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Qualitative and Quantitative Chances in Ubiquitin Transcripts during Dictyostelium mucoroides Development.

Vincent Van Rothe

Qualitative and Quantitative Changes

In Ubiquitin Transcripts during

Dictyostelium mucoroides Development

A Thesis

Presented to the

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and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

Vincent Van Rothe

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THESIS ACCEPTANCE

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Committee Member   Department

William E. Jappuch   Biology

Michal B. Zimmt   CHEMISTRY

Chairperson _A. T. Thompson Woodn___

Date _January 7, 2000___

ABSTRACT

The slime mold *Dictyostelium mucoroides* is an effective model for studying eukaryotic development because it exists as both unicellular and multicellular entities depending on the stage of their life cycle. Development is regulated by a number of proteins. During the transition from single amebas to a multicellular entity, developmentally regulated proteins appear. In the case of the macrocysts, produced during sexual reproduction, proteins not only function in the formation of the cell walls, but also aid in the fusion of gametes, selective phagocytosis, cell to cell contact, and make up lysosomal enzymes. We have cloned a developmentally regulated gene whose sequence shows homology to the gene encoding the protein ubiquitin. Ubiquitin is known to act as a selective marker of proteins for degradation. In other organisms, ubiquitin is involved in the regulation of the cell cycle and controlling development. The isolated gene was used as a probe to determine the number of copies of this gene in the genome of *D. mucoroides* and any changes in ubiquitin messenger RNA during development. The results of the Southern blot analysis showed that in *D. mucoroides* there are two copies of the ubiquitin gene. These are arranged in one chromosome and 800bp from each other. The RNA analysis identified four bands that were identical in size and quantity for each stage of development examined in both the macrocyst and sorocarp life cycles. The results therefore show that in *D. mucoroides* the ubiquitin gene is not developmentally regulated.

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Funding for this project was partially provided by the University Committee on Research, and the Biology department. This funding is without a doubt vital in order to conduct this kind of research which utilizes very expensive equipment and reagents. Their support is greatly appreciated.

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INTRODUCTION

Development at all levels is a direct or indirect result of synthetic activities within cells. It is generally accepted that the basis for generating cell diversity and cytodifferentiation is the formation of different gene products in different cells at different times (Carlson 1996). One of the underlying principles of molecular cell biology is that the actions and properties of each cell type are determined by the proteins it contains. The quality and quantity of various proteins that characterize a cell type are determined by the concentration of each protein's corresponding mRNA, the efficiency with which the mRNA is translated into protein and the stability of the protein itself. The concentration of a specific mRNA is determined by the efficiency with which the gene is transcribed. The observation that differences in the RNA and protein content of different tissues are not paralleled by significant differences in their DNA content suggest that transcription must be one level at which gene expression is regulated in eukaryotes (Nevins, 1983). Thus transcription is also a means of regulating development.

In bacterial systems, gene control serves mainly to allow a single cell to adjust to changes in its nutritional environment so that growth and division can be optimized. Gene control in eukaryotes is more complex and even though in some cases it involves responses to environmental changes, its most characteristic and important purpose is the regulation of the genetic program which results in development and differentiation (Lodish et al., 1995).

The quantity of gene product present in a cell can be regulated at several levels, such as transcription, translation and mRNA and protein degradation. As is the case for transcriptional regulation, control of gene expression at the level of translation often takes place at the initiation step. Gene products that require posttranslational modification may be regulated at each level of their synthesis (Meyers 1995). Studies have shown, however, that in higher eukaryotes the primary control of gene expression is at the level of transcription (Latchman, 1990).

Dictyostelium is an excellent model organism for the study of eukaryotic developmental processes. It is unique in that the multicellular form arises from aggregation of individual cells rather than division of a zygote. It has a small haploid genome of approximately 34 Mb (Kuspa and Loomis, 1996) arranged in six chromosomes. Because of its simple developmental cycle and relatively small genome, *Dictyostelium* offers the opportunity to carry out developmental, genetic, and molecular research which would be impossible with more complicated organisms.

Cellular slime molds were first discovered in 1869, when Brefeld identified *Dictyostelium mucoroides*, however, their value as models for eukaryotic development was not realized until 1940 (Bonner, 1982). Collectively grouped as social amebas, they do not belong to the genus ameba, and are not slime molds. The difficulty in classifying them lies in the fact that although they resemble both, they are neither plants nor animals. They are not fungi or protozoan but something in between. They are eukaryotic and exist as both unicellular and multicellular entities depending on the stage of their life cycle (Bonner, 1982; Devreotes, 1989; Waterfall, 1983).

Most species of the genus *Dictyostelium* are capable of development along two alternate life cycles: sexual and asexual. When the conditions are right and food is abundant, the haploid, uninucleate amebas forage on bacteria in the upper soil layer and beneath the rotting leaves of the forest floor (Bonner, 1982; Waterfall, 1983). Cell division at this stage takes place by binary fission. The name social amebas refers to the ability of the organism to enter its next step of development, aggregation, and become what appears to be a multicellular animal (Waterfall, 1983).

Both developmental life cycles are brought about by the depletion of bacteria and therefore starvation (Bonner, 1982; Devreotes, 1989; Loomis, 1975; Mutzel, 1995; O'Day, 1979; Waterfall, 1983). Nickerson and Raper (1973a) have shown that environmental conditions are the determining factors for the type of reproduction followed by the aggregated amebas. The sexual cycle is favored by conditions of low light, low phosphate, high humidity, and higher temperatures. During sexual development haploid cells fuse to form a zygote. In heterothallic strains such as *D. discoideum* opposite mating types are required. The macrocysts produced by these strains are difficult to obtain in large numbers and do not germinate very efficiently. Homothallic strains, such as *D. mucoroides*, do not require opposite mating types and produce fairly high numbers of macrocysts which are easier to germinate in the laboratory (O'Day, 1989). Figure 1 shows the life cycle of *D. mucoroides*. When the haploid cells fuse, they form a larger diploid cell known as the zygote giant cell. The giant cells secrete cAMP as a chemoattractant for other amebas (O'Day, 1989; O'Day, 1981). The giant cells ingest the surrounding amebas and enclose them each in a vacuole

(Erdos at al., 1972; North and Cotter, 1991). Engulfment may occur at several locations of the forming macrocyst. As more amebas continue to aggregate and are ingested, the macrocyst increases in size. These cells become surrounded by a fibrillar sheath which has been termed the primary wall. After the available amebas have been phagocytized, the giant cell forms a cellulosic secondary cell wall inside the primary wall (Erdos et al., 1972; O'Day 1979).

During the formation of the secondary wall, the endocytes begin to shrink and disappear in a process that resembles the digestion of food vacuoles in vegetative cells (Erdos et al., 1972; North and Cotter, 1991). O'Day and Lewis (1981) suggest that the amebas that are phagacytosed are used as a food source during development. A third wall is then formed inside the secondary wall. This wall is more flexible than the secondary wall and is composed of three layers, an outer, middle and inner layer (Erdos et al., 1972; O'Day, 1981). The macrocyst will mature and remain as a resistant structure until conditions become favorable for germination. These conditions seem to be somewhat specific for each strain. The end result is the release of new haploid vegetative amebas whose fate will be determined by the environmental conditions (Nickerson and Raper 1973b).

The asexual cycle begins when up to 10^5 single amebas aggregate and form a multicellular structure which migrates. This structure resembles a garden slug (Devreotes, 1989; O'Day, 1981; Mutzel, 1995). More properly it is referred to as a migrating pseudoplasmodium (Waterfall, 1983). Aggregation, which initiates the asexual cycle, is also induced by starvation and also involves chemotaxis as in macrocyst

Figure 1. Life cycle of *Dictyostelium mucoroides* showing both the asexual (sorocarp) and sexual (macrocyst) cycles.

meiosis

primary
secondary
tertiary
} walls

Macrocyst
Formation &
Germination

Appearance
of amebas
in cyst

Giant cell
engulfing
amebas

Rupture of
primary and
secondary walls

Formation of
giant cell

Rupture of tertiary
wall and liberation
of amebas

Dark, moisture
higher temp
low phosphate

Germination

Interphase

Spore

Vegetative
growth

Aggregation
7-10hr

Fruiting Body
Formation

Aggregate 12hr
10^5 cells

formation. The amebas stream into central collection points in a process typically mediated by cyclic AMP, which can orient the cells in a constant gradient, or by pulses which radiate outward. The anterior cells in the pseudoplasmodium will become stalk cells and the posterior three-quarters will become spores (Bonner, 1982). The stalk raises the pre-spore cell mass into the air, forming what is known as a sorocarp. From each spore will emerge an ameba that is competent to repeat the life cycle (Bonner, 1982; Devreotes, 1989; Mutzel, 1995; Waterfall, 1983).

