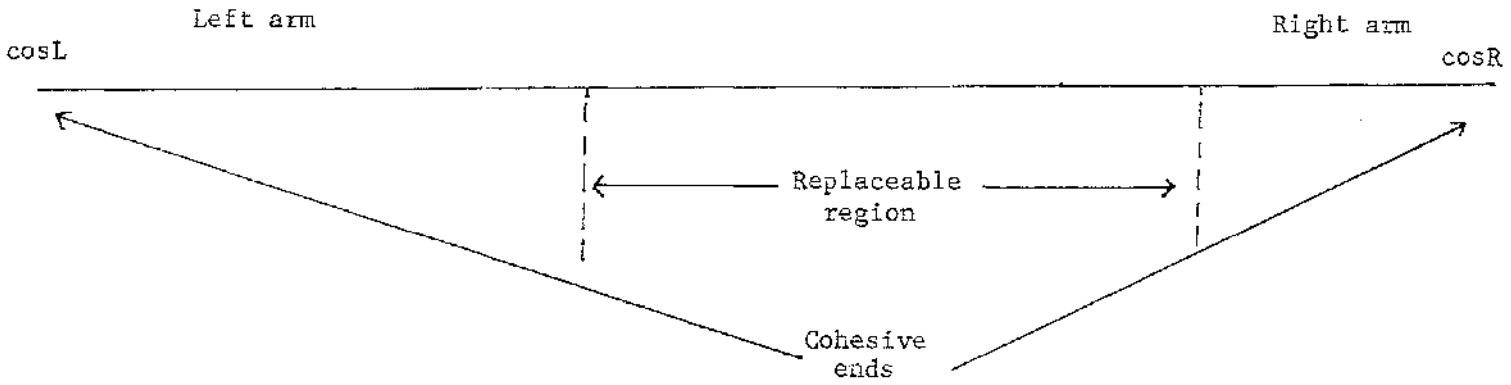
b) The organisation of the Lambda genome

A genetic map of phage λ is shown in Figure 4. The genome can be divided into three parts, the left arm, the right arm and the central region. Each section of the λ genome contains specific genes which control different phage functions. The left arm contains the genes which code for capsid components and packaging of λ DNA. The right arm of the phage contains genes which direct the replication of λ DNA and the lysis of the E. coli host cell. All genes that are required for the lysogenic mode of propagation are localized on the central fragment. This means that all the genes which are necessary for the phage to complete its lytic cycle are located on the left and right arms of the genome. The central region which contains the genes which are dispensible for lytic growth has been called the replaceable region. It is this piece of DNA that can be replaced by the foreign DNA of interest and so create recombinant DNA molecules.

Before λ could be used as a vector several in vivo and in vitro recombination events were necessary. It was essential that the phage DNA had sites for endonuclease cleavage located only in the region not required for lytic growth. These fragments of DNA must also have a total length shorter than that of the wild type λ DNA and be able to be packaged after insertion of foreign DNA (Rambach & Tiollais, 1974; Thomas et al, 1974; Murray & Murray, 1974; Tiemeier et al, 1976; Enquist et al, 1976). After the initial isolation of λ vectors, new λ phage were constructed that made it easier to distinguish the recombinant phage of interest (Murray et al, 1977; Blattner et al, 1977).

Figure 4. The Lambda Genome


capsid components
'b' region
Chromosome Integration
recomb-
ination
immunity
DNA rep-
lication
lysis
Genome
functional
groupings
20
40
60
80
100 % λ
Physical
map
distance
10
20
30
40
50 kb


Left arm
cosL
Right arm
cosR
Replaceable
region
Cohesive
ends

c) 'In vitro' packaging

The development of an in vitro packaging system made the process of recovering recombinant DNA molecules more efficient (Hohn & Murray, 1977; Sternberg et al, 1977).

In this system the DNA to be packaged is mixed together with concentrated lysates from two cultures; one of E. coli which is undergoing an infection with a phage which can not mature because of a mutation in gene D and the other in which the infecting phage has a mutation in gene E. In vitro the two lysates complement each other and concatomers of λ DNA are packaged and mature phage particles are produced. The packaging extracts are made from E. coli strains which carry a λ phage as a prophage. These phage have a temperature-sensitive repressor. When cells are grown at 30°C, the lysogenic state is maintained. Vegetative phage growth is initiated by thermo-induction and subsequent growth at 37°C. The packaging mixtures are stored at -70°C and thawed on ice before use.

A good packaging system can yield as many as 10^8 plaque forming units per μg of DNA.

d) Detection of recombinant phage

When the phage particles are plated on E. coli it is then neccssary to detect the plaques whose phage carry segments of foreign DNA. Several selection procedures have been developed such as size selection (Cameron et al, 1977; Philippsen et al, 1978), visual screens (Murray et al, 1977; Sanzey et al, 1976; Blattner et al, 1978), immuno-detection (Skalka & Shapiro, 1976; Broome & Gilbert, 1978) and restriction mapping (Cameron et al, 1977; Maniatis et al, 1978). One

selection procedure which has become increasingly popular is genetic selection.

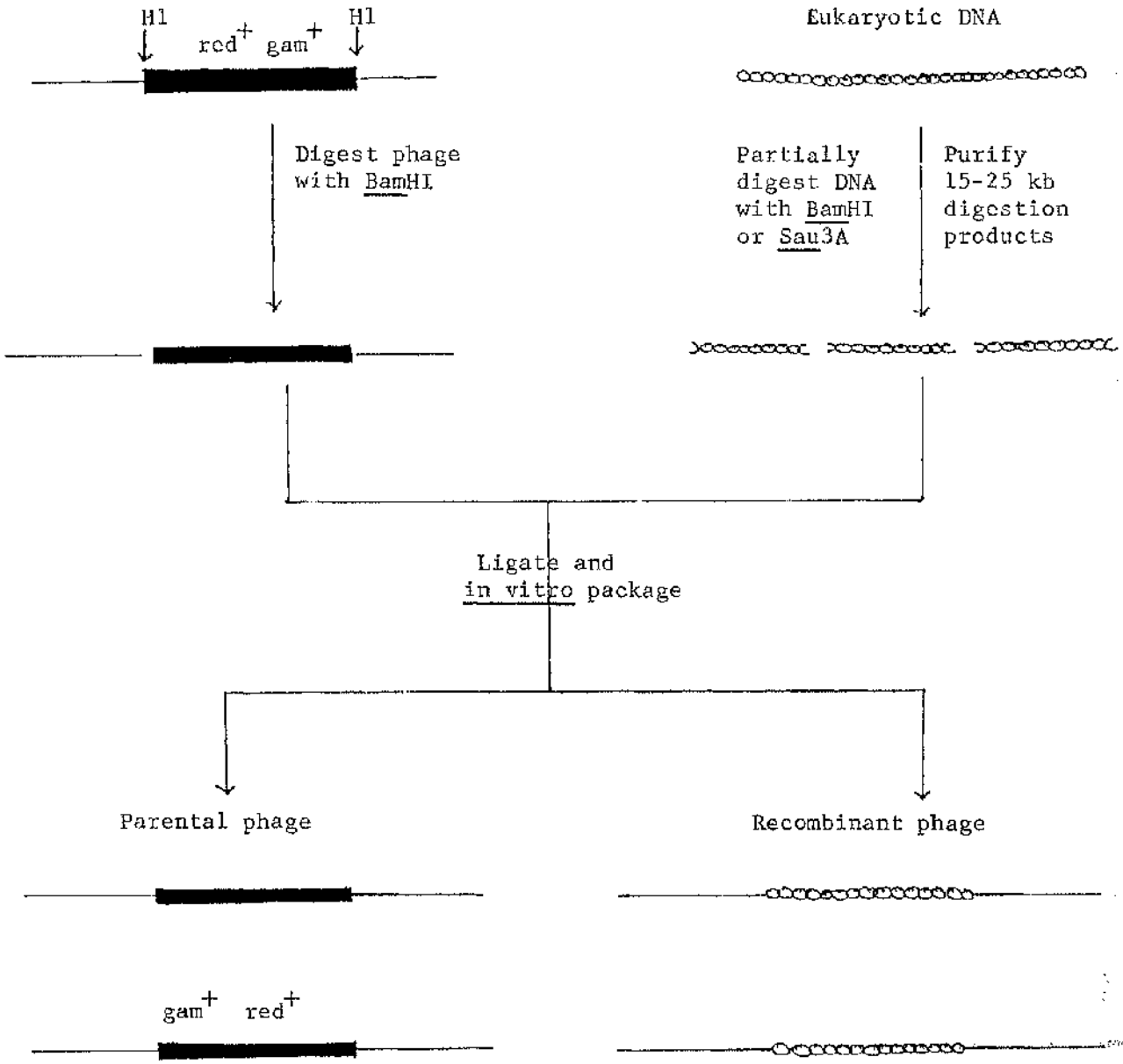
A positive selection available in λ is based on the 'Spi' phenotype (sensitivity to phage P2 inhibition) (Zissler et al, 1971). It is the selection method used in the work reported here.

Any λ phage which contains either the red gene or the gam gene can not form plaques on E. coli strains lysogenic for the temperate phage P2. These genes lie between 65 and 68% on the physical map of wild type λ. Using this system the total plaque forming units per ml can be determined by plating on a non-lysogenic E. coli; recombinant phage are titred when the lysate is plaqued on the lysogenic strain of E. coli. One such cloning vector is λ1059 (Karn et al, 1980). A schematic diagram outlining the strategy for cloning in λ1059 is shown in Figure 5. The use of the spi phenotype allows construction of recombinants in λ without separation of the phage arms. A permanent collection of recombinant phage is then established by allowing the phage harbouring inserts to amplify through several generations of growth on a strain that restricts the growth of the parental phage (Karn et al, 1983). Some red$^-$ phage grow slowly and this must be kept in mind during the amplification as reconstituted vectors may have a growth advantage over vectors containing foreign DNA (Philippsen et al, 1978; Cameron & Davis, 1977).

Since this work began a new phage vector has been described (Frischauf et al, 1983). The vector EMBL4 is a derivative of λ1059 and the cloning principle used with this vector is essentially the same as λ1059 in that EMBL4 carries the genes for the spi phenotype on a central fragment. One disadvantage of using λ1059 is that in 3 out of 4 cases

Figure 5. Cloning Strategy in λ1059

(From Karn et al, 1980)


Hl
red+ gam+
Hl
Eukaryotic DNA
Digest phage with BamHI
Partially digest DNA with BamHI or Sau3A
Purify 15-25 kb digestion products
Ligate and in vitro package
Parental phage
Recombinant phage
gam+ red+
Phage express red and gam genes. Growth is restricted on P2 lysogen.
Phage deleted in red and gam genes. Phage grow on P2 lysogen.

the BamH1 site is eliminated after the ligation of Sau3A cleaved foreign DNA into the BamH1 cleaved phage arms. This means that it is impossible to digest the recombinant phage DNA with BamH1 to recover the intact piece of cloned DNA. EMBL4 overcomes this disadvantage. The original BamH1 sites have been replaced by polylinker sequences which contain the restriction sites for EcoRI, BamH1 and Sal1. These restriction sites, next to the BamH1 sites allow digestion of the phage DNA and the recovery of the complete piece of cloned DNA.

e) Hybridization

Several procedures have been described in which labelled RNA probes or complementary DNA (cDNA) probes are used to screen hybrid plasmids or bacteriophage for the presence of a particular gene. Jones & Murray (1975) reported the use of conventional *in situ* hybridization techniques (John *et al*, 1969; Gall & Pardue, 1969) for screening hybrid phage. They cut plaques of various λ derivatives from *E. coli* lawns, fixed them onto glass slides and released and denatured the DNA with acid. They then hybridized with [^{3}H] labelled λ RNA and showed that a sequence of 1 kilobase or less could be detected. An estimated 30 plaques an hour could be screened by this method. Olivera & Bonhoeffer (1974) showed that bacterial colonies can be grown on nitrocellulose filters, then lysed and tested *in situ* for DNA polymerase 1 activity. Grunstein & Hogness (1975) used a similar technique in which DNA of plasmid containing colonies is denatured and fixed *in situ* for hybridization. This technique of growing colonies on nitrocellulose filters was extended and used for λ (Kramer *et al*, 1976). Plaques of hybrid phage are formed on *E. coli* lawns on nitrocellulose filters and their DNA released, denatured and fused directly for hybridization with radioactive probes.

Benton & Davis (1977) found that a single plaque contains enough phage DNA for detectable hybridization to complementary labelled RNA and that this DNA can be fixed to nitrocellulose filters by making direct contact between plaque and filter. This method has several advantages over previous methods: i) Physical containment is easier; ii) At least 2×10^4 plaques can be screened per hour (Kramer et al, 1976); iii) Because up to 2×10^4 phage can be screened on a single petri dish and nitrocellulose filter, the method requires smaller amounts of material including the radioactive probe. The method is given in detail by Benton & Davis (1977) and also in Methods, Section 8a.

Since the advancement of in situ hybridization techniques it has become the most widely used of all the screening techniques and has allowed the identification of hybrid vectors containing; mouse ribosomal gene fragments (Hemeier et al 1977); chorion gene sequence of a silk moth, β-globin genes from a rabbit genome (Maniatis et al, 1978); the nematode unc-54 myosin heavy chain gene (Karn et al, 1980) and many more genes that are of interest to workers in a variety of research areas.

f) 'In vitro' gene manipulation and eukaryotic differentiation

The question which now arises is "Has gene manipulation helped in understanding the genetic control behind cellular differentiation?"

Several groups of workers have already used recombinant DNA techniques in their work with a number of experimental systems.

i) Saccharomyces cerevisiae

Sporulation in the yeast S. cerevisiae can be viewed as a simple developmental process. This differentiation process is under the control of the mating type locus (MAT). Haploid cells can be one of

two mating types a and α each of which can mate only with the other to produce a α/a zygote. This diploid can no longer mate but is competent to undergo meiosis and produce two α and two a haploid spores.

The α and a mating types behave as if they are determined by a pair of alleles (MATa and MATα). They are however unlike ordinary alleles. Each allele appears to control the expression of a different set of functions. MATa and MATα are co-dominant, a MATa/MATα diploid possesses many properties absent in both haploid parents. In strains carrying the homothallism allele, HO, cells arising from germinating spores can switch their mating type at a frequency of almost once per cell division (Hicks & Herskowitz, 1976). It has been proposed that mating type switching is effected by transposition of silent copies of the α or a specific sequence from elsewhere in the genome to the mating type locus (Hicks & Herskowitz, 1977). Two sites for the proposed silent copies have been identified. One, HMLα, situated near the left arm of chromosome III, is necessary for the a - α conversion. The other, HMRa, situated near the end of the right arm of the same chromosome is necessary for the reverse process (Harashima et al, 1974). The present model of the mating type locus has been called the Cassette hypothesis (Hicks et al, 1977). It likens the transposition of a or α specific instructions from the HML or HMR loci into the mating type locus to that of a cassette being placed into a tape recorder. The cassettes are unexpressed, ie silent unless plugged into the MAT locus. The model has been supported by cloning and characterisation of the mating type genes (Nasmyth & Tatchell, 1980).

Clancy et al (1983) performed a differential plaque hybridization screen of a yeast DNA library. They estimated that they had identified 15 different sporulation specific genes. They also carried

out detailed analysis of several of these clones and found evidence that some of the sporulation specific genes may be clustered.

In a separate study at least 14 genes were found to be expressed preferentially during sporulation (Percival-Smith & Segall, 1984). They first constructed a partial Sau3A yeast DNA library in the plasmid pBR322 and then performed a differential hybridization screen with [^{32}P] labelled cDNA probes representing the poly(A)$^+$ RNA of aα and αα cells at various times after transfer to sporulation medium. Since only the aα cells undergo sporulation, clones showing enhanced hybridization with the aα probe relative to the αα probe contained potential sporulation specific genes. This approach, although simple, allowed the identification of colonies which contained sporulation specific genes.

Another approach was used by Weir-Thomson & Dawes (1984). They isolated poly(A)$^+$ RNA from a/α, a/a and α/α at different times after transfer to sporulation medium. The message was in vitro translated in a messenger-dependent cell free rabbit reticulocyte lysate and the products separated by two-dimensional gel electrophoresis. They found that 43 of the 750 mRNA species detected underwent alterations only during sporulation in the a/α strain, whereas 36 changes were common to all three strains and one mRNA specific to α/α vegetative cells was detected. Of the 43 sporulation specific changes only 4 were due to de novo synthesis of translatable species, and two of these became predominant species of the total population. The majority of the specific changes were due to either permanent or transient increases in the concentration of individual mRNA species.

ii) Aspergillus nidulans

Asexual reproduction in the Ascomycete fungus A. nidulans

involves the differentiation of multicellular spore-producing structures called conidiophores and uninucleate spores called conidia. Evidence pointed to the process being under the control of several genes as many single mutations either blocked conidiophore development at a specific stage or resulted in the formation of abnormal conidophores or conidia (Clutterbuck, 1969; Martinelli & Clutterbuck, 1971).

Timberlake (1980) isolated poly(A)$^+$ RNA from somatic hyphae, conidiating cultures and purified conidia. These messages were then used to direct the synthesis of cDNA. DNA hybridization experiments were then carried out. cDNA hybridization experiments indicated that a significant proportion of the poly(A)$^+$ RNA isolated from either conidiating cultures or spores consisted of sequences which were absent from somatic hyphae. He then isolated cDNAs which were complementary to poly(A)$^+$ RNA from conidiating cultures. Hybridization of the cDNAs with poly(A)$^+$ RNA from conidiating cultures showed that around 18.5% of the poly(A)+ RNA comprised around 1300 diverse sequences not present in somatic cells. of these about 300 were present only in conidia.

Zimmerman et al (1980) prepared a genomic library in the λ vector Charon 4A. EcoRI was used to generate random complete and partial digestion products. These were then selected for fragments longer than 12 kb and the DNA was ligated into the EcoRI digested phage arms. Radioactive probe was then prepared by cascade hybridization (Timberlake, 1980). ^{32}P-cDNA was synthesized using poly(A)$^+$ RNA from sporulating cells as a template. The cDNA was hybridized sequentially with 20, 50 and 100 fold mass excess of poly(A)$^+$ RNA from somatic cells. After each reaction hybrids were removed by chromatography on hydroxyapatite at 68°C and the unreacted DNA was collected by ultracentrifugation. Finally the

cDNA was hybridized to a 25 mass excess of poly(A)$^+$ RNA from sporulating cultures and chromatographed on HAP as above. The bound fraction was eluted and treated with NaOH to remove the RNA. This cDNA was then used to screen the DNA library. They found over 350 clones which contained sporulation specific genes. Detailed analysis of several of the clones showed that some code for multiple developmentally regulated poly(A)$^+$ RNAs and indicate that the developmental regulating regions may not be randomly dispersed in the genome.

One of the above clones was studied in greater detail by Timberlake & Barnard (1981) to try and learn how the developmental genes are organised in the genome. By hybridizing whole phage and digested phage DNA to poly(A)$^+$ RNA from conidiating cultures and spores they were able to show that the clone λAnSpoC1 contained six discrete genes all expressed specifically in conidia.

30 clones from the CH4A Library were further analysed by Orr & Timberlake (1982). Again they hybridized labelled λ DNAs to poly(A)$^+$ RNA from the three types of cells. They found that 69 spore-specific genes were contained in the 30 cloned sequences. More than 50% of the clones contained two or more spore specific genes. In a second set of experiments plasmid library clones were used. Here sample clones were hybridized with spore specific cDNAs to determine the fraction of clones, with different insert sizes, that did not contain spore-specific coding regions. Fewer clones yielded positive hybridization signals with spore-specific cDNA than predicted if the sequences were randomly dispersed.

From all the evidence available it appears that the spore-specific genes are not randomly dispersed in the A. nidulans genome but rather occur in close association with one another.

iii) Dictyostelium discoideum

Another lower eukaryotic organism that undergoes differentiation is the cellular slime mould Dictyostelium discoideum. The developmental pathway of this organism has been described in a previous section.

Blumberg & Lodish (1980) investigated the changes that occur in the messenger RNA population during differentiation of D. discoideum. They isolated mRNA from vegetative cells, cells 6 hours after the induction of development and cells 13 hours after the induction of development. Those mRNAs were used as templates to direct the synthesis of cDNA in vitro. The cDNAs were used in RNA excess hybridization reactions. They found that there is only one time period in the developmental sequence when the initiation of expression of a significant number of genes occurs. This point was found to be mid-way in the developmental cycle, coincident with the time multicellularity occurs and just before the onset of differentiation of spore and stalk cells. They also found that the number of mRNA species does not change significantly by 6 hours of differentiation. The polysomes of postaggregation cells contained 7000 individual mRNA species which represented 30% of the single copy genome. The majority of the mRNA species present before aggregation remain in the cells during development, although the average abundance of these mRNAs decreases with time. Cross hybridization experiments could not detect any difference between the mRNA population in postaggregation cells and culminating cells.

Rowenkamp & Firtel (1980) tried to estimate the number of developmentally regulated genes in D. discoideum. They isolated mRNA from two stages of development. The first was at 6 hours, just prior to aggregation, the second at 15 hours during the grex stage. cDNAs were

then made using the mRNAs as templates and then cloned into plasmid pBR322. Approximately 4000 clones from each library were then screened by the colony hybridization technique. Three categories of genes were identified: i) genes which are turned off during development, ii) genes which are turned on during development, and iii) genes that show relatively constant activity.

This type of approach was used again by Mangiarotti et al (1981). In this research a gene bank of Dictyostelium was prepared in the lambda vector λgtwes. Cytoplasmic RNA was then isolated from vegetative cells and developing cells. Poly(A)$^+$ RNA from developing cells was isolated by chromatography on an oligo(dT) cellulose column. The poly(A)$^+$ RNA was labelled in vitro with [^{32}P]ATP and then used in hybridization experiments. Clones which contained developmentally regulated genes were detected by hybridizing a filter replica of plaques with labelled mRNA from developing cells in the presence of a several hundred-fold excess of RNA prepared from vegetative cells. Many of the clones isolated encoded multiple species of mRNA, some of which were developmentally regulated. They also found that a significant fraction of cloned DNA fragments contained both constitutive genes and genes specific for development. This suggests that the two kinds of genes are interspaced in the Dictyostelium genome.

Sixty clones from this library were taken and analysed further. This was done by identifying and qualitating the homologous mRNAs by RNA gel analysis. About one third of the mRNAs encoded by these clones were present only in aggregated cells (Mangiarotti et al, 1983).

Zuker & Lodish (1981) screened a λ genomic library and found a clone that contained a specific set of repetitive DNA sequences that are

found interspersed in the D. discoideum genome. They found that when these sequences are expressed they are linked only to developmentally regulated mRNAs and suggest that the sequence may play an important regulatory role in the developmental programme of D. discoideum.

A D. discoideum genomic library was prepared in plasmid pBR322 (Julien et al, 1982). D. discoideum DNA was partially digested with Sau3A and then ligated into pBR322. The insert sizes were 3-10 kb long. 2×10^4 clones were isolated and screened by the colony hybridization method. Three cDNA probes were used which corresponded to vegetative mRNA, preculminating mRNA and spore mRNA. Seven clones were found to hybridize specifically to spore cDNA. One clone was analysed further and was found to contain a unique sequence flanked by repeated sequences.

In principle, these techniques can be used to screen any library of cloned cDNA or genomic DNA for genes expressed only in a given condition during the life cycle of any cell or organism.

Physarum polycephalum has a great deal to offer as an experimental model system for differentiation. The cell cycle of Physarum is synchronous not only during growth but also during differentiation. Differentiation is a process that can be controlled by the worker in the laboratory and is itself naturally synchronous. This synchrony coupled with in vitro genetic manipulation techniques offers a unique chance to study the timing of expression of sporulation specific genes.

One theory that has been proposed to explain the control of differentiation is that the transcription of a gene may be dependent on its time of replication. It is not known whether specific genes are replicated at the same time in growth and differentiation. These questions could be answered by gene manipulation using Physarum as it has

been shown that a sequence of DNA is replicated at the same time during each cell cycle (Braun *et al*, 1965). It has already been shown that the *Physarum* genome can be cloned (Sakaki *et al*, 1981; McLachlan & Hardman, 1982; Peoples & Hardman, 1983; Peoples *et al*, 1983; Gerrie *et al*, 1983) and this is a strong indication that the procedures of *in vitro* gene manipulation may be of use with *Physarum polycephalum* as the model system and could be fruitful in clarifying the genetic control which governs the process of sporulation.

OBJECT OF RESEARCH

Objects of the Research

The synchrony of cell cycle events in *Physarum* offers an opportunity to correlate gene expression specific to differentiation with particular stages of the cell cycle. With this in mind the immediate objects of this research were:

1. To determine from which stage of the cell cycle the starved plasmodium embarked on sporulation.

2. To assess, by noting the effects on DNA replication following inhibition of mitosis, whether a G1 phase of the cell cycle could be detected in starved plasmodia.

3. To prepare a genomic library of *P. polycephalum* DNA in a lambda vector.

4. To isolate poly(A)$^+$ RNA, suitably undegraded, for preparation of copy DNA for use as a probe for the detection of sporulation-specific DNA sequences in the genomic library.

The longer-term objective was to gain a better understanding of the biochemical control of differentiation by applying *in vitro* genetic techniques to the cellular changes in a primitive eukaryote.

MATERIALS AND METHODS

1) Maintenance and Culture of Physarum polycephalum

a) Physarum polycephalum CL was used throughout this research. This strain was obtained from T. Laffler, McArdle Laboratory for Cancer Research, Madison, Wisconsin, USA, as a derivative of the original Colonia Leicester (CL) strain (Cooke & Dee, 1975).

b) Culture of microplasmodia

The organism was maintained as a shaken suspension of microplasmodia. To subculture, 2 ml of a 72 h old culture was used to inoculate 20 ml of semi-defined growth medium (GM, see Appendix 1) (Daniel & Baldwin, 1964) plus 0.2 ml of hemin solution (see Appendix 1) contained in a 250 ml dimpled conical flask. This was incubated in the dark at 26°C and shaken at 100 rpm on a Gallenkamp orbital shaker.

c) Surface culture of plasmodia

Microplasmodia (5 ml) were collected from an exponentially growing culture, 24 h after subculture, and harvested by centrifugation at 500xg for 2 min at room temperature in a MSE Super Minor bench centrifuge. The microplasmodia were resuspended in 0.1 ml of sterile distilled water and were then pipetted in a circle onto a sterile Whatman 50 filter paper supported by a wire mesh in a 9 cm petri dish. Approximately 20 ml of GM was introduced under the filter paper until the paper was soaked, but not flooded. Cultures were incubated in the dark at 26°C.

d) Sporulation of P. polycephalum

Plasmodia were grown as described in Section 1(c) above. After 48 h of growth the plasmodia were transferred to fresh petri dishes.

Sporulation medium (SpoM, see Appendix 1) was then introduced under the filter paper. Plates were incubated in the dark at 26°C. After 72 h of starvation, sporulation was induced by exposing the plasmodia to light for 1 h. Surface cultures were incubated and illuminated in a Gallenkamp cooled incubator. Illumination was from 8W cool white fluorescent lights 15 to 20 cm from the plasmodia.

2) Miscellaneous Techniques

a) Measurement of DNA synthesis

Uptake of [methyl-^{3}H] thymidine (50-70 Ci mmol^{-1}, Amersham) into growing and starving plasmodia was followed. Small pieces of filter paper supporting the plasmodia were cut out with sterile scissors and transferred to a wire grid in a petri dish containing 20 ml of GM or SpoM with thymidine added to a final concentration of 0.2μ Ciml^{-1}. These were incubated at 26°C for 30 min after which time they were immediately plunged into 5 ml of ice-cold 1:1 (v/v) TCA (8% (w/v))/acetone. This treatment removed all the pigment from the plasmodium. The precipitates were collected by centrifugation at 1000xg in an MSE bench centrifuge for 5 min at room temperature. The absorbance of the supernatant was determined at 400 nm. This was used to estimate the amount of plasmodial material collected so that the final radioactive count could be standardized. The precipitates were washed twice in 0.25M perchloric acid containing 100 μg ml^{-1} thymidine and finally solubilised in 1 ml of 0.4M NaOH. Toluene based scintillant (see Appendix 10) was then added to 0.5 ml of the sample and the radioactivity counted in a Packard TRI-CARB 300C scintillation counter.

b) Microscopic observation of nuclei

i) Light microscopic observation

A small piece of plasmodium was cut with sterile scissors and scraped onto a glass microscope slide. The piece of plasmodium was crushed and spread across the slide using a second microscope slide. The plasmodium was fixed in absolute ethanol for 15 sec. A drop of 1:1 (v/v) glycerol/alcohol was then added to the preparation and a coverslip placed on top. Nuclei were observed at x1000 magnification on a light microscope (Vickers Instruments).

ii) Preparation of samples for electron microscopic observation

The method used was that of Herbert et al (1980). Samples of plasmodia were cut and fixed for 1 h in 2.5% (v/v) glutaraldehyde in 0.1M Na-cacodylate buffer (pH 7.2) containing 8% (w/v) tannic acid and 0.003M $CaCl_2$. After fixation, samples were washed in Na-cacodylate buffer (pH 7.2) four times for 10 min each wash, then post-fixed in 2% (w/v) osmium tetroxide for 2 h in the dark. The samples were washed three times in distilled water to remove all traces of the osmium. The samples were embedded in 1.5% (w/v) purified agar and stained for 2 h in 0.5% uranyl acetate at room temperature after which the samples were dehydrated in a graded propylene oxide series. After dehydration the samples were embedded in Epon resin and sectioned using an LKB ultratome III. Electron microscopic observations and photography were performed on a Philips EB 300 operated at 60 kV.

3) Maintenance and Growth of E. coli Strains

a) Three E. coli strains were used in the preparation and maintenance of

genomic libraries. *E. coli* DP50 *supF* (F^-, *dapD8*, *lacY*, Δ[*gal-uurB*] Δ *thy A*, *nalA*, *hsdS* SuII, SuIII) was obtained from F. Blattner, University of Wisconsin, Laboratory of Genetics, Madison, Wisconsin, USA.

E. coli Q358 (r^-k, m_k^+, SuII, 80^R) and *E. coli* Q359 (r^-k, m_k^+, SuII, 80^R, P2) were obtained from J. Karn, Medical Research Council Laboratory of Molecular Biology, Cambridge, England.

E. coli BHB2688 (λ*imm*434, cI_{ts}, *b2*, *red3* *Eam4*, Sam7)λ, and *E. coli* BHB2690 (λ*imm*434, cI_{ts}, *b2*, *red3* Dam IS, Sam7)λ (Sternberg *et al*, 1977)) were used in the preparation of *in vitro* packaging extracts. They were obtained from P. Anderson, Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow.

b) Stock cultures

Stock cultures of all *E. coli* strains were maintained on CY or NZ agar plates (see Appendix 2) and were subcultured every month. Cultures were replaced every three months from stocks kept in 50% glycerol at -20°C.

c) Growth of *E. coli* DP50 *supF*, *E. coli* Q358 and *E. coli* Q359

E. coli DP50 *supF* was grown in NZ broth supplemented with 100 μg ml^{-1} thymidine and 100 μg ml^{-1} diaminopimelic acid. *E. coli* Q358 and *E. coli* Q359 were grown in CY broth.

A single colony of bacteria from a stock plate was used to inoculate 10 ml of the relevant growth medium + 2 mg ml^{-1} maltose in a 250 ml dimpled conical flask. This was incubated overnight at 37°C on an orbital shaker operating at 150 rpm.

d) Growth of *E. coli* BHB2688 and *E. coli* BHB2690

Bacteria were streaked out on an NZ agar plate and incubated

overnight at 30°C. A single colony was then used to inoculate 20 ml of NZ broth in a 250 ml dimpled conical flask which was incubated at 30°C overnight on an orbital shaker operating at 150 rpm.

4) Growth of Lambda Phage

Charon 4AP was obtained from R. Losick, Biological Laboratories, Harvard University, Boston, Mass., USA. λ1059 was obtained from J. Karn, Medical Research Council Laboratory of Molecular Biology, Cambridge, England.

a) Plate lysates

Phage were diluted to the correct concentration in plaque storage buffer + gelatin (see Appendix 3).

A bacterial suspension was prepared by adding 1 ml of an overnight culture of the appropriate E. coli strain to 4 ml of growth medium. 5.0 ml of CaMg solution was then added (see Appendix 3). Maltose (20% (w/v)) was then added to a final concentration of 0.2%. 0.2 ml of E. coli suspension was added to 0.1 ml of phage dilution. This was mixed and incubated statically at 37°C for 20 min to allow phage absorption. 3 ml of molten CY or NZ agarose + 2 mg ml^{-1} maltose (50°C) was then added. The mixture was well mixed and then poured as an overlay onto an NZ or a CY agar plate. Plates were incubated at 37°C overnight.

b) Liquid lysates

An aliquot (100 µl) of an overnight culture of the appropriate E. coli strain was taken and the cells were collected by centrifugation in a Sarstedt microfuge for 5 min at room temperature. The bacterial

pellet was well drained then the cells were taken up in 100 µl of 0.01M $MgCl_2$.

A phage plaque was removed from an agar plate using a sterile pasteur pipette. The plaque was placed in the bacterial suspension which was incubated statically at 37°C for 20 min to allow phage adsorption. The plaque and the bacterial suspension were then added to 5 ml of the appropriate growth medium containing 2 mg ml^{-1} maltose and incubated at 37°C with vigorous shaking for 4 to 6 h until cell lysis had occurred.

5. Isolation of Nucleic Acids

a) Bacteriophage DNA

The phage Charon 4AP (Ferrari *et al*, 1981) was grown as a liquid lysate on *E. coli* DP50 *supF* in NZ broth + 100 µg ml^{-1} thymidine, + 100 µg ml^{-1} diaminopimelic acid + 2 mg ml^{-1} maltose. The phage λ1059 (Karn *et al*, 1980) were grown as liquid lysates on *E. coli* Q358 in CY broth + 2 mg ml^{-1} maltose.

The method used is a slight modification of the one described by Yamamoto *et al* (1970).

Bacteria and phage were mixed together at the correct multiplicity of infection (see Results Section 2a) and incubated statically at 37°C for 20 min. This was then used to inoculate 500 ml of prewarmed broth containing 2 mg ml^{-1} maltose in a 2 litre dimpled flask. Cultures were incubated for 4 h at 37°C with vigorous shaking until cell lysis occurred. 60 g l^{-1} of NaCl was added and the cell debris was collected by centrifugation in a MSE High Speed 21 centrifuge at 11,500xg for 15 min at 4°C. The supernatant was decanted off and the phage were

precipitated by the addition of 70 g l^{-1} polyethylene glycol 8000 (Sigma). The phage precipitate was collected by centrifugation in a MSE High Speed 21 centrifuge at 9000xg for 45 min at 4°C and after being well drained it was dissolved in 4 ml of plaque storage buffer + gelatin (PSB + gel).

Bovine pancreas ribonuclease A (RNase A, Sigma; 0.1% (w/v) in 0.15M NaCl, pH 5.0) and bovine pancreas deoxyribonuclease II (DNaseII, Sigma; 0.1% (w/v) in 0.15M NaCl, pH 5.0) were each added to a final concentration of 50 μg ml^{-1} and the concentrated phage suspension was incubated at 37°C for 60 min.

Phage were purified by Caesium chloride (CsCl) density gradient centrifugation. Saturated CsCl (BDH, Analar Grade, 99.9-100%) was added (1.1 ml CsCl per ml of phage suspension) and the mixture was centrifuged in a MSE Superspeed 65 ultracentrifuge at 110,000xg for 16 h at 4°C in 10 ml polycarbonate tubes in a MSE 8 x 35 ml fixed titanium rotor with 10 ml adaptors.

After centrifugation phage particles were visible as a distinct blue band within the gradient. They were removed from the gradient using a 5 ml syringe and a 19 gauge needle and then dialysed for 72 h against 3 litres of TSM buffer (see Appendix 4) at 4°C. Phage heads were disrupted by adding a one-hundredth volume of 0.5M EDTA (pH 8.0) and a one-tenth volume of 10% (w/v) SDS. This mixture was incubated at 70°C for 15 min. The lysate was then mixed with an equal volume of buffer-saturated phenol (see Appendix 4) and centrifuged at 2000xg for 10 min in a MSE bench centrifuge. The upper aqueous layer was removed and retreated with buffer-saturated phenol. After centrifugation the aqueous layer was removed, mixed with an equal volume of buffer-saturated ether (see Appendix 4) and centrifuged as before. A second treatment with

buffer-saturated ether was carried out and the aqueous layer was then treated with t-amyl alcohol. After centrifugation the aqueous layer was removed and sodium acetate (3M, pH 7.2) was added to a final concentration of 0.3M, 3 volumes of ice-cold absolute ethanol were added and after mixing the DNA was precipitated at -20°C overnight. The DNA was collected by centrifugation in a MSE bench centrifuge operating at top speed for 10 min. The pellets were well drained and dissolved in 0.5 ml TE buffer (pH 8.0) (see Appendix 4). The DNA was generally stored at a concentration of 1.0 mg ml^{-1}.

b) Physarum DNA

i) Isolation of nuclei

Nuclei were isolated using a slight modification of the method described by Mohberg & Rusch (1971). Microplasmodia were collected from 20 ml of an exponentially growing culture and harvested by centrifugation at 500xg for 2 min at room temperature in a MSE Super Minor bench centrifuge. The pellet was washed with 10 ml of Tris/sucrose buffer pH 8.0 (see Appendix 4) and resuspended in 5 ml of the same buffer. The microplasmodia were then homogenized by passage 10 times through a 21 gauge needle. The homogenate was centrifuged at 50xg in a MSE bench centrifuge at room temperature for 5 min. The supernatant was removed and centrifuged at 1000xg for 5 min. The nuclear pellet was washed twice in 5 ml of Tris/sucrose buffer (pH 8.0).

ii) Lysis of nuclei

The method used is a modification of that described by Hardman & Jack (1978).

The nuclei were resuspended in 0.8 ml of Tris/EDTA buffer pH 8.0 (see Appendix 4). Sodium lauryl sarcosinate was added to a final

concentration of 1% (w/v) and bovine pancreas ribonuclease A (RNase A, Sigma; 0.1% (w/v) in 0.15M NaCl, pH 5.0) and Aspergillus oryzae ribonuclease T_1 (RNase T_1; Sigma, 1000 U ml^{-1} in 0.15M NaCl, pH 5.0) were added to a final concentration of 50 µg ml^{-1} and 100 U ml^{-1} respectively. (Both RNases were incubated at 80°C for 10 min to denature any contaminating DNase). The nuclei were incubated at 37°C for 30 min when protease XI (Tritirachium album, Sigma; 0.2% (w/v) in Tris/EDTA buffer pH 8.0) was added to a final concentration of 200 µg ml^{-1}. Incubation was continued for a further 60 min at 37°C.

iii) Purification of DNA

Method 1

The viscous lysate was mixed with an equal volume of buffer saturated phenol (see Appendix 4) and centrifuged in a MSE bench centrifuge for 10 min at room temperature. The aqueous layer was removed and retreated with buffer saturated phenol. After centrifugation the aqueous layer was treated with an equal volume of buffer-saturated ether and centrifuged as before. A second treatment with buffer-saturated ether was carried out and the aqueous layer was then mixed with an equal volume of t-amyl alcohol and centrifuged for 10 min. The aqueous layer was removed and the DNA was precipitated by adding sodium acetate (3M) to a final concentration of 0.3M and 2-3 volumes of absolute ethanol.

The DNA was then purified on a CsCl density gradient. A partially preformed gradient was prepared using three different concentrations of CsCl. The preformed gradient consisted of three 2 ml layers of densities 1.65, 1.72 and 1.79 g ml^{-1} of CsCl made up in distilled water. The densities of the CsCl solutions were checked by measurement of the refractive index at 20°C using a refractometer (Bellingham & Stanley Ltd).

The Physarum DNA sample was layered on top of the preformed gradient and 10 ml polycarbonate tubes filled with liquid paraffin (BDH) to prevent the tubes collapsing. Centrifugation was carried out in a MSE Superspeed 65 Ultracentrifuge at 110,000xg for 24 h at 4°C using a MSE 8x35 fixed angle titanium rotor with 10 ml adaptors.

After centrifugation the tubes were pierced using a MSE tube piercer and 0.1 ml fractions were collected. The fractions which contained the Physarum DNA were found using the method of Blin et al (1975). 2 µl of each fraction was spotted onto a glass fibre filter (Whatman GF/A) dried and stained for 5 min in an ethidium bromide solution (0.5 µg ml^{-1} in distilled water). The filters were then viewed on a Chromato vue transilluminator C-60 (Ultraviolet Products Inc., San Gabriel, California USA). The fractions containing the DNA were pooled and dialysed for 72 h against 3 litres of TE buffer (pH 8.0) at 4°C. After dialysis the DNA was precipitated by the addition of sodium acetate (3M) to a final concentration of 0.3M and 3 volumes of absolute ethanol. The DNA pellet was collected by centrifugation, well drained then dissolved in TE buffer (pH 8.0) to give a concentration of 1.0 mg ml^{-1}.

Method 2

This method was described by Hardman & Jack (1978). Isolation and lysis of nuclei were as above. The viscous lysate was made up to 4 ml with Tris/EDTA buffer pH 8.0. Solid CsCl (BDH, Analar Grade 99.9 - 100%) was added 1 g ml^{-1} of lysate) and dissolved thoroughly. Ethidium bromide (5 mg ml^{-1} in distilled water) was added to a final concentration of 0.5 mg ml^{-1} and this was then placed in 10 ml polycarbonate centrifuge tubes. Liquid paraffin was used to completely fill the tubes in case of collapse. Centrifugation conditions were as described above.

After centrifugation the DNA was visualised by ethidium bromide fluorescence over a UV transilluminator (Chromata vue C-60) and removed from the gradient using a 5 ml syringe and a wide-bore needle. Six isopropanol extractions were then carried out to remove all traces of ethidium bromide and the DNA was then dialysed for 72 h against 3 litres of TE buffer (pH 8.0).

The DNA was treated with buffer-saturated phenol, buffer-saturated ether and t-amyl alcohol as described above. The DNA was precipitated by the addition of sodium acetate (3M) to a final concentration of 0.3M and 3 volumes of absolute ethanol. The pellet was collected by centrifugation, well drained and dissolved in TE buffer pH 8.0. The DNA was stored at a concentration of 1.0 mg ml^{-1}.

c) Physarum RNA

i) Cytoplasmic RNA preparation

Three different polysome buffers were used (see Appendix 5) in attempts to isolate polysomes from which RNA could be prepared. All buffers and solutions used in preparing RNA were autoclaved before use.

Method 1

A surface plasmodium was grown as described in Section 1c. The plasmodium was submerged for 30 seconds in 100 ml of ice-cold polysome buffer. The plasmodium was then scraped into 1.0 ml of ice-cold polysome buffer and homogenised with 10 strokes in a Potter-Elvehjem glass homogeniser. The homogenate was then centrifuged in a Beckman microfuge at 12,000 rpm for 15 min at $4^{o}C$ to pellet the debris. 0.2 ml of the supernatant was removed and kept for a polysome profile. The remaining supernatant was mixed with an equal volume of phenol and an equal volume of chloroform. This was centrifuged in a Beckman

microfuge at 12,000 rpm for 10 min at 4°C. The aqueous layer was removed and treated with phenol/chloroform twice more. The aqueous layer was made 0.3M with sodium acetate (pH 6.0) and 2 volumes of absolute alcohol were added. The solution was mixed and incubated at -20°C for 3 h. The precipitate was collected by centrifugation in a Beckman microfuge. The pellets were well drained and dissolved in 1.0 ml of TE buffer (pH 8.0). 0.05 ml of 10% (w/v) SDS (heated at 65°C for 1 h) and 0.1 ml fungal proteinase K (BRL; 1% (w/v) in TE buffer (pH 8.0)) were added. The mixture was incubated at 37°C for 45 min, then extracted with an equal volume of phenol plus an equal volume of chloroform until a clear interphase was obtained. The aqueous phase was removed and the RNA was precipitated by adding sodium acetate (3M, pH 6.0) to a final concentration of 0.3M and 2 volumes of absolute alcohol. This was mixed and incubated at -20°C overnight.

Cetyl trimethyl ammonium bromide precipitation

The method used was that described by Bellamy & Ralph (1968). The -20°C precipitate was collected by centrifugation and the pellets were well drained before being dissolved in 0.25 ml cold 0.025M Tris, 0.025M NaCl (pH 8.1). At 4°C 0.25 ml cold 2.5M phosphate buffer (pH 8.0) (see Appendix 5) and 0.25 ml 2-methoxyethanol were added. This was whirlimixed for 1 min before being centrifuged in a MSE bench centrifuge for 5 min at room temperature. The upper layer was removed and 0.2 ml sodium acetate was added to give a final concentration of 0.1M. One half volume of cetyltrimethylammonium bromide (CTAB; BDH, 1% (w/v) in sterile distilled water) was added. This was mixed and incubated at 4°C overnight. The RNA was collected by centrifugation in a Sarstedt microfuge at 20,000 rpm at 4°C. The pellet was washed twice with 1.0 ml

of 70% ethanol made 0.1M with sodium acetate and then with 1.0 ml of 95% ethanol. The pellet was drained well, dried and dissolved in 50 µl sterile distilled water.

Method 2

The plasmodium was grown and lysed as described in Method 1 above. The homogenate was centrifuged in a Beckman microfuge at 12,000 rpm for 15 min at 4°C to pellet the debris. The supernatant was then layered on top of a solution of 1.7M sucrose made up in polysome buffer. This was then centrifuged on a MSE Superspeed 65 ultracentrifuge at 55,000 rpm for 90 min at 4°C. Clear pellets were obtained and these were taken up in 1.0 ml of TE buffer (pH 8.0). 0.05 ml of 10% (w/v) SDS (heated at 65°C for 1 h) and 0.1 ml fungal proteinase K (BRL; 1% (w/v) in TE buffer, pH 8.0) were added. This was incubated at 37°C for 45 min, then extracted with phenol/chloroform until a clear interphase was obtained. The aqueous phase was removed and the RNA was precipitated by adding sodium acetate (pH 6.0) to a final concentration of 0.3M and 2 volumes of absolute alcohol. This was mixed and incubated at -20°C overnight. The RNA was collected by centrifugation and a CTAB precipitation was carried out. The RNA was finally dissolved in 50 µl of sterile distilled water.

Polysome profile

0.2 ml of supernatant (see Section C(i)) was layered on a 12.5-50% sucrose gradient (see Appendix 5). The gradients were centrifuged in a MSE Super speed 65 Ultracentrifuge at 37,000 rpm for 75 min at 4°C using a MSE 8 x 35 ml fixed angle titanium rotor with 10 ml adaptors. 25 drop fractions were collected by piercing the bottom of the tubes using an MSE tube piercer and passed through an LKB Uvicord with an automatic readout at 260 nm.

Ribonuclease inhibitors

These were included in some experiments in an attempt to prevent degradation of RNA. Vanadyl-ribonucleoside complex (VRC) was used at a final concentration of 10 mM which creates optimal conditions for nuclease inhibition (BRL information). RNasin (human placental, P + S Biochemicals Ltd., Liverpool, England) was used at a final concentration of 1000 units ml^{-1}.

ii) Total RNA preparation

Four plasmodia were grown as described in Section 1(c). Each plasmodium was then treated in the following way. The filter was removed from the GM and drained well. In early experiments the plasmodium was scraped from the filter paper into 1.5 ml guanidine thiocyanate solution (see Appendix 5) in a Potter-Elvehjem homogeniser. The plasmodium was then homogenised by 10 strokes at 60°C. 0.75 ml of sodium acetate (pH 5.2), preheated to 60°C was then added to the homogenate. 2 ml of buffer-saturated phenol (preheated to 60°C) and 2 ml of chloroform were then added and the mixture was again homogenised at 60°C. The homogenate was cooled on ice before being centrifuged in a MSE bench centrifuge for 10 min at room temperature. The aqueous layer was removed and re-extracted with the phenol/chloroform until a clear interphase was obtained. The aqueous layer was removed and the RNA was precipitated by adding sodium acetate (3M) to a final concentration of 0.3M and 2 volumes of absolute alcohol. This was left overnight at -20°C. The RNA was recovered by centrifugation in a MSE bench centrifuge for 30 min at room temperature. The pellets were well drained and dissolved in 50 µl of sterile distilled water.

iii) Preparation of poly(A)$^+$ RNA from total RNA

The method used was a modification of that described by Aviv & Leder (1972).

1 g of oligo (dT) cellulose (BRL) was treated with 5 ml of sterile distilled water before being packaged into a mini-column (5 ml syringe). The oligo (dT) cellulose was washed with 5 ml of 0.1M NaOH, 0.005M EDTA, 5 ml of sterile distilled water and finally with 3 x 5 ml of sterile binding buffer (see Appendix 5).

The RNA preparation was incubated at 65^{o}C for 5 min and then an equal volume of 2x binding buffer was added and mixed. This was then applied to the oligo (dT) cellulose column. The flow through was collected and incubated at 65^{o}C for 5 min, cooled and reapplied to the column. 3 x 5 ml of sterile binding buffer was then passed through the column, collected and reapplied to the column as 3 x 5 ml.

The poly(A)$^+$ RNA was eluted by washing the column with 5 ml fractions of sterile elution buffer (see Appendix 5). The E_{260nm} of each fraction was read on a Unicam SP500 spectrophotometer. The poly(A)$^+$ RNA was precipitated from fractions showing an E_{260nm} reading by adding sodium acetate (3M) to a final concentration of 0.3M and 2 volumes of absolute ethanol. This was mixed and left overnight at -20^{o}C.

The poly(A)$^+$ RNA was recovered by centrifugation in a Sarstedt microfuge for 10 min, the pellets were well drained and dissolved in 50 µl of sterile distilled water.

(iv) Single step isolation of poly(A)$^+$ RNA

The method used was that described by Cox & Smulian (1983).

Poly (U) sepharose 4B (Pharmacia) was treated with 1M NaCl at 4^{o}C overnight to swell the gel. Once swollen the sepharose was

washed thoroughly with sterile eluting buffer (see Appendix 5) and then equilibrated with wash buffer (see Appendix 5).

Plasmodia were grown as described in Section 1(c) and 1(d). In initial experiments the plasmodia were scraped into pre-cooled lysis buffer (10 ml per plasmodium) but in later experiments the plasmodia were lysed in situ by submerging the filter in precooled lysis buffer (see Appendix 5). The lysate was centrifuged at 1,200xg in a MSE bench centrifuge at room temperature for 10 min and the supernatant was decanted into a 250 ml conical flask. 100 mg dry weight of prepared poly(U) sepharose 4B per plasmodium was then added to the lysate and this was allowed to shake at $4^{o}C$ overnight.

The poly(U) sepharose was collected by centrifugation at 1,200xg in a MSE bench centrifuge at room temperature for 10 min. The gel was washed extensively with wash buffer before being packaged into a mini-column (5 ml syringe). The column was washed with wash buffer until the E_{260nm} of the eluate was <0.002 and then washed again with storage buffer (0.01M Tris/HCl, pH 7.0).

The poly(A)$^{+}$ RNA was eluted from the column by treatment with elution buffer. The poly(A)$^{+}$ RNA was then precipitated from the eluate by adding potassium acetate (5M) to a final concentration of 0.05M and 2-3 volumes of absolute ethanol. This was left overnight at $-20^{o}C$. The RNA was recovered by centrifugation in a Sarstedt microfuge, dried then dissolved in sterile water and stored at $-20^{o}C$.

d) Bacteriophage DNA minipreparation

The method used was that described by Cameron et al (1977).

A 5 ml phage lysate was prepared as described in Section 4(b). Bovine pancreatic deoxyribonuclease I (DNaseI; Sigma, 0.1% (w/v) in

0.15M NaCl, pH 5.0) was added to a final concentration of 0.5 $\mu g\ ml^{-1}$ and the phage suspension was incubated at 37°C for 1 h. 0.6 ml of phage lysate was removed and transferred to a microfuge tube. 120 µl of SDS mix (2.5% SDS, 0.5M Tris/HCl (pH 9.0), 0.25M EDTA) was added and this was then incubated at 70°C for 15 min. After cooling to room temperature potassium acetate (8M) was added to a final concentration of 1.66M and the mixture was allowed to incubate on ice for 15 min. The lysate was centrifuged for 1 min in a Sarstedt microfuge and 600 µl of supernatant was removed into a fresh microfuge tube. 360 µl of propan-2-ol was added and the tube was incubated at room temperature for 2 min. The DNA was collected by centrifugation in a Sarstedt microfuge for 1 min. The DNA pellet was washed in 70% ethanol and dissolved in 20 µl of TE buffer (pH 8.0).

e) Formaldehyde denaturing gel

A horizontal slab electrophoresis apparatus (LKB Stockholm, Sweden) with gel dimension of 25cm x 11cm was used. Slots of 20 µl capacity were made in the gel using a Biorad 16 tooth comb.

i) 1.2 g of agarose (Sigma; Type 1) was added to 75 ml of distilled water plus 10 ml of 10x running buffer (see Appendix 7). The agarose was melted and cooled to 60°C; 15 ml of 40% (v/v) formaldehyde solution was then added before pouring the gel.

ii) Sample preparation

Each sample contained 5 µl of RNA solution, 10 µl of deionised formamide (BRL, see Appendix 5), 3 µl 40% formaldehyde and 2 µl of 10x running buffer. The samples were incubated at 65°C for 10 min, then cooled in ice water. 2 µl of tracker dye (see Appendix 7) was then added and the samples were applied to the wells which were then sealed with vaseline.

The gel was run at a constant current of 10 mA for 4 h with cooling by circulated water at 4°C and stained in an ethidium bromide solution (1 μg ml^{-1}) for 2 h. The gel was destained overnight in distilled water before being viewed on a chromato vue transilluminator model C60. Photographs were taken using a Polaroid 800 camera and Polaroid type 47 film.

6. Preparation of Packaging Extracts

These were prepared according to the method of Hohn (1980).

a) Sonicated extract from induced prehead donor BHB2690

500 ml of NZ broth in a 2 litre dimpled flask was prewarmed to 30°C. This was inoculated with a 20 ml overnight culture of *E. coli* BHB2690 (see Methods, Section 3d) and incubated at 30°C in an orbital shaker until an E_{600nm} of 0.3 was reached. Phage were then induced by shaking the flask in a water bath at 45°C for 15 min. The bacteria were then allowed to grow at 37°C for 3 h under vigorous aeration.

The cells were collected by centrifugation in a MSE High Speed 21 centrifuge at 2,750xg for 10 min at 4°C. The cell pellets were well drained and resuspended in 1.0 ml cold sonication buffer (see Appendix 6). The cells were then sonicated on ice with 6 x 5 second bursts at 22 kcs^{-1} using an MSE sonibrose and the sonicate was transferred to 2 microfuge tubes. These were centrifuged in a Sarstedt microfuge for 10 min at 4°C. The supernatant was decanted off into a fresh microfuge tube. An equal volume of cold sonication buffer and a one sixth volume of packaging buffer (see Appendix 6) were then added and mixed.

15 µl aliquots were then dispensed into pre-cooled microfuge tubes and quick frozen in liquid nitrogen. These were then stored at -70°C.

b) Freeze-thaw lysate from induced packaging protein donor BHB2688

Growth and phage induction of E. coli BHB2688 were the same as those described above for E. coli BHB2690.

The cells were collected by centrifugation at 2,750xg for 10 min at 4°C. The pellets were well drained and resuspended in 1.0 ml cold sucrose solution (see Appendix 6). This was then transferred to two microfuge tubes and 25 µl of lysozyme solution (2 mg ml^{-1} in 0.25M Tris/HCl, pH 7.5) was added to each tube. These were mixed gently, frozen in liquid nitrogen and allowed to thaw in ice. 25 µl of packaging buffer (see Appendix 6) was added to each tube, mixed and the lysate was centrifuged for 15 min at 4°C in a Sarstedt microfuge. The supernatants were pooled and 10 µl aliquots were dispensed into the tubes containing the sonicated extract. The tubes were immediately frozen in liquid nitrogen and stored at -70°C.

7. Preparation of a Physarum polycephalum gene bank

a) Restriction enzyme digestion of DNA

Restriction enzyme digestions were carried out in a total of 20 µl. 5 µg of Physarum DNA was digested in a reaction mixture which contained 0.01M Tris/HCl pH 7.4, 0.01M $MgCl_2$, 0.01M β-mercaptoethanol, 0.05M NaCl (HIN buffer, made up as a 10 fold concentrate) and 0.5, 1, 2, 4 and 8 units of BamHI or Sau3A (BRL, diluted in HIN buffer).

Partial digestion of Physarum DNA with EcoRI was carried out

in a reaction mixture which contained 0.1M Tris/HCl pH 7.5, 0.005M $MgCl_2$, 0.05M NaCl (EcoRI buffer made up as a 10 fold concentrate), 5 µg of Physarum DNA and 0.25-8 units of restriction enzyme diluted in EcoRI buffer.

These were incubated for 1 h at 37°C. After digestion the reaction mixtures were pooled and the 15-25 kb fragments were isolated.

b) Isolation of 15-25 kb Physarum DNA fragments

i) Method 1 - Phenol extraction

A horizontal slab electrophoresis apparatus (LKB, Stockholm, Sweden) with gel dimensions of 25 cm x 11 cm was used. A low melting point agarose gel was prepared (1% agarose (Sigma Type 1) (w/v) in GGB buffer (see Appendix 7)). 20 µl of tracker dye (see Appendix 7) was added to the 120 µl of pooled digests. The sample was applied to 14 wells which were then sealed with vaseline. The gel was run at a constant current of 40 mA for 3 h with cooling. A slice of gel was cut and stained in an ethidium bromide solution (1 µg ml^{-1}) for 20 min and the DNA was visualized on a chromato vue transilluminator. The relevant bands were then cut from the unstained gel.

The agarose gel was melted at 70°C for 5 min and an equal volume of TE buffer (pH 8.0) was added. This was then mixed with an equal volume of buffer-saturated phenol and centrifuged at 2,000xg for 10 min in a MSE bench centrifuge. The aqueous layer was removed and re-extracted with buffer-saturated phenol. After centrifugation the aqueous layer was treated with buffer-saturated ether and centrifuged as before. A second treatment with buffer-saturated ether was performed and the aqueous layer was then treated with t-amyl alcohol. After centrifugation the aqueous layer was removed and wheat germ transfer

ribonucleic acid V (tRNA; Sigma, 1% (w/v) in sterile distilled water) was added to give a final concentration of 50 μg ml^{-1}. The DNA was then precipitated by adding sodium acetate (3M) to a final concentration of 0.3M and 3 volumes of absolute alcohol. The DNA was collected by centrifugation in a Sarstedt microfuge and after drying it was dissolved in 50 μl of sterile TE buffer (pH 8.0).

ii) Method 2 - Butanol extraction

The method used was that described by Langridge et al (1980).

A low melting point agarose gel was prepared and run as described in Section 6b(i) above. Using this method the whole gel could be stained in an ethidium bromide solution (1 μg ml^{-1}). The DNA was visualized on a transilluminator and the relevant bands cut from the gel.

The segment of agarose gel containing the DNA was melted at 70°C and the volume measured. The solution was cooled to 37°C and equal volumes of the equilibrated butanol and aqueous phases (see Appendix 8) were added. The tube was inverted 50 times and left at 37°C for the phases to separate. The butanol layer was removed, two further extractions were made with the butanol solution only and the butanol extracts were combined. A one quarter volume of NaCl (0.2M) was added to the butanol extract and the tube was inverted 50 times. The aqueous layer was removed and the salt extraction repeated. The aqueous phases were pooled and an equal volume of chloroform was added dropwise. The solution was then placed in ice for the quaternary ammonium salt (QN^+) to precipitate. The chloroform layer was removed and the residual chloroform was evaporated in a stream of air. The DNA was precipitated by adding 3 volumes of absolute alcohol to the aqueous layer and incubating the mixture at -20°C overnight. The DNA was collected by

centrifugation and the pellet was well drained before being dissolved in 20 µl of sterile TE buffer (pH 8.0).

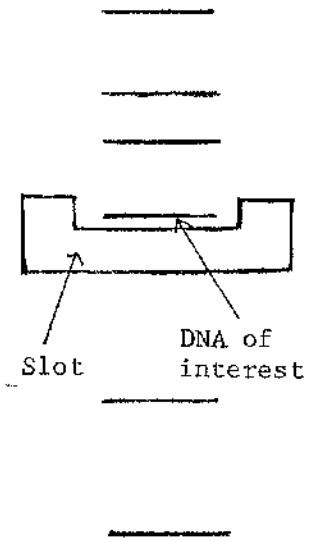
iii) Method 3 - Electroelution

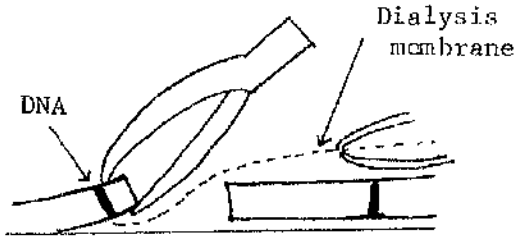
The method was described by Yang et al (1979).

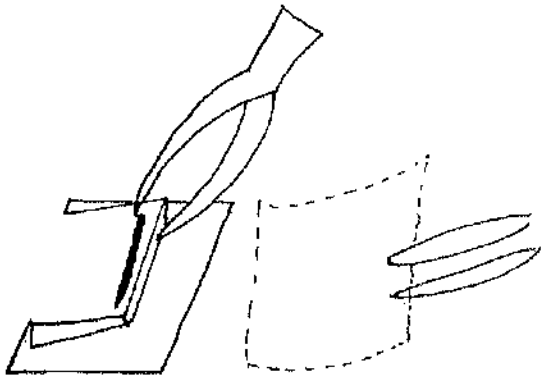
The DNA was fractionated by electrophoresis in an agarose gel (1% (w/v) in GGB buffer) containing ethidium bromide (0.5 µg ml^{-1}) at 50 mA for 3 h with cooling. After electrophoresis the DNA was visualized by UV illumination and the fractions of interest were marked by cutting a slit in front of them. Then, under room light, a U shaped slot (as shown in Figure 6) was cut out. A piece of washed dialysis membrane (see Appendix 8) was inserted so that the DNA band was surrounded with the dialysis membrane from 3 sides (see Figure 6). The slot was then filled with GGB buffer and electrophoresis was allowed to continue for 30-60 min to elute the DNA from the gel into the U shaped slot. UV light was used to check the completeness of the elution. Under the UV light the DNA could be seen loosely accumulated on the dialysis membrane. The buffer in the slot was used to rinse the DNA from the membrane and was then transferred to a microfuge tube. 200 µl of GGB buffer was used to wash the slot and recover any remaining DNA. The washes were pooled and an equal volume of buffer-saturated phenol was added. After centrifugation in a Sarstedt microfuge the aqueous layer was treated with buffer-saturated ether and centrifuged as before. The aqueous layer was then mixed with 3M Tris/HCl, pH 8.5 to give a final concentration of 0.5M. Two volumes of propan-2-ol were then added and the mixture held at -70°C for 5 min. The DNA was collected by centrifugation in the microfuge at 4°C for 10 min. The precipitation step was repeated once more to ensure the complete removal of the ethidium bromide. The DNA pellet was well drained, dried then dissolved in 20 µl of sterile TE buffer (pH 8.0).

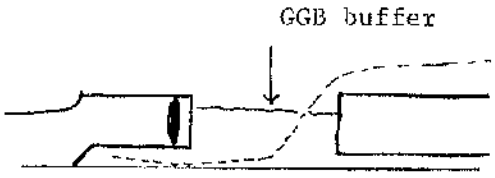
Figure 6. Electroelution of DNA from an agarose gel

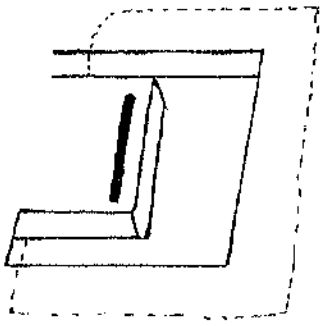
(from Yang et al, 1979)


Slot
DNA of
interest


Dialysis
membrane
DNA




GGB buffer



iv) Titre of 15-25 kb fragments

As the 15-25 kb DNA fragments were in a total volume of only 20 µl it was inconvenient to titre the fragments using the E_{260nm} reading. An alternative method was sought. The method used was described by Blin et al (1975).

A DNA solution with a concentration of 0.1 mg ml^{-1} was used to calibrate the transilluminator. Doubling dilutions of the DNA solution were made and these were spotted onto glass fibre filters (Whatman GF/A). The filters were dried and then stained for 5 min in an ethidium bromide solution (0.5 µg ml^{-1}). They were then viewed on the chromato vue transilluminator. It was found that the lowest concentration of DNA that gave a visible spot was 0.0125 µg $µl^{-1}$. This fact was used to calculate the weight of 15-25 kb Physarum DNA fragments isolated.

1 µl of the solution of DNA fragments was taken and used to prepare a series of doubling dilutions. These were spotted onto glass fibre filters which were then treated as described above.

When the filters were viewed on the transilluminator the highest dilution of the DNA which gave a visible spot was assumed to contain 0.0125 µg of DNA. Knowing the dilution factor the total weight of DNA isolated could then be calculated.

c) Digestion of bacteriophage DNA

λ1059 DNA (2 µg) was digested in a reaction mixture which contained 0.01M Tris/HCl, pH 7.4, 0.01M $MgCl_2$, 0.01M β-mercaptoethanol, 0.05M NaCl (HIN buffer made as a 10 fold concentrate) and 4 units of BamHI in a total volume of 20 µl. This was incubated at 37°C for 1 h and the reaction was terminated by incubation at 70°C for 5 min. A BamHI

digest of λ1059 results in 3 fragments, but it was not necessary to separate the λ arms as recombinant phage could be detected by plating on E. coli Q359 (see Introduction, Section 5d).

Charon 4AP DNA was digested in the same way except that the reaction mixture contained 0.1M Tris/HCl pH 7.5, 0.005M $MgCl_2$, 0.05M NaCl(EcoRI buffer made as a 10 fold concentrate)and 4 units of EcoRI.

An EcoRI digest of CH4AP results in six fragments the largest of which are the phage arms. These arms, which contain all the essential genes for phage growth, must be isolated before recombinant phage can be constructed. Therefore after digestion the phage arms were isolated using one of the three methods outlined above in Sections b(i),(ii) and (iii).

d) Construction and recovery of recombinant phage

i) Ligation of Physarum DNA and phage DNA

Aliquots (2 µg) of digested λ1059 DNA or separated arms of Ch4AP were ligated in the presence of 0-1.0 µg of 15-25 kb Physarum DNA in 20 µl reaction mixtures which contained 0.5 units of T_4 DNA ligase (BRL) and a one tenth volume of ligation buffer. The ligation buffer was made immediately before use by mixing the following together: 66 µl 1M Tris/HCl (pH 7.5), 2.5 µl 0.4M EDTA (pH 9.0), 10 µl 1M $MgCl_2$, 10 µl 1M β-mercaptoethanol, 1 µl 0.1M ATP and 10.5 µl distilled water. The ligation reactions were incubated at 9-12°C for 24 h and then the ligated DNA was precipitated by adding sodium acetate (3M) to a final concentration of 0.3M and 3 volumes of absolute alcohol (see Results Section 2b). After overnight incubation at -20°C the DNA was recovered by centrifugation in a Sarstedt microfuge. The pellets were well drained and then taken up in 5 µl of sterile TE buffer (pH 8.0).

ii) In vitro packaging of ligated DNA

The packaging extracts were removed from their storage at -70°C and allowed to thaw on ice. Once thawed the DNA to be packaged (5 µl) was added, mixed and the tubes were incubated at room temperature for 1 h. The packaging reaction was stopped by adding 250 µl of PSB + gelatin to the packaging mix. 10 µl was removed from each packaging mixture and the phage were titred on the correct indicator bacteria as described in Section 4(a).

e) Amplification and storage of recombinant phage

Phage were grown as plate lysates as described in Section 4(a). Recombinant phage were grown on E. coli Q359 to give at least 10^5 infective centres per agar plate. After overnight growth at 37°C, 2.5 ml of PSB + gelatin was added to each agar plate and the top layer of agarose was removed into a sterile glass beaker. 5 ml of chloroform was added to the agarose/phage mixture and this was then stirred at room temperature for 30 min. The phage lysate was then spun at 11,500xg in a MSE High Speed 21 centrifuge at 4°C for 15 min to pellet the agarose and bacterial debris. The supernatant was removed and stored over chloroform at 4°C. The amplified phage lysates gave titres of approximately 10^7 phage per ml.

8. Preparation of Nitrocellulose Filters

a) Transfer of phage DNA to nitrocellulose filters

The method used was described by Benton & Davis (1977).

Phage were grown on E. coli Q359 as described in Section 4(a)

to give 10^4 infective centres per agar plate. After overnight growth at 37°C the plates were chilled at 4°C for at least 1 h to allow the top agarose to harden.

Dry non-sterile nitrocellulose filters (Schleicher & Schull; BA45, 0.45 μm pore size) were cut, numbered and marked with a pencil so as to orientate them.

At room temperature, a nitrocellulose circle was placed on the surface of the top agarose so that it came directly into contact with the phage plaques. Transfer of the DNA was allowed to take place at room temperature for 5 min. The nitrocellulose filter was then peeled off the surface of the agar plate and immersed, DNA side up, in denaturing solution (1.5M NaCl, 0.5M NaOH). After 30 sec the filter was transferred to neutralizing solution (2 x SSC (see Appendix 7), 0.2M Tris/HCl pH 7.0) for 1 minute with periodic gentle agitation. The filters were then placed on Whatman 3MM paper to dry. the DNA was fixed to the filter by baking for 2 h at 80°C in vacuo.

Filters were stored wrapped in aluminium foil at room temperature.

b) Transfer of digested DNA to nitrocellulose filter paper

The method used was the Southern transfer system (Southern, 1975).

The DNA restriction fragment digest was fractionated on a 1% agarose gel (1% agarose (w/v) in GGB buffer) containing ethidium bromide (0.5 μg ml^{-1}).

After electrophoresis the gel was visualised under UV light and photographed. To aid the transfer of large fragments the portion of the gel containing these fragments was placed on the chromato vue trans-illuminator for 15 minutes. The DNA fragments were denatured by soaking

the gel in 0.2M NaOH, 0.6M NaCl for 60 min with periodic gentle agitation. The gel was then neutralized in a solution of 1M Tris/HCl (pH 7.4), 1.5M NaCl for 30 min, again with periodic agitation. The transfer system show in Fig 7a was then set up.

A support plate was mounted over a reservoir containing 10 x SSC. This was covered with a sheet of Whatman 3MM paper which had been presoaked in 10 x SSC. The piece of 3MM paper was wide enough to cover the plate and long enough to hang over the ends of the plate into the reservoir of 10 x SSC. Another piece of presoaked 3MM paper, the same size as the support plate, was then placed on top. Care was taken to remove all air bubbles. The gel was placed on top of this second sheet and spacers were placed on either side as shown in Fig 7b. The nitrocellulose filter was placed on top of the gel. A sheet of 3MM paper (presoaked in 10 x SSC) cut to the size of the nitrocellulose filter was placed on top of the nitrocellulose and then another identical sheet of dry 3MM was placed on top.

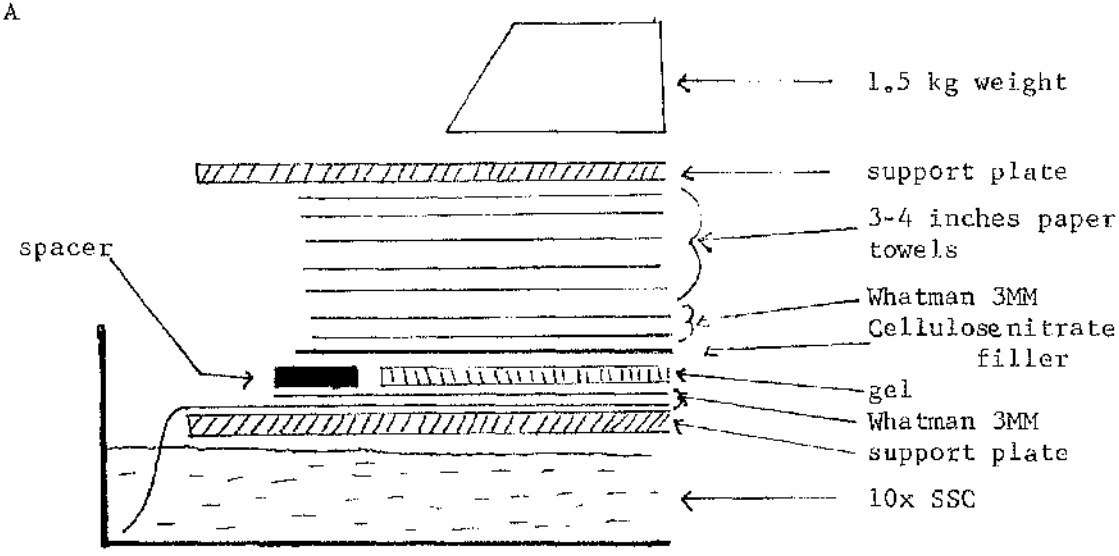
Paper towels which had been cut to the same size as the nitrocellulose filter were then placed on top to a height of 3-4 inches. A heavy weight was then placed on top of the towels and the transfer was allowed to continue for 24 h at room temperature.

After the transfer period the towels and the top sheets of 3MM paper were discarded and the nitrocellulose filter was carefully peeled off. The gel, which was stuck to the nitrocellulose filter, had shrunk considerably in size. The nitrocellulose filter and the gel were placed in 2 x SSC and the gel was then carefully removed. The nitrocellulose filter was blotted dry, allowed to air dry then baked <u>in vacuo</u> for 2 h at 80°C.

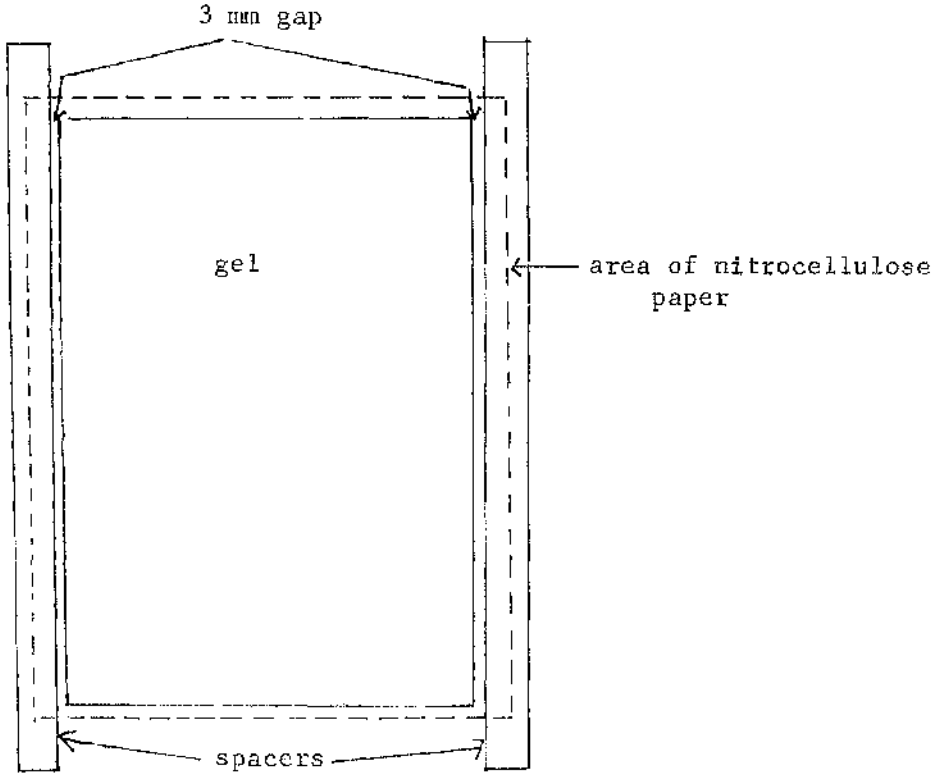
The gel was stained in a solution of ethidium bromide (0.5 $\mu g\ ml^{-1}$)

Figure 7. Transfer of DNA from an agarose gel to nitrocellulose paper according to the procedure of Southern (1975).

A


1,5 kg weight
support plate
3-4 inches paper towels
spacer
Whatman 3MM
Cellulose nitrate filler
gel
Whatman 3MM
support plate
10x SSC

B


3 mm gap
gel
area of nitrocellulose paper
spacers

for 30 min then viewed under a UV light to check the completeness of transfer of the DNA. The nitrocellulose filters were stored wrapped in aluminium foil at room temperature.

9. Preparation of Nucleic Acid Probes

a) Nick-translated DNA

The method used was similar to that described by Rigby et al (1977). The DNA was 'nicked' in a total volume of 50 µl. The reaction mixture contained 30 µl sterile distilled water, 5 µl 10x nick translation buffer (NTB, see Appendix 9), 5 µl 0.1M $MgCl_2$, 5 µg Physarum DNA and bovine pancreatic deoxyribonuclease I (DNase I; Sigma 1.5 mg ml^{-1} in 0.001M HCl) at a final concentration of 0.05 µg ml^{-1}. This was incubated at 37°C for 15 min and the reaction was stopped by heating at 65°C for 15 min.

The translation reaction was carried out in a total volume of 100 µl. To the 'nicked' DNA 5 µl of 10x NTB, 42 µl distilled water, 2 µl of dNTP mixture (10^{-4}M) and 100 µCi [^{32}P] dCTP (Amersham, specific activity 3000 Ci mM^{-1}) were added. 10 units of DNA polymerase I (BRL) were then added and the reaction mixture was well mixed and incubated at room temperature for 2 h.

After this time an equal volume of buffer-saturated phenol was added and the mixture was centrifuged in a Sarstedt microfuge. The aqueous layer was removed and the phenol phase was re-extracted with an equal volume of TE buffer (pH 8.0). After centrifugation the aqueous layers were pooled and treated with an equal volume of buffer-saturated ether and centrifuged as before. A second treatment with buffer-saturated

ether was carried out and then the aqueous layer was mixed with an equal volume of t-amyl alcohol and centrifuged again. The nick-translated DNA was precipitated by adding sodium acetate (3M) to a final concentration of 0.3M and 3 volumes of absolute ethanol.

b) i) Copy DNA (cDNA)

The reaction was carried out in a total volume of 20 µl. The reaction mixture contained 2 µl 0.4M KCl, 2 µl 0.06M $MgCl_2$, 2 µl 0.5M Tris/HCl (pH 8.3 at 42°C), 0.8 µl 0.7M β-mercaptoethanol, 2 µl dNTPs (0.005M), 3 µl oligo$(dT)_{12-18}$ (BRL, 6 units μl^{-1}), 0.5 µl RNasin (P + S Biochemicals), 20 µCi [^{32}P]dCTP (Amersham, specific activity 3000 ci mM^{-1}) and 2 µg of poly$(A)^+$ RNA. This was incubated at 42°C for 10 min to allow pairing of the oligo$(dT)_{12-18}$ with the poly(A) tails. 200 units of reverse transcriptase (BRL) were then added and the volume made up to 20 µl with sterile distilled water. The reaction was incubated at 42°C for 2 h. 2.5 µl of EDTA (0.25M) and 5 µl NaOH (0.5M) were added, mixed and this was then incubated at 60°C for 1 h. 5 µl of HCl (0.5M) was added to neutralize the reaction mixture.

ii) Isolation of cDNA

The cDNA was separated from the unreacted nucleotides by passage through a sephadex G100 column of bed volume 5 ml. The sephadex was swollen in sterile distilled water. The reaction mixture was applied to the column. 10 ml of sterile distilled water was then passed through the column and 0.5 ml fractions were collected, 15 µl from each fraction was taken and added to 4.5 ml of toluene based scintillant and the radioactivity counted in a Packard TRI-CARB 300C scintillation counter.

Two peaks of radioactivity were obtained (see Results Section 2k (v)). The first peak was due to the cDNA, the second represents the

unreacted nucleotides. The fractions which contained the cDNA were pooled and wheat germ transfer ribonucleic acid V (tRNA; Sigma, 1% (w/v) in sterile distilled water) was added to a final concentration of 50 $\mu g\ ml^{-1}$. The cDNA was precipitated by adding sodium acetate (3M) to a final concentration of 0.3M and 3 volumes of absolute ethanol. The cDNA was collected by centrifugation in a Sarstedt microfuge and the pellet was dissolved in 20 µl of sterile TE buffer (pH 8.0).

10. Hybridization Conditions

a) Conditions used with nick-translated DNA

A 50% formamide hybridization solution was used (see Appendix 9). The nitrocellulose filters were welled in the hybridization solution before being transferred to a heat sealable bag. 3-4 ml of pre-hybridization solution (see Appendix 9) was then added and the bag sealed. This was incubated at 42°C in a shaking water bath for 1-2 h. During this time the hybridization solution was prepared. 5×10^5 cpm of 'nick translated' DNA in TE buffer (pH 8.0) was boiled for 10 minutes and then chilled in an ice/water bath. The DNA was then added to 3 ml of hybridization solution.

When the prehybridization was complete the bag was taken from the water bath and the pre-hybridization solution was removed. This was replaced with the radioactive hybridization solution and the bag was resealed. This was incubated at 42°C in a shaking water bath for 24 h.

The filters were removed from the hybridization solution and washed 5 times in 2 x SSC (Standard Saline Citrate) + 0.1% SDS, for 5 minutes each wash. The filters were then placed in a solution of

1 x SSC + 0.1% SDS and incubated at 68°C with gentle agitation for 1½ h. The wash buffer was changed and the filters were reincubated at 68°C for a further 1½ h. When the washes were complete the filters were blotted dry, allowed to air dry and then mounted for autoradiography against an X-ray film (Kodak XOmat) plus an intensifying screen at -70°C.

b) Conditions used for cDNA

An aqueous hybridization solution was used (see Appendix 9). The filters were wetted in the hybridization solution and then transferred to a heat sealable bag. 3 ml of prehybridization solution (see Appendix 9) was added and the bag sealed. This was incubated at 68°C in a shaking water bath for 1-2 h.

After prehybridization, the bag was removed from the water-bath and the prehybridization solution was removed. This was replaced with 3 ml of hybridization solution. The bag was resealed and the reaction was allowed to continue at 68°C with gentle agitation for 24 h.

When the hybridization reaction was complete the filters were removed from the bag and placed immediately into 2 x SSC + 0.5% SDS. They were then transferred to 2 x SSC + 0.1% SDS and incubated at room temperature for 15 min. The filters were then washed in a solution of 0.1 x SSC + 0.5% SDS at 68°C with gentle agitation for 2 h.

After washing the filters were dried and then mounted for autoradiography as described in Section 10(a).

c) Development of autoradiographs

The cassette was removed from -70°C and allowed to warm up to room temperature. The X-ray film was then placed in developer (Kodak, Xomat) for 5 min. After this time the X-ray film was transferred to a

fixer solution (Kodak, Xomat) for a further 5 min. The plate was then washed in cold tap water for 10-15 min before being allowed to dry.

RESULTS

1. Cell Cycle Events in Growing and Starving Plasmodia

a) Cell cycle events during growth of Physarum polycephalum CL

The absence of a G1 phase in the cell cycle of a growing plasmodium of P. polycephalum has been well reported. Nygaard et al (1960) followed the incorporation of radioactively labelled orotic acid into DNA. They reported that one period of DNA synthesis took place during a cell cycle and that this S phase occurred immediately after mitosis. Incorporation of [^{3}H] thymidine into acid insoluble material showed that DNA synthesis began in telophase and lasted for around 3 h (Braun et al. 1965). Beach et al (1980) used both [^{3}H]thymidine uptake and autoradiography to determine the point of initiation of DNA synthesis. They found that the onset of S phase occurred 3 min after the majority of nuclei were in anaphase.

The fact that no G1 exists in a growing plasmodium of P. polycephalum was confirmed here for the CL strain (Fig 8). The timing of mitosis was followed by light microscopic examination of the nuclei within the plasmodium while the DNA synthesis was monitored by following the uptake of [methyl ^{3}H] thymidine into acid-insoluble material.