Marx (1992) estimates that 15% of the 300 developmentally regulated *Dictyostelium* genes have been isolated and cloned. Some of these genes code for known proteins such as actin (Kindle and Firtel, 1978), myosin (De Lozanne et al., 1985), discoidin (Williams et al., 1979), a cyclic AMP dependent protein kinase (Mann and Firtel, 1993; Gaskins et al., 1994), β-glucosidase (Bush et al., 1994), a MAP kinase (Gaskins et al., 1996), a ubiquitin conjugating enzyme (Clark et al., 1997) and ubiquitin (Muller-Taubenberger, 1988b; Giorda and Ennis, 1987). An even larger number of developmentally regulated genes that encode unknown proteins have been reported. Vegetatively growing amebas are estimated to contain 3,000 to 5,000 different mRNA species (Blumberg and Lodish 1981). Jaqiet et al. (1981) estimates that during development 700 to 900 new transcripts are present, and Blumberg and Lodish (1981) estimate between 2,000 and 3,000 new transcripts are present during development.

During the transition from single amebas to a multicellular entity, developmentally regulated proteins appear. A two-dimensional polyacrylamide gel electrophoresis study (Moriyama and Yanagisawa, 1988) on macrocyst and sorocarp

development, showed that during the first 16 hours of macrocyst development of *D. discoideum*, a total of 49 new polypeptides were synthesized. Synthesis of 27 of these started within 4 hours of development, 8 were produced by about 7 hours, 9 by 10 hours, and the remaining 5 by 13 hours. By comparing these results with those obtained during sorocarp formation, Moriyama and Yanagisawa showed that 26 of the 49 polypeptides, whose synthesis began within the first 16 hours, were specific for macrocyst development and 23 were shared by both life cycles. The function of all of these proteins is not known, however. In the case of the macrocysts, proteins not only function in the formation of the cell walls but also aid in the fusion of gametes, selective phagocytosis, cell to cell contact, and they make up lysosomal enzymes (Moriyama and Yanagisawa, 1987).

Work performed on spore germination revealed a number of genes that appear to be specifically expressed during germination of *D. discoideum* (Giorda and Ennis 1987). The amino acid sequences in two clones were almost identical to that for human ubiquitin, a 76 amino acid protein whose sequence is conserved in many organisms. Compared to the human sequence, there are two amino acid substitutions in *Dictyostelium* ubiquitin (Muller-Taubenberger et. al., 1988; Muller-Taubenberger et al., 1989). Omachi et al. (1989) and Giorda and Ennis (1987), showed that the concentration of these ubiquitin transcripts was very low throughout most of the life cycle of *D. discoideum* but their concentrations greatly increased in dormant and germinating spores. By probing Northern Blots for ubiquitin transcripts, they were able to observe bands of 1,900, 1,400, 1,100, 840, 580, and 500 nt. The 1,400 nt species was found exclusively in

spores. At 1.5 h of germination the 1,900, 1,400, 1,100 and 840 nt transcripts were

observed, whereas at 3 h the predominant species were 1,900 and 1,400 nt. In growing

cells, the 580, and 500 nt bands were observed. By using Southern blot analysis it was

determined that the cloned ubiquitin gene represented a family of six genes. Screening of

a genomic library, constructed by the insertion of fragments of 4.5 to 5.5 kilobases from a

Sau3A partial digestion of *D. discoideum* M2 DNA into the BamHI site of plasmid

pAT153, produced clones which when sequenced showed tandem repeats of the ubiquitin

sequence (Giorda and Ennis 1987).

Ubiquitin is a small protein that is probably present in all eukaryotic cells, but not

in prokaryotes (Vijay-Kumar 1987; Monia 1990). It is the most highly conserved protein

yet discovered in eukaryotes (Vijay-Kumar 1987; Monia 1990). Structurally it is a single

domain, tightly packed globular structure made up of five strands of β-pleated sheet and

three and one half turns of α-helix. It has a dense hydrophobic core as well as some

hydrophobic patches on its surface (Monia, 1990). In addition to the usual hydrogen

bonds formed in the β-sheet and α-helix, there are a number of unusual intramolecular

hydrogen bonds. The hydrophobic core and extensive hydrogen bonded secondary

structure probably explains ubiquitin's stability to thermal denaturation. The molecule

also shows three lysine residues fully exposed to the surface. These lysine residues serve

as attachment sites for polyubiquination (Vijay-Kumar, 1987). The last four residues of

the carboxy terminus protrude from the rest of the rigid molecule to form a tail. This is

critical for the function of ubiquitin since it is the C terminal glycine that attaches to the

ubiquitin activating enzyme E1 and to the target protein during the formation of ubiquitin-target conjugates (Vijay-Kumar 1987; Monia 1990).

The ubiquitin system has been shown to play a key role in a variety of cellular processes. Research has shown that it has three main functions: 1) it is covalently linked to histones H2A and H2B in regions of active chromatin transcription (Mueller-Taubenberger, 1988a). Histone proteins H2A and to a lesser extent H2B, are among the most abundant ubiquinated proteins found in cells. Most histones of the cell have been shown to be mono ubiquinated and are not degraded. The function of histone ubiquination is unclear; however, it has been proposed that it may play a role in maintaining the structure of transcriptionally active chromatin (Monia, 1990). Giorda and Ennis (1987) suggest that ubiquitin may play a role in gene expression. 2) Heat shock, cadmium treatment, starvation, and other stresses cause accumulation of ubiquitin mRNA (Muller-Taubenberger, 1988a). Genes in tandem repeats encoding polyubiquitin transcripts from a number of organisms have been shown to contain heat shock promoter elements and are inducible by stress (Omachi et al. 1989, Muller-Taubenberger et al., 1988; Monia, 1990). The current belief is that denatured proteins compete with heat-shock regulator protein for E1 activated ubiquitin. When the cells are stressed, as in the case of heat-shock, the denatured proteins will bind the ubiquitin and more regulator protein will be released. In its free form, the regulator protein activates the transcription factor (Muller-Taubenberger et al., 1988a). Work on mutants of the polyubiquitin encoding locus (UB14) in yeast, has shown that the ubiquitin dependent proteolytic pathway is essential for cell survival during conditions of stress (Monia, 1990).

3) The best studied function of ubiquitin involves ATP-dependent intracellular protein degradation (Giorda and Ennis, 1987). Ubiquitin targets proteins for degradation by covalent linkage. A model for the ubiquitin proteolytic pathway is presented in figure 2. The carboxy terminal glycine of ubiquitin is activated by ATP to a high energy thiol ester intermediate. This reaction is catalyzed by the ubiquitin-activating enzyme E1. After activation, a ubiquitin carrier protein, E2, transfers ubiquitin from E1 to a ubiquitin-protein ligase, E3. This ligase catalyzes the last step in the conjugation process, the isopeptide bond formation between ubiquitin and a specific protein. The specific binding of E3 prior to the reaction with the activated ubiquitin appears to be an important step in the selection of proteins for conjugation and degradation. After the ubiquitin-protein conjugate forms, the protein portion is degraded by a specific ATP dependent protease into free amino acids and free re-utilizable ubiquitin (Hershko, 1983; Monia, 1990; Rechsteiner, 1991; Hershko et al.1982; Chiechanover, 1994). This protease complex is known as the 26S proteasome (Chiechanover and Schwartz, 1994; Gregori et al., 1985). There are at least two ways in which preferential accumulation of proteins can occur: either by an increase in their rate of synthesis or a decrease in their rate of degradation. If a preferential decrease in protein degradation brings about an increase in accumulation, then protein turn over must be very rapid before the period of accumulation (Loomis, 1975). Giorda and Ennis (1987) and Clark et al. (1997) suggest that since ubiquitin seems to be necessary for the decay of a large number of proteins, it may function in protein degradation during developmental transitions.

E1-SH+E2-SH's   E1-SH

ATP

1

Amino
Acids

monoUb-Protein

Peptides

O
‖
Ub-C-OH
Ubiquitin

PROTEINS   2

O
‖
E2-S~C-Ub

3   E3 · Protein

ATP
5

E3's
(Types I-IV)

$(Ub)_n$ Protein
Conjugates   4
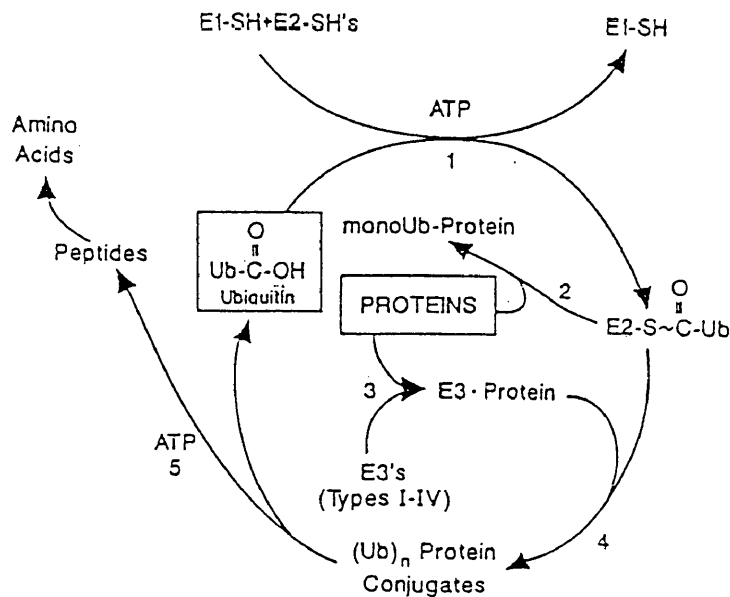
Figure 2. Proposed sequence of events in the ubiquitin degradation process. 1)
Activation of ubiquitin by E1 (ubiquitin activating enzyme) and E2 (ubiquitin carrier
protein). 2) Formation of monoubiquinated conjugates by E2. 3) Formation of E3
(ubiquitin-protein ligase-protein complex. 4) Conjugation of multiple molecules of
ubiquitin to the protein substrate. 5) ATP-dependent degradation of conjugates into
peptides and free amino acids with recycling of ubiquitin (Chiechanover and Schwartz,
1994).

Protein ubiquination can result in one of three possible outcomes. The protein may be stable and exist indefinitely as a ubiquinated molecule. The protein may be removed by ubiquitin isopeptidase enzymes, or the protein may become multiubiquinated and be destroyed by a ubiquitin-dependent protease complex (Monia, 1990). Following marking of a protein moiety, the conjugate is selectively degraded in a process that requires ATP and the ubiquitin is released intact (Chiechanover, 1994; Rechsteiner, 1991; Hershko et al., 1982).

Ubiquitin modification of some proteins has been shown to play important roles in cellular processes. One such process is regulation of the cell cycle. Cyclin, for example, activates a regulatory kinase known as cyclin dependent kinase (CDK). Part of the way through mitosis, after CDK has initiated mitosis, cyclin levels go down and the kinase becomes inactivated, allowing the cell to finish the cycle. Murray and Kirschner (Barinaga, 1995) found that by making cyclin indestructible, the inactivation of CDK did not take place and the cell became arrested in mitosis. The mutation which rendered cyclin indestructible was in the sequence for ubiquitin attachment. Protein destruction in this case would turn CDK off.

Clark et al. (1997) have shown that disruption of UbcB, a ubiquitin conjugating enzyme in *Dictyostelium*, results in developmental arrest during multicellular stages of sorocarp development. The developmental pattern of protein ubiquination is altered in *ubc*B null cells. These cells are blocked in the ability to properly undergo transition from the induction of postaggregative gene expression during mound formation to the induction of cell-type differentiation and subsequent morphogenesis. Postaggregative

gene transcripts were observed to accumulate to very high levels and did not decrease significantly with time as they do in wild-type cells. The high, extended level of expression suggests that the regulatory mechanism involved in down regulating developmental transition genes is blocked in *ubc*B null cells. The analysis of the developmental phenotypes suggests that tip formation and subsequent development requires specific protein ubiquination and possibly degradation. In *D. discoideum* it has been shown that ubiquitin is developmentally regulated in spore germination (Giorda and Ennis, 1987) and the sorocarp life cycle (Omachi et al. 1989).

This investigation on qualitative and quantitative changes in ubiquitin transcripts during *Dictyostelium mucoroides* development was initiated as a follow up to results reported by Esser (M.A. Thesis, 1995). Using differential display, Esser found a cDNA fragment specific to macrocyst development in *D. mucoroides*. The amino acid sequence for her clone was reported to have about 70% identity to *Dictyostelium discoideum* ubiquitin. The observation that the band appeared only in samples from macrocysts suggested that at least one ubiquitin transcript was developmentally regulated in *D. mucoroides* macrocyst formation.

In the work reported here, ubiquitin transcript production throughout *D. mucoroides* macrocyst development was investigated. The purpose of this study was 1) to determine, using Southern blot analysis, the number of genes encoding ubiquitin in *D. mucoroides* and 2) to determine by Northern analysis, qualitative and quantitative changes in ubiquitin transcripts during macrocyst development.

MATERIALS AND METHODS

Ubiquitin clone:

The cDNA fragment, which shared homology with a ubiquitin transcript, was

ligated into pGEM-T vector at the EcoRV site (bp 51) (figure3) by Esser (M.A. Thesis,

1995), and transformed into *E. coli* DH5-α. The ligated sequence was designated M14

A-1 and the plasmid carrying this sequence pGEM-T::M14 A-1.

The pGEM-T plasmid in *E. coli* DH5-α was isolated through a large scale

alkaline lysis procedure (Sambrook et al., 1989). *E. coli* DH5-α cells were grown

overnight at 37°C on LB agar plates with ampicillin (50 μg/mL). One colony was

transferred into 25 mL of LB broth with ampicillin (50 μg/mL) and incubated at 37°C

overnight. One milliliter of the overnight culture was transferred into 100 mL of LB with

ampicillin and incubated until the optical density (O.D) reached 0.5 to 0.6 (wavelength

600 nm). The host cells were split into two 50 mL tubes and treated identically. They

were centrifuged for 15 minutes at 4° C at 6500 rpm. The supernatant was discarded, and

as much of the remainder as possible aspirated with a glass pipette. Each pellet was

resuspended in 0.6 mL of ice cold lysis buffer (25 mM Tris, 10 mM EDTA, 50 mM

glucose) by gently vortexing and pipetting. The cell suspension was transferred to a 30

mL tube. To this, 100 μL of lysozyme solution (50 mg/mL) was added and the

suspension incubated on ice for 20 min. After the incubation, 4.8 mL of ice cold 0.2 N

Figure 3. Map of the pGEM-T vector with the M14 A-1 insert.

SphI
BstZI
NcoI
SacII
CjeI
BccI
MseI
DdeI
CjeI
BccI
SpeI
NotI
PstI
SalI
T7
SP6
M14 A-1 (170bp)

NcoI
PstI

Mult. Cl. Site

f1 ori

3000

2500

500

pGEM-T

3003 bps

2000

1000

1500

Amp. res.
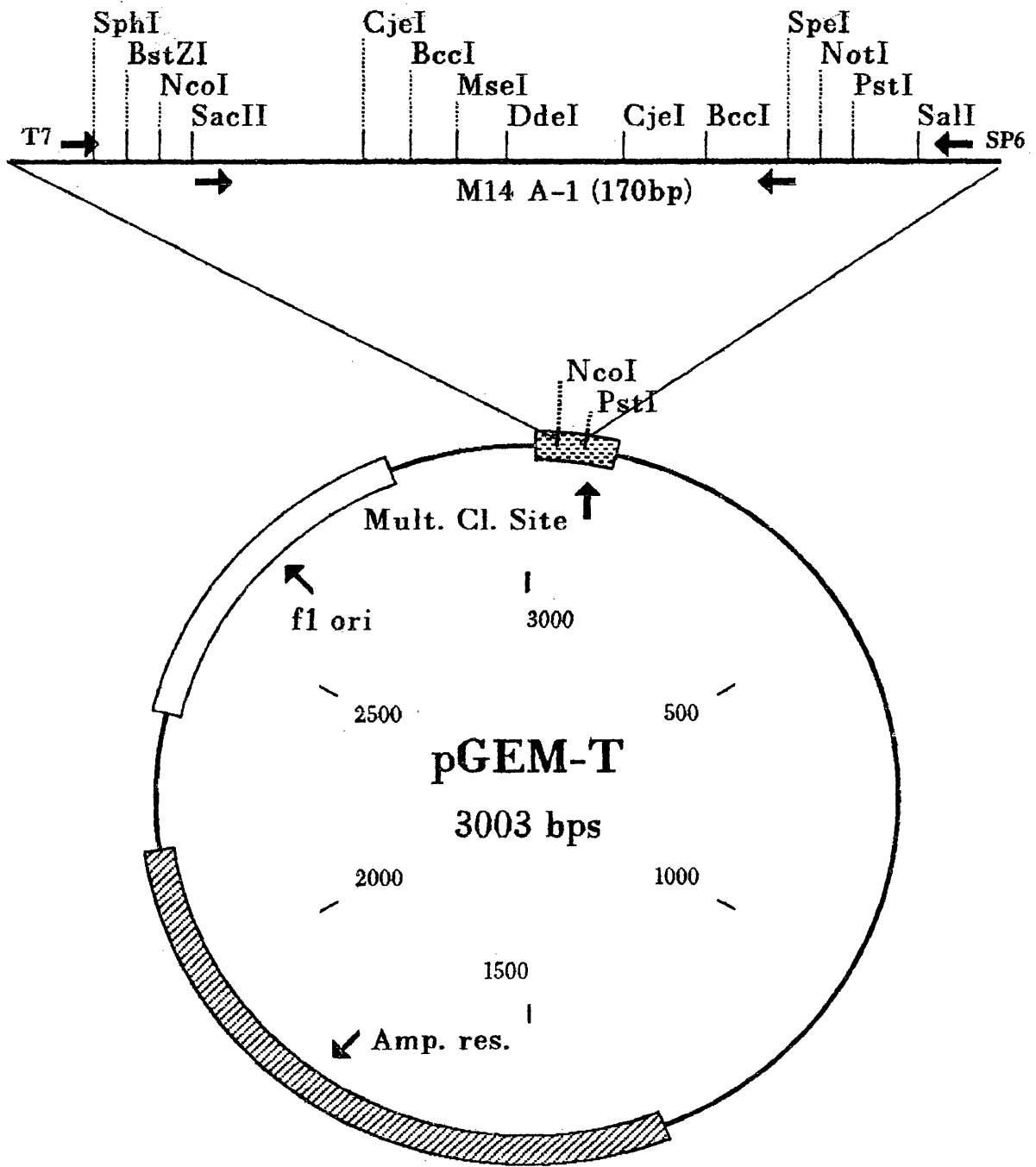
NaOH containing 1% SDS was added, mixed gently and incubated again on ice for 10