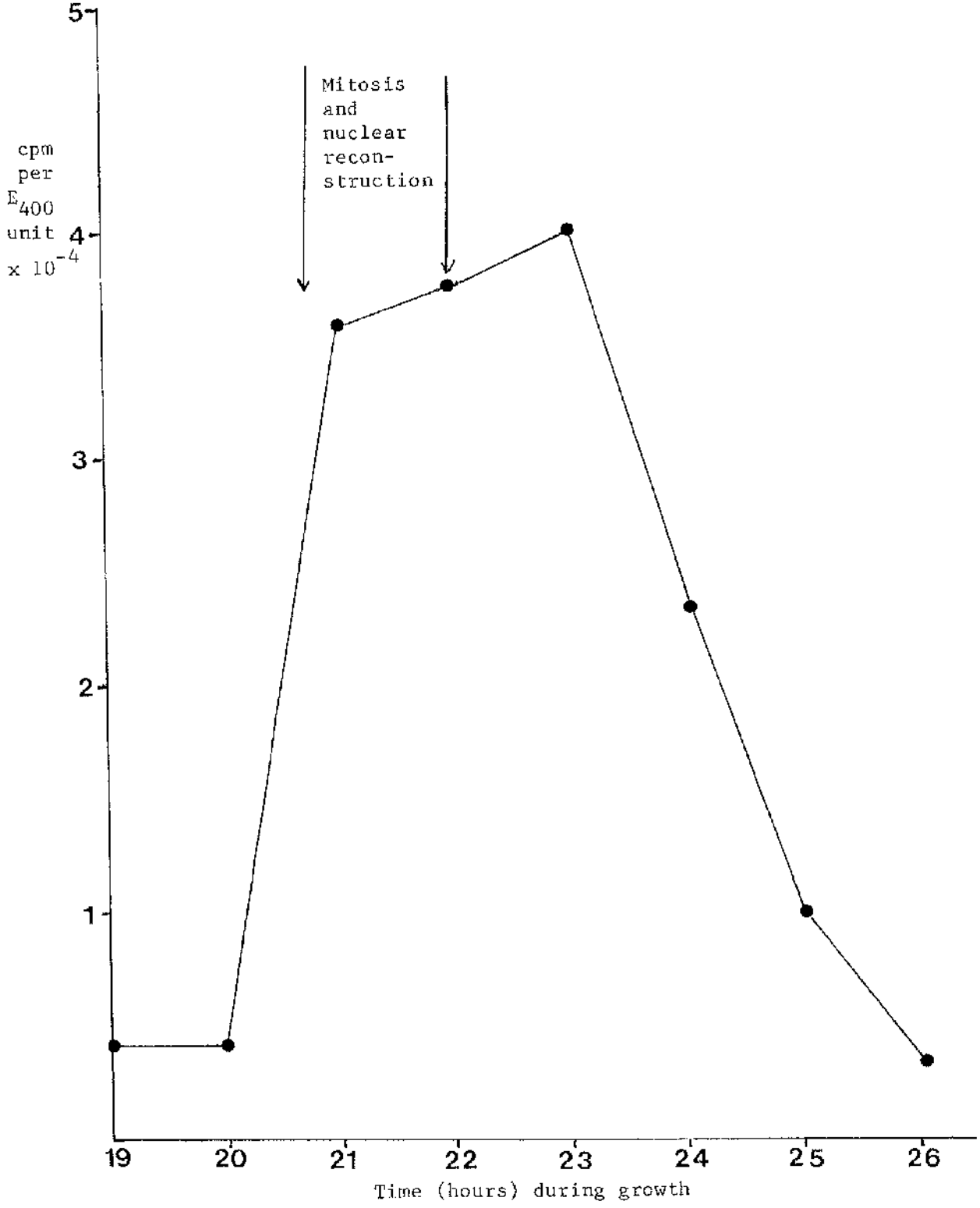
b) DNA synthesis in a plasmodium competent to sporulate

During the 72 h starvation period prior to the development of sporulation competence a nocodazole sensitive event was found to occur after 55-60 h of starvation (Chapman & Coote, 1982). It was important to discover if this event was in fact a mitosis or some other microtubule requiring event. Following mitosis by light microscopy in a starving plasmodium was found to be difficult as the plasmodial strands proved

Figure 8. The timing of mitosis and DNA synthesis in a growing plasmodium of Physarum polycephalum CL

A plasmodium was grown as described in Methods Section 1(c). The uptake of [^{3}H]thymidine into acid-insoluble material was then followed. Pieces of filter paper supporting the plasmodium were cut out with sterile scissors and transferred to radioactive GM at hourly intervals. After 30 min on the radioactive medium the pieces of plasmodium were plunged into TCA/acetone which removed all pigment from the plasmodium. The final radioactive count was standardised to 1 unit of E_{400nm} for each piece of plasmodium (see Methods Section 2(a)).

The timing of mitosis was determined by light microscopic examination of the nuclei as described in Methods Section 2(c). During mitosis samples were taken at least every 5 min.


5
4
3
2
1
cpm per E_{400} unit x 10^{-4}
Mitosis and nuclear recon-struction
19
20
21
22
23
24
25
26
Time (hours) during growth

very hard to crush (Chapman & Coote, 1982). Advantage was taken of the fact that, although nocodazole has no effect on DNA synthesis itself, blocking mitosis with the drug will prevent any subsequent round of DNA replication (Chapman & Coote, 1982). A starving plasmodium does not become committed to sporulate until 4 h after illumination (Sauer et al, 1969a; Chapman & Coote, 1982) and so transfer to fresh growth medium before this time will allow resumption of the cell cycle events associated with growth. A plasmodium which had been starved for 72 h was divided into 3 portions, one part was placed on GM alone, one part on GM + nocodazole and one part on GM + hydroxyurea. DNA synthesis began 7 h after refeeding with GM in the control portion, but was prevented in the portions of plasmodium which had been exposed to each of the drugs (Fig 9).

This suggested that the original plasmodium was in the G2 phase of the cell cycle after the 72 h starvation period. If the plasmodium had been in the G1 phase, it would first have replicated its DNA before resuming growth. As no mitosis would have preceded this, the presence of nocodazole would not have been expected to interfere with this round of DNA replication. It would therefore seem unlikely that the nocodazole-sensitive event late in starvation was an isolated mitosis.

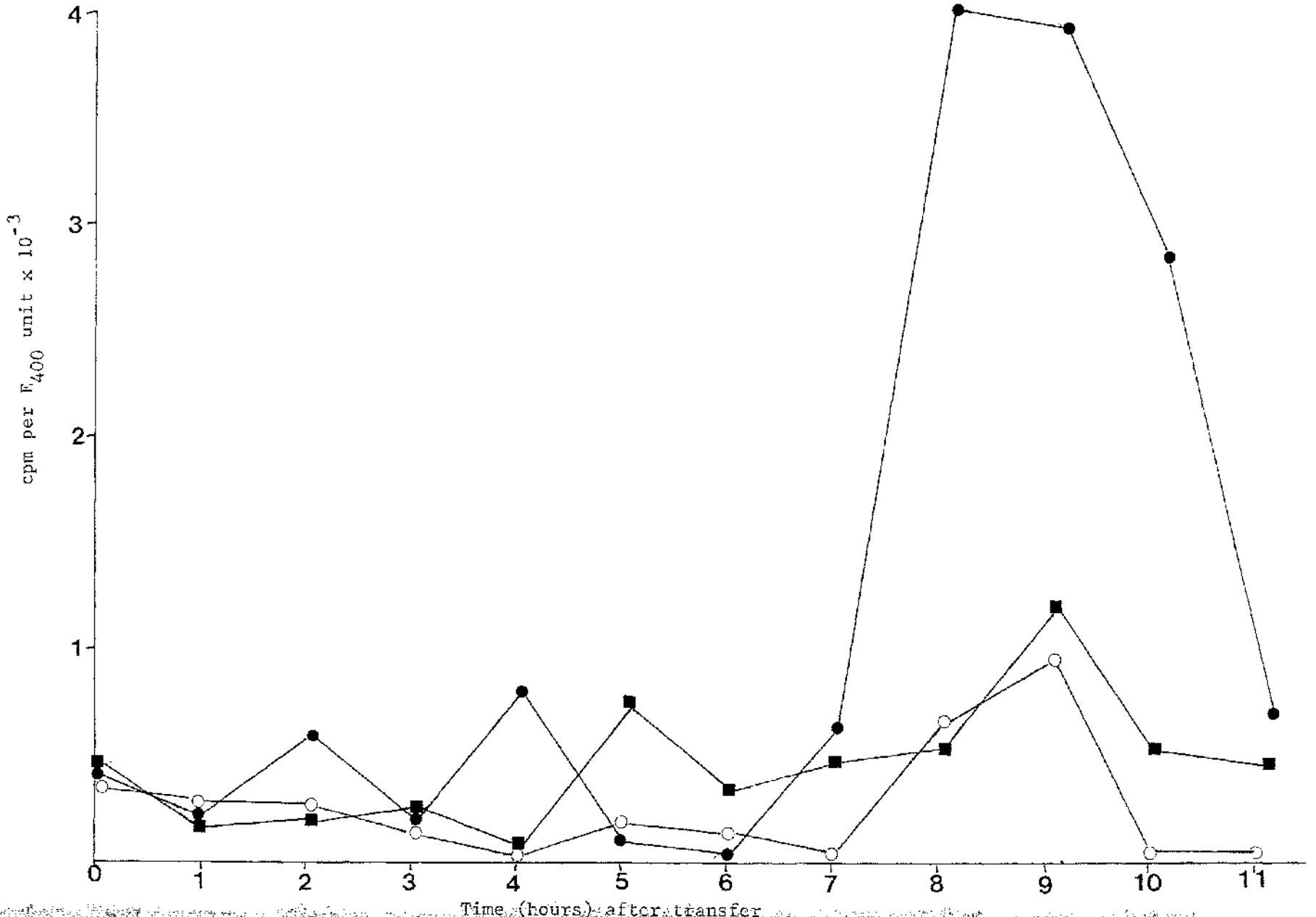
c) The cell cycle during starvation

As stated earlier the cell cycle in growing plasmodia of Physarum polycephalum has been studied by various groups of workers. The cell cycle of Physarum plasmodia during starvation has not received as much attention.

Starving plasmodia were used to find the point at which a round of DNA replication escapes the block imposed on mitosis by nocodazole.

Figure 9. DNA synthesis in a plasmodium competent to sporulate after refeeding with growth medium

A plasmodium was starved for 72 h as described in Methods Section 1(d). It was divided into three parts which were placed on growth medium alone (●), GM + nocodazole (20 μg ml^{-1}, ■) and GM + hydroxyurea (500 μg ml^{-1}, ○). The uptake of [^{3}H]thymidine into acid-insoluble material was then followed as described in the legend to Figure 8.


4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
cpm per F_{400} unit x 10^{-3}
Time (hours) after transfer

It was shown that if a plasmodium 19 h into the starvation period was exposed to nocodazole, then DNA synthesis at 25 h (detected in a control plasmodium) was prevented (Chapman & Coote, 1982). If nocodazole was added at intervals approaching mitosis there should be a point at or near mitosis where exposure to the drug should have no effect on mitosis and DNA replication should occur normally.

In two starving plasmodia, the DNA replication which began between 26 and 27 h into the starvation period was examined (Fig 10). DNA replication was prevented in portions of plasmodium which were exposed to nocodazole at 23 and 24 h. However, replication occurred at a reduced level in the plasmodial portion exposed to nocodazole at 25 h. Thus addition of the drug at 25 h, some 1 to 1.5 h before DNA replication began, allowed the plasmodium to escape the normal block on DNA replication imposed by nocodazole. During these experiments it was noted that there was a degree of asynchrony between separate plasmodia formed at the same time from the same microplasmodial culture. This asynchrony would reduce the accuracy of the experimental procedure and so a large 15 cm plasmodium was tested.

A 15 cm plasmodium was formed and starved for 23 h. The effect of nocodazole treatment on the DNA replication which began between 24 and 25 h was monitored (Fig 11). In this case DNA replication was unaffected by exposure to nocodazole at 25 h, 26 h and 27 h as would be expected. In the portions of plasmodium exposed to nocodazole at 23 and 24 h DNA replication also took place, but at a reduced level. Thus, in this experiment exposure of the plasmodium to nocodazole at 23 h had only a reduced effect on DNA replication which began 1 to 1.5 h later. The data of Figs 10 and 11 might be interpreted to suggest that a G1 period

Figure 10. Effect on DNA synthesis of exposure of portions of starved plasmodia to nocodazole

Two plasmodia were starved for 23 h as described in Methods Section 1(d).

They were then cut in half to give four parts, three of which were transferred to SpoM + nocodazole (20 μg ml^{-1}) at 23 h (■), 24 h (○) and 25 h (▲). One portion was left as a control (●).

The uptake of [methyl-^{3}H] thymidine into acid-insoluble material was then followed as described in the legend to figure 8 except that plasmodial portions were transferred to SpoM + [methyl - ^{3}H] thymidine.

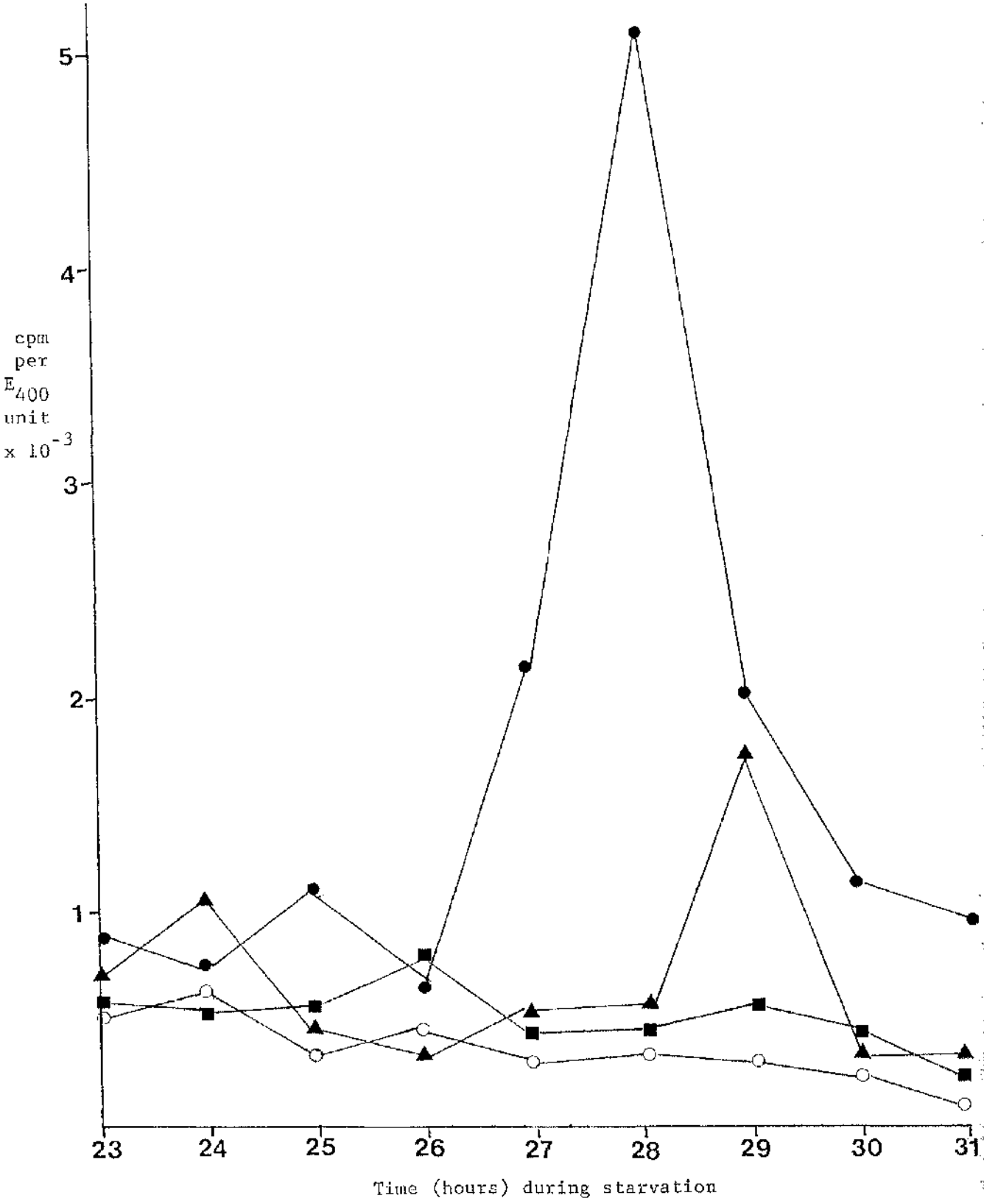
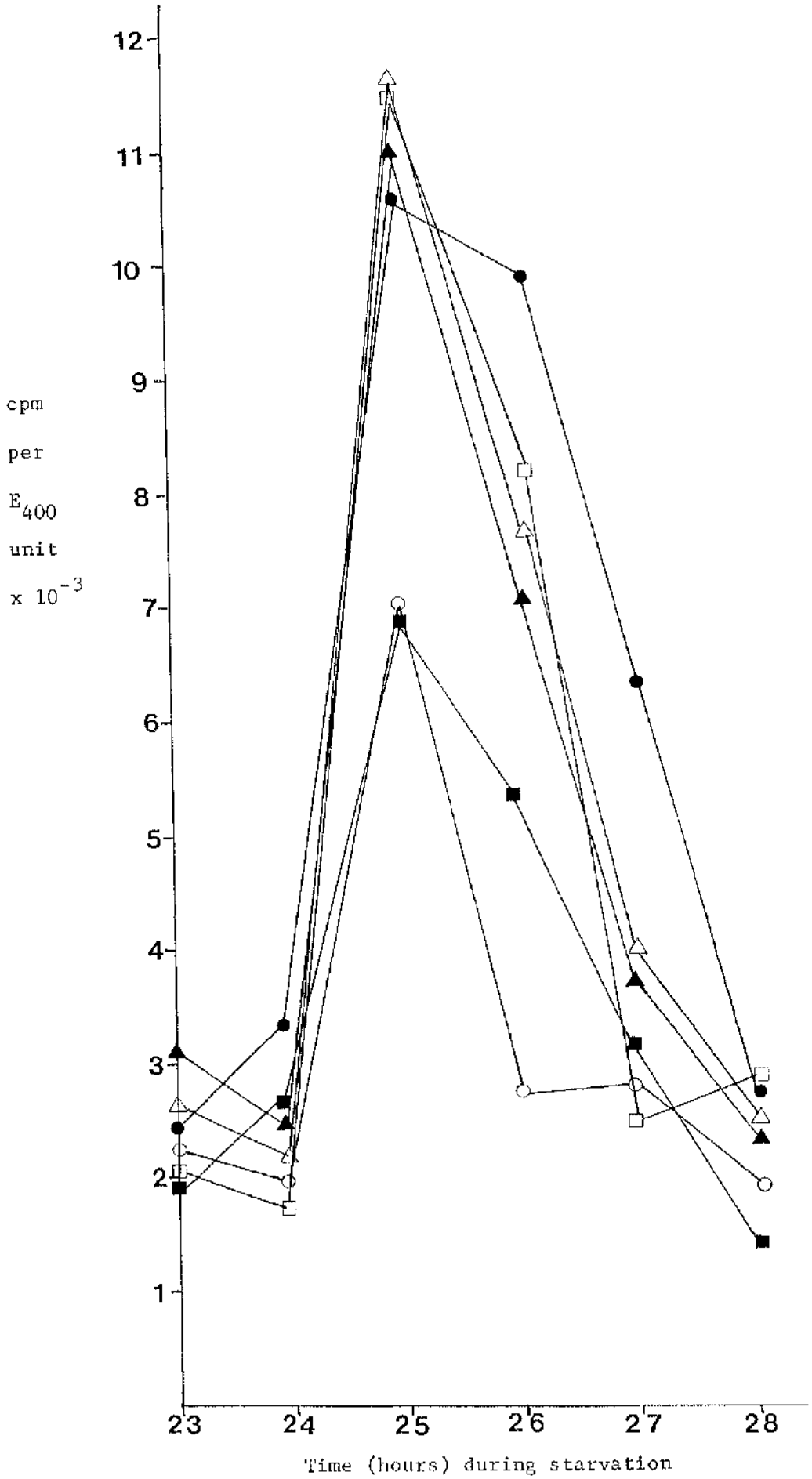

5
4
3
2
1
cpm per E_{400} unit x 10^{-3}
23
24
25
26
27
28
29
30
31
Time (hours) during starvation

Figure 11. Effect on DNA synthesis of exposure of portions of a large starved plasmodium to nocodazole

A large plasmodium (15 cm) was starved for 23 h as previously described. It was then divided into six parts, five of which were transferred to SpoM + nocodazole (20 μg ml^{-1}) at 23 h (○), 24 h (■), 25 h (□), 26 h (▲) and 27 h (△). One piece was left as a control (●). The uptake of [methyl-^{3}H] thymidine was followed as described in the legend to Figure 10.


cpm
per
E_{400}
unit
x 10^{-3}
12
11
10
9
8
7
6
5
4
3
2
1
23
24
25
26
27
28
Time (hours) during starvation

existed in the starving plasmodium and that addition of nocodazole 1 to 2 h prior to DNA replication had little effect on replication because mitosis was already in progress when the drug was added. It was therefore decided to compare the effect of nocodazole in starving plasmodia with its effect on growing plasmodia.

d) The effect on DNA synthesis in growing plasmodia of nocodazole addition

In a growing plasmodium the replication which began between 26 and 27 h after the plasmodium had been formed was used to monitor the effect of nocodazole (Figure 12). This replication was unaffected by exposure of a portion of the plasmodium to nocodazole at 27 h, as would be expected, as DNA replication had begun by this time. However, replication also occurred, at a somewhat reduced level, in plasmodial portions exposed to nocodazole at 25 and 26 h. Replication was essentially prevented by exposure at 23 and 24 h. Thus, as with starving plasmodia (Figs 10 & 11) replication was only fully prevented by nocodazole if it was added 1 to 1.5 h prior to replication, yet no G1 phase was detected in growing plasmodia (Fig 8).

In several experiments it was necessary to add nocodazole to both growing and starving plasmodia 1 to 1.5 h prior to the initiation of DNA replication in order to completely block replication (Table 1).

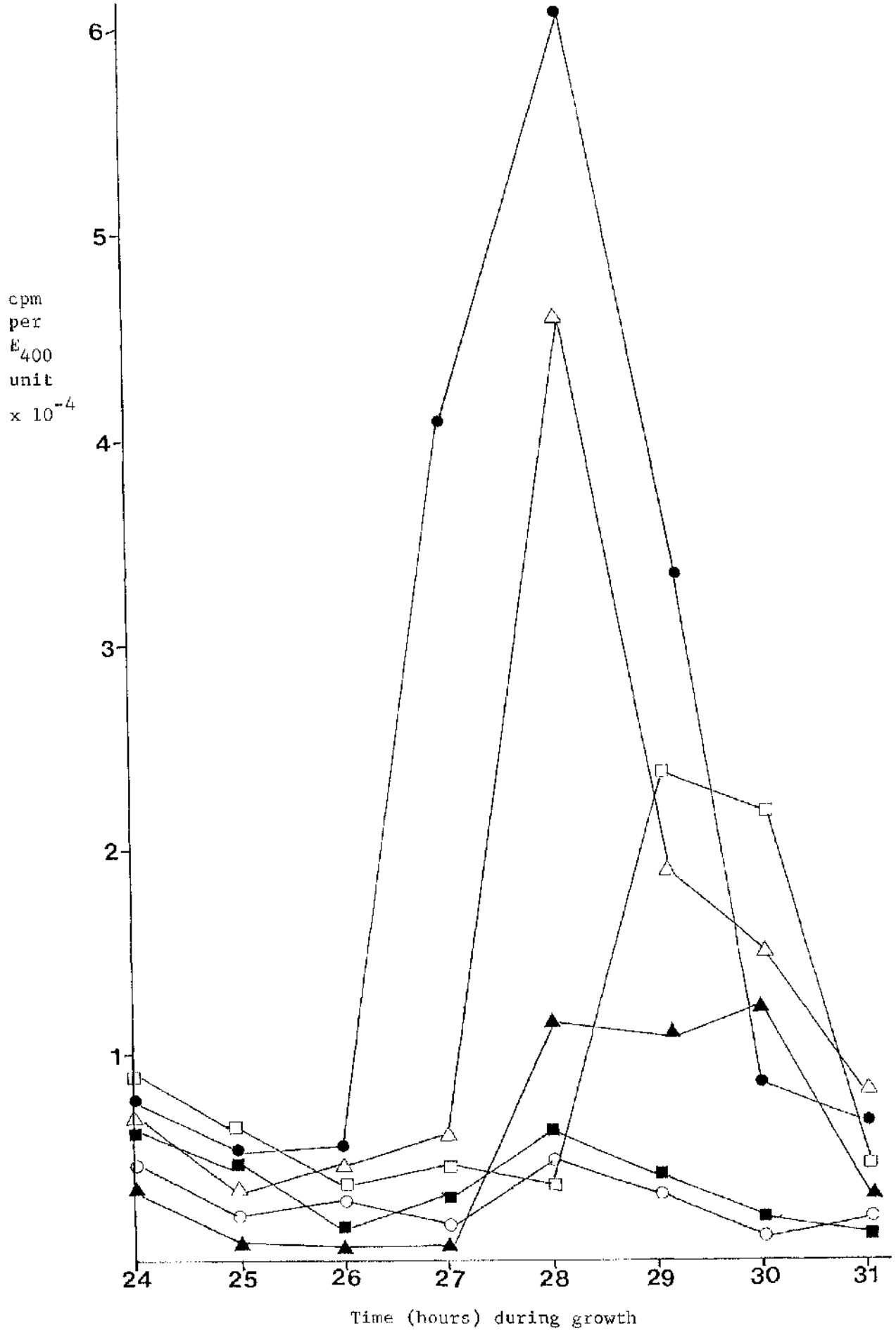
Thus similar patterns were obtained for growing and starving plasmodia. If it is assumed that nocodazole has a similar effect on mitosis in both growing and starving plasmodia then their similar behaviour with respect to nocodazole interference with the course of DNA replication appears to indicate that there is no prolonged G1 phase during starvation as well as growth.

Table 1. Points of Relief from Nocodazole Interference with DNA replication

		Time of initiation of DNA synthesis in control (h)	Point (h) at which DNA synthesis was unaffected in nocodazole treated plasmodium
Growing plasmodium	1	26-27	25
	2	26-27	25
	3	23-24	22
Starving plasmodium	1	26-27	25
	2	24-25	23
	3	26-27	25
	4	28-29	27

Figure 12. Effect on DNA synthesis of exposure of portions of a growing plasmodium to nocodazole

A large plasmodium (15 cm) was grown for 23 h as previously described. It was then divided into six parts, five of which were transferred to GM + nocodazole (20 μg ml^{-1}) at 23 h (○), 24 h (■), 25 h (□), 26 h (▲) and 27 h (△). One piece was left as a control (●). The uptake of [methyl - ^{3}H] thymidine into acid insoluble material was followed as described in the legend to Figure 8.


cpm
per
E_{400}
unit
x 10^{-4}
6
5
4
3
2
1
24
25
26
27
28
29
30
31
Time (hours) during growth

The 1 to 1.5 h that are needed prior to a mitosis for nocodazole to completely block a round of DNA replication may reflect the time taken for the drug to enter the plasmodium and reach a critical concentration. When mitosis was followed in two growing plasmodia, one treated with nocodazole, the other as a control, it was noticed that the nuclei in the treated plasmodium increased in size. In the light microscope under 1000x magnification no internal structure could be seen. Both sets of nuclei were in interphase, the only difference being their size. It was decided to study nocodazole's effect on the nuclei further using the electron microscope.

e) The effect of nocodazole on the nuclei of a growing plasmodium

An experiment was designed that would not only study the effect of nocodazole on the nuclei but it was hoped would also clarify the time it took for nocodazole to reach the critical concentration within the plasmodium necessary to block DNA replication.

Pieces of a large plasmodium were treated with nocodazole at intervals and the round of DNA replication which began between 23 and 24 h was examined. As DNA synthesis was monitored, samples were taken from the control piece of plasmodium to determine the time that mitosis began. When the nuclei were seen to be entering early prophase, samples for electron microscopic examination were taken from each of the six pieces of plasmodium. Samples were also taken when mitosis had been completed in the control piece of plasmodium.

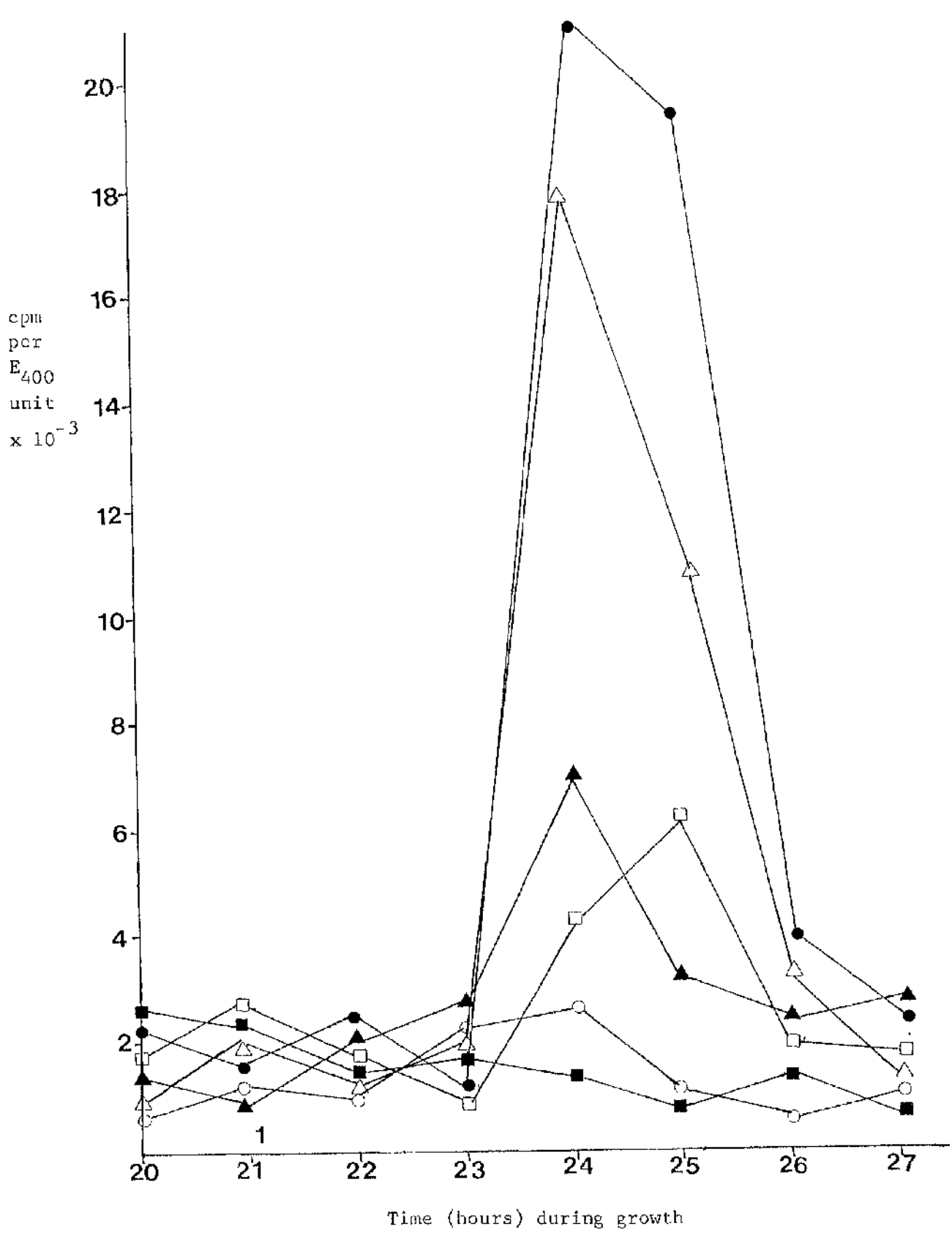
The results of the experiment are shown in Fig 13. DNA synthesis began between 23 and 24 h and was unaffected by exposure of the plasmodium to nocodazole at 24 h as expected as mitosis had already

Figure 13. Effect on DNA synthesis of exposure of portions of a growing plasmodium to nocodazole

A large plasmodium (15 cm) was grown and treated as described in the legend to Figure 12.

The uptake of [methyl - ^{3}H] thymidine was followed as described in the legend to Figure 8.

At intervals two samples were taken. One was placed into the GM + [methyl - ^{3}H] thymidine (control (●), 20 h (○), 21 h (■), 22 h (□), 23 h (▲) and 24 h (△)), the other was used for microscopic examination. Samples were also taken from each piece of plasmodium just before and just after mitosis. These samples were treated for electron microscopic examination (see Methods Section 2b(ii)).


cpm
per
E_{400}
unit
x 10^{-3}
20
18
16
14
12
10
8
6
4
2
1
20
21
22
23
24
25
26
27
Time (hours) during growth

occurred by that time. DNA replication was eliminated in the portions of plasmodia exposed to nocodazole at 20 and 21 h. Limited DNA replication took place in the plasmodial pieces exposed at 22 and 23 h. This supports the data obtained from previous experiments (Figs 10, 11 and 12). Thus DNA replication was only completely blocked if nocodazole was added some 2 h before replication began.

When the electron micrographs were studied it could be clearly seen that nocodazole was having an effect on the nuclei before mitosis. Samples taken from the portions of plasmodium transferred to nocodazole after 20 and 21 h growth showed that the nuclei appeared to be blocked in interphase. These nuclei were at least 33% bigger than control nuclei and had a much less dense cytoplasm (Fig 14).

As expected samples taken from the portion of plasmodium transferred to nocodazole at 24 h, ie 1 h after mitosis began, behaved normally. Nuclei were shown to have completed mitosis, as nuclei which were in late telophase and reconstruction could be seen (Fig 15a). Nuclei which were at a similar stage of mitosis were found in the control piece of plasmodium (Fig 15b).

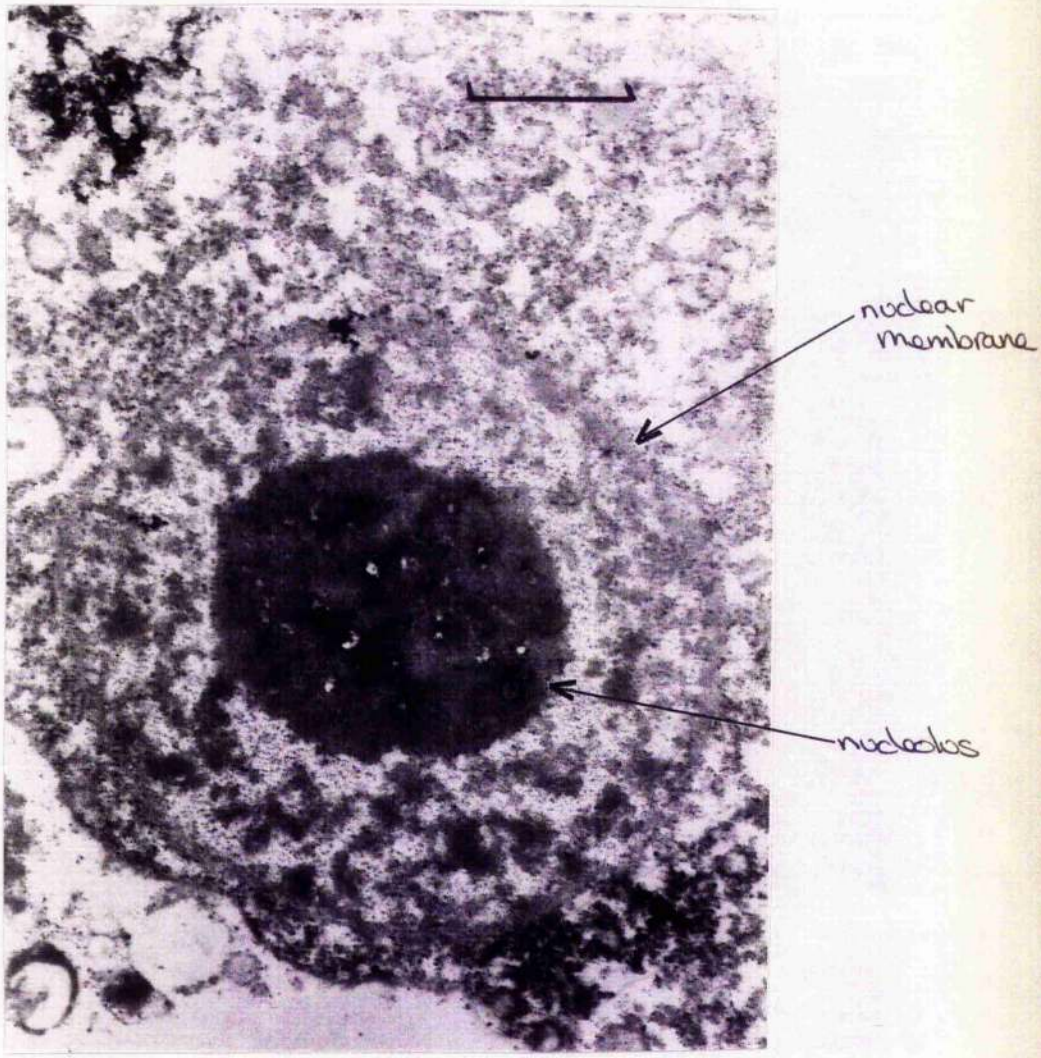
Nuclei from the plasmodial portion transferred to nocodazole just prior to mitosis showed the characteristic increase in size which occurs just before mitosis. These nuclei entered the first stages of mitosis as excentric nucleoli could be seen (Fig 16). Samples taken after mitosis had occurred in the control piece of plasmodium showed that normal mitosis had been blocked. Nuclei which were blocked in prophase were abundant (Fig 17a) as were large abnormal polynucleotate nuclei (Fig 17b).

The second plasmodial portion which replicated its DNA to

Figure 14. Electronmicrograph of nucleus treated with nocodazole 3h before mitosis

A plasmodial sample was removed from the piece of plasmodium transferred to nocodazole 3 h prior to mitosis onset after the mitosis was seen to be completed in a control portion of plasmodium. This sample was treated as described in Methods Section 2b(ii).

Bar represents 100nm.



Enlarged interphase nucleus

Figure 15. Electronmicrograph of nuclei which had completed mitosis

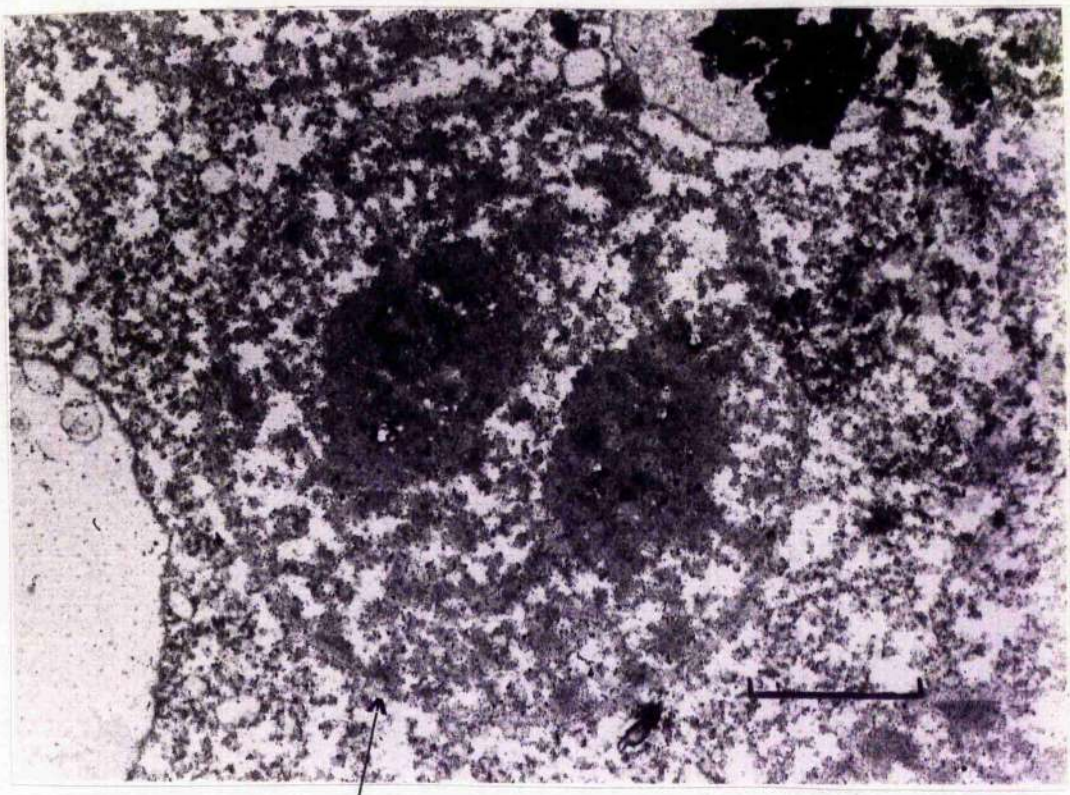
Samples were taken from the control portion of plasmodium and from the plasmodial portion exposed to nocodazole after mitosis. The samples were treated as described in Methods Section 2b(ii).

Bar represents 100nm.

A : Nucleus from plasmodium exposed to nocodazole after mitosis.

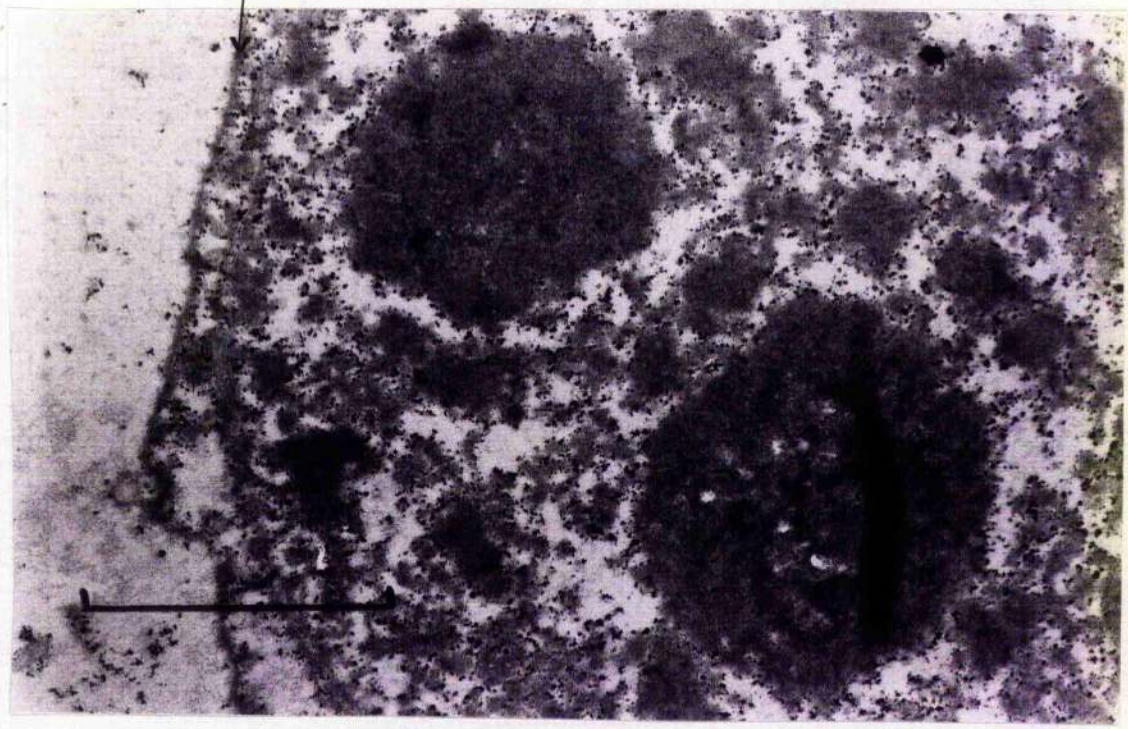
B : Nucleus from control plasmodium.