minutes. To this, 4.0 mL of potassium acetate buffer [250 g potassium acetate, 150 mL

acetic acid, 1 L distilled water (dH$_2$O)] was added and incubated on ice for 10 minutes.

This was centrifuged at 12,000 X g for 15 minutes at 4°C in a SS34 Sorvall rotor and the

supernatant was transferred to a new 30 mL tube to which 30 μL of RNAase (DNAase

free 2 mg/mL) was added. The supernatant was incubated at 37°C for 1 hour, after

which, it was phenol extracted by mixing with an equal volume of TE saturated phenol

chloroform pH 8.0. This was mixed by inversion for about 1 minute and centrifuged at

12,000 X g for 10 minutes. This phenol extraction step was repeated followed by an

extraction with one volume of chloroform: isoamyl alcohol (24:1), mixed for about 1

minute and centrifuged for 10 minutes at 12,000 X g. The upper phase was transferred to

a fresh tube and was precipitated overnight with 95% ethanol. This was centrifuged for

10 minutes at 12,000 X g at 4°C, the supernatant was carefully discarded and the pellet

was washed with 70 % ethanol. The pellet was allowed to dry and was resuspended in

200 μL of dH$_2$O.

Southern Blot Analysis:

Genomic DNA was extracted from *Dictyostelium mucoroides* DM-7 cells and

blotted on a membrane following the Southern procedure for DNA capillary transfer

(Kikkawa et al., 1992; Sambrook et al., 1989). *D. mucoroides* strain DM-7 stock cultures

were maintained on 0.2% peptone-lactose agar plates (0.2% peptone, 0.2% lactose, 1.5%

agar). Amebas were grown in liquid culture in association with *E. coli* B/r as the primary

nutrient source. Growth of the vegetative amebas was obtained by making a spore

suspension at 5×10^5 spores/mL. To this, *E. coli* B/r from an overnight culture on tryptic

soy agar (TSA) was added until milky, and 0.2 mL was used to inoculate 40 mL GYP

media (2g peptone, 1g glucose, 0.5g yeast extract, 0.8g KH_2PO_4, 0.54g Na_2HPO_4, 1L

dH_2O) in 250 mL flasks. The medium was incubated at 23°C in the dark for 56 hours.

The cells were then harvested by centrifugation in a GSA rotor for 15 minutes at 400 X g

(2000 rpm) at 4°C. The cells were washed with cold distilled water and the wash was

discarded. The pellet was resuspended in cold dH_2O. The DNA isolation was adapted

from Richardson et al. 1991. The amebas were counted after making a 10^{-2} dilution (100

µL into 9.9 mL), and 1 mL of nuclei buffer (40 mM Tris HCl pH 7.6, 15% sucrose, 0.1

mM EDTA, 6 mM $MgCl_2$, 40 mM KCl, 5 mM DTT, 0.4% NP-40) was added to dilute

the suspension to 3.5×10^8 cells / mL. The cells were then incubated on ice for 10

minutes and centrifuged at 12,000 X g for 10 minutes, after which 1 mL of nuclei buffer

was again added and the suspension was incubated on ice for 10 minutes. The cells were

then centrifuged at 10,000 X g for 10 minutes in a Sorvall SE-12 rotor. The supernatant

was discarded and the pellet resuspended in 500 µL of proteinase K buffer (10 mM Tris

pH 7.5, 5 mM EDTA, 0.5% SDS, 2U proteinase K). The cells were incubated at 65°C for

1 hour. The mixture was allowed to cool to room temperature and was extracted with an

equal volume of Tris saturated phenol. Mixing was done by gentle inversion for 3

minutes followed by centrifugation at 12,000 X g for 5 minutes. The upper phase was

transferred to a fresh tube and was then extracted with 1 volume of phenol chloroform,

mixed, and centrifuged for 5 minutes at 12,000 X g. The phenol chloroform extraction

was repeated. The final extraction was performed with one volume of

chloroform/isoamyl alcohol (24:1) and centrifuged for 5 minutes. The DNA was

precipitated in 95% ethanol overnight. The precipitate was then centrifuged for 20

minutes at 12,000 X g, washed with 70% ethanol, and allowed to air dry. The pellet was

resuspended in 100 μL of dH$_2$O.

The endonucleases chosen to cut the genomic DNA were selected so one would

cut within the insert sequence and two would not. The enzymes chosen were PstI and

PvuII, which do not cut within the insert sequence, and HincII that does cut within the

insert sequence. Five genomic digestion reactions were set up, three were single digests

and two were combination of PvuII and HincII, and PstI and HincII. In all five reactions,

30 μg of DNA were digested in a reaction containing 9 μL of dH$_2$O, 3 μL of 10 X Buffer,

15 μL of DNA concentration 2 μg/μL, 30U of enzyme. The reaction was incubated for 1

hour at 37°C after which an additional 20U of enzyme was added and incubated at 37°C

overnight. The digests were then loaded on a 60 mL 0.8% agarose gel, and the gel

electrophored at 20 V for 20 hours. The gel was soaked for 30 minutes in 0.5 N NaOH,

1.5 M NaCl, then successively 30 minutes in neutralizing buffer (0.5 M Tris-Cl, 1.5 M

NaCl) and 30 minutes in 20 X SSC (175.30g NaCl, 88.2g sodium citrate, 1L dH$_2$O). The

capillary Southern transfer was set up with 20 X SSC and allowed to go overnight. A

Zeta Probe-GT filter (Bio-Rad) was briefly soaked in dH$_2$O and soaked for 10 minutes in

20 X SSC. The membrane was briefly rinsed in 10 X SSC and UV cross linked in a

Stratagene UV Stratalinker™ 1800 Crosslinker, at 120,000 μ Joules for 1 minute.

RNA isolation:

Total RNA was isolated from each developmental stage, including zero hour. Vegetative amebas were grown as described for the genomic DNA isolation for 56-60 hours at 23°C. When the cells had reached a concentration of 3.6 X 10^6 cells/ mL, they were harvested by centrifugation in a GSA rotor at 1500 X g for 15 minutes. The supernatant was discarded and the pellet resuspended in room temperature Bonner's salt solution (BSS, 0.6 g NaCl, 0.75 g KCl, 0.3 g $CaCl_2$, 1 L dH_2O). The cells were centrifuged at 1500 RPM and resuspended 3 more times to wash them free of the *E. coli*. The pellet was then resuspended in 1 mL BSS with streptomycin (0.5 mg/mL) and the cells counted. The suspension was then diluted with 3.5 mL of BSS and streptomycin to reach a final concentration of 3.1 X 10^8 amoebas/mL, of which 0.9 mL was to be placed on each filter. The filters were contained in 50x11 mm disposable petri dishes with an adsorbent pad. To this, 1.5 mL of BSS plus streptomycin (0.5 mg/mL) was added, a "Supor 450" membrane was then place over the pad. Nine tenths milliliters of the amoeba suspension were then added, allowed to settle, and covered with another membrane filter, and 1.5 mL of BSS plus streptomycin was added. The dish was then covered with the lid and wrapped with aluminum foil for dark incubation, and placed in an incubator at a temperature of 23°C. The amebas were allowed to develop and were sampled at 0, 4, 8, 12, 18 and 24 hours.

For sorocarp development, 1.5 mL of phosphate buffer and streptomycin (0.5 mg/mL) was added, a "Supor 450" membrane filter was placed over the adsorbent pad,

and 0.5 mL of the suspension was added in a circle. The dishes were then covered with

the lid and allowed to develop in the light and sampled at 4, 8 and 12 hours.

The RNA was isolated by taking the filters from the culture dish at the specified

time and placing each in a 50 mL conical tube. With a glass pipet as much of the BSS or

phosphate buffer as possible was recovered. Ten milliliters of cold BSS was then added

to each tube and the tube was vortexed. The filters were then taken out and the tube was

centrifuged at 15,000 rpm for 15 minutes. The supernatant was discarded and the pellet

resuspended in 1.9 mL of cold dH_2O. Seventy microliters of this was transferred to a

fresh microfuge tube and 1 mL of Trizol reagent was added (GIBCO BRL). This was

then stored at $-70°C$. The rest of the sample was stored at $-20°C$. After 24 hours the

Trizol treated cells were allowed to equilibrate at room temperature for 5 minutes, and

0.2 mL of cold chloroform was added. The Trizol Reagent maintains the integrity of the

RNA while the cells are being disrupted. The addition of the chloroform after the

incubation produces an aqueous phase in which the RNA remains exclusively. The

samples were hand mixed for 15 seconds and centrifuged at 12,000 X g at $4°C$ for 15

minutes. The upper aqueous layer was transferred into a fresh tube, and 0.5 mL of

isopropyl alcohol added to precipitate the RNA. The RNA was allowed to precipitate for

10 minutes at room temperature and centrifuged at 12,000 X g for 10 minutes. The

pellets were then washed with 70% ethanol/DEPC (diethyl-pyrocarbonate) dH_2O,

allowed to dry, and resuspended in 5 μl of DEPC/ dH_2O, 5 μl of 5X formaldehyde gel

running buffer (0.1 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0).

Formaldehyde gel and Northern blot:

The RNA was separated on a 1.2% formaldehyde denaturing gel containing 10 mL of 5X running buffer (0.1 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0) 2 mL of 1.2 M formaldehyde, 0.54 g agarose, 48 mL of DEPC/ dH₂O. Each sample was prepared by adding 20 µl of a cocktail containing 7.5 µL of formaldehyde, 150 µL of formamide, and 3 µl of ethidium bromide (EtBr). The samples were heated at 65°C for 15 minutes and chilled on ice for 5 minutes.

Four microliters of RNA gel running dye (0.4% bromophenol blue, 0.4% xylene cyanol) were added to each sample and 5 µL loaded on a 1% agarose minigel. The minigel was electrophored at 80 V for 30 minutes to check the concentration of the samples. The formaldehyde denaturing gel was then electrophored at 50 V for 6 hours in 1 X running buffer after which it was observed under UV light for ribosomal RNA. A Zeta Probe-GT filter (Bio-Rad) was briefly soaked in water, then soaked in 20 X SSC. The transfer was set up with 20 X SSC made with nanopure dH₂O in baked glassware to avoid any RNAse degradation. The transfer was allowed to proceed overnight. The membrane was briefly rinsed with 5 X SSC and baked at 80°C for 2 hours in a vacuum oven.

Riboprobe synthesis:

The template DNA was prepared by linearizing the previously isolated plasmid with the M14 A-1 insert. The plasmid was linearized by incubating 32 µL of dH₂O, 10 µL of template DNA (conc. 2 mg/ml), 5 µL of 10 X buffer, 20U of SalI enzyme, for 1

hour at 37 °C. At the end of the incubation, 10U of enzyme was again added and incubated at 37 °C for 1 hour. After this, 2U of proteinase K was added and incubated at 50 °C for 1 hour, the digestion reaction was then phenol extracted twice and precipitated in 250 μL of 7 M ammonium acetate/DEPC, and 100% ethanol at -20 °C overnight. The DNA was centrifuged at 12,000 X g for 20 minutes, washed in 70% EtOH and resuspended in 10 μL of dH₂O. The probe was synthesized in a 50 μL reaction containing: 16.5 μL of dH₂O, 3.5 μL of DNA (SalI linearized M14 A-1), 2.5 μL of 10 mM ATP, 2.5 μL of 10mM CTP, 2.5 μL of 10 mM GTP, 12.5 μL of labeled P^{32} UTP, 5.0 μL of 10X Transcription Buffer, 5.0 μL of T7 polymerase (20U/μL). The reaction was incubated at 37°C for 1hr after which 2U of DNAse was added and incubated at 37 °C for 30 min. At the end of the incubation, 0.5 μL (5mg/mL) of tRNA, 100 μL of EtOH absolute and 10 μL of 7 M ammonium acetate were added and incubated at -20°C for 1hr. The probe was then centrifuged at 12,000 X g for 20 min, resuspended in 50 μL of dH₂O/DEPC, and the incorporation of the radioactive nucleotide assessed by counting in a scintillation counter.

Prehybridization of blots:

The RNA and DNA blots were pre-hybridized for 60 minues at 65 °C in 15 mL of 0.5 M Na₂HPO₄ and 7% SDS. The blots were then hybridized in 10 ml fresh pre-hybridization solution with 25 μL of the riboprobe at 65 °C overnight in a rotatory hybridization oven.

Melting temperature:

The melting temperature was determined using the formula provided in the Zeta

Probe-GT instruction manual (Bio-Rad, Hercules, CA):

Tm (DNA/DNA) = 81.5 + 16.6 X log {Na+} - 0.65 X (% formamide) + 41 (G + C)

Tm (RNA/RNA) = 79.8 + 18.5 X log {Na+} - 0.35 X (% formamide) + 58.4 X (G + C) +

11.8 X $(G + C)^2$

Tm (DNA/RNA) = approximate mean of Tm (DNA/DNA) and Tm (RNA/RNA). Used

20 – 25° C less than calculated Tm as suggested by the manufactured (Zeta Probe-GT).

The blots were each washed for 1 hour at 65°C in 50 mL of 40 mM Na_2HPO_4 pH

7.2, 5% SDS. They were washed once in 50 mL of 40 mM Na_2HPO_4 pH 7.2, 1% SDS at

65 °C for 30 minutes, and finally washed for 30 minutes in 50 mL of 10 mM Na_2HPO_4 pH

7.2, 5% SDS. They were then placed on Saran wrap and exposed to X-ray film.

Cloning of genomic DNA:

Dictyostelium mucoroides strain DM-7 DNA, previously isolated, was digested

with PstI. The DNA was incubated in two tubes containing 9 µL of dH_2O, 15 µL of

DNA (conc. 2 mg/ml), 3 µL of 10 X Buffer, 30U of enzyme for 2 hours at 37 °C. After

the two hours, 3 µL of enzyme was added and incubated at 37 °C for 2 hours. The DNA

was then loaded on a 0.8% agarose gel and the gel was run in 0.5 X TBE for 2 hours at

150 V. A band of approximately 2800 bp, which had previously been observed to

hybridize with the probe, was cut from the gel and run through a Gen Elute™ Agarose

Spin Column according to manufacturer's instructions (SUPELCO, Bellefonte,PA). The

DNA was precipitated overnight at -20 °C with 500 μL of 100% EtOH, and 70 μL of 7 M

ammonium acetate. The DNA was then centrifuged at 12,000 X g for 20 minutes at room

temperature, washed with 70% EtOH and resuspended in 20 μL of dH$_2$O.

pUC 18 plasmid vector was isolated from DH5-α host cells containing the

plasmid, following the alkaline lysis procedure described for the M14 A-1 plasmid. The

pUC 18 DNA (10μg), with a concentration of 2 mg/ml, 5 μL of Buffer 10 X and 20U of

PstI, were incubated at 37 °C for 2 hours, then 1 μL of enzyme was added and incubated

for 1 hour at 37 °C. After the incubation, 2U of protease K was added and incubated at 50

°C for 1 hour. The DNA was then allowed to precipitate overnight at -20 °C with 250 μL

of EtOH 100%, 20 μL of 7 M ammonium acetate. The DNA was centrifuged at 12,000

X g and resuspended in 10 μL of dH$_2$O.