A



nuclear membrane

B

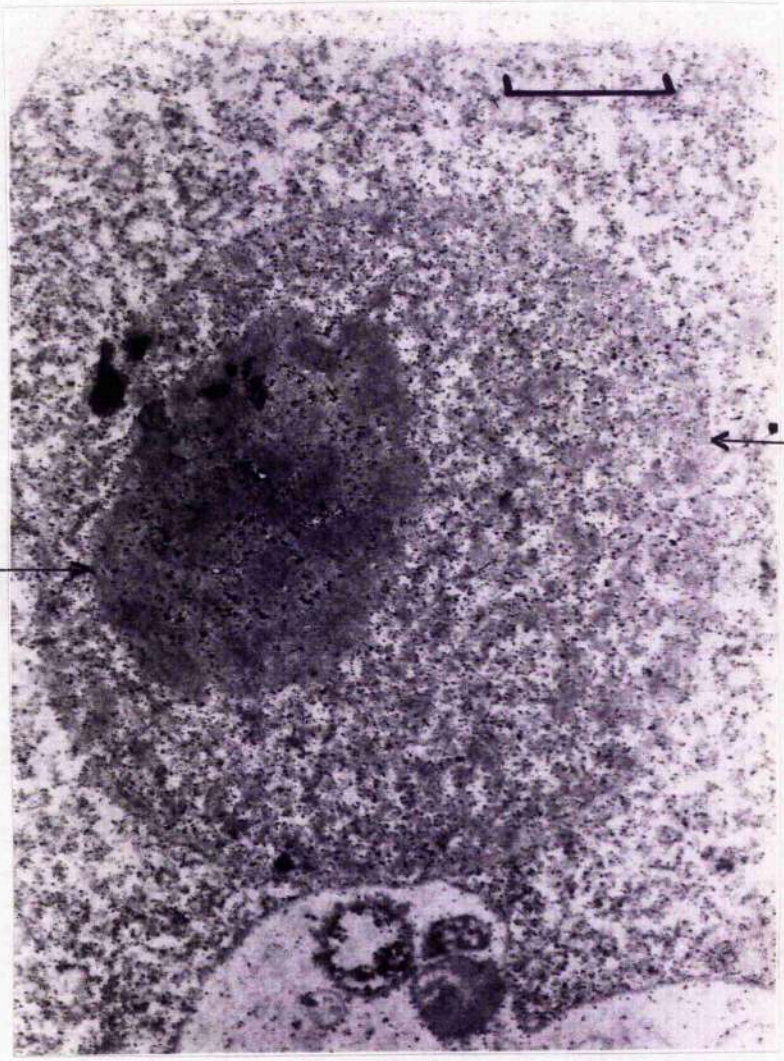


Telophase nuclei.

Figure 16. Electronmicrograph of a nucleus from a plasmodial portion exposed to nocodazole 15 min before mitosis

A sample was cut from the piece of plasmodium as mitosis was seen to be beginning in a control plasmodial portion. The sample was treated as described in Methods Section 2h(ii).

Bar represents 100nm.



Prophase nucleus

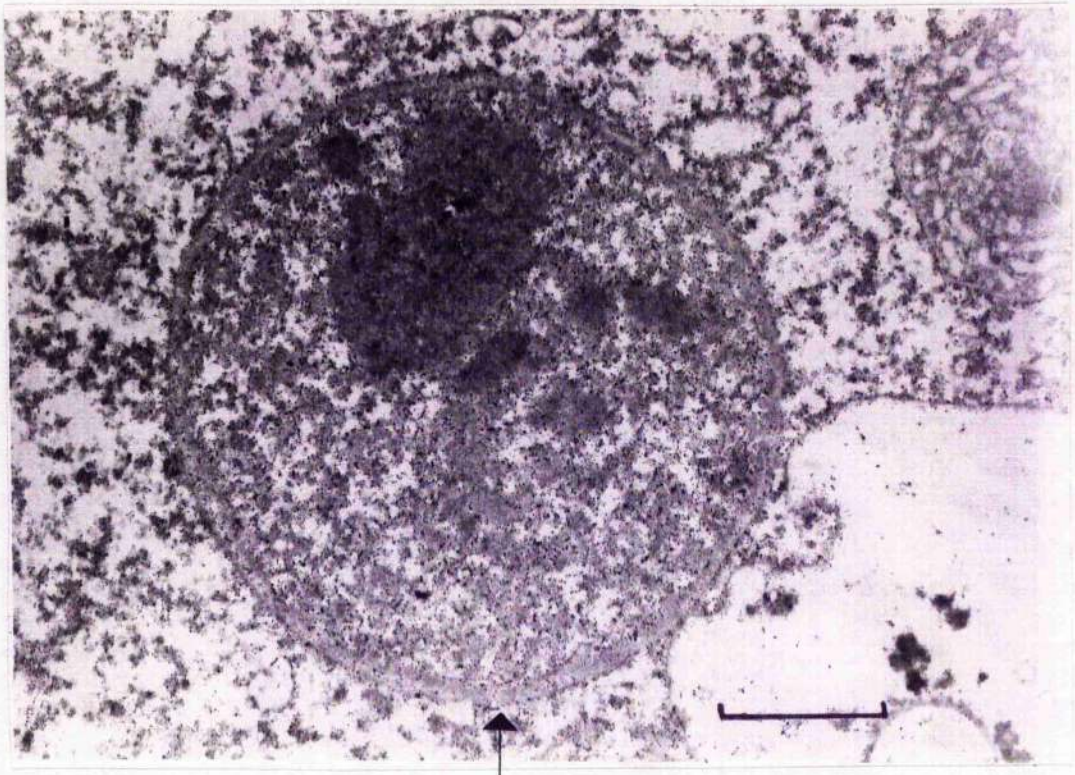
Figure 17. The effect of exposure to nocodazole 15 min before mitosis on the successful completion of that mitosis

A sample was taken from the piece of plasmodium transferred to nocodazole after 23 h of growth after mitosis was completed in a control piece of plasmodium. The sample was treated as described in Methods Section 2b(ii).

Bar represents 100nm.

Prophase nucleus

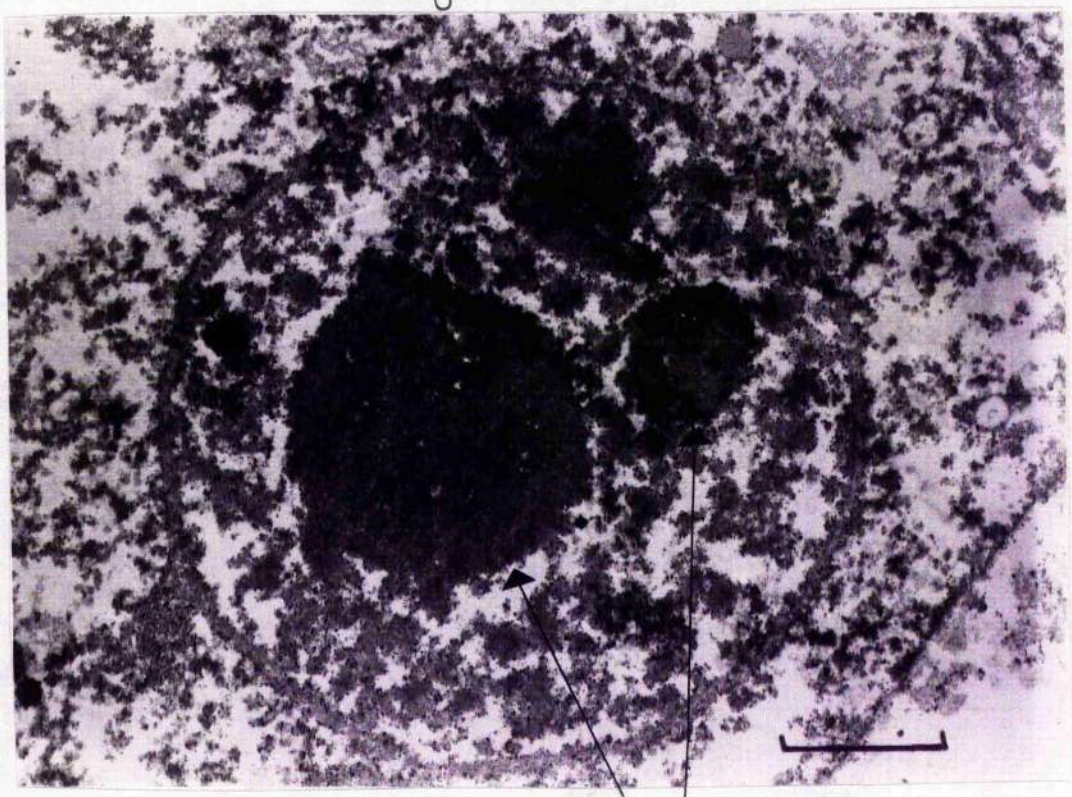
A



nuclear membrane

Polynucleolate nucleus

B



nucleoli

some degree was transferred to nocodazole around 1 h prior to the onset of mitosis. These nuclei entered the first stages of mitosis and the nucleoli were seen to be at the edge of the nuclei. Again normal mitosis was blocked in the sample taken after mitosis had occurred in the control. Some nuclei were seen blocked at metaphase (Fig 18a) while the majority were large and polynucleolate (Fig 18b).

From this experiment it can be deduced that nocodazole enters the plasmodium fairly quickly and exerts its effect almost immediately. When treated several hours before metaphase onset the nuclei become blocked in interphase. No normal nuclei could be seen in the plasmodial portions exposed to the drug before metaphase and therefore the DNA synthesis observed was not due to nuclei which escaped the effect of nocodazole and completed mitosis successfully.

f) Cell cycle events after illumination

Sauer et al (1969a) reported the occurrence of a presporangial mitosis. This mitotic division took place around 12 h postillumination and was followed by a round of DNA replication as shown by radioactive thymidine incorporation. An attempt was made to verify this observation for the CL strain.

Two plasmodia were starved for 72 h and then illuminated for 30 min (Chapman & Coote, 1982). The first 10 h postillumination were monitored in one plasmodium and the subsequent hours to melanization (17 h) in the second. Figure 19 showed that no obvious rounds of DNA replication were detected either during the first 10 h after illumination or during the last 7 h up to the completion of melanization. These findings may not indicate the lack of a presporangial round of DNA replication, but may reflect the inability of the [methyl-^{3}H]-thymidine to enter the plasmodium.

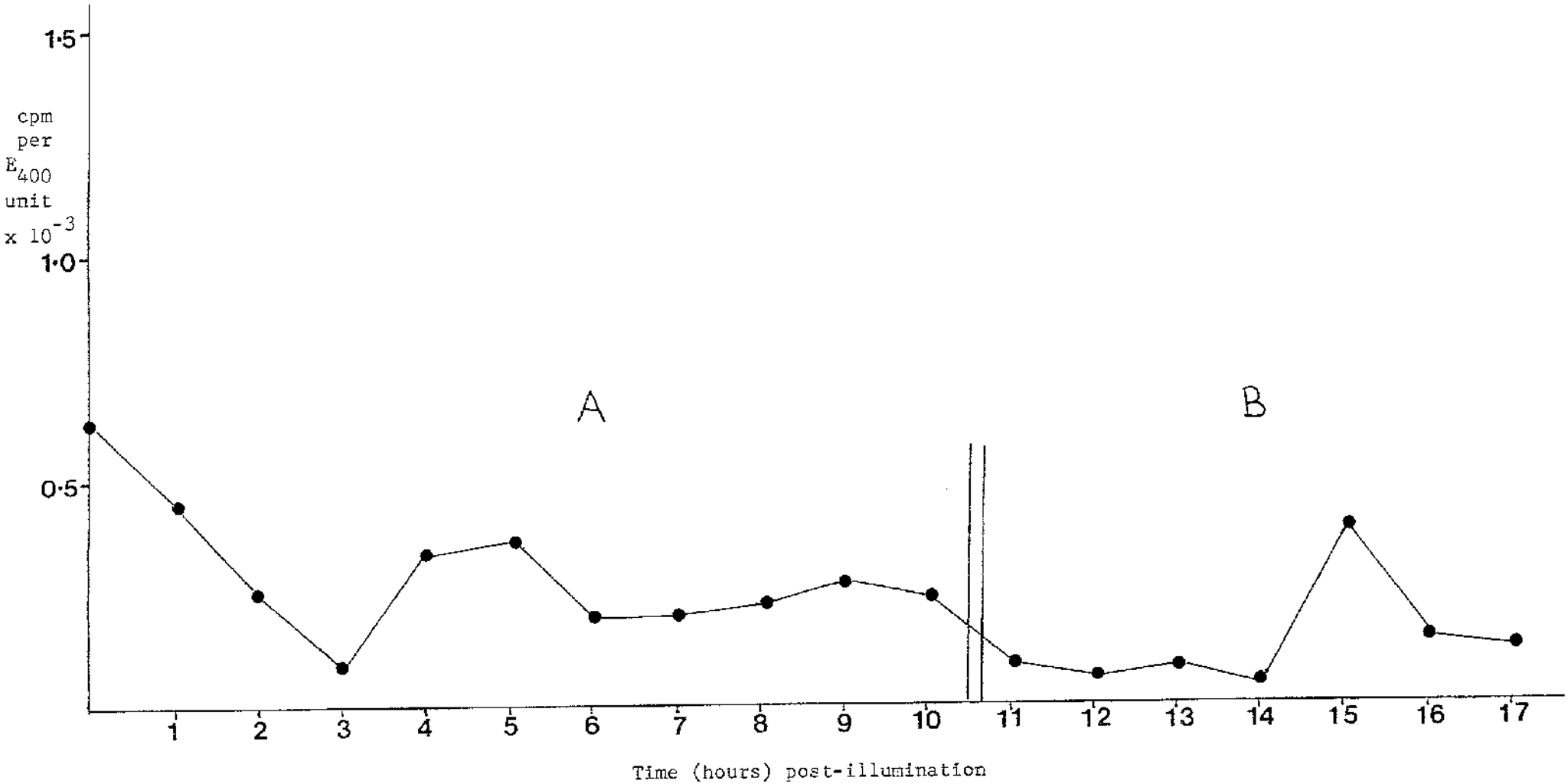
Figure 18 Effect on mitosis of exposure of a piece of plasmodium to nocodazole around 1½ h prior to mitosis

A sample was removed from the plasmodium at the end of mitosis as detected in a control piece of plasmodium. The sample was treated as described in Methods Section 2b(ii).

Bar represents 100nm.

Figure 19. DNA synthesis in a plasmodium after exposure to light after 72 h of starvation

Two starved plasmodia were prepared as described in Methods Section 1(d) and the uptake of [methyl - ^{3}H]thymidine into acid insoluble material was monitored in one plasmodium (A) over the initial 10 h post-illumination and in the second (B) over the remaining 7 h up to the melanization of the sporangia. The piece of plasmodium from A that was left went on to sporulate successfully.


1·5
cpm per E_{400} unit x 10^{-3}
1·0
0·5
A
B
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Time (hours) post-illumination

2. Preparation and Analysis of a *Physarum* Genomic Library

a) Isolation of phage vector DNA

Two different phage vectors were available for use in this research, λ1059, a vector which allowed the identification of recombinant phage by genetic selection (see Introduction Section 5d) and Charon4AP which relied on size selection for the isolation of recombinant phage.

In order to maximise the yield of bacteriophage DNA the best conditions for phage growth were sought. Phage DNA was isolated from concentrated phage preparations grown as large liquid lysates (Yamamoto *et al*, 1970) and one crucial factor which determined the phage titre obtained in a liquid lysate was the multiplicity of infection (MOI).

In initial experiments the MOI was varied from 1:500 to 1:50 for λ1059 and for Charon4AP (Table 2). For the bacteriophage λ1059 the multiplicity of infection which gave the highest titre was 1:500. When the MOI was varied for Ch4AP the phage titres varied by a factor of 10^2; 1:200 was the best MOI for this particular phage.

These MOIs were used when the liquid lysates were grown in subsequent experiments for preparation of phage DNA. The yield of DNA varied from preparation to preparation and ranged from 84-170 μg of vecto DNA.

b) In vitro packaging of DNA

During the preparation of a genomic library one important step is the *in vitro* packaging of the recombinant DNA molecules. Packaging extracts were prepared as described in Methods Section 6. Each time the extracts were prepared they varied in their ability to package λ DNA.

Table 2. Effect of varying the multiplicity of infection on the phage titre

Phage were grown as liquid lysates as described in Methods Section 4b. After cell lysis the phage titre of each lysate was determined as described in Methods Section 4a.

Phage	Multiplicity of infection	Phage titre per ml
λ1059	1 : 500	2.2×10^{10}
	1 : 200	6×10^{9}
	1 : 100	3.2×10^{9}
	1 : 50	3.5×10^{8}
CH4AP	1 : 500	3.5×10^{9}
	1 : 200	1.9×10^{10}
	1 : 100	6.5×10^{9}
	1 : 50	4.3×10^{8}

The efficiency of the prepared extracts ranged from 10^5 to 10^8 pfus per μg of vector DNA.

Before attempting to package the recombinant phage DNA molecules prepared *in vitro* the ability of the packaging extracts to distinguish between ligated and unligated phage DNA was tested (Table 3). As expected, unligated DNA was not packaged with any efficiency. However, very low titres were obtained for the ligated DNA molecules. Phage titres ranged from 10^2-10^3 pfu ml^{-1} giving packaging efficiencies of between 10^3 and 10^4 pfu μg^{-1} of phage DNA. This indicated that something was inhibiting the packaging reaction and so in an attempt to free the DNA from the inhibitor the ligated DNA was ethanol precipitated and redissolved in 5 μl of TE buffer before being added to the packaging extract. This treatment increased the number of pfu ml^{-1} obtained by a factor of 10^2 (Table 3). The efficiency of the extracts was 10^5-10^6 pfu μg^{-1} of phage DNA.

From these results it was concluded that a component in the ligation buffer was inhibitory to the *in vitro* packaging of the phage DNA.

c) Insertion of *Physarum* DNA into phage vectors

Phage vectors allow the insertion of foreign DNA with an average size of 20 kb. The isolation of such fragments is time-consuming and costly in that 100 μg of DNA may be used to recover only a few μg of sized DNA fragments.

It was decided to try and create recombinant phage by simply ligating *Bam*H1 cleaved λ1059 DNA and *Bam*H1 partially digested *Physarum* DNA. No size selection was carried out before the *in vitro* ligation reactions. Again ethanol precipitated and non-precipitated DNAs were tested and the

Table 3 Effect of ligation and ethanol precipitation of digested λ1059 DNA on phage titre

λ1059 DNA was digested with BamHI as described in Methods Section 7(c). The DNA was then treated as shown below before being *in vitro* packaged (see Methods Section 7(dii) and titred on *E. coli* Q358 (see Methods Section 4(a)).

Experiment	Treatment of BamHI digested λ1059 DNA	Phage Titre on *E. coli* Q358 per packaging extract*
1	unligated	$< 10^1$
	ligated	10^3
	ligated + ethanol precipitated	4.2×10^5
2	unligated	0
	ligated	10^2
	ligated + ethanol precipitated	1.2×10^4
3	unligated	$< 10^1$
	ligated	4.5×10^2
	ligated + ethanol precipitated	4×10^4

*packaging extracts contain a total volume of 280 µl.

results are shown in Table 4. Packaging extracts were titred on *E. coli* Q358 to give the total number of phage and on *E. coli* Q359 to give the number of recombinant phage generated. Only parental phage were recovered. No prospective recombinant phage could be isolated from any packaging extract. Again only ligated DNA that had been ethanol precipitated before packaging yielded viable phage. This supported the previous finding (Table 3) that the ligation buffer was inhibitory to the packaging reactions. All DNA to be packaged was subsequently ethanol precipitated after *in vitro* ligation.

The results suggested that before a genomic library could be made, size fractionation of digested *Physarum* DNA was necessary.

d) Isolation of 15-25 kb *Physarum* DNA fragments

Three methods of size fractionation of the *Physarum* DNA were tried in an attempt to isolate 15-25 kb fragments.

Method 1 - Phenol Extraction

The DNA was isolated from a low melting-point agarose gel by phenol extraction. After the digested DNA had been separated through a 1.0% (w/v) low melting-point agarose gel a strip was cut from the gel and stained with ethidium bromide. The position of the DNA of interest was marked with small nicks. The strip was replaced and the DNA was located and cut from the remainder of the gel. The gel was melted, diluted 1 in 2 with TE buffer (pH 8.0) and then phenol extracted. This proved to be a very inefficient method of isolation. In many cases the DNA samples did not run straight across the gel and as a result most of the important DNA was left in the gel. It also proved difficult to precipitate the DNA from the large aqueous volume which resulted from the various extractions.

Table 4. Ligation of digested λ1059 DNA with digested *Physarum* DNA

λ1059 DNA was digested with BamHI as previously described. *Physarum* DNA was digested with BamHI as described in Methods Section 7(a). No size selection of restriction fragments was carried out. The DNA was treated as shown below and then *in vitro* packaged as previously described. The phage titres on *E. coli* Q358 and Q359 were then determined.

Experiment	Treatment of digested λ1059 and *Physarum* DNAs	Titre on *E.coli* Q358 per extract*	Titre on *E.coli* Q359 per extract*
1	ligated	$< 10^1$	0
	ligated + ethanol precipitated	4×10^2	0
2	ligated	$< 10^1$	0
	ligated + ethanol precipitated	10^3	0

*: Packaging extracts contain a total volume of 280 µl.

Transfer RNA (50 μg ml^{-1}) was added to act as a carrier to aid the precipitation of the DNA, but this did not improve the yields of DNA obtained. The method was found to be successful in about 1 in 10 cases. The weight of DNA isolated was too low to calculate. One of the disadvantages of this method is that it was impossible to visualise the DNA in the complete gel. This problem was overcome using Method 2.

Method 2 - Butanol Extraction

This method also involved separating the DNA fragments through a 1% (w/v) low melting-point agarose gel. Here however the whole gel could be stained in ethidium bromide as this was removed from the DNA in subsequent steps. The reliability and efficiency of the method were investigated using 'nick translated' λ DNA (a gift from Dr. Peggy Anderson, Beatson Institute, Garscube Estate, Bearsden, Glasgow). The bands were cut from the gel and isolated as described in Methods Section 7b(ii). Samples were taken at each step of the isolation and the radioactivity was monitored. Although DNA was isolated in 3 out of 4 cases, only 8% of the total DNA was recovered from the gel. 30-40% of the DNA appeared to remain in the ethanol phase. By adding transfer RNA as a carrier (50 μg ml^{-1}) before the ethanol precipitation step, 20-25% of the total DNA could be recovered leaving 20-30% still in the ethanol phase. Although this method was more reliable than the first the yields of DNA recovered were variable and sometimes very poor.

Method 3 - Electroelution

This method was described by Yang et al (1979) and involves electroeluting the DNA from the gel into a buffer filled slot. The DNA is stained with ethidium bromide and can therefore be seen during the isolation.

This method proved to be the most reliable with a 90-95% success rate giving 40-50% recovery of the DNA. DNA isolated by this method was found to ligate efficiently *in vitro*. It was therefore decided to use this method to isolate the *Physarum* DNA fragments which were to be used in preparing the gene bank.

A 0.7% (w/v) agarose gel was found to be the best from which to isolate the DNA fragments. After restriction enzyme digestion, *Physarum* DNA fragments were electrophoresed on gels of various agarose concentrations. Each gel contained an *EcoRI* digest of Ch4AP as molecular weight markers. The 15-25 kb fragments were isolated and then separated through a 0.7% (w/v) agarose gel (Figure 20). DNA fragments isolated from a 1% (w/v) agarose gel were found to contain a proportion of contaminating fragments which were both larger and smaller than the selected size. A 0.7% (w/v) gel allowed the isolation of fragments which were of the correct size and contained very little contaminating DNA.

A 0.7% (w/v) agarose gel was used for all subsequent isolations of DNA.

e) *Isolation of Physarum and vector DNAs to be used to generate recombinant phage*

Physarum DNA, λ1059 DNA and Ch4AP DNA were digested as described in Methods Section 7(a) and the fragments of interest were isolated by electroelution. An aliquot of each DNA was separated on a 0.7% (w/v) agarose gel and the results are shown in Figure 21. The isolated Ch4AP 'arms' appear to be free of any contaminating internal fragments. Both the *EcoRI* and the *BamHI* generated *Physarum* DNA fragments are of the correct size with little contaminating DNA. These samples of DNA were therefore used in *in vitro* ligation reactions.

Figure 20. Effect of varying the agarose concentration on the range of restriction fragments obtained

Physarum DNA was digested with BamHI as described previously. 15-25 kb restriction fragments were then isolated by electro-elution (See Methods Section 7b(iii)) from 1.0% (w/v) and 0.7% (w/v) agarose gels. After precipitation the restriction fragments were separated on a 0.7% agarose gel.

Lane 1 : Fragments from a 1% agarose gel

Lane 2 : Fragments from a 0.7% agarose gel.

Lane 3 : Fragments from a 1.0% agarose gel.

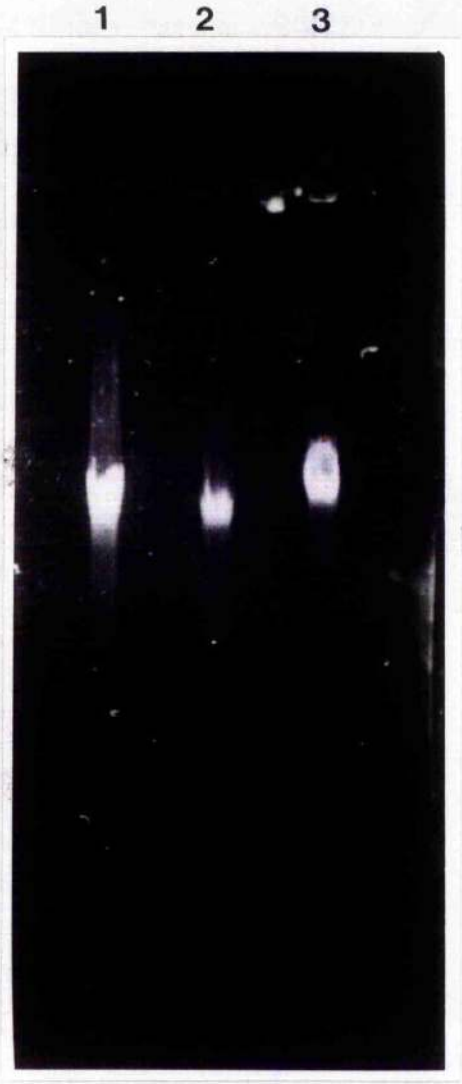

1
2
3

Figure 21 Restriction fragments of lambda and Physarum DNAs

λ1059 was digested with BamHI as previously described. Charon 4AP DNA was digested with EcoRI and the phage arms were isolated by electroelution (see Methods Section 7b(iii)). Physarum DNA was digested with BamHI or EcoRI and the 15-25 kb restriction fragments were isolated by electroelution.

A 0.7% agarose (w/v) gel was prepared and the fragments were separated as described previously.

Lane 1 : Charon 4AP 'arms' (12 and 19 kb)

Lane 2 : EcoRI Physarum restriction fragments (15-25 kb)

Lane 3 : EcoRI digested Charon 4AP DNA

Lane 4 : BamHI Physarum restriction fragments (15-25 kb)

Lane 5 : BamHI digested λ1059 DNA.


1
2
3
4
5

f) Insertion of Physarum DNA into Ch4AP "arms"

2 μg of Ch4AP "arms" was ligated alone and in the presence of 1 μg of Physarum 15-25 kb DNA fragments. The DNA was then ethanol precipitated, in vitro packaged and the extracts were titred on E. coli DP50 supF (Table 5). The left and right arms of Ch4AP contain all the information necessary to provide viable phage. However, when the arms are ligated on their own no viable phage should be generated as the arms are too short to be packaged inside phage heads with any degree of efficiency. In both the experiments this did not seem to be the case. When 2 μg of Ch4AP "arms" were ligated a total of 2.2×10^4 viable phage were created. When 1 μg of 15-25 kb Physarum DNA fragments were present in the reaction mixture only 3.3×10^4 phage were generated. In a second experiment Ch4AP "arms" also yielded a total of 1.68×10^4 phage while phage "arms" plus Physarum DNA generated a total of 4.2×10^4 viable phage.

These results showed that when λ "arms" were ligated in the presence of Physarum DNA fragments as many as two thirds of the phage isolated were probably not recombinant phage.

g) Insertion of Physarum DNA into λ1059

2 μg aliquots of BamHIdigested λ1059 DNA were religated with T_4 DNA ligase in the presence of 0-1.0 μg of 15-25 kb fragments produced by Bam HI cleavage of Physarum DNA. After in vitro packaging the total number of phage generated by the ligation reaction was determined by plating on E. coli Q358; the number of phage containing Physarum DNA was calculated by plating on E. coli Q359, a P2 lysogen of Q358 (Figure 22).

Cleavage and religation of λ1059 in the absence of Physarum

Table 5. Insertion of *Physarum* DNA into Charon 4AP 'arms'

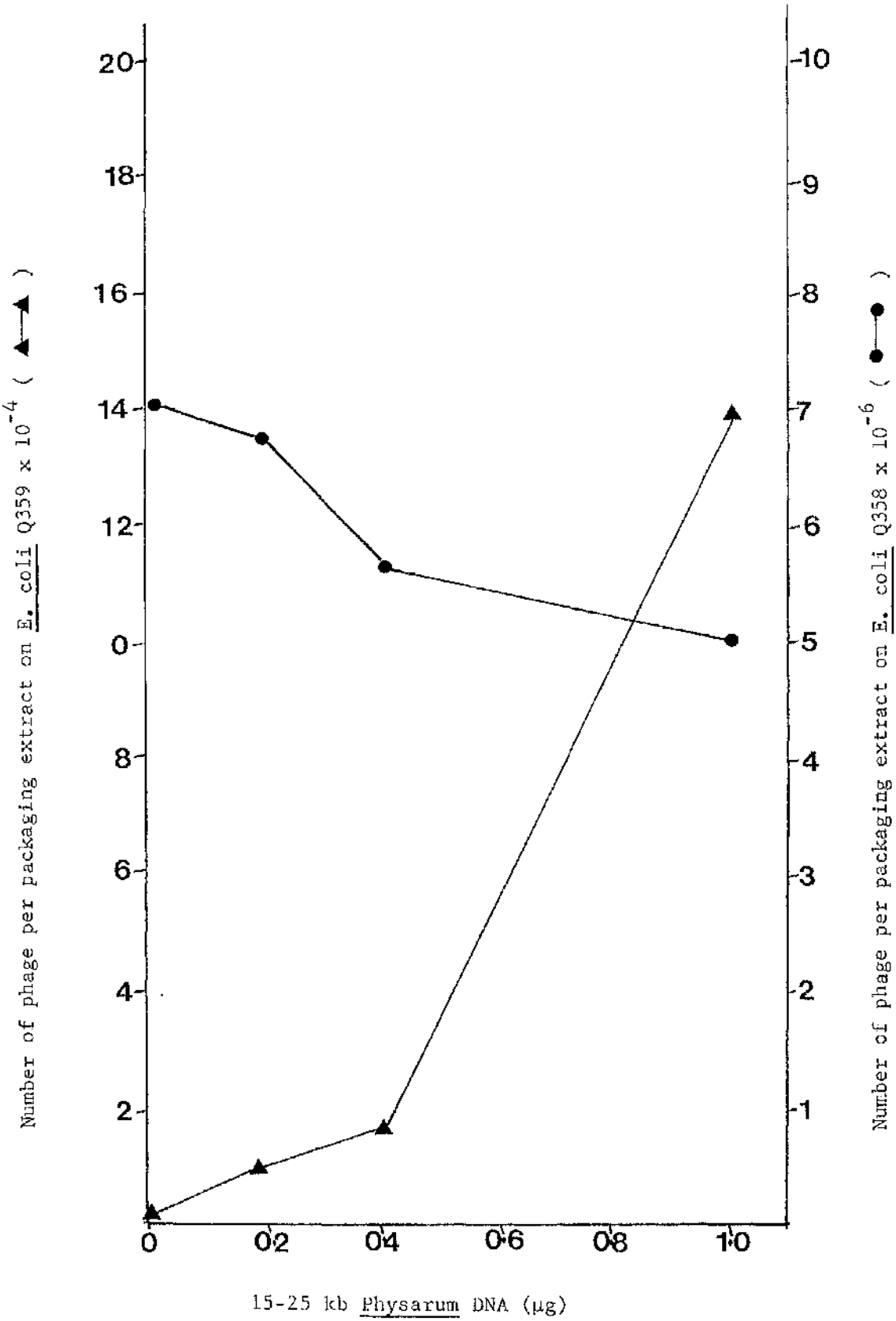
Charon 4AP and *Physarum* DNA were digested with *EcoRI*. Charon 4AP 'arms' and *Physarum* DNA restriction fragments were isolated as described in Methods Section 7b(iii). The DNAs were ligated in a ratio of 2:1, 'arms' : *Physarum* DNA. The DNAs were ethanol precipitated, *in vitro* packaged and titred on *E. coli* DP50 *supF*.

Experiment	DNA ligated	Phage titre on *E.coli* DP50*supF* per packaging extract*
1	Ch4AP 'arms'	2.2×10^4
	Ch4AP 'arms' + *Physarum* DNA	3.3×10^4
2	Ch4AP 'arms'	1.68×10^4
	Ch4AP 'arms' + *Physarum* DNA	4.2×10^4

*packaging extracts contain a total of 280 µl

Figure 22. Insertion of Physarum DNA prepared by BamH1 digestion into λ1059

2 µg aliquots of BamH1 digested λ1059 DNA were ligated in the presence of various amounts of Physarum DNA digested with BamH1. After ethanol precipitation, the DNAs were *in vitro* packaged and titred on *E. coli* Q358 (to give total phage) and *E. coli* Q359 (to give recombinant phage).


Number of phage per packaging extract on E. coli Q359 x 10^{-4} (▲—▲)
Number of phage per packaging extract on E. coli Q358 x 10^{-6} (●—●)
20
18
16
14
12
0
8
6
4
2
10
9
8
7
6
5
4
3
2
1
0
0·2
0·4
0·6
0·8
1·0
15-25 kb Physarum DNA (µg)

DNA produced more than 3 x 10^6 phage particles per μg of phage DNA. These phage grew well on E. coli Q358 but only 2.1 x 10^3 pfu were detected on Q359. A detailed analysis of the experiment is shown in Table 6. Cleavage and ligation of λ1059 in the presence of Physarum DNA produced recombinant phage which were selected by plating on E. coli Q359. The yield of recombinant phage was proportional to the amount of Physarum DNA added. The reactions yielded 0.49-1.4 x 10^5 recombinant phage per μg of 15-25 kb Physarum DNA. This is in agreement with the yield reported by Karn et al (1980) who obtained 2.4-5.4 x 10^5 recombinants per μg of nematode DNA. The total number of phage obtained decreased when Physarum DNA was added to the ligation reaction from 7 to 5 x 10^6 pfu. Recombinant phage were then amplified as described in Methods Section 7(e) and stored over chloroform at 4^oC.

As the ligation reactions with Ch4AP "arms" alone generated so many phage which were not recombinant phage and would therefore dilute a gene library, it was decided to concentrate on the library prepared in λ1059.

Genomic DNA that is suitable for insertion into λ1059 may be prepared with various restriction enzymes.

BamH1 cleaves at the sequence G↓GATCC and represents a group of enzymes that leave GATC as a 5' tetranucleotide "sticky" end. Other members of the group include Bgl(A↓GATCT), Bcl1(T↓GATCA) and Sau3A(↓GATC).

When genomic DNA is partially digested with Sau3A a nearly random population of high molecular weight DNA fragments is created as the tetranucleotide recognition sequence should occur once every 256 bp in DNA with a 50% G+C ratio. It was therefore decided to prepare a gene bank using Sau3A restricted Physarum DNA. BamH1 digested Physarum DNA was used in a parallel experiment.

Table 6. Analysis of gene bank prepared with BamH1 cleaved Physarum DNA in λ1059

Restriction Enzyme used	Weight of Physarum DNA added (μg)	Total no. of p.f.u.s x 10^{-6}*	Number of recombinant phage x 10^{-4} *	Percentage recombinant phage	Number of recombinant phage per μg of Physarum DNA x10^{-4}
BamH1	0	7	0.2	-	-
BamH1	0.2	6.7	0.98	0.14	4.9
BamH1	0.4	5.6	1.7	0.3	4.25
BamH1	1.0	5.04	14.0	2.8	14.0

*Total number of phage generated in packaging extract with a final volume of 280 μl.

h) Insertion Sau3A generated and BamHl generated Physarum DNA fragments into λ 1059

To reduce the chance of an abnormal distribution of DNA fragments that may result from a single digestion, the Physarum DNA was digested in 5 separate reactions in which the enzyme concentration was varied over a 16 fold range.

2 µg aliquots of BamHl cleaved λ1059 DNA were ligated in the presence of varying amounts of Physarum DNA. After ethanol precipitation the DNAs were in vitro packaged and the phage particles were titred on E. coli Q358 and E. coli Q359. The results are shown in Figure 23. In this experiment cleavage and religation of λ1059 DNA yielded around 1.5×10^6 phage per µg of DNA. When these phage were titred on E. coli Q359 only 2.0×10^3 pfu were detected. An analysis of the gene banks is shown in Table 7.

In both experiments the total yield of phage decreased when Physarum DNA was present in the ligation reaction mixtures. The number of recombinant phage recovered was proportional to the total amount of Physarum DNA present in the reaction mixtures. The BamHl cleaved DNA fragments yielded $1.4\text{-}2.2 \times 10^5$ recombinant phage per µg of Physarum DNA, while the Sau3A generated fragments gave $1\text{-}2.4 \times 10^5$ recombinants per µg of eukaryotic DNA. The percentage of recombinant phage generated in each ligation reaction varied from 0.79 to 6.24. Recombinant phage were amplified as described in Methods Section 7(e) and stored over chloroform at $4^{o}C$.

i) Analysis of recombinant phage

i) Restriction analysis

Figure 23. Insertion of *Physarum* into λ1059

2 μg aliquots of *Bam*H1 cleaved λ1059 DNA were ligated in the presence of various amounts of 15-25 kb *Physarum* DNA generated by digestion with *Bam*H1 (A) or *Sau*3A (B) (see Methods Section 7(a)). After ethanol precipitation, the ligated DNAs were packaged *in vitro* and titred on *E. coli* Q358 (to give the total phage) and *E. coli* Q359 (to give recombinant phage).

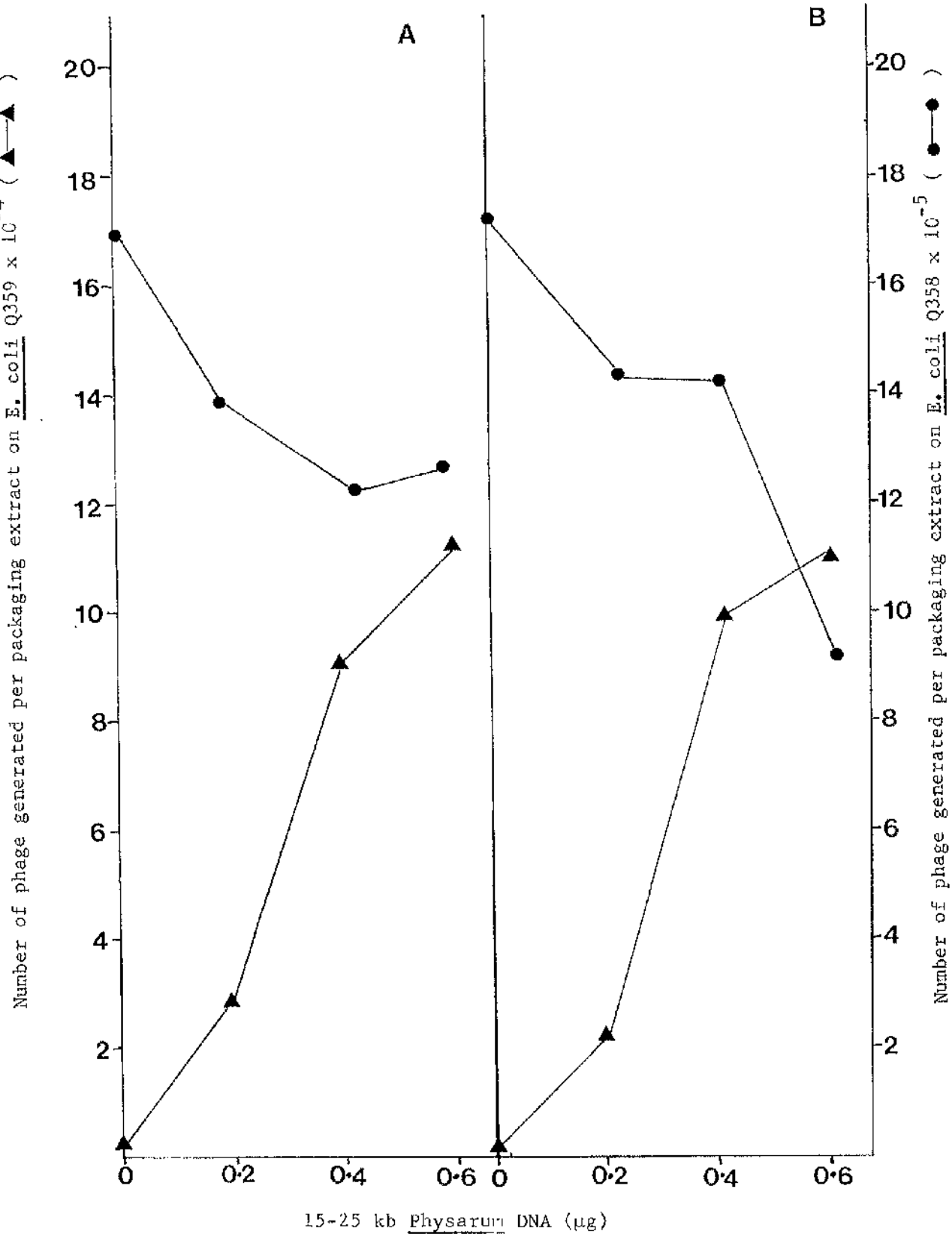

A
B
20
18
16
14
12
10
8
6
4
2
0
0·2
0·4
0·6
Number of phage generated per packaging extract on E. coli Q359 x 10⁻⁴ (▲—▲)
Number of phage generated per packaging extract on E. coli Q358 x 10⁻⁵ (●—●)
15-25 kb Physarum DNA (µg)

Table 7 Analysis of gene banks prepared with BamH1 and Sau3A cleaved Physarum DNA

Restriction Enzyme used to digest Physarum DNA	Weight (µg) of 15-25kb Physarum DNA added	Total no. of p.f.u. x 10^{-5}*	No. of recombinant p.f.u. x 10^{-4}*	Percentage recombinant phage	Number of recombinant phage per µg Physarum DNA x 10^{-5}
-	0	33.6	0.25	-	-
BamH1	0.2	27.0	2.8	1.04	1.4
BamH1	0.4	24.0	8.9	3.71	2.23
BamH1	0.6	25.0	11.2	4.48	1.87
Sau3A	0.2	28.0	2.2	0.79	1.1
Sau3A	0.4	28.0	9.8	3.5	2.45
Sau3A	0.6	17.0	10.6	6.24	1.77

*Total number of phage generated in packaging extract with a final volume of 280 µl.

To ensure that a comprehensive genomic library had been constructed it was necessary to screen a fairly large number of phage. This was done using the mini preparation described by Cameron et al (1977) (see Methods Section 5(d)). Phage from the Sau3A collection were grown on E. coli Q359 and several clones were isolated and their DNA purified. The phage DNA was digested with EcoRI and the products were separated by electrophoresis through a 0.7% (w/v) agarose gel. Samples of the restriction patterns obtained are shown in Figure 24, Lanes 7-11. Identical restriction patterns were obtained for each phage DNA tested. The EcoRI digestion of the DNA resulted in 6 restriction fragments.

These findings raised three questions;

1) Are the phage in other collections similar to the one found in the Sau3A clone collection?

2) Has one phage had such an efficient growth rate that it has overgrown all other recombinant phage?

3) Is this a contaminating phage which entered the system before or after packaging of the ligated DNAs?

Before any further work could be carried out these questions had to be answered.

Phage from the other two clone collections, which had been made with BamHl generated fragments, were grown on E. coli Q359. A dozen of these phage were picked and their DNA was isolated. After EcoRI cleavage the restriction fragments were separated through a 0.7% (w/v) agarose gel. All phage screened were found to give identical restriction patterns to the patterns obtained with phage from the Sau3A collection. The six restriction fragment pattern was always produced after EcoRI digestion. These results suggested that the phage isolated were not due

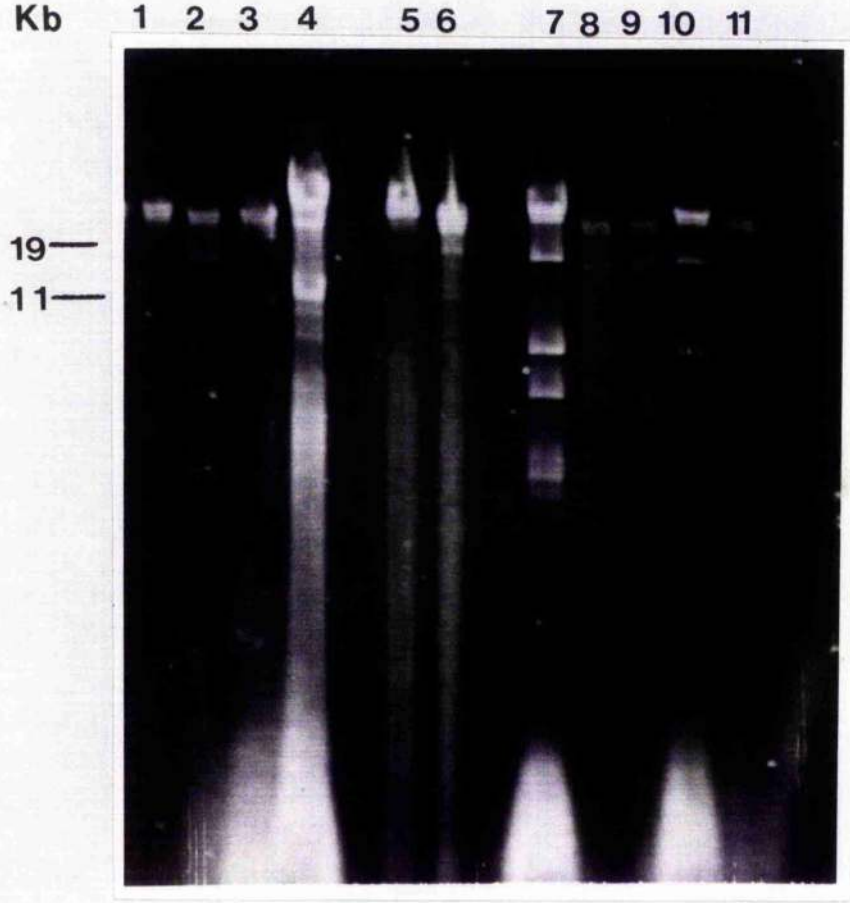
Figure 24. Restriction analysis of presumptive recombinant phage

Phage were grown as plate lysates as described in Methods Section 4(a). Individual phage plaques were picked and a 5 ml liquid lysate was prepared (see Methods Section 4(b)). DNA was isolated from the phage minipreparations as described by Cameron et al (1977) (see Methods Section 5(d)). The DNA was then digested with EcoRI and the fragments were separated through a 0.7% (w/v) agarose gel under a constant current of 40 mA.

Lanes 1-4: DNA from unamplified phage grown on E.coli Q359.

Lanes 5 & 6 : DNA from λ1059

Lanes 7-11: DNA from amplified recombinant phage grown on E. coli Q359.


Kb
1 2 3 4 5 6 7 8 9 10 11
19
11

to a source of contamination as it is highly unlikely that gene libraries prepared at different times would be contaminated with the same phage.

In order to establish if the gene banks had been overgrown by a single phage during the amplification step recombinant phage were isolated directly from the remaining packaging extracts. Phage were grown on E. coli Q359 and six were chosen for screening. The DNA was cleaved with EcoRIand a sample of the restriction pattern obtained is shown in Fig 24 lane 2. EcoRI digestion of the DNA resulted in six restriction fragments in a pattern that was identical to all other restriction patterns obtained. The digestion of the DNAs shown in Lanes 1, 3 and 4 was incomplete. It was therefore unlikely that a single phage overgrew all the others during the amplification of the library.

ii) Identification of the foreign DNA inserted into λ1059

It was of great importantance to establish whether these similar phage (sim phage) contained any Physarum DNA. A hybridization reaction between the sim phage DNA and the Physarum DNA which had been used to generate the phage was carried out. Sim phage DNA was isolated and "nick-translated" in vitro. Samples of Physarum DNA were spotted onto nitrocellulose filter paper, denatured and fixed by baking in vacuo. The sim phage DNA was used as a probe in a hybridization reaction against the Physarum DNA (Fig 25). A strong positive hybridization signal was obtained. This result showed that the 'sim' phage did contain fragments of the Physarum DNA used in their preparation.

iii) Analysis of Physarum DNA

The previous results suggested that it may have been the Physarum DNA itself that was at fault. A second method of DNA isolation was then sought. Hardman & Jack (1978) described a method for obtaining

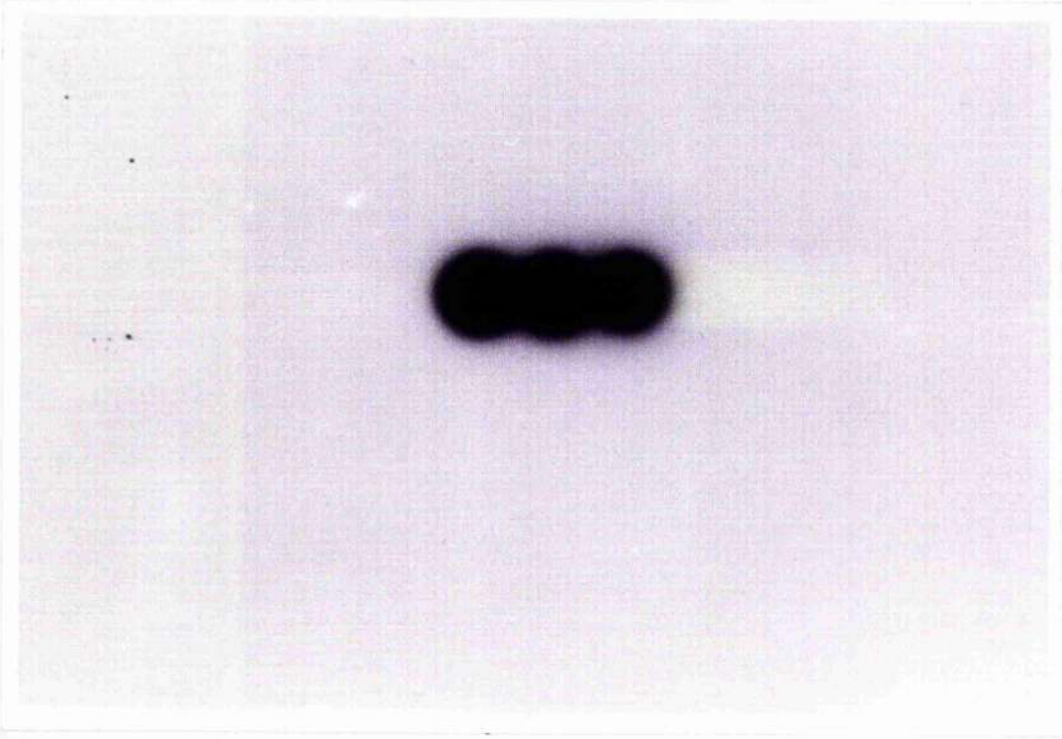
Figure 25. Hybridization of *Sim* Phage DNA to *Physarum* DNA isolated by Method 1

Physarum DNA isolated by method 1 was blotted onto nitrocellulose filter paper. The DNA was denatured and fixed as described in Methods Section 8. DNA from a *Sim* phage was isolated as described in Methods Section 5(a) and "nick-translated" as described in Methods Section 9(a).

The nick-translated *Sim* phage DNA was used as a probe in a hybridization reaction with *Physarum* DNA.

The hybridization conditions were those described in Methods Section 10(a).

Physarum DNA.



Actual size

Physarum chromosomal DNA which isolated the DNA on a CsCl density gradient directly from the nuclear lysate. When this method was tried it proved to be much more successful than the previous method in that the yield of Physarum DNA was increased by a factor of 2.

A CsCl density gradient analysis of the DNA obtained using each method was carried out. The result is shown in Fig 26. The DNA isolated by Method 1 was found to be much lighter than the DNA isolated in Method 2. It is likely that the DNA isolated by Method 1 may have been extrachromosomal in nature.

The DNA isolated by Method 2 was used to prepare another gene bank.

j) Insertion of Physarum DNA into λ1059

i) Preparation of recombinant phage

Physarum DNA isolated by method 2, was partially digested with Sau3A as previously described. 2 μg aliquots of BamH1 cleaved λ1059 DNA were ligated in the presence of 0-1.0 μg of 15-25 kb fragments produced by Sau3A cleavage of Physarum DNA. The ligated DNAs were ethanol precipitated before being in vitro packaged. The phage were titred on E. coli Q358 and E. coli Q359 (Fig 27). Cleavage and religation of λ 1059 DNA produced more than 10^6 phage particles per μg of DNA. Although these phage grew well on E. coli Q358 they had only a titre of 2.8×10^3 when grown on E. coli Q359. An analysis of the experiment is shown in Table 8. As before the yield of recombinant phage was proportional to the amount of Physarum DNA added. The total number of phage generated decreased when Physarum DNA was added to the system. The percentage of recombinant phage in each reaction varied from 2.67-14.36%. The reactions yielded $1.68\text{-}2.24 \times 10^5$ recombinant phage per μg of 15-25 kb Physarum DNA fragments.

Figure 26. Caesium chloride gradient analysis of Physarum DNA

Physarum polycephalum DNA was isolated using Methods 1 and 2 (see Methods Section 5b(iii). In both cases the DNA lysate was made up to 4 ml with buffer before solid caesium chloride and ethidium bromide solution were added.

The gradients were spun at 110,000 x g for 24 h at 4°C and then viewed under a U.V. light.

Gradient A : DNA isolated by Method 1.

Gradient B : DNA isolated by Method 2.

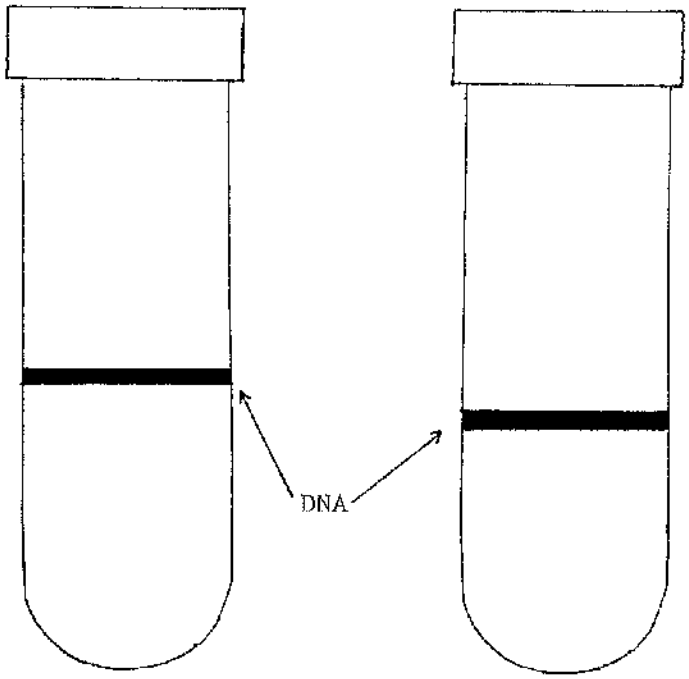

DNA
A
B

Figure 27. Insertion of Physarum DNA prepared using Method 2 into λ1059

2 µg aliquots of BamH1 digested λ1059 were ligated in the presence of various amounts of 15-25 kb Physarum DNA digested with Sau3A. After ethanol precipitation the ligated DNAs were in vitro packaged and titred on E.coli Q358 and E.coli Q359.

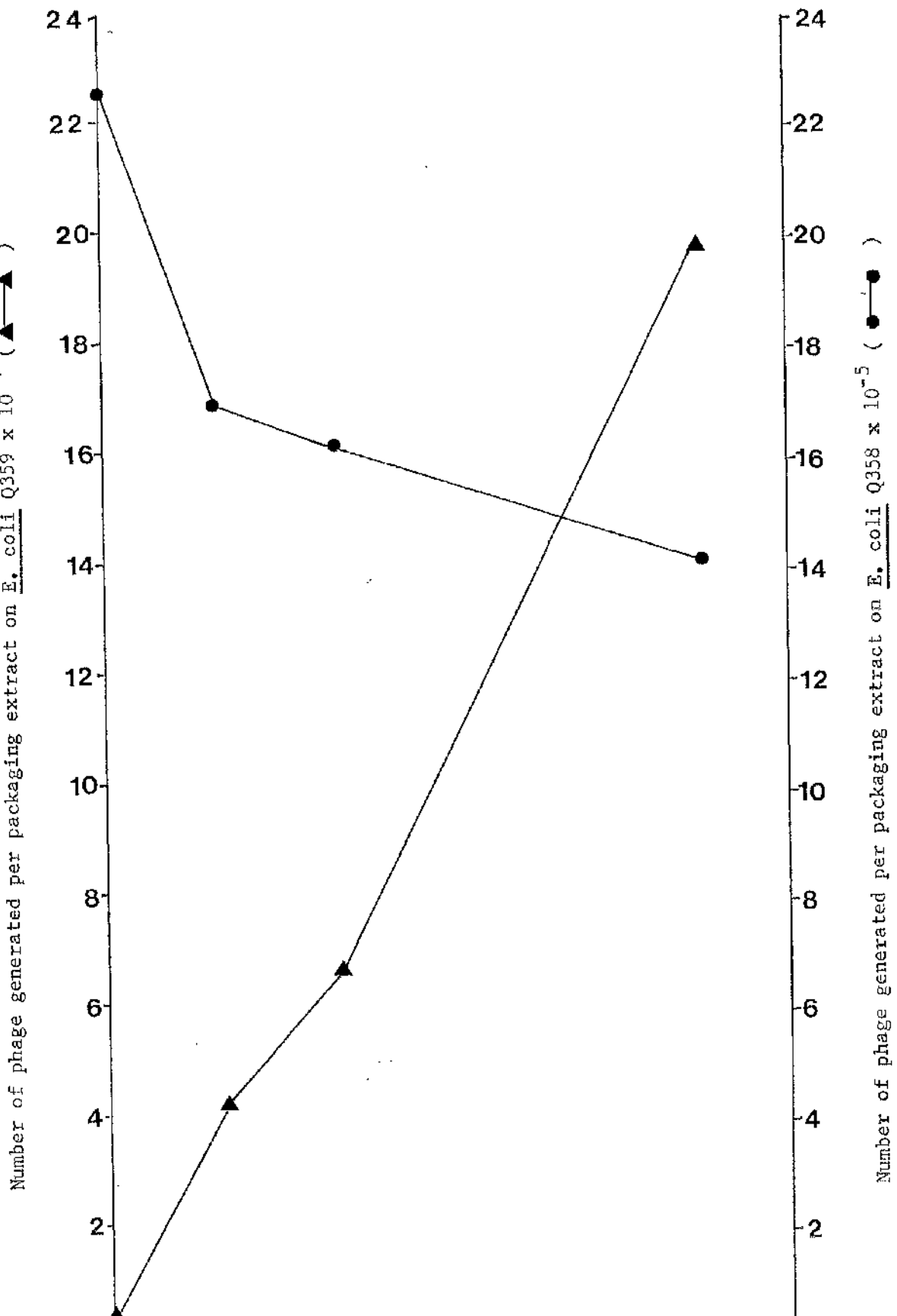

24
22
20
18
16
14
12
10
8
6
4
2
0·2
0·4
0·6
0·8
1·0
15-25 kb Physarum DNA (μg)
Number of phage generated per packaging extract on E. coli Q359 x 10^{-4} (▲—▲)
Number of phage generated per packaging extract on E. coli Q358 x 10^{-5} (●—●)

Table 8. Analysis of Gene bank Prepared with Sau3A Cleaved *Physarum* DNA

Restriction Enzyme used to cleave *Physarum* DNA	Weight(μg) of 15-25kb *Physarum* DNA added	Total no. of pfu $\times 10^{-5}$ *	No. of recombinant pfu $\times 10^{-4}$ *	Percentage recombinant phage	Number of recombinant phage per μg *Physarum* DNA $\times 10^{-5}$
-	0	22.4	0.28	-	-
Sau3A	0.2	16.8	4.44	2.67	2.24
Sau3A	0.4	16.0	6.72	4.2	1.68
Sau3A	1.0	14.0	20.1	14.36	2.01

* Total number of phage generated in packaging extract with final volume of 280 μl.

These results are in agreement with those described by Karn et al (1980) for the insertion of nematode DNA into λ1059.

The presumptive recombinant phage were amplified and stored as described in Methods Section 7(e).

ii) Restriction analysis of recombinant phage

In order to ensure that a comprehensive genomic library had been constructed it was decided to carry out restriction analysis of DNA isolated from recombinant phage.

Four recombinant phage were plaque purified and their DNA was isolated. After EcoRI digestion the restriction fragments were electrophoresed through a 0.7% (w/v) agarose gel (Fig 28). All restriction patterns were found to contain a small fragment of around 3 kb in length. This is caused by the EcoRI recognition site which is located very near to the end of the right 'arm' of λ1059. This shows that all phage were derived from the vector. EcoRI digestion of phage 1 DNA resulted in a restriction pattern very similar to the one obtained when λ1059 DNA was digested with the restriction enzyme. The restriction pattern obtained for phage 2 indicated that the DNA contained only one EcoRI recognition site. The DNA isolated from phage 3 had two recognition sites for EcoRI and three restriction fragments were obtained, one around 19-20 kb in length. EcoRI digestion of phage 4 DNA resulted in the production of a different restriction pattern which showed the presence of two EcoRI recognition sites within the phage DNA.

From the restriction patterns obtained for the four phage it was deduced that phage 1 and phage 2 may not have been recombinants. The pattern obtained for phage 2 might have been caused by the DNA being made of only the phage 'arms' of λ 1059. As viable phage were generated when

Figure 28. E.coRI digest of presumptive recombinant phage

Phage were taken from the amplified library and grown as a plate lysate (see Methods Section 4a.). A 5 ml liquid lysate was then prepared from 4 plaques (see Methods Section 4b) and the phage DNA was isolated as previously described. A sample of each DNA was digested with E.coRI and the restriction fragments were separated on a 0.7% (w/v) agarose gel.

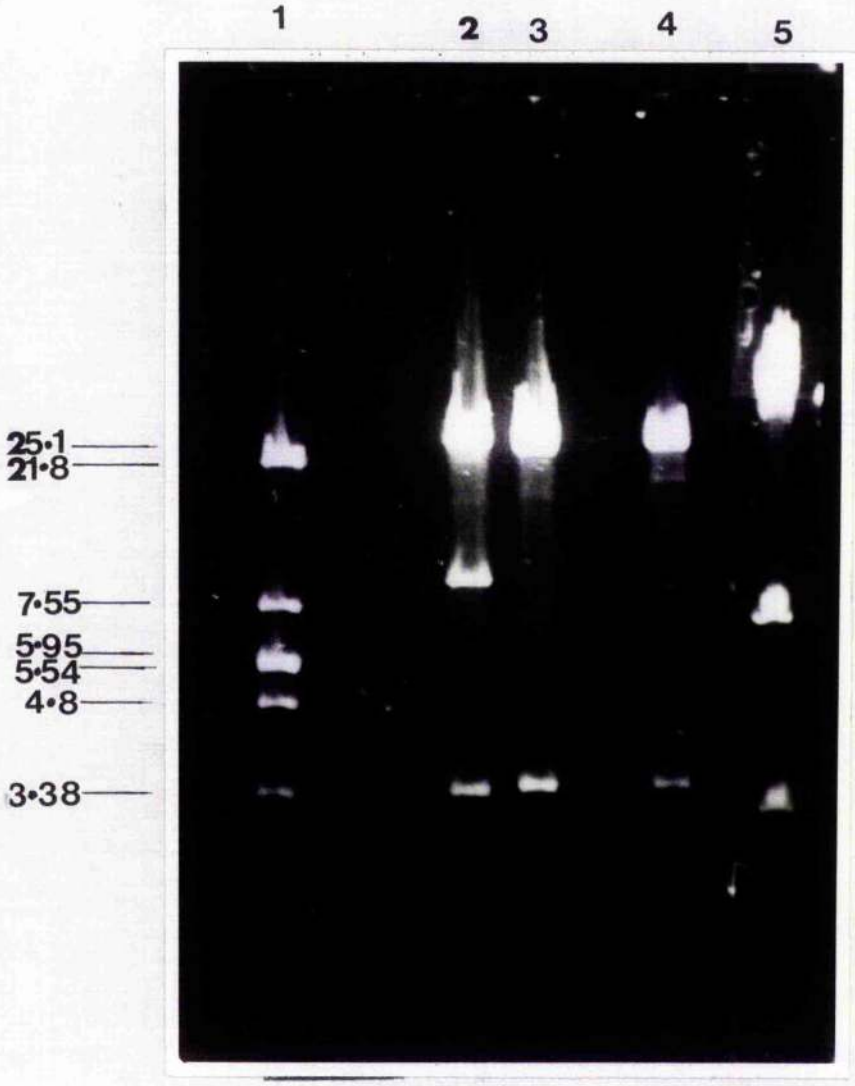
Lane 1 : E.coRI digest of λDNA (BRL) included as molecular weight markers (kb)

Lane 2 : E.coRI digest of phage 1 DNA

Lane 3 : E.coRI digest of phage 2 DNA

Lane 4 : E.coRI digest of phage 3 DNA

Lane 5 : E.coRI digest of phage 4 DNA


1
2
3
4
5
25·1
21·8
7·55
5·95
5·54
4·8
3·38

the 'arms' of Ch4AP had been ligated together it was important to establish if the same thing had happened when the library in λ1059 was made.

The phage DNAs were digested with BamH1 and the restriction fragments were separated through a 0.7% (w/v) agarose gel. In addition undigested phage DNA was also included in the gel (Fig 29). The undigested phage DNAs all migrated about the same distance into the gel as the marker λ DNA (BRL) indicating that they were all of similar molecular weight. A BamH1 digest of phage 1 DNA resulted in a restriction pattern different to the one generated when λ1059 DNA was digested with BamH1. This showed that phage 1 was indeed a recombinant phage and not regenerated λ1059. If phage 2 had been created by the ligation of two phage arms BamH1 digestion would have resulted in the production of two restriction fragments of similar molecular weight. This was not the case. BamH1 digestion of phage 2 DNA generated three fragments of around 8, 17 and 20 kb in length. Phage 3 was again confirmed as a recombinant phage.

These results showed that all four phage isolated were recombinant phage presumably carrying Physarum DNA. Phage 2 contained a fragment of foreign DNA which did not have an EcoRI recognition site.

Several more recombinant phage were isolated and their DNA was analysed by EcoRI digestion. A sample of the restriction patterns obtained is shown diagrammatically in Fig 30. Again all phage were derived from λ1059 as the 3 kb fragment was always generated.

The restriction patterns showed that all phage tested were recombinant phage. No parental phage were isolated and no phage were found which contained only phage arms.

iii) Hybridization of ^{32}[P]-'nick-translated' Physarum DNA against recombinant phage plaques

Figure 29. Restriction analysis of recombinant phage DNA to verify the presence of inserted DNA fragments

Phage DNA was isolated as described in the legend to Figure 28 The DNAs were digested with BamH1 and the restriction fragments were separated through a 0.7% (w/v) agarose gel. A BamH1 digest of λ phage DNA (BRL) was included on the gel as molecular weight markers.

Lane 1 : BamH1 digest of λ phage DNA (BRL) as molecular weight markers (kb)

Lane 2 : BamH1 digest of phage 1 DNA

Lane 3 : BamH1 digest of phage 2 DNA

Lane 4 : BamH1 digest of phage 3 DNA

Lane 5 : undigested phage 1 DNA

Lane 6 : undigested phage 2 DNA

Lane 7 : undigested λ phage (BRL)

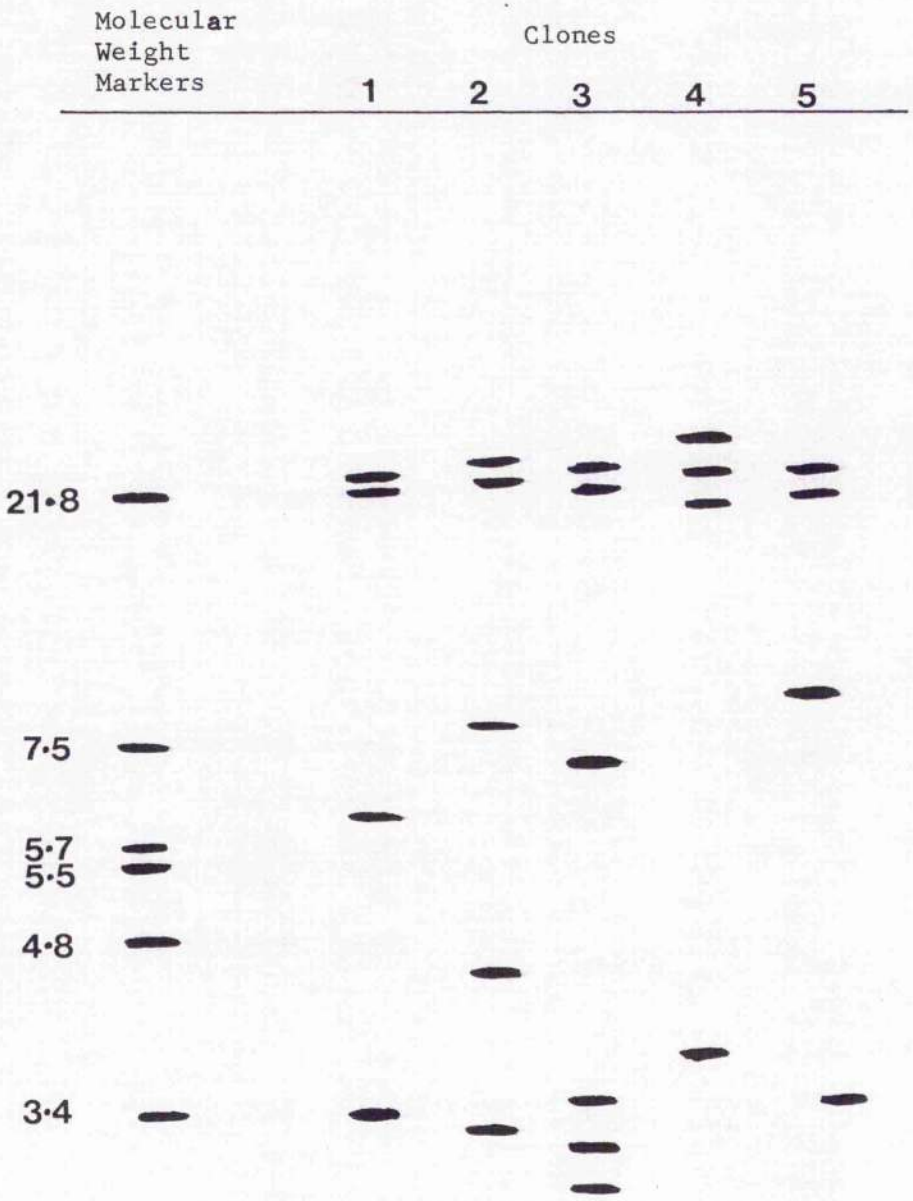
Lane 8 : undigested phage 3 DNA


1
2
3
4
5
6
7
8
49
17·4
7·3
6·6
5·54

Figure 30. Electrophoretic pattern of phage DNA after restriction endonuclease digestion

Recombinant phage DNA was isolated from mini lysates as described in Methods Section 5d. The DNAs were digested with E.coRI as described in Methods Section 6c.

The restriction fragments were separated by electrophoresis through a 0.7% (w/v) agarose gel.


Molecular Weight Markers
Clones
1
2
3
4
5
21·8
7·5
5·7
5·5
4·8
3·4

Another method that was used to verify the presence of Physarum DNA in the recombinant phage was to carry out hybridization reactions with ^{32}[P] nick-translated Physarum DNA against phage plaque replicas prepared by the method of Benton & Davis (1977).

^{32}[P] nick-translated λ DNA was used as a probe in several hybridization reactions against recombinant phage replicas to test the conditions used. Figure 31 shows a representative autoradiograph of plaque filter replicas hybridized to nick-translated λ DNA. A strong hybridization signal was obtained after 5 days exposure. During the course of this work it was noted that tryptone in the phage growth medium inhibited the DNA pick up from the phage plaques to the nitrocellulose filters.

^{32}[P] nick-translated Physarum DNA was used as a probe in hybridization reactions against plaque filter replicas. Examples of the autoradiographs obtained are shown in Figure 32 and Figure 33. The filter shown in Fig 32 was prepared from a plate on which recombinant phage had been grown to give a final plaque count of 5×10^3. Around 7% of the plaques gave a positive signal after 5 days exposure. When a recombinant phage titre of 5×10^2 per plate was used 10% of the plaques produced a positive hybridization signal (Fig 33). These percentages are low as around 20% of the phage plaques were expected to give positive hybridization signals after a fairly short exposure time due to the repeat sequences present in the Physarum genome (Peoples & Hardman, 1983). When the autoradiographs were studied closely 3 levels of hybridization signal could be seen, the very strong signal, the slightly weaker signal and the very weak positive signal which was just visible in the background. All of these signals could be aligned with phage plaques on the master plates.

Figure 31. Hybridization of [^{32}P] "nick-translated" λDNA against recombinant phage plaques

λ1059 was isolated as described in Methods Section 5a. 2 μg of λ1059 DNA was "nick-translated" as described in Methods Section 9a. Phage plaques were picked up onto a nitrocellulose filter as described in Methods Section 8a. These were then used to set up a hybridization reaction with conditions as described in Methods Section 10a. 10^5 cpm of [^{32}P] λDNA were used in the reaction.

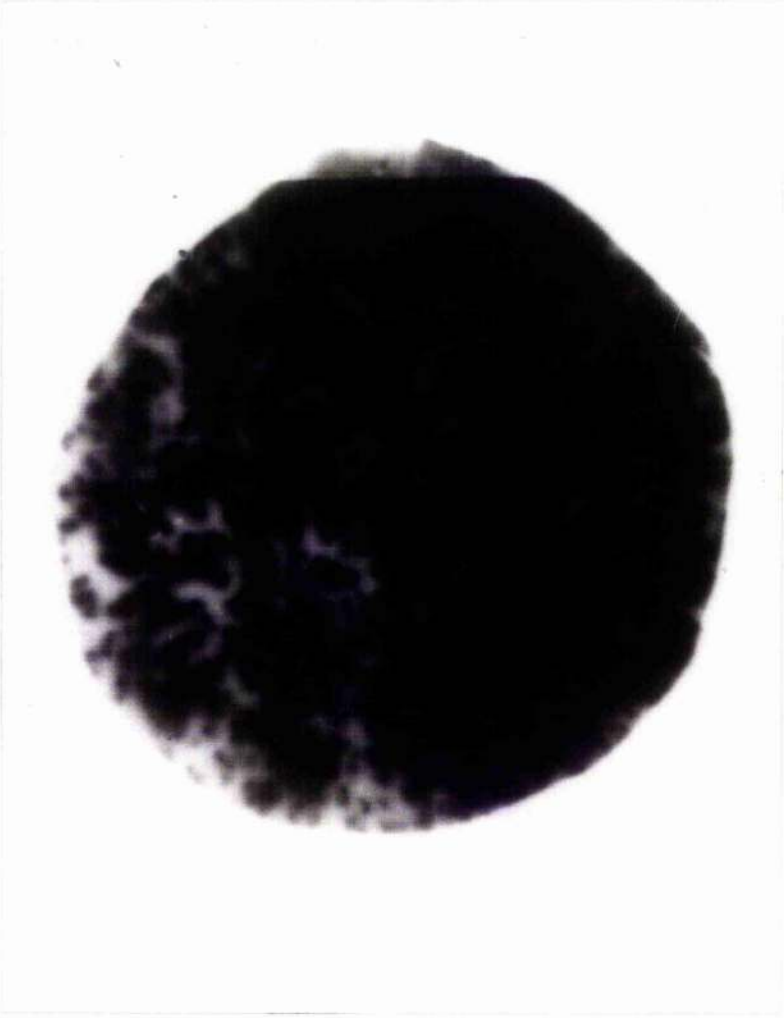


Figure 32. Hybridization of [^{32}P] "nick-translated" *Physarum* DNA against recombinant phage plaques

Physarum DNA was isolated as described in Methods Section 5b, Method 2. 5 µg was "nick-translated" as described in Methods Section 9a. Hybridization filters were prepared as previously described. Hybridization conditions were as described in Methods Section 10a. 10^5 cpm [^{32}P] *Physarum* DNA were used in the reaction.

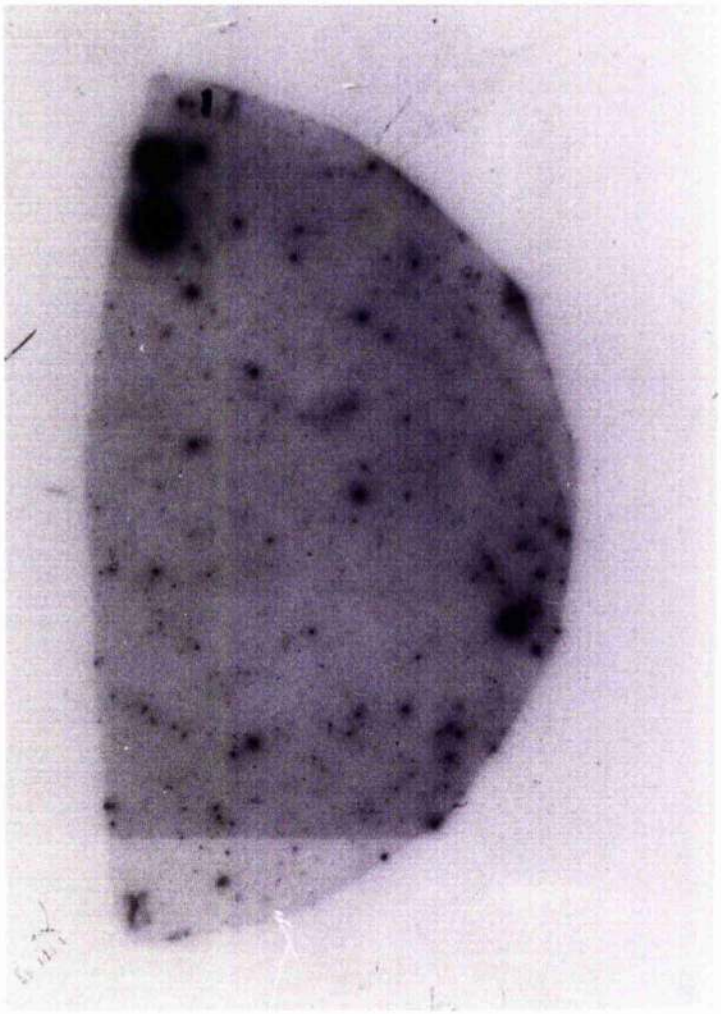
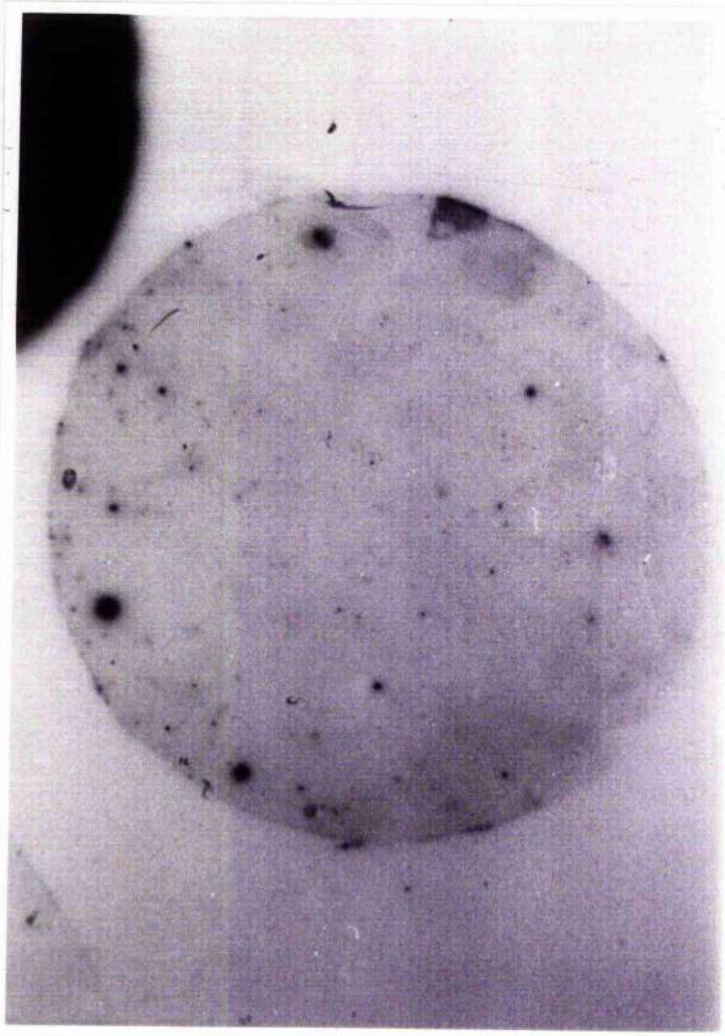


Figure 33. Hybridization of ^{32}[P] "nick-translated" *Physarum* DNA to recombinant phage plaques plated at 5×10^2 pfu per plate

The experimental procedure was as described in Methods Section 10a.



The cause of the differential hybridization reaction is unclear but it may be an indication that the washing conditions that were employed were too stringent.

k) Isolation of RNA from Physarum polycephalum

As a genomic library had apparently been successfully constructed it was necessary to isolate from the plasmodium the messenger RNA which would be used to direct the in vitro synthesis of the copy DNA (cDNA) which was to be used to screen the gene bank.

i) Polysome isolation

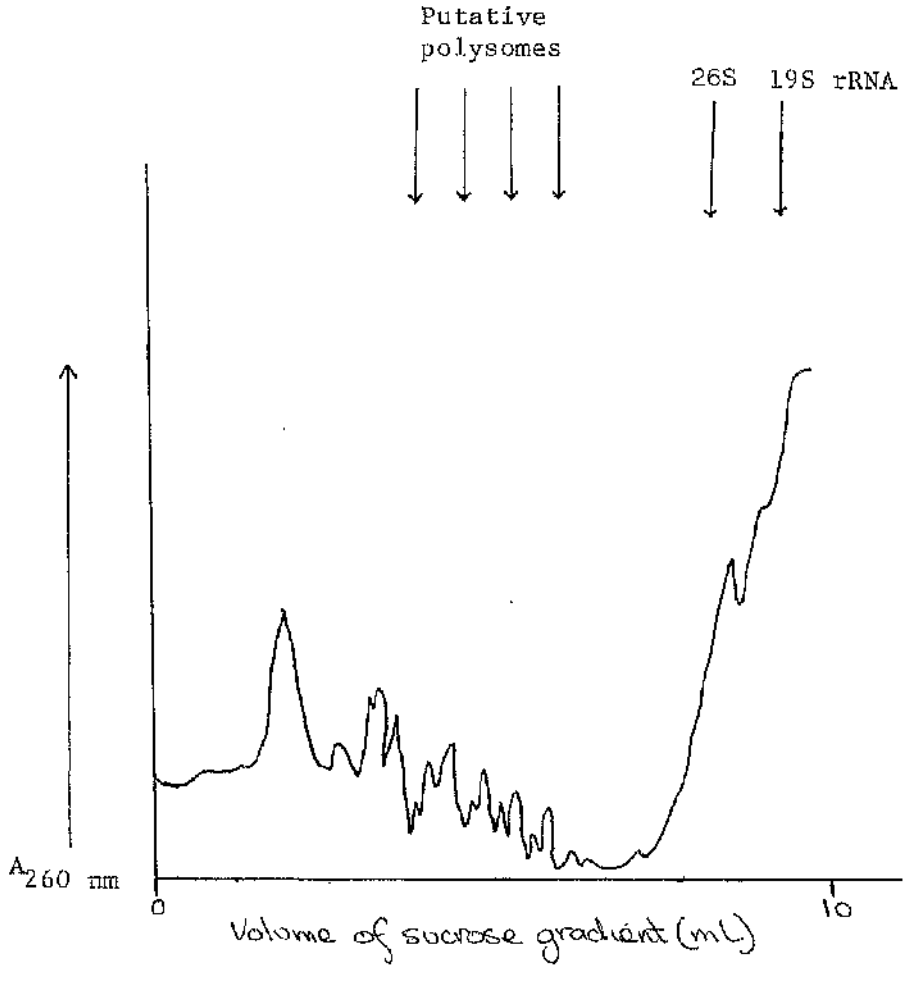
In order to isolate mRNA that was being actively translated at the time of isolation several attempts were made to isolate intact polysomes.