The ligation reaction of the DM-7 DNA into pUC 18 was carried out by

incubating 2 μL of 10 X Buffer, 16 μL of dH$_2$O, 1 μL of 1:25 dilution of PstI linearized

(1μg/μL) pUC18, 2 μL of genomic DNA digested with PstI, and 1 μL of a 1:10 dilution

of ligase, at 16 °C overnight.

Transformation of DH5-α competent cells:

Twenty five milliliters of LB medium were inoculated with 250 μL of an

overnight culture of *E. coli* DH5-α and allowed to grow at 37 °C for 2.5 hours (O.D. 0.5).

A 5 mL sample of this was chilled on ice for 20 minutes. The cells were then centrifuged

at 3000 rpm in GLC-2 Sorvall for 15 minutes and the pellet was resuspended in 0.5 ml of

TSS (85mL LB, 5mL DMSO, 10g PEG (8000), 50mM MgCl2 pH 6.5). From the TSS

suspension, 100 µL was transferred to a microfuge tube, and 10 µL of linearized pUC 18

plasmid solution (previously isolated) was added. The suspensions were placed on ice

for 20 minutes and then incubated at 42 °C for 1 minute. One milliliter of LB without

ampicillin was added and incubated for 1 hour with gentle shaking at 37 °C. After the

incubation, 10 µL, 100 µL, 250 µL were then plated on LB with ampicillin (60 mg/ml) to

which x-gal had been added and incubated at 37 °C overnight.

Clone selection:

The pUC18 cloning vector has two characteristics which aid in clone selection. It

contains an ampicillin resistance gene and a segment of the β-galactosidase gene (lacZ')

of the lactose operon of *E.coli*. The multicloning site incorporates the insert into this

segment. When cells transformed with pUC18 are grown in the presence of

isopropylthiogalactoside (IPTG) and x-gal (5-bromo-4-chloro indolyl-b-galactoside) the

nonrecombinant colonies (the cells which can synthesisze β-galactosidase) will appear

blue, whereas recombinants with disrupted lac Z' gene and unable to make β-

galactosidase will be white (Glick and Pasternack, 1994).

The plasmid was isolated from nine white colonies, using the Promega Wizard

Miniprep Kit. To isolate the plasmids, 5 milliliters of LB with ampicillin (50 mg/ml)

were inoculated and incubated overnight at 37 °C in a shaking water bath.

After the incubation, 1.5 ml of the culture was transferred to a microfuge tube and centrifuged at 12,000 X g for 1 minute. The supernatant was discarded and 1.5 ml of media transferred again into the microfuge tube. The media was centrifuged for 1 minute at 12,000 X g and the supernatant was discarded (per manufacturer's instructions). The pellets were resuspended in 200 μL of resuspension solution and 200 μL of lysis solution was added to each tube. The tubes were mixed by inversion four times and allowed to incubate for five minutes, after which 200 μL of neutralization solution was added and mixed by inversion. The tubes were then centrifuged at 12,000 X g for 10 minutes and the aqueous layer transferred to a column (syringe) to which 1 ml of resin had been added. Each column was vacuum filtered, and washed with 2 ml of wash solution. The tubes were centrifuged for 2 minutes to remove the excess wash, and the DNA eluted into fresh microfuge tubes with 50 μL of dH₂O. The nine samples were electrophored in a 1 % agarose minigel and from this, the concentration of plasmid DNA to be digested was estimated.

Restriction digest of extracted DNA plasmids:

In order to verify that the ligation reactions were successful, the plasmids from each of the transformed clones was digested with PstI enzyme. This enzyme was used originally to linearize the plasmid before the ligation reaction. The plasmid DNA from clones 1, 3, 4, and 10, was digested by incubating 12 μL of dH₂O, 5 μL of DNA, 2 μL of 10 X Buffer D and 10U of PstI enzyme. The DNA from clones 2, 5, 6, 8, was digested by incubating 15.5 μL of dH₂O, 1.5 μL ofDNA, 2 μL of 10 X Buffer, 10U of PstI

enzyme. Clone 9 was digested by incubating 7 µL of dH_2O, 10 µL of DNA, 2 µL of 10

X Buffer, 10U of PstI enzyme. The digestion reactions were then loaded on an agarose

gel, transferred to a membrane and probed with the sequence for the ubiquitin gene. All

reactions had a total volume of 20 µL, were incubated at 37 °C for 2 hours, and digested

with PstI. At the end of the digest the samples were loaded on a 60 ml 0.8% agarose gel

and electrophored at 150V for 1.5 hours, then at 50 V for 2 hours and visualized under

UV light. Each linearized plasmid was loaded on the lane adjacent to the uncut plasmid

from each clone. The gels were then soaked in 0.5 N NaOH, 1.5 m NaCl, for 30 minutes.

They were then soaked for 30 minutes in 1.5 m NaCl, 0.5 mM Tris-Cl pH 8.0 for 30

minutes, and finally in 10 X SSC for 30 minutes. After the last soaking, the gels were

assembled for overnight capillary transfer following the southern procedure on a Zeta

Probe GT membrane filter (Bio-Rad).

Clone selection for sequencing:

A riboprobe was synthesized as previously described for the Genomic Southern

and Northern blots. The blots were pre-hybridized at 65 °C for 1 hour in 15 ml of 0.5 M

Na_2HPO_4, pH 7.2 and 7% SDS. At the end of the pre hybridization, the solution was

discarded and new hybridization solution was added with 25 µL of the probe. The blot

was incubated at 65 °C overnight, washed for 1 hour at 65 °C with 50 ml 5% SDS, 40 mM

Na_2HPO_4 pH 7.2, and washed for 30 minutes at 65 °C in fresh 50 ml of the same solution.

Finally, the blot was washed for 30 minutes at 65 °C in 1% SDS, 40 mM Na_2HPO_4, pH

7.2 and exposed to X-ray film. From the results of the agarose gel, one of the clones was

selected to be sequenced at the UNMC sequencing core facility. The cloned DNA showing the highest concentration was selected for sequencing.

RESULTS

Timing of Growth and Development.

Vegetative amebas from *D. mucoroides* to be induced into development as macrocysts or

sorocarps were harvested at fifty-six hours of growth as they entered stationary phase, to

monitor progression of cells through development. The amebas were washed and placed

on filters to initiate development into multicellular structures and their development was

stopped at precise developmental stages in the sorocarp and macrocyst pathway.

At zero hours for both sorocarp and macrocyst development, the cells were in the

ameba form. By four hours, the cells in the sorocarp development conditions began to

show aggregation. The once uniform lawn of cells showed a stippled appearance. After

eight hours, the cells had fully reached the "Mexican hat" stage, and by twelve hours they

had initiated spore formation (figure 4).

The ameba development into macrocysts showed the pattern of aggregation

characteristic of this part of the life cycle. At four hours the mass of cells appeared

stippled. Microscopically the cells appeared to be aggregating into large clumps. The

clumps appeared larger after eight and twelve hours, with rounding of the massive

aggregations by twelve hours. After developing for eighteen hours the mass of cells

separated into clusters and microscopically macrocysts had formed. By twenty-four

hours the walls surrounding the macrocysts were evident (figure 5). The amebas were

Figure 4. Pictures showing the sorocarp developmental cycle. (a) Zero hour

amebas [400X], (b) four hours, the cells began to show aggregation [1.8X], (c and

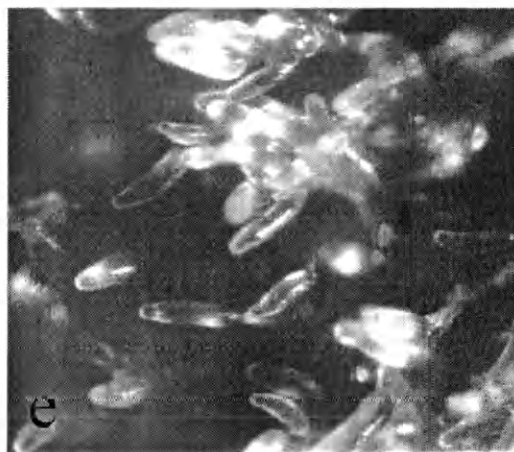d) eight hours ("Mexican hat" stage) [2.4X] and [1.8X], (e)twelve hours spore