A) Jackson & Larkins (1976) described buffers which were shown to improve the isolation of intact polysomes from tobacco leaves. These buffers had both a high pH and a high ionic strength, two factors shown to be crucial for good polysome preparations. In addition, ethylene glycol bis(βamino ethyl ether)NN'tetraacetic acid (EGTA) was added to each buffer. EGTA is a divalent cation chelator with a high affinity for Ca^{2+} and Zn^{2+}, two metals known to activate RNase activity and in low concentrations to cause aggregation of polysomes.

These buffers were used to try and isolate intact polysomes from Physarum plasmodia. Plasmodia were scraped into the isolation buffer. 0.2 ml of the cytoplasmic fraction was then layered onto a sucrose gradient. After centrifugation the gradient was dripped and the samples were passed through a Uvicord with an automatic readout. The profile obtained is shown in Figure 34. This profile shows that there has been severe

Figure 34. Absorbance profile of polysomes isolated from a growing plasmodium

Polysomes were prepared as described in Methods Section 5c(i). The buffers of Jackson & Larkin (1976) were used in the isolation (see Appendix 5). No vanadylribonucleoside complex (VRC) or RNasin was added to the homogenising buffer.


Putative
polysomes
26S
19S rRNA
A_{260} nm
0
10
Volume of sucrose gradient (mL)

degradation of the polysomes during their isolation. Although polysomes appear to be present, the yield is poor. Vanadyl ribonucleoside complex (VRC) and RNasin, later used as inhibitors of ribonuclease activity, were not added to the buffer during this isolation procedure.

B) A second buffer used for the isolation of polysomes was described by Duncan & Humphreys (1981). This buffer had been found to be suitable for the isolation of sea urchin polysomes. The buffer has a lower pH than that described by Jackson & Larkins, but it again has a high ionic strength. The buffer was used to isolate polysomes from a growing plasmodium of P. polycephalum. EGTA was included in the buffer to chelate any divalent cations and VRC and RNasin were added to give a final concentration of 10 mM and 1000 units ml^{-1} respectively. The isolation procedure was the same as that described above. The polysome profile obtained is shown in Fig 35.

Again the isolation procedure has not been successful. It is very difficult to distinguish peaks which clearly represent polysomes within the gradient. It appeared that even in the presence of the two ribonuclease inhibitors, severe degradation of RNA occurred during the isolation procedure.

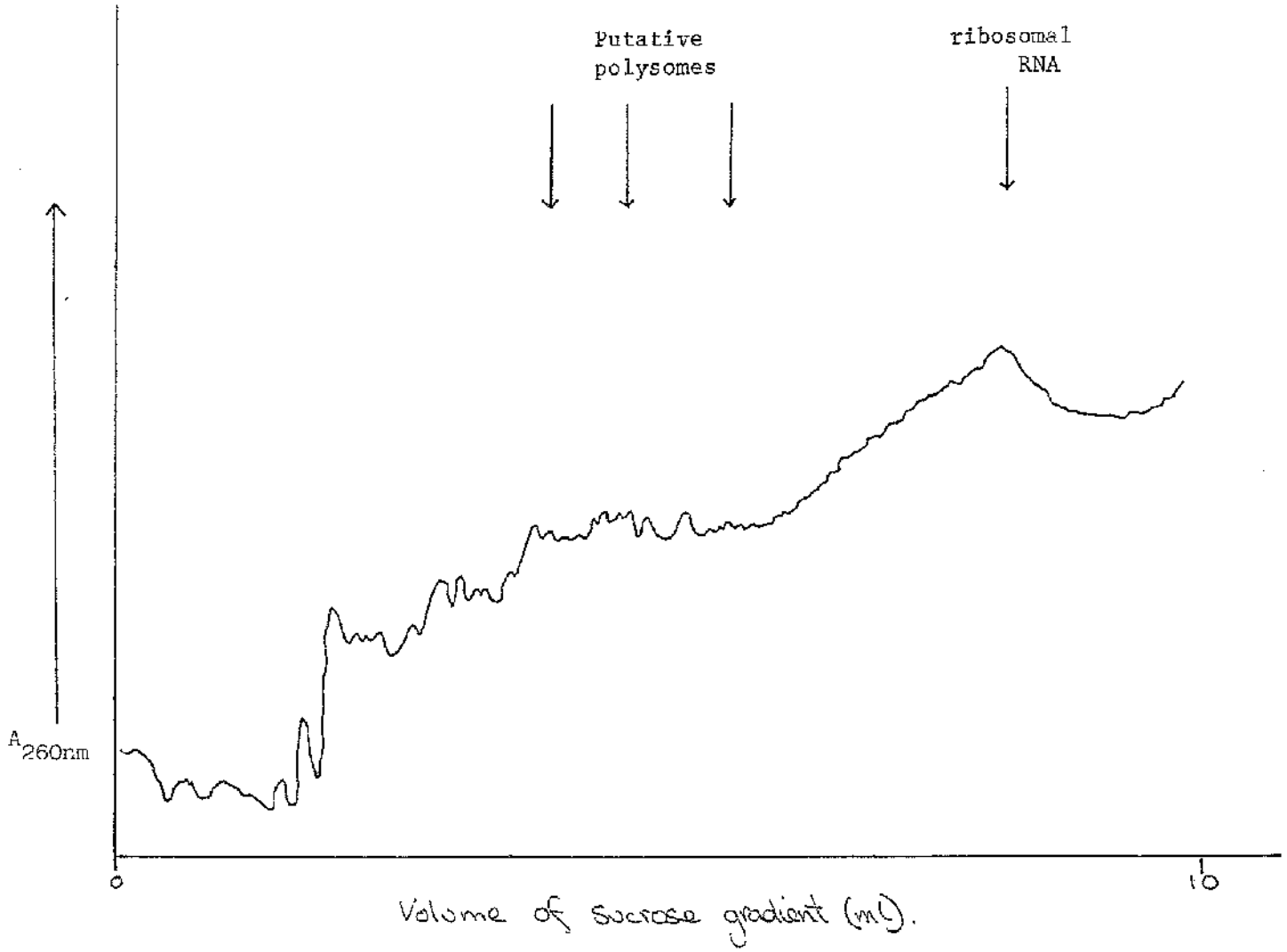
C) Adams et al (1980) reported the successful isolation of polysomes from the post-mitochondrial lysates of P. polycephalum. The buffer they described has both a low ionic strength and a low pH. It did however contain high levels of $MgCl_2$ and EGTA. When this buffer was used to try and isolate polysomes from plasmodia of P. polycephalum CL no profiles were obtained (which indicated the presence of intact polysomes).

This lack of success in preparing in good yield intact polysomes from the plasmodia of Physarum prevented the preparation of RNA

Figure 35. Absorbance profile of polysomes isolated from a growing plasmodium in a buffer with a high ionic strength and neutral pH

Polysomes were isolated as previously described. The buffer of Duncan & Humphreys (1981) (see Appendix 5) was used in the isolation.

VRC and RNasin were added to the homogenising buffer to give final concentrations of 10 mM and 1000 U ml^{-1} respectively.


Putative
polysomes
ribosomal
RNA
A260nm
0
10
Volume of Sucrose gradient (ml).

that was being translated at the time of preparation. It was therefore decided to try and isolate total cytoplasmic RNA (extranuclear RNA) as the next best choice.

ii) Cytoplasmic RNA isolation

A) The initial stage of isolation of cytoplasmic RNA was the same as that used to isolate polysomes. After the removal of the nuclear material the cytoplasmic fraction was extracted with a 1:1 mixture of phenol/chloroform. The phenol/chloroform extraction was repeated 3 times and the RNA was ethanol precipitated. The RNA was then treated with SDS and proteinase k to remove any contaminating protein. The RNA was re-extracted with the phenol/chloroform mixture then ethanol precipitated once more.

Cytoplasmic RNA was isolated by this method using the buffers of Jackson & Larkins (1976) and Adams et al (1980). A sample of each RNA was separated through a formaldehyde denaturing gel (Fig 36, Lanes 1 & 2). In each case the RNA was unable to penetrate the gel and stayed in the wells. This suggested that the RNA was contaminated with an impurity which was not removed during the treatments described above.

B) Cetyltrimethylammonium bromide precipitation

Nucleic acids isolated by phenol extraction are often found to be contaminated with polysaccharides, sugar phosphates or nucleoside mono-di and triphosphates so that a further purification step is necessary. In order to eliminate these materials from the nucleic acid, a cetyltrimethylammonium bromide precipitation was carried out (Bellamy & Ralph, 1968). This involves the precipitation of the nucleic acids from a dilute sodium chloride solution as cetyltrimethylammonium salts.

Figure 36. Cytoplasmic RNA preparations

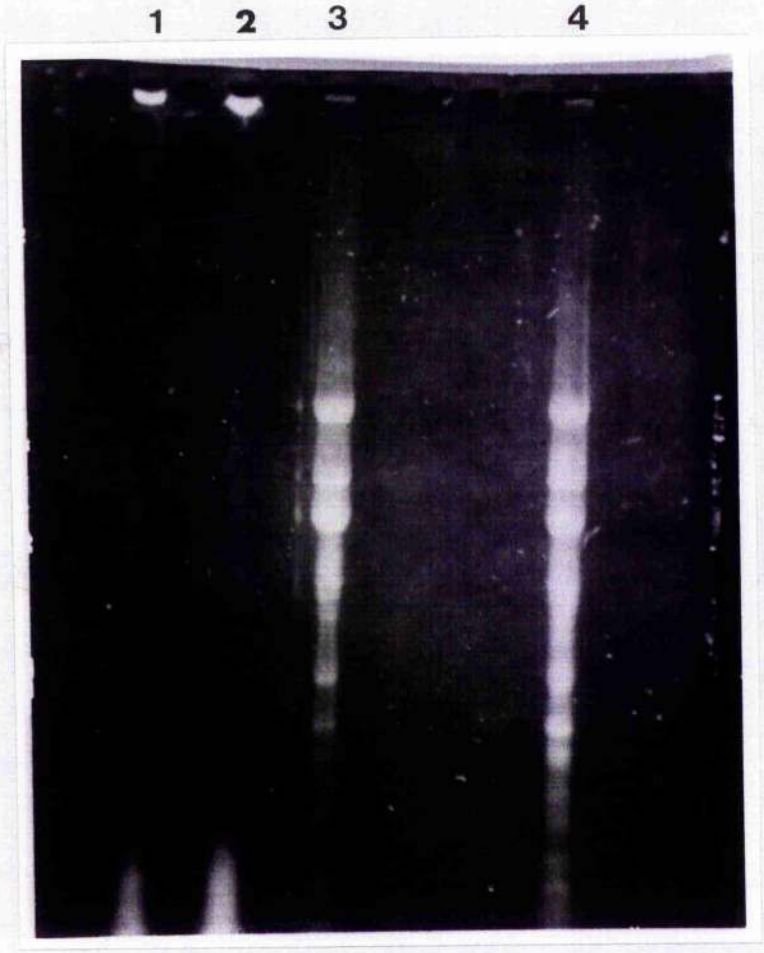
Cytoplasmic RNA was isolated as described in Methods Section 5c(i). RNA was isolated using the buffers of Jackson & Larkins (1976) and Adams *et al* (1980) (see Appendix 5). The RNA was further treated with a CTAB precipitation (see Methods Section 5c(i)) and the samples were electrophoresed on a formaldehyde gel as described in Methods Section 5e.

Lane 1 : RNA isolated in Jackson & Larkins buffer
No CTAB precipitation.

Lane 2 : RNA isolated in Adams *et al* buffers.
No CTAB precipitation.

Lane 3 : RNA isolated in Jackson & Larkins buffers
plus a CTAB precipitation.

Lane 4 : RNA isolated in Adams *et al* buffers
plus a CTAB precipitation.


1
2
3
4

As Physarum is known to contain a large amount of polysaccharide material a CTAB precipitation was carried out on the RNA isolated in the buffers of Jackson & Larkins (1976) and Adams et al (1980). After the CTAB precipitation a sample of RNA was separated through a formaldehyde denaturing gel. An example of the pattern obtained is shown in Fig 36, Lanes 3 and 4. The CTAB precipitation has allowed the samples to be separated through the formaldehyde gel. There are many low molecular weight degradation produces of RNA present in both preparations and it is not clear which bands, if any, represent the 26S and 19S ribosomal RNA that should be visible under those conditions. There is not a great deal of difference between the RNA preparations obtained using either buffer, although the buffer of Adams et al (1980) apparently gave the greatest degradation.

iii) Polysomes isolated through a sucrose cushion

In order to try and minimize this degradation an attempt was made to prepare polysomal RNA by pelleting the polysomes through a 1.7m sucrose cushion. After the centrifugation step, the polysomes were treated with SDS + proteinase k. The RNA was then phenol/chloroform extracted, alcohol precipitated and then CTAB precipitated.

As the RNA isolated in Jackson & Larkins buffer showed the least signs of degradation the same buffer was used here. Two preparations were isolated, one in the presence of VRC and RNasin, the other in the presence of VRC alone. Samples of the RNA were separated through a formaldehyde denaturing gel and the results are shown in Fig 37. Also included as a size marker was an EcoRI digest of pAT153 (Twigg & Sherratt, 1980). This DNA (kindly supplied by Dr. J. Milner, Botany Department, Glasgow University) has a single EcoRI site and produces a linear molecule of 24.5S (See Fig 38).

Figure 37. RNA isolated from polysomes pelleted through a sucrose cushion

Polysomal RNA was prepared by pelleting the RNA through a 1.7M sucrose cushion as described in Methods section 5c(i). RNA was prepared in Jackson & Larkins (1976) buffers plus VRC and RNasin, and Jackson & Larkins (1976) buffers plus VRC alone.

Samples were electrophoresed on a formaldehyde gel as previously described.

Lane 1 : Jackson & Larkins buffer plus VRC and RNasin.

Lane 2 : Jackson & Larkins buffer plus VRC.

Lane 3 : EcoRI digest of plasmid pAT153 DNA (see Fig 38).

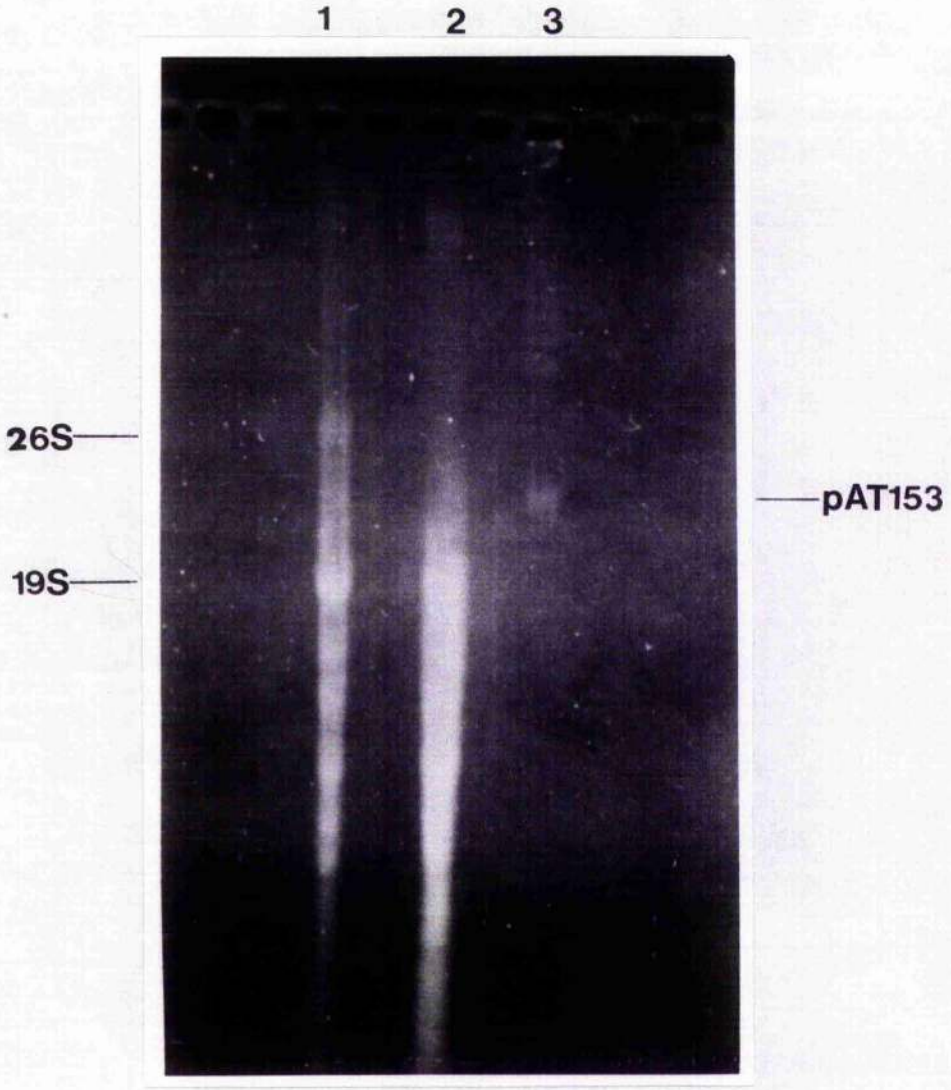
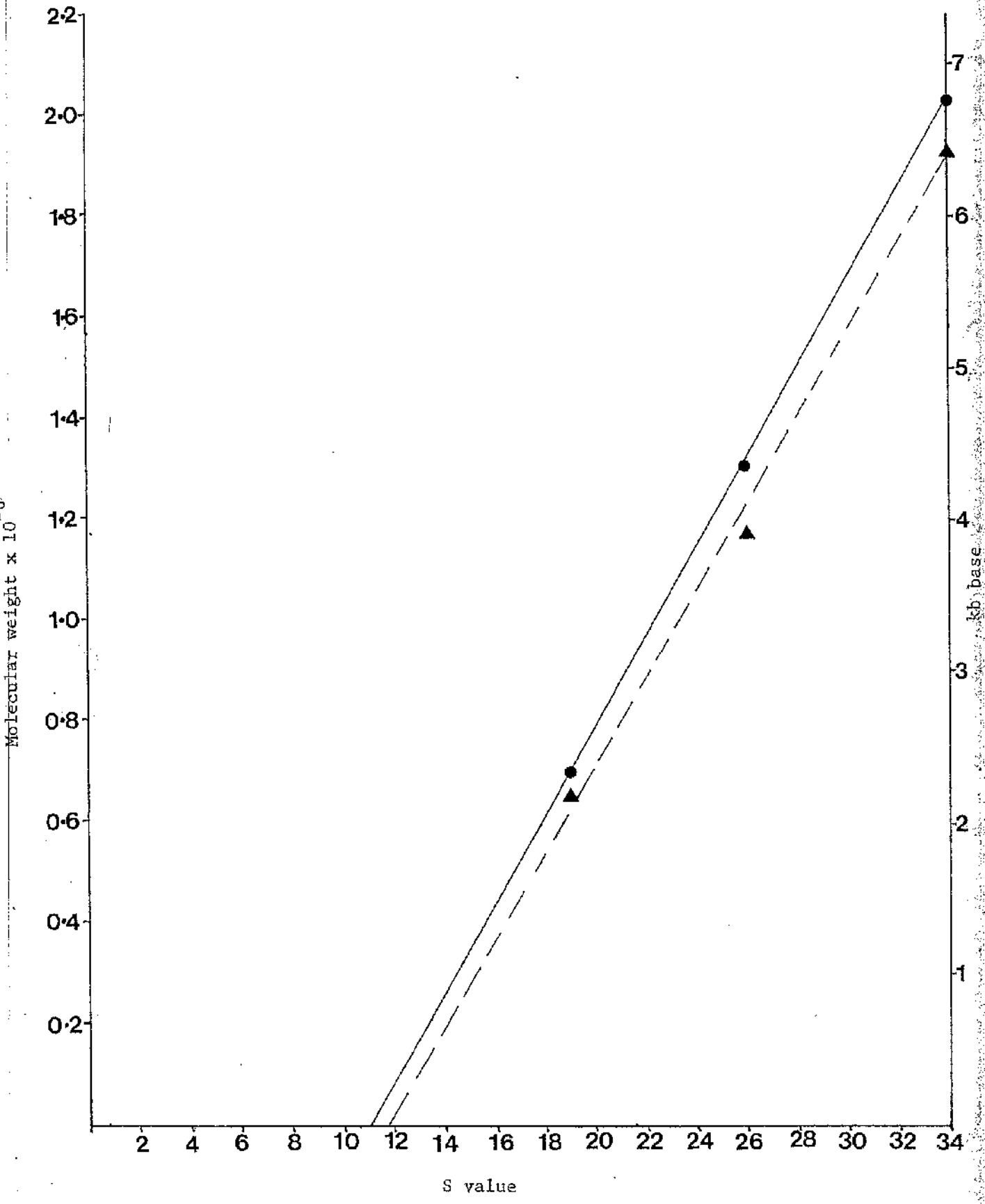

1
2
3
26S
19S
pAT153

Figure 38 Calculation of the S value of pAT153

The length and S value of known nucleic acids were used to draw the figure. TMV (Tobacco Mosaic Virus) has a length of 6.4 kb and an S value of 34. The 26SrRNA of Physarum polycephalum has a length of 3.9 kb and the 19SrRNA is 2.1 kb long. From the graph pAT153 has an S value of 24.5 as it is 3.6 kb long.

● : Molecular weight versus S value

▲ : Length versus S value.


2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Molecular weight x 10^{-6}
7
6
5
4
3
2
1
kb base
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
S value

When VRC and RNasin were both present during the isolation the 26S and 19S ribosomal RNAs can be seen but many low molecular weight bands are also present. In the presence of VRC alone degradation of the RNA has been more extensive. More degradation of the RNA has occurred during this preparation than during the cytoplasmic RNA preparation where the phenol/chloroform extraction of the homogenate was carried out immediately.

It appeared that even using EGTA, VRC and RNasin to minimize RNase activity a substantial amount of degradation of the RNA still occurred. An alternative method of RNA preparation was therefore sought.

iv) Total RNA preparation

A) Guanidine thiocyanate is known to inactivate ribonuclease and to disrupt cellular and subcellular structures (Chirgwin et al, 1979) and so should be suitable for use in isolating RNA.

A buffer containing 4M guanidine thiocyanate was used to isolate the total RNA from a growing plasmodium. Two total RNA preparations were carried out in parallel but only one included an SDS + proteinase k treatment. In each case, after the initial ethanol precipitation step, the RNA was CTAB precipitated.

Two samples of each total RNA preparation were separated through a formaldehyde denaturing gel. In order to compare the cytoplasmic and total RNA preparations samples of cytoplasmic RNA isolated in the buffer of Duncan & Humphreys (1981) were included in the gel. The results are shown in Fig 39.

The SDS + proteinase k treatment did not seem to be beneficial as the patterns obtained for RNA isolated with and without this treatment were essentially identical. There were more RNA bands visible when the

Figure 39. A comparison of a total RNA preparation and a cytoplasmic RNA preparation

Cytoplasmic RNA was isolated as described previously using Duncan & Humphreys buffers (1981).

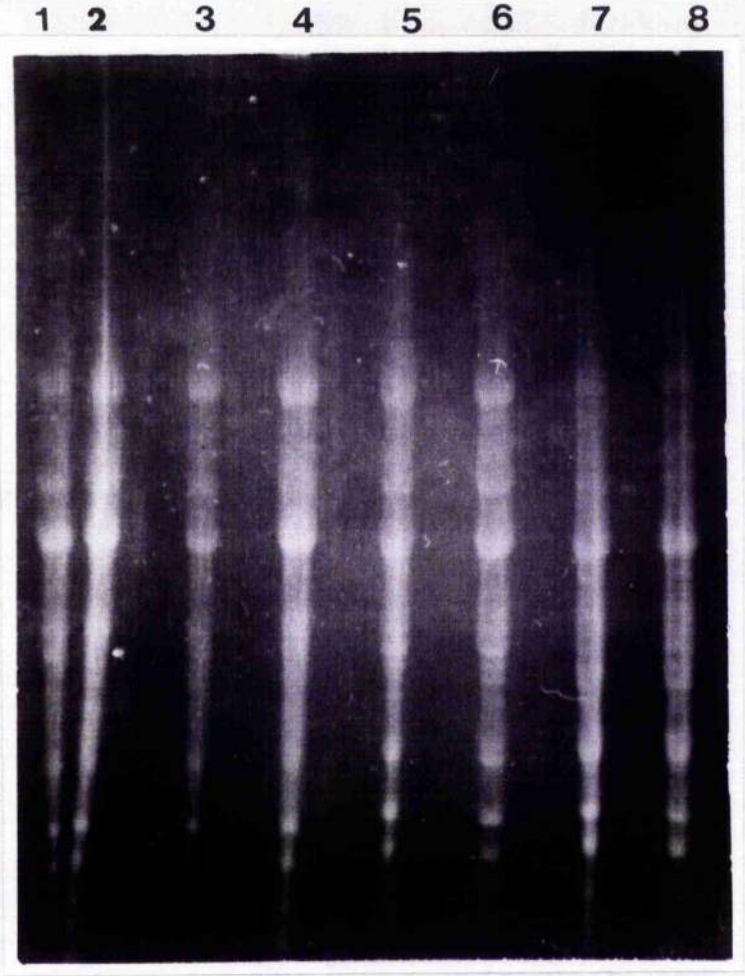
Total RNA was isolated as described in Methods Section 5c(ii). One preparation of total RNA was treated with SDS + protease k (see Methods 5c(i)) before ethanol precipitation and CTAB precipitation. Samples were separated on a formaldehyde gel as previously described.

Lanes 1 & 2 : Guanidine thiocyanate total RNA preparation (no protease k treatment)

Lanes 3 & 4 : Guanidine thiocyanate total RNA preparation + protease k treatment

Lanes 5 & 6 : Cytoplasmic RNA preparation in Duncan & Humphreys buffers + VRC + RNasin

Lanes 7 & 8 : Cytoplasmic RNA preparation in Duncan & Humphreys buffers + RNasin.


1 2 3 4 5 6 7 8

cytoplasmic RNA was separated through the formaldehyde gel which indicated that the guanidine thiocyanate was preventing some degradation of the RNA. It is interesting to note that the presence of VRC in the Duncan & Humphreys buffer had little effect in stopping degradation as the separation patterns in Lanes 5 and 6 are essentially the same as Lanes 7 and 8. Isolation of cytoplasmic RNA in the Duncan & Humphreys (1981) buffer, containing VRC and RNasin led to a separation pattern that was very similar to that obtained when RNA that had been isolated in Jackson & Larkins (1976) buffer, containing both inhibitors, was separated through a formaldehyde denaturing gel (Fig 37, Lane 1).

A direct comparison of these three methods of preparation was attempted and the results are shown in Fig 40. The total RNA preparations, using the guanidine thiocyanate buffer, showed less degradation than the total RNA preparations shown in Fig 40. Again the separation pattern of the cytoplasmic RNA prepared in Jackson & Larkins (1976) buffer containing both inhibitors was similar to the pattern obtained with cytoplasmic RNA prepared in Duncan & Humphreys (1981) buffer plus both inhibitors (Fig 39, Lanes 5 & 6). The cytoplasmic RNA preparation using Duncan & Humphreys buffer (1981) plus VRC and RNasin gave a poor yield and so it is difficult to see anything clearly in this particular case (Lane 5). A degradation product slightly longer than the 19S RNA molecule was present in the RNA preparations.

B) A second buffer containing a high concentration of guanidine was more recently described by Cox & Smulian (1983). This lysis buffer contained 6M guanidine HCl, and was used to isolate total RNA from growing plasmodia.

Figure 40. A direct comparison of total RNA and cytoplasmic RNA preparations

Total RNA was isolated from plasmodia as described in the legend to figure 39. Protease k treatment was carried out before ethanol precipitation and CTAB precipitation.

Cytoplasmic RNA was isolated as described previously.

Samples were electrophoresed on a formaldehyde gel as previously described.

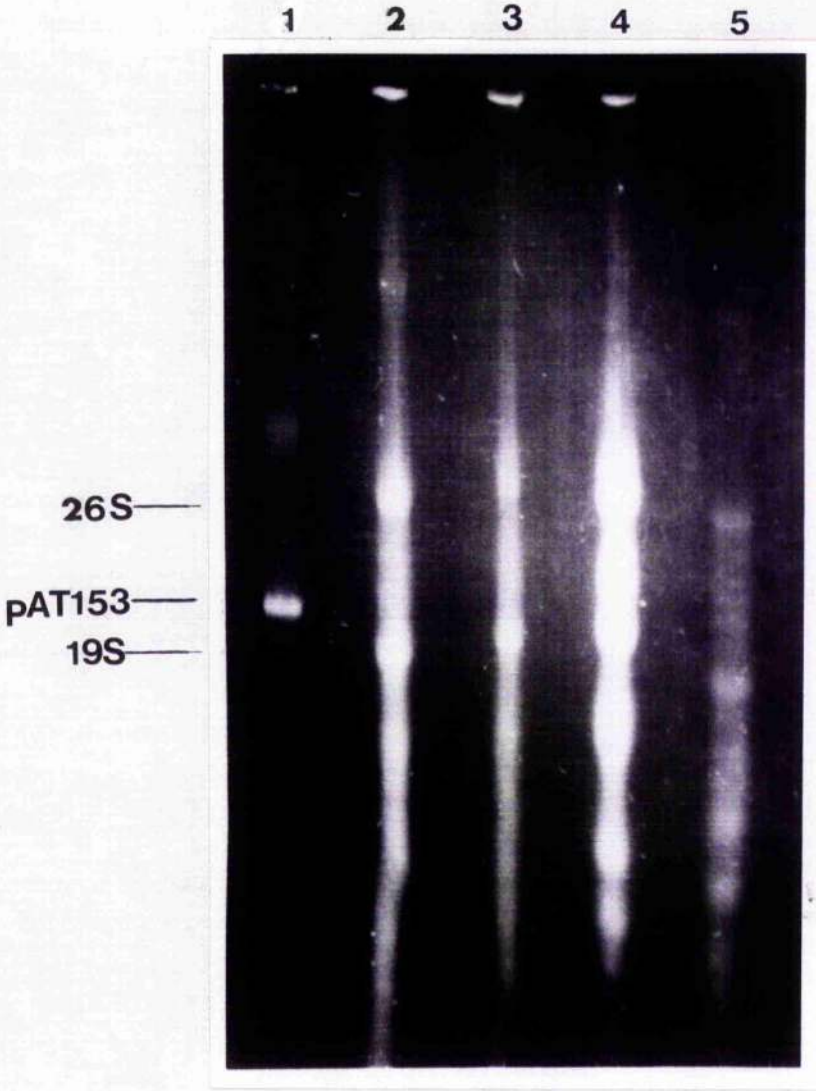
Lane 1 : EcoRI digest of pAT153 DNA

Lane 2 : Total RNA preparation

Lane 3 : Total RNA preparation

Lane 4 : Cytoplasmic RNA prepared in Jackson & Larkins buffers + VRC + RNasin

Lane 5 : Cytoplasmic RNA prepared in Duncan & Humphreys buffers + VRC + RNasin.


1
2
3
4
5
26S
pAT153
19S

A sample of the RNA was separated through a formaldehyde denaturing gel (Fig 41). The 19S and 26S ribosomal RNA bands are clearly visible, but there is still evidence of RNA degradation occurring during the isolation procedure. Again the degradation product slightly longer than the 19S RNA was present.

The total RNA isolated using the guanidine thiocyanate buffer shows the least amount of degradation of the RNA during the procedure.

v) Preparation of the copy DNA hybridization probe

Polyadenylate containing [Poly(A)$^+$] RNA was isolated from the total RNA preparation (isolated in the guanidine thiocyanate buffer) by oligo-deoxythymidylate cellulose chromatography (see Methods Section 5c(iii)). This poly(A)$^+$ RNA was then used to direct the synthesis of copy DNA (cDNA) in vitro. The reaction was carried out in a total volume of 20 µl and contained RNasin, a 0.5 mM pool of nucleotides, 10-15 µCi [^{32}P] dCTP, oligo(dT)$_{12-18}$ as a primer for DNA synthesis and 100 units of RNA dependent reverse transcriptase. After hydrolysis of the poly(A)$^+$ RNA at 65^{o}C for 1 h, the cDNA was purified by chromatography on Sephadex G-100. A typical elution profile is shown in Fig 42. The first peak represents the cDNA, the second peak is due to the unreacted radionucleotides.

The cDNA had a specific activity of 5.2 x 10^3 cpm per µg of cDNA and when sized on an agarose gel was found to consist of very short DNA fragments.

When the cDNA was tested in a hybridization reaction against whole Physarum DNA a very weak positive hybridization signal was obtained.

The specific activity of the cDNA was very low as values as high as 10^6-10^7 cpm µg^{-1} DNA have been reported. The length of cDNA prepared was very short as mRNA in Physarum has been shown to be as long as 1100 nucleotides (Brown & Hardman, 1980) and cDNA of 400-500 nucleotides in length has been reported (Timberlake, 1980).

Figure 41. Total RNA preparation using guanidine HCl lysis buffer

Plasmodia were lysed in guanidine HCl buffer described by Cox & Smulian (1983) (seeAppendix 5). The total RNA was isolated as described previously. Samples were separated on a formaldehyde gel as previously described.

Lane 1 : EcoRI digest of pAT153

Lane 2 : Total RNA isolated in guanidine HCl buffer

Lanes 3 & 4 : Dilutions of total RNA preparation.

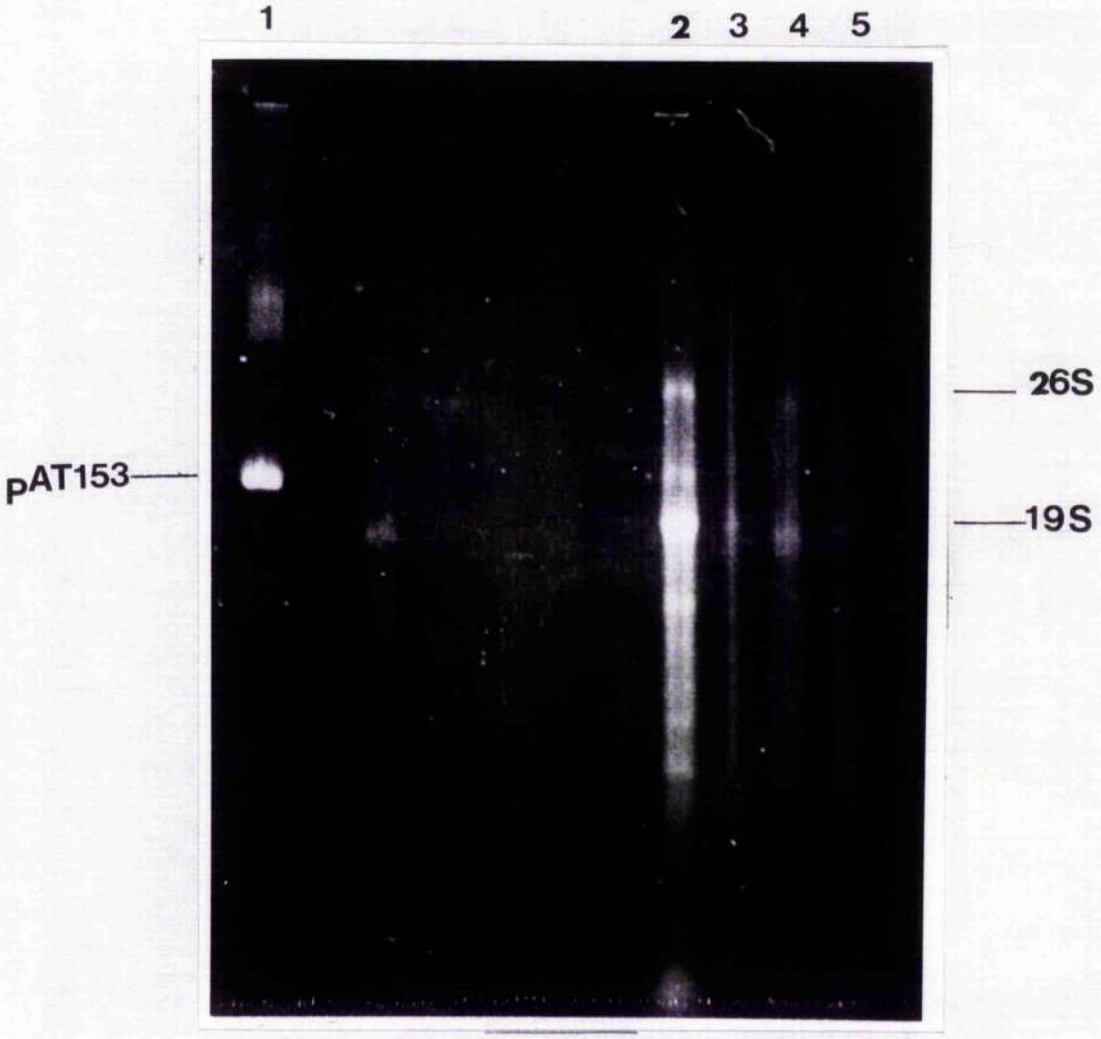
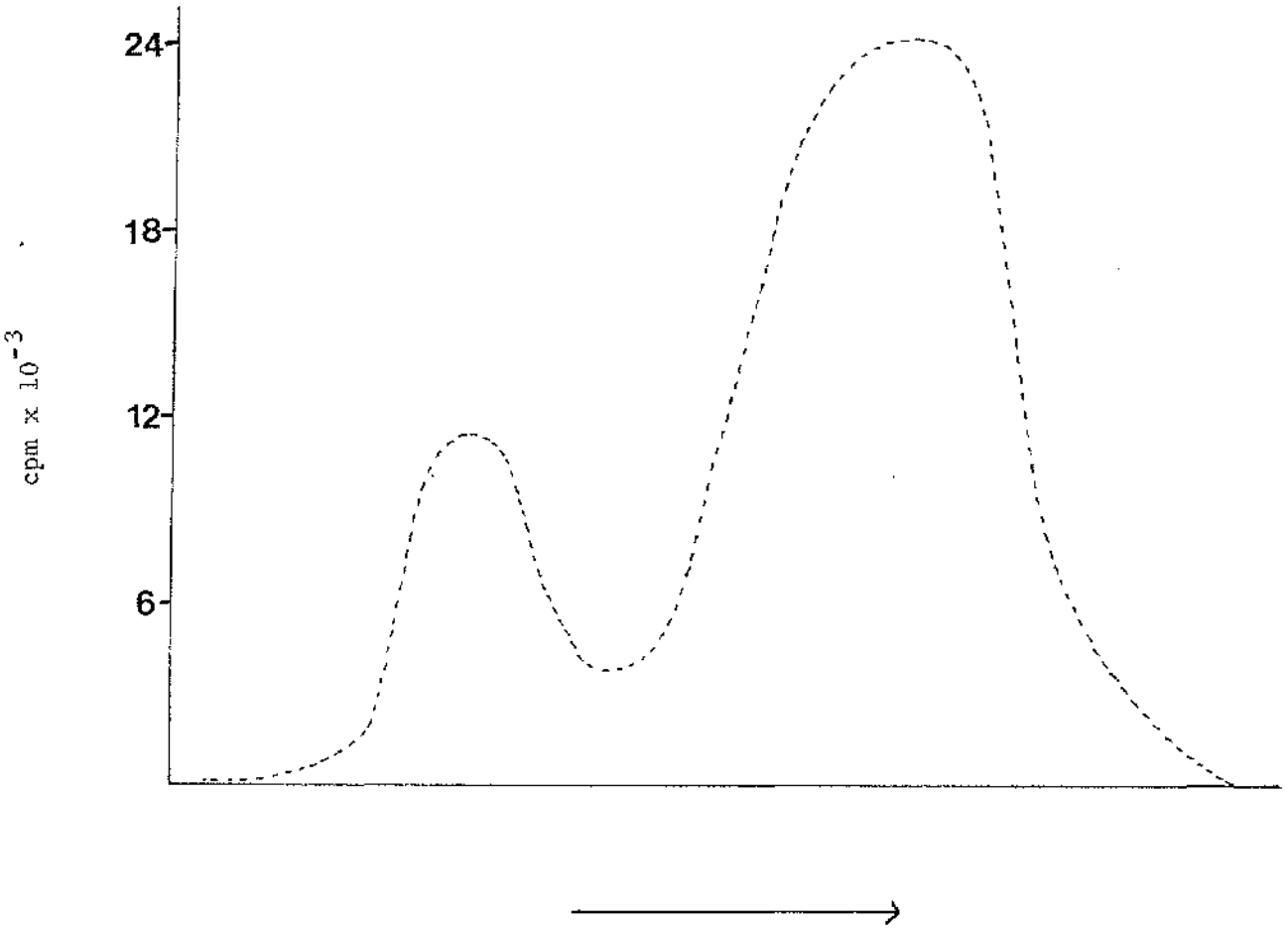

1
2
3
4
5
pAT153
26S
19S

Figure 42. Elution profile of copy DNA from a Sephadex G-100 column

Copy DNA was prepared as described in Methods Section 8b(i), using poly(A)$^+$ RNA isolated by oligo (dT) cellulose chromatography as a template. The cDNA was isolated by passage through a Sephadex G-100 column (see Methods Section 9b(ii)).


24
18
12
6
cpm x 10^{-3}
Fraction number

One problem in the cDNA synthesis may be caused by the presence of shortened poly(A)$^+$ RNA due to degradation. It was shown that the RNA isolated had undergone a substantial amount of degradation so an alternative isolation method was attempted.

vi) Direct isolation of poly(A)$^+$ RNA

Cox & Smulian (1983) described a single step procedure for the isolation of poly(A)$^+$ RNA from crude plasmodial lysates. The method involves lysing the plasmodium in a buffer containing 6M guanidine HCl. The poly(A)$^+$ RNA is then isolated directly from the lysate by the affinity of the poly(A)$^+$ tails for poly(U) linked to a sepharose bead.

The method was used to isolate poly(A)$^+$ RNA from growing plasmodia of P. polycephalum. After the initial precipitation from the elution buffer the poly(A)$^+$ RNA was extracted with a 1:1 mixture of phenol/chloroform and ethanol precipitated once more.

The poly(A)$^+$ RNA was used to direct the synthesis of cDNA in vitro. After isolation by sepharose G-100 chromatography a sample of the cDNA was separated through a 1.5% (w/v) agarose gel. A HaeIII digest of ØX174 DNA (BRL) was included as molecular weight markers. After staining with ethidium bromide the positions of the molecular weight markers were noted and the gel was then exposed against an Xray film. The result is shown in Fig 43.

This showed that cDNA of various lengths had been made. The majority of the cDNA fragments ranged from 200 to around 500 nucleotides in length. The specific activity of the cDNA was around 10^4 cpm μg^{-1} DNA. The poly(A)$^+$ RNA was used to direct the synthesis of cDNA in several experiments. An analysis of each synthesis is shown in Table 9.

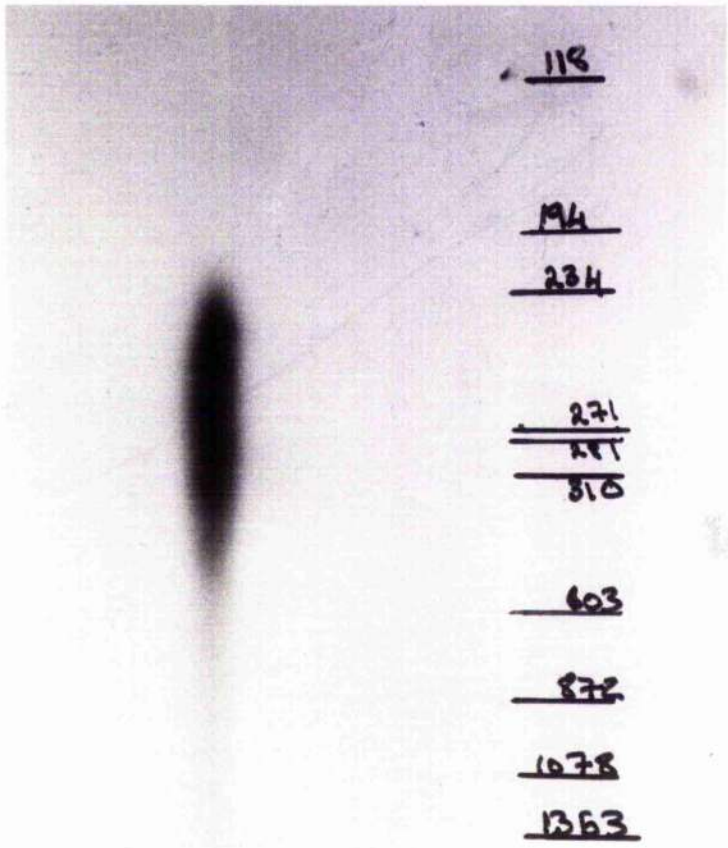
Figure 43. Sizing of copy DNA prepared using poly(A)$^+$ RNA isolated directly from a plasmodial lysate

Physarum polycephalum poly(A)$^+$ RNA was isolated as described in Methods Section 5c(iv)).

After the initial precipitation from the elution buffer the poly(A)$^+$ RNA was phenol/chloroform extracted and precipitated once more.

The cDNA was prepared as described previously and 20,000 cpm were taken and the cDNA was separated on a 1.5% (w/v) agarose gel alongside a HaeIII digest of ØX174 RF DNA (BRL) as molecular weight markers (bp). The position of the marker DNA was noted and the gel was then placed in 2 changes of 7% TCA for 30 min before being placed against an Xray plate.
The exposure was for 48 h.

Molecular weight markers (bp) from agarose gel.



Direction of electrophoresis

Table 9. Effect of various treatments of poly(A)$^+$ RNA on the weight of cDNA made and the percentage of poly(A)$^+$ RNA copied

Treatment of poly(A)$^+$ RNA	Percentage incorporation of radio-active label	Weight of cDNA made (ng) *	Percentage of poly(A)$^+$ RNA copied **
Total RNA preparation then oligo(dT) cellulose chromato-graphy	0.2	26.4	1.76
	0.7	92.4	2.16
	0.5	66	4.4
	0.3	39.6	2.64
Poly(U) Sepharose	13.9	1834.8	39.8
	5.3	708.2	26.3
	8.9	1174	43.5
	2.0	264	48.8
Poly(U) Sepharose then oligo(dT) cellulose	1.99	262.68	13.2
	1.48	196.17	9.5
	1.85	243.8	11.8
	2.5	330	15.1
Poly(U) Sepharose + CTAB precipitation	1.65	218.16	12.12
	1.23	162.6	9.04
	0.8	105.9	5.8
	6.12	807.2	33.6
	4.04	533.4	23.8
Poly(U) Sepharose + CTAB + phenol/ chloroform extraction	3.76	496.74	22.35
	6.2	818.4	34.1
	2.8	369.6	15.4
	2.7	364.4	20.24

*Calculated using the equation:-

$$\text{wt of cDNA made} = \text{fraction of radiolabel incorporated} \times \text{conc. of dCTP} \times \text{wt of a nucleotide} \times 4$$

**Calculated using the equation:-

$$\text{Percentage of poly(A)}^+ \text{ copied} = \frac{\text{wt of cDNA made}}{\text{wt of poly(A)}^+ \text{ RNA added}} \times \frac{100}{1}$$

The incorporation of the radionucleotide added to each reaction varied from 2.0 to 13.9%. As a result the weight of cDNA made varied from 264 ng to 1834.8 ng. The percentage of the poly(A)$^+$ RNA copied ranged from 26.3 to 49. These values are very high as a value of between 15 and 20% is normal. From the sizing gel it appeared that a large proportion of the cDNA made was short copy having lengths of only a few hundred nucleotides.

This cDNA hybridized to Physarum DNA but very weak positive signals were obtained.

Disturbance of the plasmodium during the initial stages of isolation of the RNA, eg by scraping into the lysis buffer, had been found to cause a considerable amount of degradation of the RNA (T.H. Schrekenbach, personal communication). In all the experiments described so far the plasmodium had been scraped from the filter paper into the buffer used and it would seem likely that this will have caused activation of RNase activity. This degradation would mean that the poly(A)$^+$ RNA used to direct the synthesis of the cDNA was short which would lead to short cDNA being made as was shown on the sizing gel.

1) In situ lysis of the plasmodium

Here the plasmodia were lysed in situ by plunging the plasmodia + filter paper directly into the lysis buffer. After the initial isolation of the poly(A)$^+$ RNA no further purification step was carried out. The poly(A)$^+$ RNA was used to direct the synthesis of cDNA in vitro, a sample of which was sized on a 1.5% agarose gel. The result is shown in Fig 44. The cDNA made in this synthesis ranged in length from just under 100 nucleotides to just over 310 nucleotides. This cDNA was in fact shorter than the cDNA made on previous occasions.

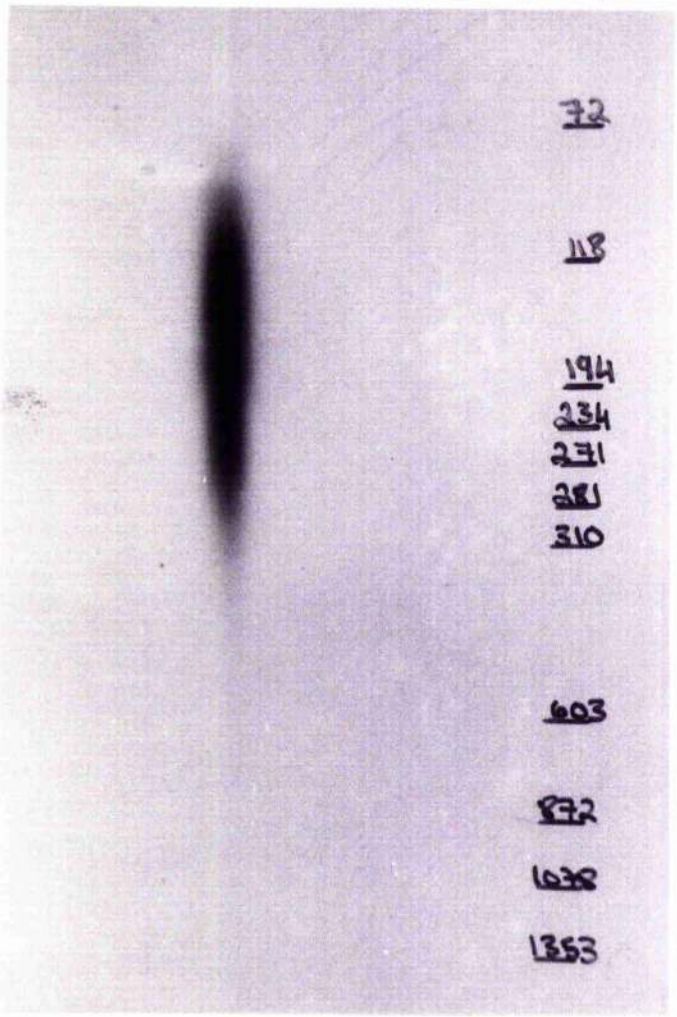
Figure 44. Sizing of cDNA prepared using poly(A)+ RNA isolated from a plasmodium lysed in situ

Poly(A)$^+$ RNA was isolated as described in Methods Section 5c(iv) except that the plasmodium was lysed in situ. This was done by plunging the plasmodium into lysis solution while still on the supporting filter paper.

No further purification steps were carried out after the initial precipitation from the elution buffer.

The experimental procedure was as described in the legend to figure 43.

Molecular weight markers (bp) from agarose gel.



Direction of electrophoresis

One explanation for this might be that a contaminating substance was inhibiting the synthesis of cDNA as the length of the poly(A)$^+$ RNA used here should have been longer than before.

2) Oligo(dT) cellulose chromatography

The poly(A)$^+$ RNA was passed through an oligo(dT) cellulose column in an attempt to remove any contaminating substances. The poly(A)$^+$ RNA was then used to direct the synthesis of cDNA *in vitro*. The results of several experiments are shown in Table 9.

Using the poly(A)$^+$ RNA that had been passed through the oligo(dT) cellulose column a more consistent set of results were obtained. Between 1.5 and 2.5% of the radioactive [^{32}P] dCTP added to the reaction mixtures had been incorporated into the cDNA and 200-330 ng of DNA were made. These results meant that between 10 and 15% of the poly(A)$^+$ RNA was being copied. When the cDNA from those experiments was sized on a 1.5% agarose gel it was found to be the same length as before. The specific activity of the cDNA was 5-6 x 10^4 cpm μg^{-1} DNA. It still appeared as if something was inhibiting the poly(A)$^+$ RNA being copied by the reverse transcriptase.

3) CTAB precipitation

As a CTAB precipitation had cleaned up the total and cytoplasmic RNA isolated earlier it was decided to use this precipitation in an attempt to rid the RNA of the contaminant.

The plasmodia were grown again and lysed *in situ* and the poly(A)$^+$ RNA was isolated as before. After the ethanol precipitation step the poly(A)$^+$ RNA was CTAB precipitated. The RNA was then used to direct the synthesis of cDNA. The results of several experiments are shown in Table 9.

0.8 to 6% of the added radionucleotide was incorporated into the cDNA. The weight of cDNA made in these reactions varied between 105.9 ng and 807.2 ng. 5.8 to 33.6% of the poly(A)$^+$ RNA added to the reactions was copied. The cDNA had an average specific activity of 10^5 cpm μg^{-1} DNA which is a power of 10 higher than previously obtained.

The cDNA was sized on a 1.5% agarose gel as described previously (Fig 45). The length of the cDNA was found to be between 200 and 1353 nucleotides long with the majority of the fragments being between 200 and 600 nucleotides long. This cDNA was used as a probe in hybridization experiments with whole Physarum DNA, a southern blot of EcoRI digested Physarum DNA and a southern blot of undigested DNA from a recombinant phage chosen at random (Fig 46). A fairly strong hybridization signal was obtained for both the whole and digested Physarum DNA. The hybridization signal was weak against the southern blot of a recombinant phage's DNA. However the signal was positive showing that the recombinant phage contained a fragment of Physarum DNA which is transcribed during growth.

4) CTAB precipitation and phenol extraction

In previous experiments both CTAB precipitation and phenol extraction helped to clean up the poly(A)$^+$ RNA and allow longer cDNA to be made. It was therefore decided to combine the two purification steps.

Plasmodia were lysed in situ as before and the poly(A)$^+$ RNA was isolated using poly(U) sepharose. After the ethanol precipitation step a CTAB precipitation was carried out. The poly(A)$^+$ RNA was then phenol/chloroform extracted before being ethanol precipitated once more.

The poly(A)$^+$ RNA was used to prepare cDNA in vitro. Several experiments were carried out and the results are analysed in Table 9.

Figure 45. Sizing of cDNA prepared from poly(A)$^+$ RNA isolated from plasmodia lysed in situ and purified with a cetyltrimethylammonium bromide precipitation

Poly(A)$^+$ RNA was isolated from P. polycephalum as described in the legend to figure 43.

After the initial precipitation from the elution buffer the poly(A)$^+$ RNA was further purified by a cetyltrimethylammonium bromide precipitation (see Methods Section 5c(i)).

The preparation and treatment of the cDNA was as previously described.

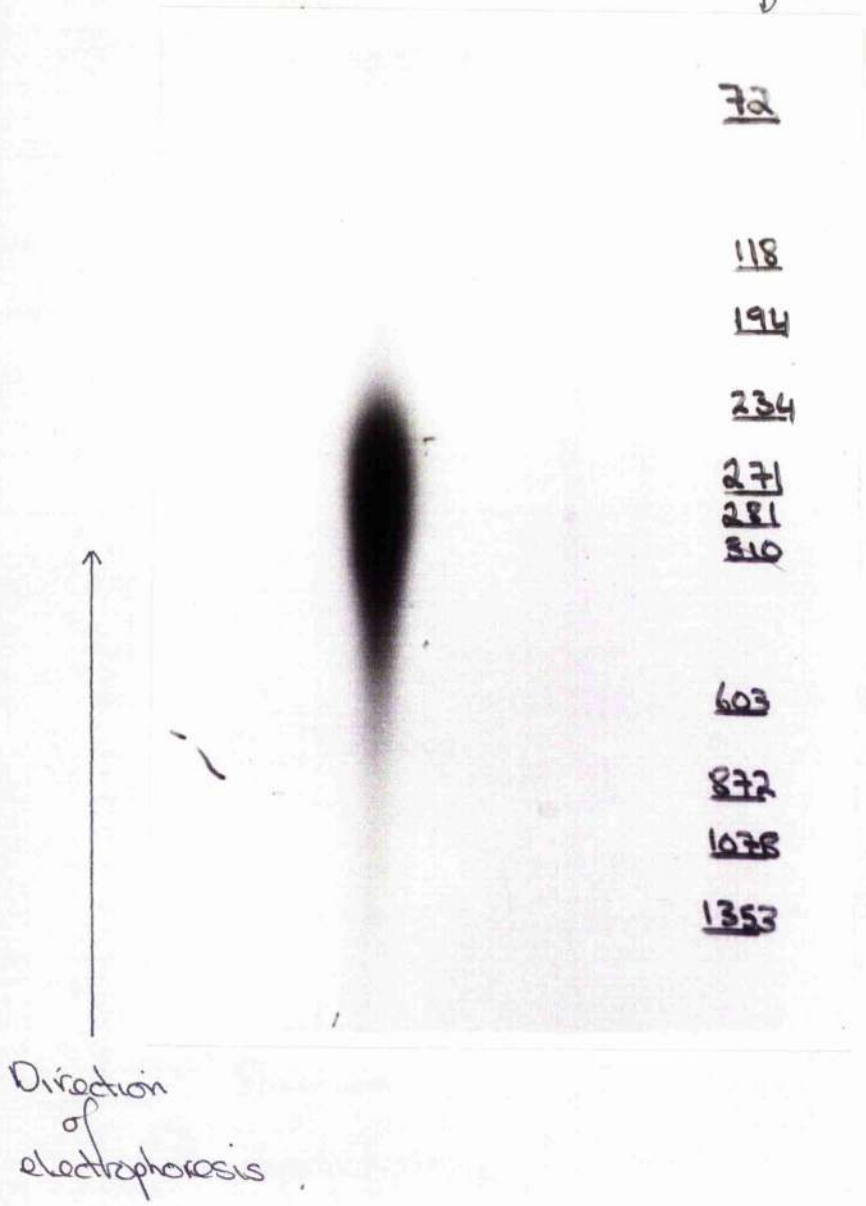

Molecular weight markers (bp) from agarose gel
72
118
194
234
271
281
310
603
872
1078
1353
Direction of electrophoresis

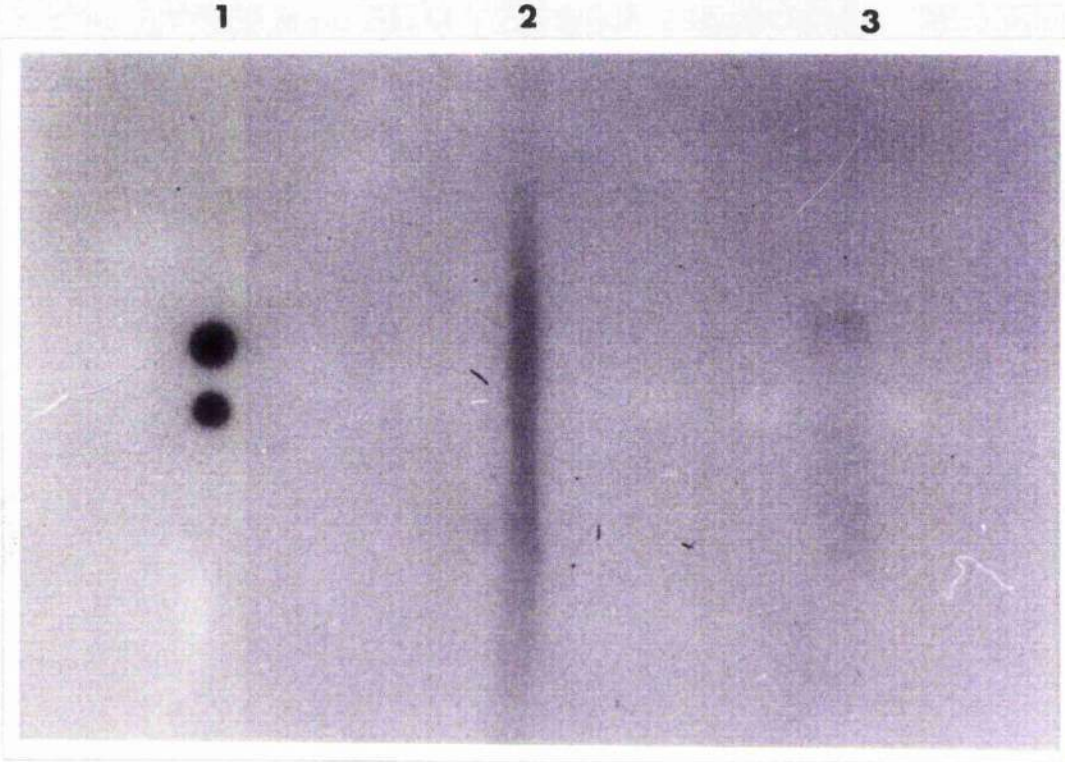
Figure 46. Hybridization of cDNA to undigested Physarum DNA, EcoRI digested Physarum DNA and undigested recombinant phage DNA

Southern blots of EcoRI digested Physarum DNA and whole recombinant phage DNA were prepared as described in Methods Section 8b. Whole Physarum DNA was blotted onto nitrocellulose filter paper, allowed to dry and then treated as a Southern blot. A hybridization reaction was carried out as described in Methods Section 10b.

1 : whole Physarum DNA.

2 : EcoRI digested Physarum DNA

3 : Recombinant phage DNA.


1
2
3

Radionucleotide incorporation varied from 2.7 to 6.2% of the total added. The weight of cDNA made ranged from 364 ng to 818 ng. These figures show that between 15 and 34% of the poly(A)$^+$ RNA added to the reaction mixtures was being copied by the reverse transcriptase. The specific activity of the cDNA ranged from 10^6-10^7 cpm μg^{-1} DNA.

When the cDNA was sized on a 1.5% agarose gel it was shown to be the longest copy DNA made during this work (Fig 47). The DNA ranged in size from 200 nucleotides to around 900 nucleotides.

As this cDNA was the longest made and had the expected specific activity it was decided to use the cDNA as a probe in a hybridization reaction against recombinant phage plaques.

L) Hybridization of cDNA against recombinant phage plaque replicas

Recombinant phage were grown as a plate lysate to give a final plaque number of 3×10^3. Nitrocellulose filter replicas were prepared by the method of Benton & Davis (1977). CopyDNA was made *in vitro* as described in Section k(vi)4 above. The cDNA was then used as a probe in hybridization reactions against recombinant phage plaque replicas as described in Methods Section 10b.

Figure 48 shows an example of an autoradiograph of plaque filter replicas hybridized to growing plasmodial cDNA. Only a few plaques gave a positive hybridization signal after 5 days exposure. A differential hybridization reaction was found to have occurred during these experiments as it had when [^{32}P] nick-translated *Physarum* DNA had been used as a probe. Again this may reflect the possibility that too stringent washing conditions were being used.

This experiment showed that the cDNA prepared *in vitro* would

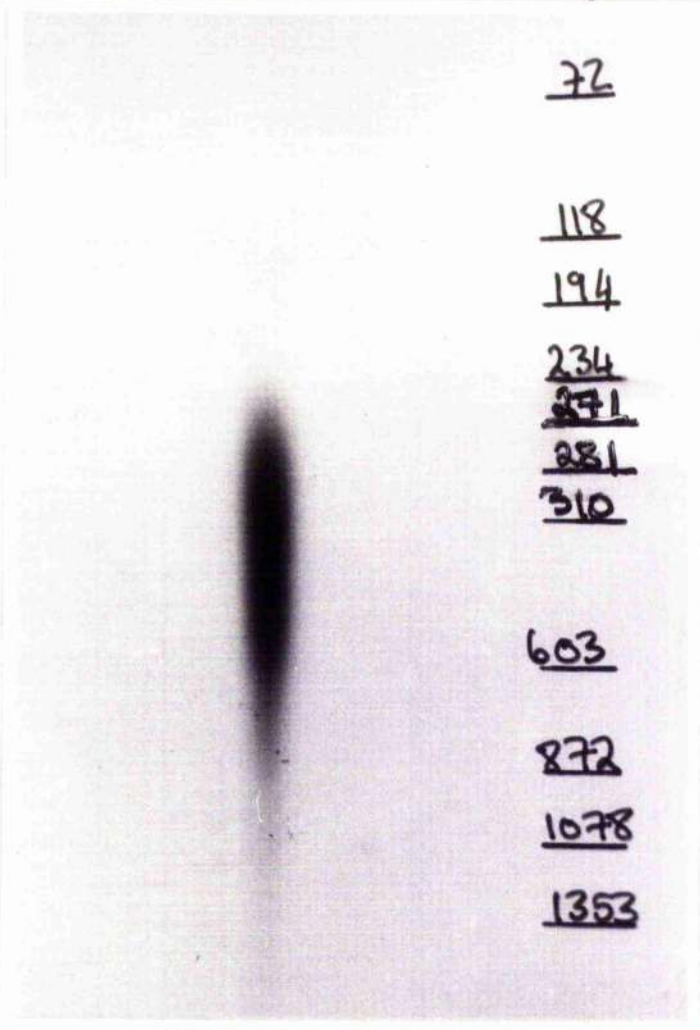
Figure 47. Sizing of copyDNA prepared from poly(A)$^+$ RNA isolated from plasmodia lysed in situ and purified by CTABprecipitation and phenol/chloroform extraction

Poly(A)$^+$ RNA was isolated and treated as described in the legend to Figure 43.

After the CTAB precipitation the poly(A)$^+$ RNA was phenol/chloroform extracted then precipitated with ethanol.

Copy DNA was prepared and treated as previously described.

Molecular weight markers (bp) from agarose gel.



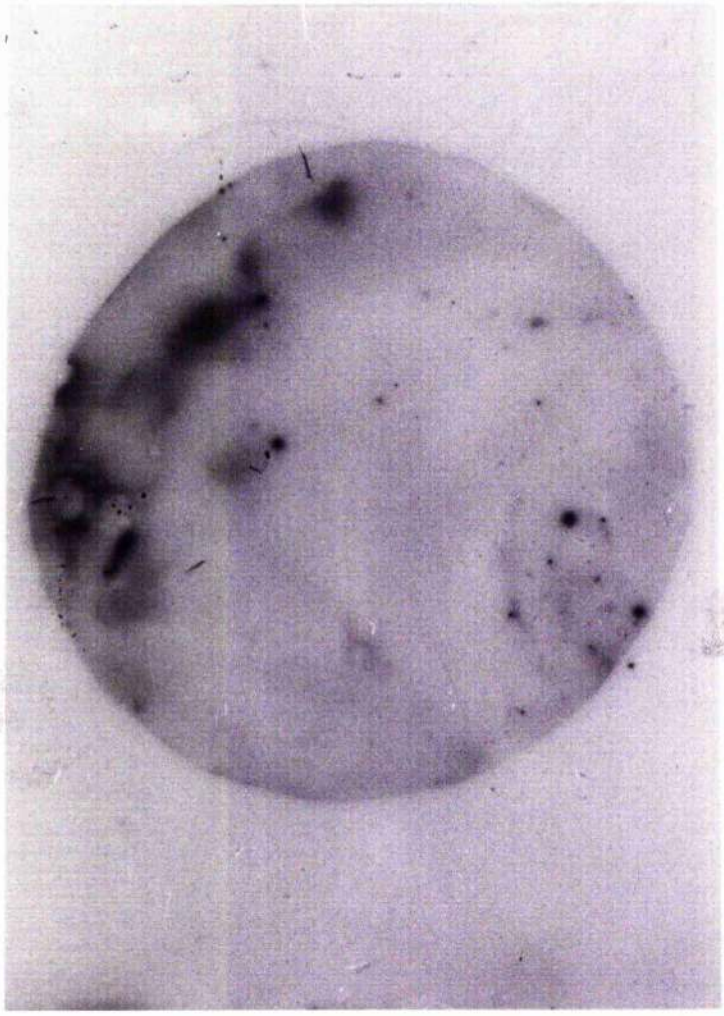
Direction of electrophoresis

Figure 48. Hybridization of ^{32}[P] cDNA prepared with poly(A)$^{+}$ RNA isolated from plasmodia lysed in situ and then CTAB and phenol/chloroform treated against recombinant phage plaques

Poly(A)$^{+}$ RNA was isolated and treated as described in the legend to Figure 47. It was then used to direct the synthesis of cDNA in vitro. Hybridization filters were prepared as previously described.

A hybridization reaction was set up as described in the Methods Section 10b.

10^{6} cpm of ^{32}[P] cDNA were used in the hybridization reaction.



hybridize to phage plaque replicas and that several recombinant phage carried fragments of Physarum DNA that were transcribed during growth of the slime mould.

DISCUSSION

1. The Cell Cycle During Differentiation of Physarum polycephalum

a) The cell cycle after 72 h starvation

P. polycephalum is a simple eukaryote with a life cycle that can be described as an alteration between two cellular forms; the amoebae and the plasmodium. These two cell types differentiate into each other by characteristic pathways. The macroscopic plasmodium is flat, disc-like and consists of protoplasm that exhibits a rapid to and fro streaming. In this stage of the life cycle growth occurs in the absence of cell division giving rise to a large multinucleate structure. When the food supply is exhausted the plasmodium enters a synchronous developmental cycle which results in the formation of uninucleate spores. Under favourable growth conditions the spores germinate and release amoebae. The plasmodial stage of the life cycle is re-established by differentiation withinan amoebal population (Howard, 1931a). Structurally, and because of the mitotic synchrony, the plasmodium can be regarded for biochemical purposes as one giant cell. This organism can therefore be thought of as a good model for developmental studies and in particular for investigating how cell cycle events are coupled with differentiation.

A starving plasmodium was found to be in the G2 phase of the cell cycle when it became competent to sporulate (Fig 9). The lack of DNA replication in the presence of nocodazole indicated that before the plasmodium could resume growth a mitosis was necessary before any DNA replication could take place.

The fact that a plasmodium of P. polycephalum differentiates from the G2 phase of a cell cycle contrasts with evidence obtained from other eukaryotic model systems. The yeast Saccharomyces cerevisiae has

been exploited in studies of the relationship between the cell cycle and differentiation because of its well characterised genetic system (Mortimer & Hawthorn, 1969). Cell cycle mutants are easily isolated and it has been shown that 32 gene products are necessary for the successful completion of a cell cycle (Hartwell et al, 1970; Hartwell et al, 1973). Several temperature-sensitive cell cycle mutants were studied and a point called 'start' early in G1 was found (Hirschberg & Simchen, 1977; Hartwell et al, 1974; Nurse, 1981). This is the earliest known gene controlled event in the cell cycle. When cells are past 'start' in the cell cycle they are committed to the completion of the cell cycle and must go through mitosis before being able to differentiate. Cells which are at a stage in the cell cycle before 'start', when they are exposed to conditions which induce differentiation, can go directly into the differentiation pathway without first undergoing a mitosis. Thus 'start' defines the point in the cell cycle from which S. cerevisiae cells differentiate. A similar point in the G1 phase of the cell cycle was found in the yeast Schizosaccharomyces pombe. In more complex eukaryotic organisms it has been shown that some types of cell development are linked to aspects of the cell cycle (VanderHaar & Topper, 1974; Torrey & Fosket, 1970). The G1 period of the cell cycle again seems to be important as it is from this point that cell fusion during myogenesis occurs and friend erythroleukemia cells undergo differentiation and become capable of synthesising haemoglobin (Okazaki & Holtzer, 1966; Conkie et al, 1981). When the cellular slime mould Dictyostelium discoideum was used in cell cycle studies, however, a much more complicated picture emerged. When chemical inhibitors of the processes of mitosis and cell division were used to disrupt the cell cycle it was reported that development and therefore

successful differentiation was dependent on the completion of these cell cycle events (Cappucinelli & Ashworth, 1976). A later study showed that mitosis could be prevented and differentiation would still take place (Cappucinelli et al, 1979). D. discoideum amoebae are in the G2 phase of the cell cycle during aggregation (Katz & Bourguignon, 1974). Starving amoebae are also arrested in the G2 phase of the cell cycle (Soll et al, 1976). These starving cells can go directly into the developmental programme without any preaggregation division (Zada-Hames & Ashworth, 1978). It may be therefore that both cellular and acellular slime moulds differentiate from the G2 phase of a cell cycle.

An isolated nocodazole-sensitive event during starvation was detected by Chapman and Coote (1982) who found that plasmodia transferred to nocodazole after 55-60 h could escape the inhibitory effect of the drug and were able to complete the sporulation process. Nocodazole is known to inhibit microtubule assembly (Quinlan et al, 1981) and as the plasmodium was found to be in the G2 phase of the cell cycle when differentiation is initiated this event can not be an isolated mitosis but some other event necessary for morphogenesis that requires functional microtubules.

A similar cytoplasmic microtubule requiring event has been suggested to occur during the development of D. discoideum. White et al (1981) used the drug CIPC, to interfere with microtubule function. Mutants which were resistant to the drug during growth were found to be sensitive to its action during development. The authors interpreted this finding as indicating that CIPC did not inhibit development by blocking mitosis but that it interfered with some other event which was necessary for development and required functional microtubules. The only microtubules so far detected in the plasmodium of P. polycephalum are those of

the mitotic spindle (Burland et al, 1984; 6th European Physarum Workshop). Since these studies were carried out using growing plasmodia it is possible that cytoplasmic microtubules may be present in starving plasmodia and remain as yet undetected.

An attempt was made to verify the findings of Sauer et al (1969a) who reported the occurrence of a presporangial mitosis and a round of DNA replication 12 h post-illumination. Two plasmodia were starved for 72 h and the uptake of [methyl-^{3}H] thymidine into acid-insoluble material was monitored from the time of illumination until melanization of the spores 17 h later (Fig 19). No obvious rounds of DNA replication were detected. These findings may not have been the result of the absence of a period of DNA synthesis, but may reflect the inability of the [methyl-^{3}H] thymidine to enter the starved plasmodia. The impermeability of the starved plasmodium is thought to have been the cause of the differences in the results of two groups of workers who tried to estimate the time of transcription of the last mRNA essential for sporulation.

Sauer et al (1969b) relied on the uptake of actinomycin D into the starved plasmodium to block RNA synthesis. They found that sporulation was unaffected when the drug was added any time after 3 h post-illumination and deduced that the last mRNA required for sporulation was transcribed around that time. More recently mRNA has been isolated from induced plasmodia at different times and translated in vitro. Using these new techniques the first point of synthesis of new mRNA species was found to be nine hours post-illumination (T.H. Schrechenbach, 1984; 6th European Physarum Workshop).

b) The cell cycle during the initial 24 h starvation and growth

Having established that the plasmodia of P. polycephalum differ-

entiate from the G2 phase of the cell cycle attention was turned to the three rounds of DNA replication which were reported to occur during the initial 24 h of starvation (Chapman & Coote, 1982). Although the absence of a G1 phase in growing plasmodia has been well documented little or nothing is known about the cell cycle during starvation in P. polycephalum (Nygaard et al, 1960; Braun et al, 1965; Beach et al, 1980). An experiment was designed to try and determine if a G1 phase exists in a starving plasmodium. Although nocodazole has no effect on DNA synthesis itself, blocking mitosis with the drug will prevent any subsequent round of DNA replication (Chapman & Coote, 1982). The round of DNA replication which took place between 24 and 26 h of the starvation period was studied. Pieces of starving plasmodium were transferred to SpoM + nocodazole at hourly intervals before the initiation of the round of DNA replication. If a G1 phase existed in the plasmodium there should be a point at or near mitosis when the plasmodium escaped the effect of nocodazole and DNA replication occurred. The plasmodia began to escape the block on DNA replication imposed by nocodazole between 1 and 1.5 h prior to the initiation of the DNA synthesis (Fig 10).

It had been reported that separate plasmodia prepared at the same time from a single culture of microplasmodia underwent DNA synthesis with almost perfect synchrony (Chapman & Coote, 1982). However, during these experiments it was noticed that the onset of DNA replication in plasmodia prepared at the same time and from the same microplasmodial culture could vary by up to 2 h. As this asynchrony could affect the accuracy of the results a plasmodium large enough to be divided into 6 pieces was subsequently used.

Several of these experiments were carried out and in each case

nocodazole had to be present between 1 and 2 h prior to the initiation of a round of DNA replication to completely block the synthesis of DNA (Table 1). This suggested that a G1 phase may be present in the cell cycle of starving plasmodia and that it was at least 1 h in length and perhaps as long as 2 h. When these experiments were repeated with a growing plasmodium, which had been shown to lack a G1 phase (Fig 8), a similar pattern was obtained. Nocodazole was required at least 2 h prior to the initiation of a round of DNA replication to completely block the synthesis of DNA (Table 1). If it is assumed that nocodazole works in the same way in both types of plasmodia the results show that no prolonged G1 period exists in starving plasmodia.

The 1 to 2 h required for the complete elimination of DNA synthesis may reflect the time taken for the drug to enter the plasmodium and reach an effective concentration. Nocodazole was found to have a marked effect on the nuclei of a treated plasmodium. Exposure to the drug caused a swelling of the nuclei resulting in abnormally large interphase nuclei being seen in the light microscope under 1000x magnification. No internal structures could be seen. It was hoped that a series of electron micrographs would clarify nocodazole's effect on the nuclei and also help to estimate the time taken for the drug to accumulate in the plasmodium.

Pieces of a large growing plasmodium were treated with nocodazole at hourly intervals approaching the round of DNA replication which began between 23 and 24 h. DNA synthesis in each plasmodial portion was monitored by following the uptake of [^{3}H] thymidine into acid-insoluble material. Samples were taken for electron microscopic examination at times just before and just after mitosis in the control. The microtubule

disrupting drug had to be present 1-1½ h before the initiation of a round of DNA replication for the synthesis to be completely blocked. If added later some DNA replication took place (Fig 13).

By studying the electron micrographs it was seen that nocodazole entered the plasmodium and exerted its effect fairly rapidly. The electron micrographs of nuclei from plasmodial portions which replicated their DNA to some extent showed that the nuclei did enter the initial stages of mitosis (Fig 16). However, samples which were taken later (after the completion of mitosis in the control portion of plasmodium) showed that the nocodazole treated nuclei did not complete mitosis successfully. Large nuclei were seen to be blocked in prophase, those having attempted mitosis being grossly abnormal and polynucleolate (Fig 17a, 17b & 18). No normal nuclei were found which eliminates the possibility that the limited DNA replication, found in plasmodia exposed to nocodazole 1-2 h prior to the onset of replication, was due to a few nuclei within the plasmodium behaving normally. It seems that nocodazole treatment had uncoupled the cell cycle events of mitosis and the initiation of DNA synthesis by blocking mitosis yet allowing a low degree of DNA synthesis to occur.

As DNA synthesis begins immediately after nuclear division in Physarum it was suggested that the replication of DNA was somehow triggered by an event in the mitotic sequence. Guttes & Guttes (1968) transferred nuclei from S phase plasmodia to G2 plasmodia and vice versa. The transfer was done by allowing two plasmodia to fuse for 15 min then separating them. When S phase nuclei were transferred to a G2 phase cytoplasm they completed DNA synthesis normally and did not induce DNA replication in the host plasmodium's nuclei. G2 nuclei when placed in

S phase cytoplasm did not begin DNA synthesis but underwent a synchronous mitosis with the host nuclei and started DNA replication normally. These results lead to the notion that DNA replication was dependent on a successful mitosis. Since then the two events have been uncoupled by several treatments. Brewer & Rusch (1968) exposed plasmodia to an elevated temperature just before metaphase. These nuclei were found to be polyploid. Only anaphase and telophase were aborted by the heat treatment. DNA synthesis was delayed and less intense than in control nuclei. However, DNA replication was eventually completed leading to a doubling of the ploidy level (Brewer & Rusch, 1968; Wolf et al, 1979). Griseofulvin is an antibiotic which binds to tubulin heterodimers and prevents their polymerization into complete microtubules (Herbert et al, 1980). Continuous treatment of P. polycephalum macroplasmodia with griseofulvin induced mitotic delays dependent on the phase of the plasmodium with delays of up to 6 h when the drug was added in early G2. Little or no delay occurred when the drug was added within 1 h of metaphase. When a plasmodium of P. polycephalum was treated with the drug several hours before metaphase onset the nuclei showed an abnormal metaphase with the chromosomes forming a ring structure which persisted for 2 h. The chromosomes then decondensed without karyokinesis. The resulting interphase nuclei were larger than normal and were polyploid indicating that although mitosis was blocked at metaphase, DNA replication took place (Gull & Trinci, 1974; Herbert et al, 1980). Electron microscopic examination of these nuclei showed the presence of microtubule crystals within the cytoplasm of the nuclei. Such structures were not visible in the electron micrographs of nocodazole treated nuclei (Fig 17a & 17b). More recently the effect of platinum antitumour compounds has been

studied in P. polycephalum (Wright et al, 1984). Cisplatin is an active antitumour compound against leukaemia. When a plasmodium was treated with 0.1 mM cisplatin abnormally large nuclei were observed. Mitosis was studied in detail using a series of electron micrographs and cisplatin was found to inhibit the final stage of nuclear division, ie chromosome separation. This resulted in large nuclei which contained 2 or more nucleoli and a cytoplasmic mass of microtubules. The abnormal nuclei were found to be polyploid which indicates that cisplatin inhibits the final stage of mitosis without inhibiting the replication of DNA.

The results obtained after nocodazole treatment may be explained with reference to a heat-sensitive pathway described by Wright & Tollon (1979). These workers studied the effects of a temperature shift from 22°C to 32°C on cell cycle events. They found that mitosis was always associated with an increase in thymidine kinase synthesis. This increase could occur without mitosis when the temperature shift was applied between 90 and 15 min before metaphase. Wright & Tollon (1979) postulated that the two events lay on the same regulatory pathway and that both were under the control of the same regulatory substance (Fig 49a). The results obtained in this study could be explained if nocodazole was having an effect on this heat-sensitive pathway. Addition of nocodazole more than 1½ h before the initiation of a round of DNA replication might block both the increase in synthesis of thymidine kinase and metaphase onset. Treatment with the drug 1-1½ h before DNA synthesis would allow some de novo synthesis of thymidine kinase, and thus limited DNA replication, but block metaphase onset. A schematic diagram is shown in Fig 49b.

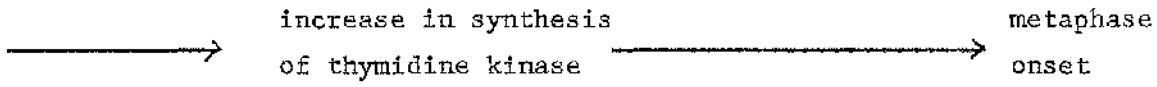
This theory is supported by the fact that heat shock was also

Figure 49.

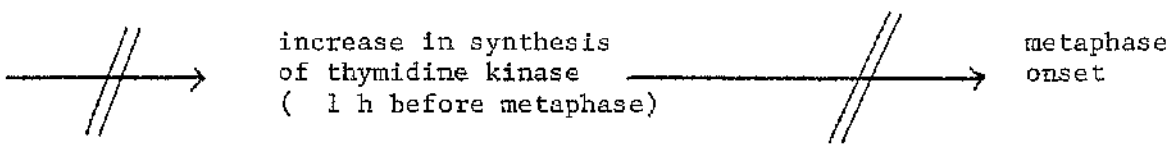
A : Temperature sensitive pathway of Wright & Tollon (1979)

B : Proposed mechanism of nocodazole action.

A



B



Pathway blocked by adding nocodazole more than 1-1½ h before initiation of DNA synthesis

Pathway blocked by nocodazole treatment less than 1-1½ h prior to initiation of DNA synthesis

effective only on mitosis and not DNA replication less than 90 min before metaphase (Wright & Tollon, 1979) and that the de novo synthesis of thymidine kinase is known to begin around 1 h prior to metaphase (Sachsenmaier et al, 1967; Pahlic & Tyson, 1983b). Before the theory can be confirmed the thymidine kinase activity during a similar experiment using nocodazole will have to be studied.

If the model was confirmed it would be interesting from the point of view that a microtubule disrupting drug was exerting an effect on an unrelated biochemical event. A similar effect was observed when the action of amphidicolin on P. polycephalum was studied (Eon-Gerhardt et al, 1981). Amphidicolin is known to interfere with DNA synthesis by its action on polymerase α. This drug however also delays mitosis in P. polycephalum when added 3 h prior to metaphase without interfering with the increase in thymidine kinase synthesis.