formation [2.6X].
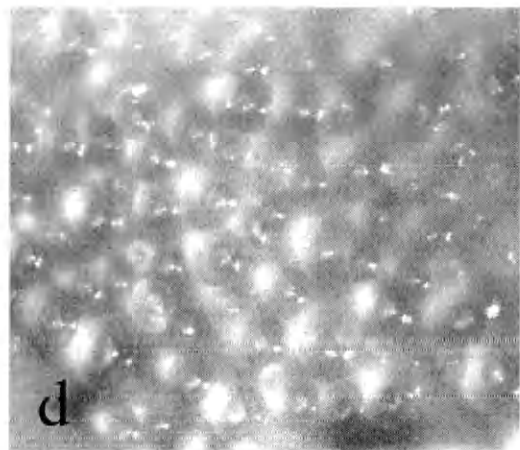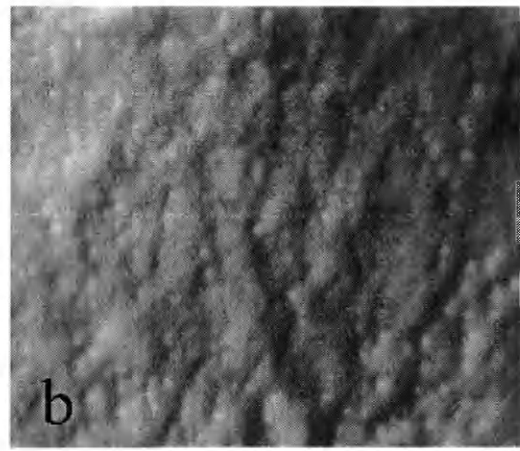
Figure 5. Pictures showing the macrocyst life cycle. (a) Zero hour amebas
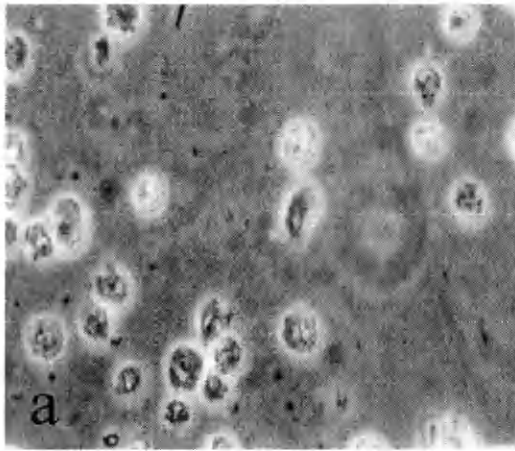
[400X], (b) eight hour aggregation [160X], (c) Twelve hour aggregation [160X],

(d) eighteen hour, macrocysts become distinguishable [160X], (e) twenty four

hour mature macrocysts [160X], (f) twenty four hour mature macrocysts [400X].
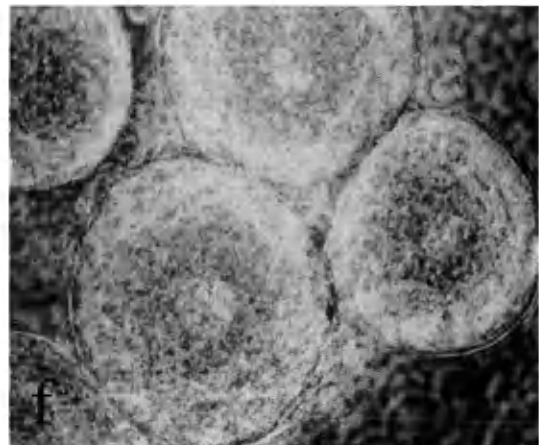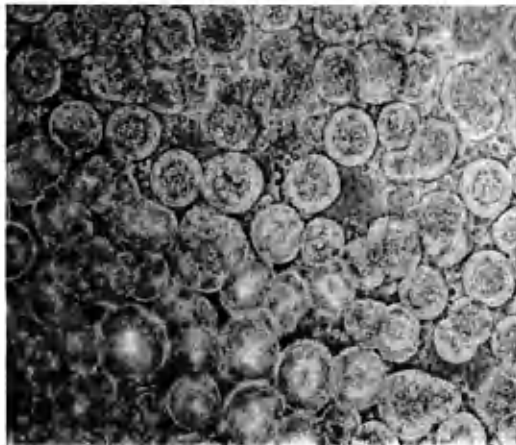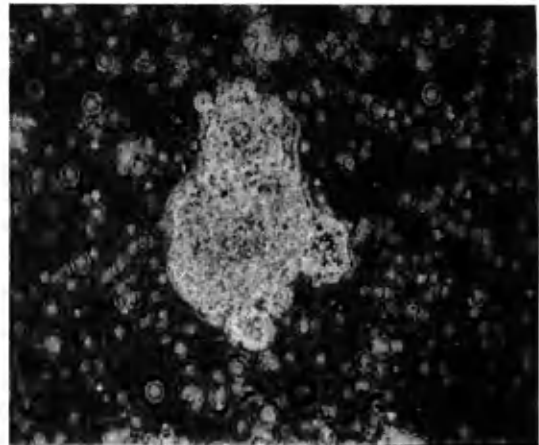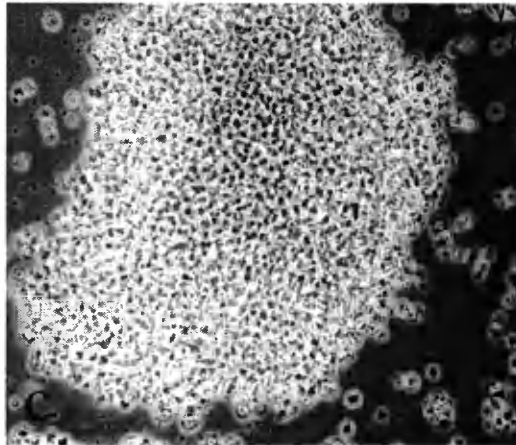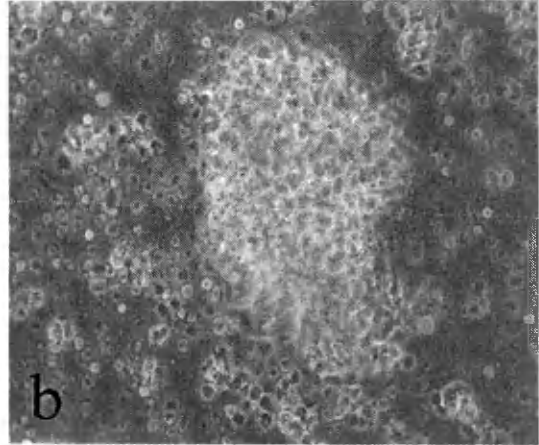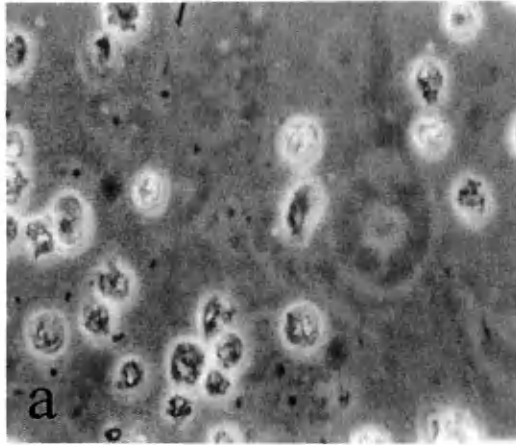
harvested for RNA extraction at 0, 4, 8, 12hr for the sorocarp and 0, 4, 8, 12, 18, 24hr for the macrocyst lifecycles.

Southern Blot:

A genomic Southern blot was used to determine the gene copy number. The restriction-enzyme digested genomic DNA, was visualized on an EtBr stained agarose gel. The PstI digest showed some incomplete digestion, as indicated by the amount of high molecular weight DNA, but there was distinct banding after the 6557 bp marker. The PvuII, HincII, PstI and HincII double digest and the PvuII and HincII double digest lanes showed very distinct banding indicative of complete digestion patterns (Figure 6).

The DNA was blotted onto a nylon membrane and probed with a riboprobe for the M14 A-1 ubiquitin sequence. The blot was then exposed to x-ray film for 10 days. The radiograph showed a single band (a) of 13,000 bp for the PstI digest. The lane with the PvuII digest showed a dark band (b) of 5100 bp, and a faint band (c) of 2700 bp. The HincII digest produced two distinct bands, one (d) at 3200bp and the second (e) at 1000 bp. The PstI and HincII double digests showed two bands at 3200bp (f) and 1000bp (g). The PvuII and HincII double digest lane showed a single band (h) of 3200 bp (figure 7).

A restriction map compatible with these results is shown in figure 8. This map provides evidence for at least two copies of the ubiquitin gene in tandem on the chromosome.

Figure 6. Genomic DNA digested with restriction enzymes and separated on a 1%

agarose gel. Lane 1 and 7 show the molecular weight marker (HindIII cut λ

DNA). Lane 2 shows the PstI single digest, lane 3 the PvuII single digest, lane

four the HincII single digest. Lane 5 shows the PstI and HincII double digest, and

lane 6 shows the PvuII and HincII double digest.