These results show that although DNA replication can occur in the absence of a successful mitosis the two events are closely coupled in the cell cycle of Physarum.

Knowing that the plasmodium was in a G2 phase of the cell cycle when it became competent to sporulate lead to questions concerning the timing of gene transcription during the G2 phase.

The number of genes specific for differentiation has been estimated for the cellular slime mould D. discoideum (Mangiorotti et al, 1981). This was done by competitive screening of a genomic library of D. discoideum prepared in a lambda vector. Radioactively labelled poly(A)$^+$ RNA from differentiating cells was used as a probe in hybridization reactions against filter replicas of phage in the presence of excess unlabelled poly(A)$^+$ RNA from vegetative cells.

It was hoped that a similar approach might prove successful in elucidating the genetic control of sporulation in Physarum polycephalum. An attempt was made to prepare a comprehensive genomic library of P. polycephalum CL in a lambda vector.

2. Preparation of a Physarum Genomic Library

Two phage vectors were available at the start of this research, Charon 4AP (Ferrari et al, 1981) which is Ch4A with a B. subtilis insert and λ1059 a phage described by Karn et al (1980).

It was originally hoped that recombinant phage could be recovered by simply ligating partially digested Physarum DNA and vector DNA together. This was tested using BamHI digested λ1059 DNA and BamHI partially digested Physarum DNA. This simple method would have avoided the step of sizing the DNA to be cloned. No recombinant phage could be isolated this way, presumably because the fragments of Physarum DNA which were ligated into the λ1059 arms generated phage that were either too long or too short to be packaged (Table 4). A quick and reliable method of size fractioning the digested Physarum DNA was sought.

a) Size fractionation of digested Physarum DNA

The first method tried involved separating the digestion products through a 1% (w/v) low melting point gel. The DNA was then extracted from the gel by phenol extraction. This method was very inefficient as in most cases the important DNA was left behind in the portion of the gel which was unstained because the samples did not run straight. When the DNA had been extracted from the gel it was nearly impossible to precipitate the μg quantities from the large aqueous volume which resulted from the various extraction procedures.

The second method which was attempted eliminated the problem of not being able to visualise the DNA in the complete gel. Again however it proved very difficult to precipitate the DNA from a large aqueous volume. In this case as much as 40% of the extracted DNA remained in the ethanol solution. The addition of t-RNA, as a carrier, reduced this to 20-30% allowing 20-25% of the DNA to be precipitated. The method was described by Langridge et al (1980). They tested the method using a derivative of a ColE1 plasmid with a molecular weight of 7×10^6 and obtained a recovery value of 94%. When they tested the efficiency of the method with varying sizes of plasmid they found that the recovery of small plasmids was essentially quantitative, but that the value falls to around 30% with larger plasmids with molecular weights of $2.5\text{-}4.6 \times 10^7$. This may explain the low recovery yields obtained with Physarum DNA as the largest piece of DNA to be extracted had a molecular weight of 1.7×10^7.

The third method which was tested was described by Yang et al (1979) who claimed a 90% recovery of DNA with sizes ranging from 1.9-23.7 kb. The method involved electroelution of the DNA from the gel into a buffer-filled well. When this method was tested using Physarum DNA it proved to be very successful giving at least 40% recovery of the DNA.

The DNA isolated in this way was found to ligate efficiently and so this method was used routinely to isolate size fractionated Physarum DNA and Ch4AP 'arms'.

b) Comparison of Ch4AP and λ1059 as cloning vectors

i) The cloning strategy used with Ch4AP is such that the phage 'arms', which contain all the information necessary to generate viable phage, must be isolated from the other DNA fragments after EcoRI digestion. The

phage 'arms' were then ligated alone and in the presence of 15-25 kb Physarum EcoRI generated DNA fragments. Viable phage were recovered by in vitro packaging and detected by plating on E.coli DP50 SupF.

When λ4AP 'arms' were ligated alone a significant number of viable phage were generated (Table 5). Viable phage should not be created as the left and right arms of Ch4AP together are only 31 kb long which is theoretically too short to be efficiently packaged into phage heads (Williams & Blattner, 1980). When two sets of 'arms' are ligated together the resulting DNA molecule is around 8 kb too long to be packaged by the in vitro system. If any other DNA had been present in the Ch4AP 'arms' preparation it may have been ligated with the 'arms' and thus allow viable phage to be isolated. A sample of the Ch4AP 'arms' preparation was electrophoresed through a 0.7% (w/v) agarose gel and no contaminating DNA could be seen (Fig 21). The results of the experiment showed that as many as 66% of the phage generated may not contain Physarum DNA (Table 5).

A recombinant DNA library of Aspergillus nidulans was made in the vector λ Ch4A (Zimmerman et al, 1980). They estimated that 95% of the phage which were generated by the ligation of Ch4A 'arms' with A. nidulans DNA were recombinant phage. This estimate was revised when the genomic library was used to study the clustering of spore specific genes (Orr & Timberlake, 1982). These workers estimated that 70% of the phage in the library were recombinants. Thus 30% of the viable phage produced during the ligation reaction did not contain A. nidulans DNA.

A second attempt was made to produce a representative gene library in Ch4AP (Table 5). In this case 60% of the phage generated when phage 'arms' and Physarum DNA were ligated were presumptive recombin-

ants. The identity and origin of the remaining 40% of the phage are unknown but they are presumably caused by the ligation of phage 'arms' alone which are somehow packaged in the *in vitro* system giving rise to viable phage.

ii) The cloning strategy of the phage λ1059 eliminates the necessity of isolating the two phage 'arms' before *in vitro* ligation reactions can take place (see Introduction Section 5d). When *Bam*HI cleaved λ1059 DNA was ligated in the presence of varying amounts of 15-25 kb *Bam*HI generated *Physarum* DNA fragments the results indicated that around 99% of the phage generated were presumptive recombinant phage (Table 6). This was in agreement with the results reported by Karn *et al* (1980). These workers used λ1059 as a vector to prepare a genomic library of nematode DNA and found that 0.75% of the phage generated were non-recombinants.

When Ch4AP 'arms' are ligated together they produce viable phage which would dilute a genomic library and therefore make screening the library a more difficult task. λ1059 on the other hand has a preliminary screening method, ie plating the phage on *E. coli* Q359, which reduces the number of non-recombinant phage present to around 1% of the total phage. Thus from these experiments it seemed that λ1059 was the better vector for preparing a genomic library than Ch4AP. It was therefore decided to concentrate on the gene library prepared in λ1059.

Two other genomic libraries were prepared in λ1059, one from *Sau*3A generated 15-25 kb fragments and the other using fragments created by *Bam*HI digestion of *Physarum* DNA. These libraries were found to contain 2.29×10^5 and 2.26×10^5 presumptive recombinant phage respectively (Fig 23 & Table 7). Using the relationship of Clarke & Carbon (1976) and presuming that the average insert size would be around 20 kb in length it

was calculated that 1.3×10^5 phage were required so that the probability of a given sequence being represented in the library was 0.99. All genomic libraries prepared in λ1059 contained more than the estimated number of phage which were required to ensure that the entire Physarum genome was represented. As the libraries were apparently complete, analysis of the phage was undertaken.

c) Analysis of recombinant phage

Restriction analyses of the phage showed that although they were recombinant phage they were all identical (Fig 24). Analysis of the first three genomic libraries constructed revealed that only one type of phage was present in them all. The phage was not a contaminant and was shown to contain fragments of the Physarum DNA that had been used to prepare the libraries (Fig 25). When analysed on a CsCl density gradient the DNA used for the library preparation was found to be lighter than the chromosomal DNA isolated by the method of Hardman & Jack (1978) (Fig 26).

Physarum polycephalum contains three types of DNA; chromosomal DNA with a buoyant density of approximately 1.700 g $(cm^3)^{-1}$ (Braun et al, 1965), ribosomal DNA, which is located in the nucleolus and has a buoyant density of 1.714 g $(cm^3)^{-1}$ (Hall & Braun, 1977), and mitochondrial DNA (Guttes & Guttes, 1964) and has a buoyant density of 1.686 g $(cm^3)^{-1}$ (Holt & Gurney, 1969). This suggested that the DNA used to generate the recombinant phage had been enriched in mitochondrial DNA. This enrichment may have occurred during the phenol extractions carried out on the nuclear lysate as a very broad interphase was present and only the aqueous phase was removed for further treatment. After restriction enzyme digestion and electrophoresis the size fractionated DNA probably contained a preponderence of a mitochondrial DNA fragment which was then preferentially

cloned into the 'arms' of λ1059. This resulted in the same phage being produced after every ligation reaction.

The difficulty in isolating chromosomal DNA free from mitochondrial DNA has been reported for the yeast Candida albicans (Wills et al, 1984). They tried several methods in order to isolate nuclear DNA free from mitochondrial DNA. Nuclei were isolated from spheroplasts and treated in one of three ways. A portion of the crude nuclear preparation was used directly for DNA isolation, and the remaining nuclei were further purified by one of two ways, discontinuous sucrose gradient purification or differential centrifugation through a dense medium containing glycerol. DNA was then isolated from the purified nuclei. The extracted DNAs were treated with EcoRI, electrophoresed and transferred to nitrocellulose. The electrophoretic patterns obtained were identical with one another and with other reported patterns. No visible sign of mitochondrial DNA contamination was present. When these DNA restriction patterns were hybridized to nick translated ^{32}[P] mitochondrial DNA the results showed that all the nuclear DNA digests contained EcoRI fragments identical to the mitochondrial DNA fragments. Thus all three methods of purification failed to eliminate the mitochondrial DNA. It seems likely that the same thing was happening when the Physarum DNA was EcoRI digested. The 15-25 kb fraction contained a mitochondrial DNA fragment generated by EcoRI treatment.

This problem appeared to be overcome when Physarum DNA was isolated by the method of Hardman & Jack (1978). The DNA was restricted with Sau3A and the 15-25 kb fragments were isolated by electroelution. The fragments were then used in a series of in vitro ligation reactions with BamHI digested λ1059 DNA (Fig 27). These reactions yielded a total of 3.1×10^5 presumptive recombinant phage (Table 8). This number of phage

was 1.8×10^5 more than was theoretically required in order to ensure that the entire Physarum genome was represented in the library.

Restriction analysis of the phage showed that all tested were derived from λ1059 and were different recombinant phage (Figs 28, 29 & 30).

3. Isolation of RNA from Physarum polycephalum

a) Isolation of polysomes

One major problem encountered during the course of this research was the difficulty in isolating undegraded RNA. Poly(A)$^+$ RNA, further purified from this RNA, was to act as a template for copy DNA (cDNA) synthesis in vitro which was then to be used as a probe to screen the gene library.

Preparation of intact polysomes from the macroplasmodia of P. polycephalum had proved difficult in the past (Mittermayer et al, 1966a). A method which had been developed for the isolation of polysomes from Physarum microplasmodia (Brewer, 1972) was adapted for use with macroplasmodia (Schwarzler & Braun, 1977). This method relied on high levels of Mg^{2+} and EDTA and although some polysomes were isolated most were lost due to degradation and EDTA dissociation. Adams et al (1980) reported the development of an improved method for the isolation of polysomes from synchronous macroplasmodia. This method involved preincubation and homogenization in a high ionic strength buffer supplemented with EGTA. One problem in isolating RNA from Physarum is that the plasmodium produces around 12 intracellular RNases, some of which are activated by divalent cations (Hiramaru et al, 1968; cited by Brewer, 1972). As EGTA is a potent chelator of divalent cations its presence in the isolation buffer

should reduce the RNase activity allowing the successful isolation of polysomes.

Adams et al (1980) reported the isolation of more than 90% of the macroplasmodial single ribosome population in polysomes. However, when this method was tested here for the macroplasmodia of P. polycephalum CL no intact polysomes were detected within the sucrose gradients showing that extensive RNA degradation was occurring during the isolation procedure. Three factors were shown to be important for the isolation of intact polysomes from tobacco leaves; high ionic strength; high pH and chelation of divalent metal ions (Jackson & Larkins, 1976). The buffer described by Adams et al (1980) did have a high ionic strength and a chelator of divalent cations but it only had a pH of 7.6. This may have been the cause of the disruption of the polysomes which occurred when the buffer was tested.

Polysomes were isolated from macroplasmodia when the buffer described by Jackson & Larkins (1976) was used. The isolation procedure produced a low yield of polysomes showing again that a considerable amount of RNA degradation had occurred (Fig 34). Another isolation buffer was described for the isolation of polysomes from sea urchin embryos (Duncan & Humphreys, 1981). This buffer again had a high ionic strength, contained EGTA to chelate any divalent cations, but had a pH of near neutral. An attempt was made to isolate polysomes from P. polycephalum macroplasmodia using this buffer. In addition the extraction buffer contained RNasin and Vanadyl ribonucleoside complex (VRC), two inhibitors of RNase activity. Again extensive degradation occurred leading to a very poor yield of polysomes (Fig 35). Free Mg^{2+} ions in the extraction buffer are essential for polysome stability (Brewer, 1972; Schwarzler & Braun, 1977; Adams

et al, 1980). As a result the concentration of Mg^{2+} ions in the isolation buffer must exceed the concentration of EGTA (Jackson & Larkins, 1976). The buffer described by Duncan & Humphreys (1981) contained 50 mM EGTA and only 25 mM $MgCl_2$. It may be therefore that the low yield of polysomes obtained by this method was not due to excessive RNase activity but because of the polysome instability caused by the low level of Mg^{2+} ions in the isolation buffer.

It had been hoped to isolate messenger RNA that was being actively translated at the time of isolation. The lack of success in preparing polysomes with any degree of efficiency precluded this and so an alternative method was tried.

b) Isolation of cytoplasmic RNA from macroplasmodia of P. polycephalum

Using a differential extraction procedure which was shown to yield one nucleic acid fraction enriched in cytoplasmic RNA and another enriched in nuclear RNA, two poly adenylated RNA populations were isolated (Melera et al, 1979). They identified the polyadenylated RNA isolated from the fraction enriched in cytoplasmic nucleic acid as Physarum poly(A) containing mRNA. It was therefore decided to isolate cytoplasmic RNA from P. polycephalum CL and then to selectively enrich for poly(A)$^+$ RNA by oligo(dT) cellulose chromatography.

Cytoplasmic RNA was isolated from P. polycephalum by phenol/chloroform extraction after disruption of the macroplasmodia by homogenisation. RNA isolated by this method was so highly contaminated with an impurity that it was unable to penetrate a formaldehyde denaturing gel (Fig 36). A similar result was reported by Melera et al (1978). These workers used a slightly different extraction method than the one used here. Phenol extractions were carried out at 4°C, 25°C and 45°C and then the RNA

was collected by ethanol precipitation. They found that the RNA extracted at 25°C and 45°C contained a significant proportion of molecules that were unable to penetrate a formaldehyde gel even after denaturation. This problem had not been reported for other eukaryotic systems. Weir-Thompson & Dawes (1984) isolated cellular RNA from the yeast Saccharomyces cerevisiae by simple phenol/chloroform extraction of a cellular homogenate. After ethanol precipitation, the RNA could be successfully electrophoresed on denaturing gels. RNA isolated in a similar manner from the cellular slime mould D. discoideum and the fungus Aspergillus nidulans were also able to penetrate formaldehyde denaturing gels (Palatnik et al, 1979; Timberlake & Barnard, 1981). After the extensive phenol extractions which were carried out on the cellular homogenate of P. polycephalum it seemed unlikely that the contaminant was proteinaceous. As P. polycephalum produces a large amount of polysaccharide material, both extracellularly and intracellularly, it was possible that the contaminant of the RNA was a carbohydrate. Nucleic acids which have been isolated by phenol extractions can often be contaminated by polysaccharide material. The nucleic acid can be freed from these contaminants by a cetyltrimethylammonium bromide precipitation step (Bellamy & Ralph, 1968). When the cytoplasmic RNA isolated from P. polycephalum in the buffers of Jackson & Larkins (1976) and Adams et al (1980) was further purified by a CTAB precipitation it was able to penetrate the formaldehyde gel (Fig 36). Having isolated total RNA from P. polycephalum CL macroplasmodia by phenol extraction, Baeckmann & Sauer (1982) used a CTAB precipitation to further purify the nucleic acid. These authors did not present data on the extent of degradation in their preparations. Cytoplasmic RNA was isolated in all three buffers described for polysome isolation. The RNA isolated in the

buffers of Jackson & Larkins (1976) and Duncan & Humphreys (1981) had very similar separation patterns when electrophoresed on a formaldehyde gel (Fig 36, Lane 3 & Fig 39, Lanes 5 & 6). Both showed signs of degradation as many low molecular weight RNA products were present. In this experiment the high pH of the Jackson & Larkins buffer did not seem to be beneficial. This however was not the case when the buffers of Jackson & Larkins (1976) and Adams et al (1980) were compared for their ability to allow the isolation of undegraded RNA (Fig 36). Although degradation had occurred during the isolation of both samples many more degradation products were present in the sample isolated in the buffer of Adams et al (1980). In this case the high pH appeared to have an advantageous effect. It should, however, be noted that the level of RNA degradation which occurred varied even when the same conditions of isolation were used.

Two inhibitors of RNase activity were added to the isolation buffer in several experiments. Although both were used at their optimum concentration, RNase activity was still high. In one case more RNA degradation occurred in the presence of both inhibitors than in its absence (Fig 36, Lane 3 & Fig 37, Lane 1). Of the two inhibitors RNasin appeared to be the most effective in eliminating the activity of the ribonuclease enzymes (Figs 37 & 39).

As substantial RNA degradation was occurring even in the samples which contained both RNase inhibitors a third method of RNA isolation was tested.

c) Isolation of total RNA from P. polycephalum

This method involved the isolation of total RNA from a plasmodium which had been disrupted in a buffer containing 4M guanidine

thiocyanate, a known inactivator of RNase activity. This method had been used successfully in the isolation of total RNA from the microplasmodia of a white mutant of P. polycephalum (Putzer et al, 1983). The hot phenol extraction of the RNA was sufficient to remove any contaminating protein as additional treatment with SDS and proteinase k did not allow any better separation of the RNA on a denaturing gel (Fig 39). Total RNA isolated in the 4M guanidine thiocyanate buffer was less degraded than the cytoplasmic RNA which had been isolated in any one of the other three buffers used (Figs 39 & 40). This may be partly due to the immediate phenol extraction carried out after disruption of the plasmodium in the guanidine thiocyanate buffer. A similar effect was seen when cytoplasmic RNA was compared with RNA isolated from polysomes which had first been pelleted through a sucrose cushion (Fig 37). RNA isolated from the polysomes showed much more degradation than the cytoplasmic RNA. It appeared that the shorter the period was between the disruption of the plasmodium and the first phenol/chloroform extraction the less degradation of the RNA occurred. This was probably due to the shorter time the ribonucleases had to act on the nucleic acid.

Poly(A)$^+$ RNA was isolated from the total RNA preparation by oligo(dT) cellulose chromatography and was used to direct the synthesis of cDNA in vitro. The cDNA was found to be made up of short DNA fragments of 100 nucleotides or less.

The length of poly(A)$^+$RNA in P. polycephalum has been estimated by several groups of workers all using different isolation procedures. Melera et al (1979) isolated total RNA from lyophilized microplasmodia using a three step phenol/chloroform extraction procedure. RNA isolated by extraction at 4^{o}C was found to be enriched in cytoplasmic poly(A)$^+$RNA

and the RNA isolated by phenol/chloroform extraction at 45°C was rich in nuclear polyadenylated RNA. The RNA was sized on formamide/sucrose density gradients. These workers estimated that the cytoplasmic mRNA was on average 1339 nucleotides long and that the mRNA isolated at 45°C had a mean length of 1533 nucleotides. Brown & Hardman (1980) isolated polysomal and post-polysomal fractions from microplasmodia of P. polycephalum as described by Brewer (1972). After phenol/chloroform extraction of the RNA, the poly(A)$^+$RNA was isolated by poly(U) sepharose chromatography. The poly(A)$^+$RNA was separated through a 1.25% (w/v) denaturing agarose gel. They found a size distribution which showed a maximum at 13S which corresponds to approximately 1100 nucleotide residues. No RNA was found which was less than 4S. Total RNA was isolated from lyophilized macroplasmodia of P. polycephalum CL (Baeckmann & Sauer, 1982). Poly(A)$^+$ RNA was isolated from the purified RNA preparation by affinity chromatography on oligo(dT) cellulose. This material was analysed on 4% (w/v) polyacrylamide gels in 99% formamide and a broad peak ranging from 30S to 4S was detected. When the poly(A)$^+$ RNA was sized on formamide density gradients values consistent with the data of Melera et al (1979) were obtained.

The differences in these results may be due to the different methods of sizing the poly(A)$^+$ RNA. There seems to be some agreement in that the smallest detected RNA was 4S, however, the longest RNA may be between 1100 and 1340 nucleotides long. It seems unlikely that the poly(A)$^+$ RNA could be over 3000 nucleotides long as was suggested by the data of Baeckmann & Sauer (1982). Thus the length of cDNA prepared from the poly(A)$^+$RNA isolated from the total RNA preparation was exceptionally short and could be less than 10% of the length of poly(A)$^+$ RNA from

Physarum. The production of short cDNA was thought to be due to the presence of degradation products of poly(A)$^+$RNA present in the sample. As this poly(A)$^+$RNA had been isolated from total RNA which showed the least degradation of all the methods tested it was clear that an alternative method of RNA isolation was required.

d) Direct isolation of poly(A)$^+$RNA from plasmodial lysates

The method itself was simple, a plasmodium was lysed in a buffer containing 6M guanidine HCl and the poly(A)$^+$ RNA is isolated directly from the lysate by its affinity for poly(U) tails immobilized on sepharose beads. The method claimed to be effective because it minimized the risk of damage to the mRNA by the action of RNases (Cox & Smulian, 1983). These workers found that poly(A)$^+$ RNA isolated by this method was functional in directing the synthesis of proteins in a rabbit reticulocyte lysate system.

During experiments involving this procedure the length of the cDNA prepared and its specific activity were used to measure the purity of the RNA and the level of degradation which had occurred during the isolation procedure. During the initial experiments the plasmodia had been scraped from the filter into the isolation buffer and the same experimental procedure was employed when poly(A)$^+$ RNA was first isolated directly from plasmodial lysates by the method of Cox & Smulian (1983). An additional phenol extraction was carried out after the precipitation of the RNA from the elution buffer (J. Milner, personal communication). The poly(A)$^+$ RNA isolated by this method was functional when used as a template to direct the synthesis of cDNA in vitro (Table 9). Between 25 and 50% of the poly(A)$^+$ RNA was calculated to have been copied in the reverse transcription reaction. Most of the cDNA was however composed

of fairly short DNA fragments ranging from 200-500 nucleotides in length (Fig 43). This meant that the cDNA was still only between one third to one half of the length of the poly(A)$^+$ RNA found in Physarum (Melera et al, 1979; Brown & Hardman, 1980).

Very slight perturbation of the plasmodium during the initial stages of an isolation procedure had been found to cause degradation of RNA by the action of RNases released from the plasmodium (T.H. Schreckenbach, personal communication). It was obvious therefore that scraping the plasmodium from the filter might lead to the isolation of degraded poly(A)$^+$RNA which would inevitably lead to short cDNA being synthesised in vitro.

Poly(A)$^+$ RNA was next isolated from plasmodia lysed in situ on the filters and no phenol/chloroform extraction was carried out after the precipitation of the poly(A)$^+$RNA from the elution buffer. When this RNA was used as a template for in vitro synthesis of cDNA very short DNA fragments were produced (Fig 44). As this poly(A)$^+$ RNA should in theory have been longer than that prepared in earlier experiments it was assumed that an impurity was contaminating the poly(A)$^+$ RNA. Cox & Smulian (1983) did not report the necessity of a further purification step after the precipitation of the RNA from the elution buffer. This RNA was then capable of directing protein synthesis in vitro. This study isolated the poly(A)$^+$ RNA from microplasmodia of P. polycephalum. Microplasmodia are easier to work with than macroplasmodia in that they are not so difficult to handle and do not produce as much slime as macroplasmodia. The microplasmodia could easily be washed free of any polysaccharide slime before being lysed in the pre-cooled lysis buffer.

Several attempts were made to free the poly(A)$^+$ RNA from the impurity and so to increase both the length and specific activity of the

cDNA (Figs 45 & 46). Of all the treatments tried after *in situ* lysis of the plasmodium a combination of a CTAB precipitation and a phenol/chloroform extraction allowed the isolation of the longest and purest poly(A)$^+$ RNA. This was shown by the fact that cDNA prepared from this RNA was between 200 and 900 nucleotides long (Fig 47) and had a specific activity of 1.4×10^7 cpm. The cDNA prepared here was found to be longer than the cDNA prepared from poly(A)$^+$ RNA isolated from *A. nidulans* (400-500 nucleotides) and cDNA prepared from poly(A)$^+$ RNA from the basidiomycete *Schizophyllum commune* (600 nucleotides) (Timberlake, 1980; Dons *et al*, 1984). Thus the method developed during this work for isolating poly(A)$^+$ RNA from macroplasmodia was perhaps as efficient as could be achieved.

Isolation of poly(A)$^+$ RNA from *Physarum* appeared to be much more difficult than its isolation from other organisms. Not only was the RNA subject to extensive degradation by RNases, even in the presence of the enzyme's inhibitors, it was also heavily contaminated with protein and polysaccharide material. The results obtained from cDNA synthesis show that poly(A)$^+$ RNA molecules from *Physarum polycephalum* are at least 900 nucleotides long.

4. Hybridization of Radioactively labelled DNA Probes to Replica Filters of Recombinant Phage

Nick-translated *Physarum* DNA was used as a probe in hybridization reactions which were carried out in a 50% formamide solution + 5x SSC. The washing conditions were those recommended by Maniatis *et al* (1982) and involved washing the filters in a solution of 1x SSC at

68°C for 3 h. Under those conditions, three degrees of positive hybridization could be detected on the autoradiograph. Between 7 and 10% of the plaques screened gave a strong positive signal. The second degree of positive hybridization was visible as small 'pin-head' points on the autoradiograph. The weakest of all the signals was only just visible in the background of the filter showing that a very weak reaction had taken place. The Physarum genome contains a substantial amount of repeat sequences and the percentage of plaques giving a strong positive signal was theoretically expected to be around 20% (Peoples & Hardman, 1983). When a plasmid genomic library was screened using a total Physarum DNA probe 13% of the colonies were found to give a strong positive signal (Gerrie et al, 1983). The lower percentage of positive signals obtained here indicated that the phage library contained a significant number of non-recombinant phage. This, however, was not confirmed by the restriction analysis carried out on a random sample of phage from the library (Figs 28, 29 & 30). No phage were detected which showed the parental restriction pattern. If the genomic library had contained a significant number of parental phage it would have been likely that at least one would have been picked when such a random sample of phage were taken. This together with the fact that the three distinct levels of hybridization could be aligned with phage plaques on the original plates, suggested that the problem might have lain in the washing conditions used.

It is a combination of both the temperature and the concentration of the salt solution used which governs the stringency of the washing conditions. Under the conditions used here the washing temperature was 17°C below the T_m of the Physarum DNA (Holt, 1980). In other systems, similar hybridizations were used but the washing conditions

were less stringent (Zucker & Lodish, 1981; Mangiarotti et al, 1981). The conditions employed by both sets of workers meant that the washing temperature was around 20°C below the T_m of D. discoideum DNA.

All the positive signals seen on the autoradiograph appeared to be due to the true hybridization of the [^{32}P] labelled DNA probe with the phage DNA. The stringency of the washing conditions used may have meant that unless the DNAs were perfectly matched most would be washed from the filters after the hybridization reaction leading to weak signals being produced. Thus the problem may be eliminated if less stringent washing conditions were used.

A similar effect was seen when ^{32}[P] cDNA was used as a hybridization probe against replica filters of recombinant phage. Only a few plaques gave a strong positive hybridization signal. The washing conditions used here were those recommended by Maniatis et al (1982) to give stringent washing conditions after a hybridization reaction carried out in an aqueous solution. The cDNA failed to hybridize to whole Physarum DNA when tested in a 50% formamide solution. Perhaps these conditions were too harsh for the cDNA. Again when compared with other systems the washing conditions were extremely stringent. Although reported conditions use an SSC concentration as low as 0.2X SSC, the washing is carried out at room temperature (Percival-Smith & Segall, 1984).

It appeared that the washing conditions employed after the hybridization reactions may have been too stringent and that the results obtained would have been improved if either the SSC concentration had been increased or the temperature at which the washing took place was lowered.

The results show that the poly(A)$^+$ RNA isolated by a modification

of the method described by Cox & Smulian (1983) can direct the synthesis of long copy DNA *in vitro*. This cDNA will hybridize to replica filters of recombinant phage. Once the stringency of the washing conditions has been optimised it seems likely that the cDNA will produce stronger hybridization signals.

By screening two filter replicas of recombinant phage, one with cDNA made from poly(A)$^+$ RNA isolated from vegetative plasmodia, and one using cDNA prepared from poly(A)$^+$ RNA from sporulating plasmodia as a probe it should be possible to identify those phage which contain DNA preferentially transcribed during sporulation in *Physarum*. Thus it may be possible to identify a number of spore specific genes and ultimately the time of their transcription.

The overall conclusion from this work is that the molecular genetical approach used in this study offers a chance to investigate, at the molecular level, the sequence of events which lead to successful sporulation in *Physarum polycephalum*.

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APPENDICES

Appendix 1

Semi-defined growth medium (GM)

Tryptone (Oxoid)	20 g
Yeast extract (Difco)	3 g
D-glucose	20 g
KH_2PO_4	4 g
N + C salts (see below)	240 ml

Each of the above was dissolved and made up to 2 litres in distilled water. The pH was adjusted to 4.6 with 40% (w/v) KoH and the medium autoclaved at 15 lb $(in^2)^{-1}$ for 15 min. Hemin solution (see below) was added immediately before use, 10 ml per litre of medium.

N + C Salts

Citric acid	67.3 g
$FeCl_2.4H_2O$	1.0 g
$MgSO_4.7H_2O$	10.0 g
$CaCl_2.2H_2O$	10.0 g
$MnCl_2.4H_2O$	1.4 g
$ZnSO_4.7H_2O$	0.56 g

Each of the above was dissolved and made up to 2 litres in distilled water and autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Hemin solution

Hemin (Sigma) equine type III crystalline was prepared by adding 1 g of NaOH to 100 ml distilled water and then adding 0.1 g hemin. The hemin solution was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Sporulation medium (SpoM)

$CuCl_2$	0.024 g
KH_2PO_4	0.4 g
Salts (see below)	120 ml

Each of the above was dissolved in and made up to 1 litre in distilled water and autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Sporulation Salts

A.	Citric acid	4 g
	$CaCl_2.2H_2O$	5 g
	$MgSO_4.7H_2O$	5 g

Each of the above was dissolved in 600 ml of distilled water to which 0.5 ml conc. HCl had been added.

B.	$FeCl_2.4H_2O$	0.5g
	$MnCl_2.4H_2O$	0.7 g
	$ZnSO_4.7H_2O$	0.28 g

Each of these was dissolved in 50 ml of distilled water.

Solutions A and B were mixed and the volume made up to 1 litre with distilled water. This was then autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Immediately before use 1 ml sterile 10% (w/v) $CaCO_3$ and 1 ml sterile 1% nicotinamide (niacinamide, Sigma) were added per 100 ml of medium. The nicotinamide was made as a 1% (w/v) solution immediately before use.

Appendix 2

NZ broth

NaCl	10 g
$MgSO_4 \cdot 7H_2O$	5 g
Tryptone (Oxoid)	10 g
Yeast extract (Difco)	5 g
Casamino acids (Difco)	1 g

The above were dissolved and made up to 1 litre in distilled water. The pH was adjusted to 7.2 with 40% (w/v) NaOH and the medium autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

NZ agar

15 g of purified agar was added to 1 litre of broth. This was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

NZ agarose

7.5 g of agarose (Sigma, Type 1) was added per litre of NZ broth. This was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

CY broth

Casamino acids (Difco)	10 g
Yeast extract (Difco)	5 g
NaCl	3 g
KCl	2 g
Tris	3 g
$MgCl_2 \cdot 7H_2O$	2 g

Each of these was dissolved in and made up to 1 litre in distilled water. The pH was adjusted to 7.0 with conc. HCl and the medium autoclaved at 15 lb $(in^2)^{-1}$ for 15min.

CY agar

15 g of purified agar was added to 1 litre of CY broth. This was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

CY agarose

7.5 g of agarose (Sigma, Type 1) was added per litre of CY broth. This was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Appendix 3

Plaque storage buffer (PSB)

0.01M Tris

0.1M NaCl

0.01M $MgCl_2.6H_2O$

The pH was adjusted to 7.4 with conc. HCl and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

PSB + gelatin

50 ml of 1% (w/v) gelatin (autoclaved at 15 lb $(in^2)^{-1}$ for 15 min) was added to 1 litre of PSB. This was then autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Ca/Mg Solution

0.01M $CaCl_2.2H_2O$

0.01M $MgCl_2.6H_2O$

This was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Appendix 4

Tris/EDTA buffer (TE)

0.01M Tris

0.001M EDTA

The pH was adjusted to 8.0 with conc. HCl and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Tris/EDTA/Mg buffer (TSM)

0.05M Tris

0.01M NaCl

0.01M $MgCl_2 \cdot 6H_2O$

The pH was adjusted to 7.4 with conc. HCl

Tris/NaCl/EDTA buffer (TSE)

0.01M Tris

0.005M NaCl

0.001M EDTA

The pH was adjusted to 8.0 with conc. HCl.

Tris/sucrose buffer

0.25M sucrose

0.01M $CaCl_2 \cdot 2H_2O$

0.25M NaCl

0.1% Triton X-100

0.01M Tris

The pH was adjusted to 8.0 with conc. HCl and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Tris/EDTA buffer

0.05M Tris

0.01M EDTA

0.25M NaCl

The pH was adjusted to 8.0 with conc. HCl and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Buffer saturated phenol

Phenol	500 g
m-cresol	50 ml
8-hydroxyquinoline	0.5 g

The phenol was saturated with TE buffer (pH 8.0), before the m-cresol and 8-hydroxyquinoline were added.

Buffer saturated ether

Diethyl ether saturated with TE buffer (pH 8.0).

Appendix 5

Polysome buffers

i) Buffer of Jackson & Larkins (1976)

Tris	24.2 g
KCl	29.8 g
$MgCl_2.6H_2O$	7.11 g
Sucrose (Sigma)	68.46 g
β-mercaptoethanol	64 µl

Each of the above was dissolved and made up to 200 ml in distilled water. 9.51 g of EGTA was dissolved in 250 ml of distilled water containing a small amount of 40% (w/v) NaOH. The two solutions were mixed and the pH was adjusted to 9.0. The volume was made up to 500 ml with distilled water and the buffer was autoclaved at 10 lb $(in^2)^{-1}$ for 10 min.

Sucrose Solutions for Polysome Profile

Tris	0.242 g
KCl	0.745 g
$MgCl_2.6H_2O$	0.305 g
EGTA	0.095 g

The above were dissolved and made up to 50 ml in distilled water and the pH was adjusted to 8.5.

Four sucrose solutions were used to make the gradients. These were 50% (w/v) sucrose, 37.5% (w/v) sucrose, 25% (w/v) sucrose and 12.5% (w/v) sucrose. Each sucrose solution was made up in the buffer described above and the gradients were allowed to equilibrate overnight at 4°C before use.

ii) Buffer of Duncan & Humphreys (1981)

Tris	3.02 g
NaCl	23.37 g
$MgCl_2.6H_2O$	5.08 g
Sucrose (Sigma)	68.46 g

Each of the above was dissolved and made up to 200 ml with distilled water. 19.02 g of EGTA was dissolved in 250 ml of distilled water containing a small amount of 40% NaOH. The two solutions were mixed and the pH was adjusted to 7.1. The volume was made up to 500 ml with distilled water and the buffer was sterilized by autoclaving at 10 lb $(in^2)^{-1}$ for 10 min.

Sucrose Solutions for Polysome profile

The same 4 concentrations of sucrose were used. The sucrose solutions were made up in the above buffer minus sucrose.

iii) Buffer of Adams et al (1980)

Tris	6.05 g
NaCl	5.84 g
$MgCl_2.6H_2O$	20.33 g
Sucrose (Sigma)	68.46 g
β-mercaptoethanol	64 µl

Each of the above was dissolved and made up to 200 ml in distilled water. 28.5 g of EGTA was dissolved in 250 ml of distilled water containing a small amount of 40% (w/v) NaOH. The two solutions were mixed and the pH adjusted to 7.6. The volume was made up to 500 ml with distilled water and the buffer was sterilized by autoclaving at 10 lb $(in^2)^{-1}$ for 10 min.

Sucrose solutions for Polysome Profile

The same concentration of sucrose solutions was used as before. The solutions were made up in the above buffer minus sucrose.

Guanidine thiocyanate solution

1. 1M Tris/HCl pH 7.6
2. 0.2M EDTA

These two solutions were made and autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

10 g of guanidine thiocyanate was added to 10 ml of sterile distilled

water. 1.06 ml of each of the above solutions was then added and this was warmed to 60°C.

2.12 ml of 20% (w/v) sodium lauryl sarcosinate and 0.2 ml β-mercaptoethanol were added. The volume was adjusted to 21 ml with sterile distilled water and the solution was stored at 4°C.

Binding buffer used with oligo(dT) cellulose

0.02M Hepes

0.5M NaCl

0.001M EDTA

The pH was adjusted to 7.6 with 40% (w/v) NaOH and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min. 10% (w/v) SDS was then added to a final concentration of 0.1%. The SDS solution was heated to 60°C for 60 min before addition.

Elution buffer used with oligo(dT) cellulose

0.01M Hepes

0.001M EDTA

The pH was adjusted to 7.5 with 40% (w/v) NaOH and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min. 10% (w/v) SDS (preheated to 60°C for 60 min) was then added to a final concentration of 0.05%.

Lysis buffer

6M guanidine HCl (BRL)

0.001M β-mercaptoethanol

0.015M Tris

The pH was adjusted to 7.5 with conc. HCl and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Wash buffer

4M guanidine HCl

0.001M β-mercaptoethanol

0.015M Tris

The pH was adjusted to 7.5 with conc. HCl and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Elution buffer used with Poly(U) sepharose 4B

90% (v/v) deionized formamide (BRL, see below)

0.001M EDTA

0.015M Tris

The Tris/EDTA buffer was made and the pH was adjusted to 7.5 with conc. HCl before the formamide was added. The buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Deionized formamide

Formamide (BRL) was mixed with 10% (w/v) mixed bed ion-exchange resin (Bio-Rad, AG 501-X8, 20-50 mesh). This was stirred at room temperature for 30 min, then filtered through Whatman No. 1 filter paper twice. This was then stored at -20°C.

Phosphate buffer

The following two solutions were prepared

1. 2.5 M K_2HPO_4
2. 88% phosphoric acid

2 ml of K_2HPO_4 were added to 100 ml of 88% phosphoric acid. The pH was adjusted to 8.0 and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Appendix 6

Sonication buffer

0.02M Tris

0.001M EDTA

0.003M $MgCl_2 \cdot 6H_2O$

0.005M β-mercaptoethanol

The pH was adjusted to 8.0 with conc. HCl and the buffer was dispensed into 10 ml aliquots. It was then autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Packaging buffer

0.006M Tris

0.05M spermidine (Sigma)

0.05M putrescine (Sigma)

0.02M $MgCl_2 \cdot 6H_2O$

0.03M ATP

0.03M β-mercaptoethanol

The pH was adjusted to 8.0 with conc. HCl and the buffer was filter sterilized.

Sucrose Solution

1 g of sucrose was dissolved in 0.05M Tris/HCl pH 7.5 and the solution was filter sterilized.

Appendix 7

GGB X 20 Electrophoresis buffer

0.8M Tris

0.4M Sodium acetate

0.015M EDTA

The pH was adjusted to 8.3 with conc. acetic acid and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Tracker dye

GGB buffer	4 ml
Glycerol	1 ml
Bromophenol blue	0.005 g

The tracker dye was stored at $4^{o}C$.

Standard Saline Citrate (SSC X 20)

3M NaCl

0.3M Sodium citrate

The pH was adjusted to 7.0 with 40% (w/v) NaOH and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Formaldehyde Gel Running Buffer X10

0.2 M Hepes

0.01M EDTA

The pH was adjusted to 7.8 with 40% (w/v) NaOH and the buffer was autoclaved at 15 lb $(in^2)^{-1}$ for 15 min.

Formaldehyde Gel Tracker Dye

Sterile glycerol	5 ml
10X running buffer	1 ml
Sterile distilled water	4 ml
Bromophenol blue	0.01 g

The tracker dye was stored at $4^{o}C$.

Appendix 8

Butanol and Aqueous Solutions

n-Butanol	150 ml
Distilled water	150 ml

The above were mixed together and vigorously shaken and then the phases were allowed to separate at 37°C. 1 g of hexadecyltrimethylammonium bromide was dissolved in 100 ml of the butanol fraction which was shaken with 100 ml of the equilibrated aqueous fraction (from above). Antifoam A (Sigma) (50 µl) was added to reduce the formation of emulsions. The solution was left overnight for the phases to separate. Each phase was bottled separately and stored at 37°C.

Dialysis Membrane for Electroelution

The dialysis membrane was washed by boiling in 10% (w/v) Na_2CO_3 solution, rinsed extensively with distilled water containing 0.001M EDTA and stored in 60% (v/v) ethanol. Before use the membrane was rinsed with sterile distilled water.

Appendix 9

Formaldehyde prehybridization solution

50% (v/v) deionized formamide

5X Denhardt's solution (see below)

5 X SSC

0.1% sodium dodecylsulphate

100 $\mu g ml^{-1}$ denatured salmon sperm DNA (BRL)

Denhardt's Solution (X50)

Ficol (Sigma)	1 g
Polyvinylpyrrolidone (Sigma)	1 g
Bovine serum albumin (Pentax fraction V, Sigma)	1 g

Each of the above was dissolved in 100 ml of distilled water. The solution was filter sterilized and dispensed into 20 ml aliquots and stored at $-20^{\circ}C$.

Hybridization Solution

50% (v/v) deionized formamide

5X Denhardt's solution

5X SSC

0.1% sodium dodecyl sulphate

Aqueous prehybridization solution

6X SSC

5X Denhardt's solution

0.5% sodium dodecylsulphate

100 $\mu g ml^{-1}$ denatured salmon sperm DNA (BRL)

Aqueous hybridization solution

6X SSC

5X Denhardt's solution

0.5% sodium dodecylsulphate

0.01M EDTA.