Lane 1

Lane 2 - PstI

Lane 3 - PvuII

Lane 4 - HincII

Lane 5 - PstI + HincII

Lane 6 - PvuII + HincII

Lane 7

Figure 7. Autoradiogram of a Southern blot of the genomic DNA digest probed with the M14 A-1 sequence.
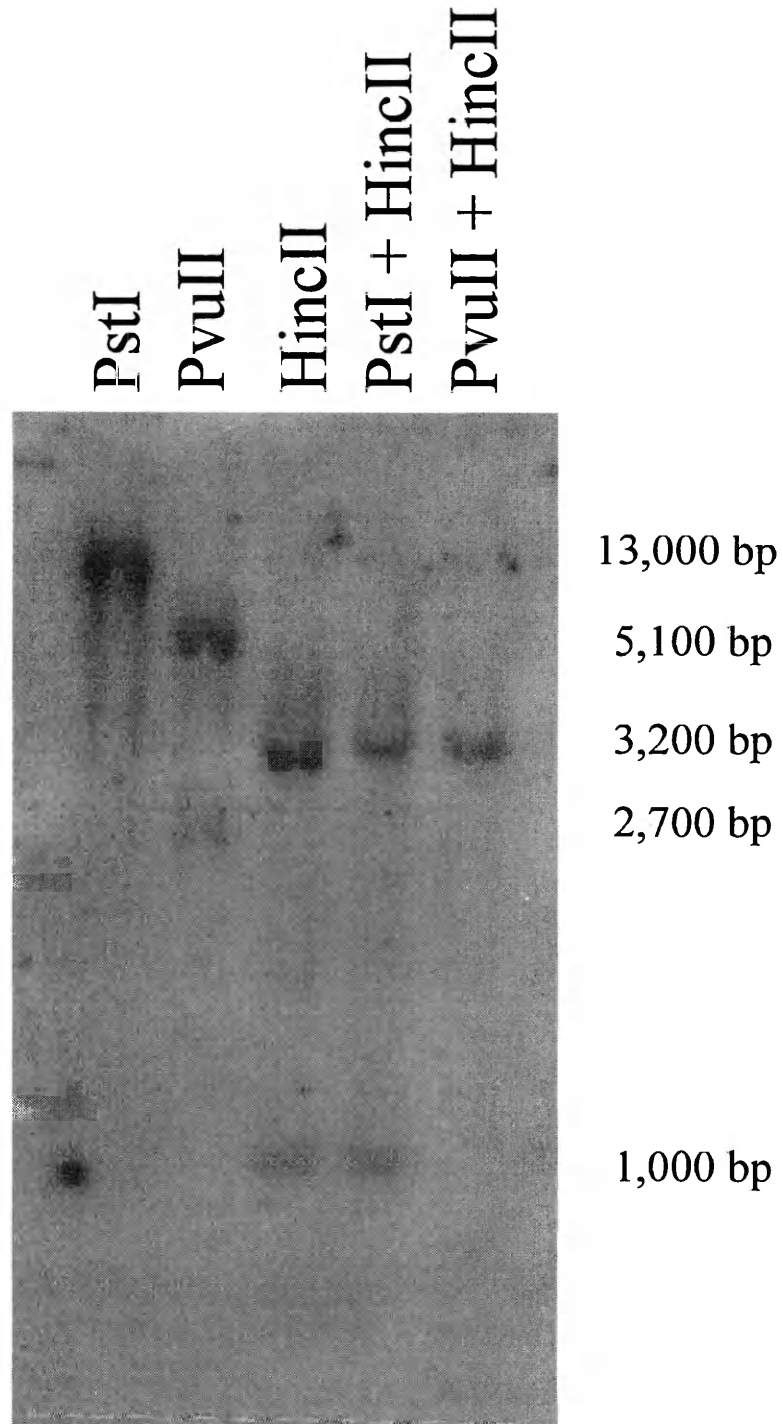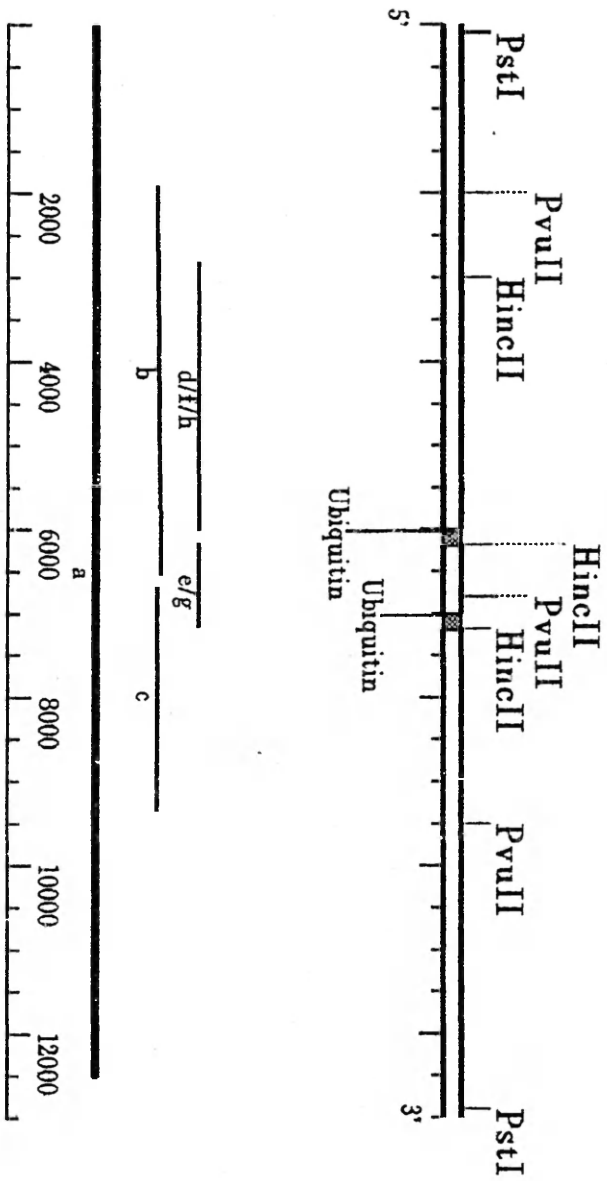
Figure 8. Proposed map of the organization of the ubiquitin genes *in D. mucoroides* based on restriction digests with PstI, PvuII and HincII enzymes.

Proposed Map

(13000 bps)

5'

PstI

PvuII
HincII

Ubiquitin
Ubiquitin

HincII
PvuII
HincII

PvuII

3'

PstI

d/f/h

b

a

e/g

c

2000   4000   6000   8000   10000   12000

Northern Blot:

For use in northern blot analysis, RNA samples were extracted from selected

developmental stages, separated on a 1% formaldehyde denaturing gel, stained with EtBr,

and viewed under UV light. Although the lanes appear to contain slightly different

concentrations of RNA, all the lanes showed the expected 26S and 17S ribosomal RNA

bands, indicating that the RNA was not noticeably degraded (figure 9). The RNA in the

gel was then blotted onto a nylon membrane and hybridized with a riboprobe of the M14

A-1 ubiquitin sequence. The radiograph showed four bands in every developmental stage

(figure 10). The four bands appeared to be the same size in all of the sorocarp and

macrocyst samples. The largest transcript was approximated to be 3000 bp long. Next

was a 2300 bp band which showed the highest intensity, likely to represent the largest

quantity. The third band was 1800bp and was the second most intense band in all the

samples. The last band appeared faint throughout and was about 1100 bp. Even after

prolonged exposure to film, in an attempt to detect faint signals, the blot did not produce

any more bands. An identical banding pattern for the macrocysts and sorocarp samples

was observed when the experiment was duplicated.

Clone Selection:

To clone the DNA containing the ubiquitin coding region, genomic DNA was digested

with PstI and separated on a 0.8% agarose gel. There are no PstI sites within the gene.

Figure 9. Representative picture of a 1% denaturing gel with the developmental

RNA samples. Lanes 1-6 show the macrocyst samples for the 0, 4, 8, 12, 18, 24hr

stages of development. Lanes 7-8 are the 4 and 8hr sorocarp samples. Lane 9

shows the molecular weight standard.

Figure 10. Northern blot of the RNA extracted at each developmental stage of

sorocarp and macrocyst development.

| | 0 hr M | 4 hr M | 8 hr M | 12 hr M | 18 hr M | 24 hr M | 4 hr S | 8 hr S |

3,000 b
2,300 b
1,800 b

1,100 b
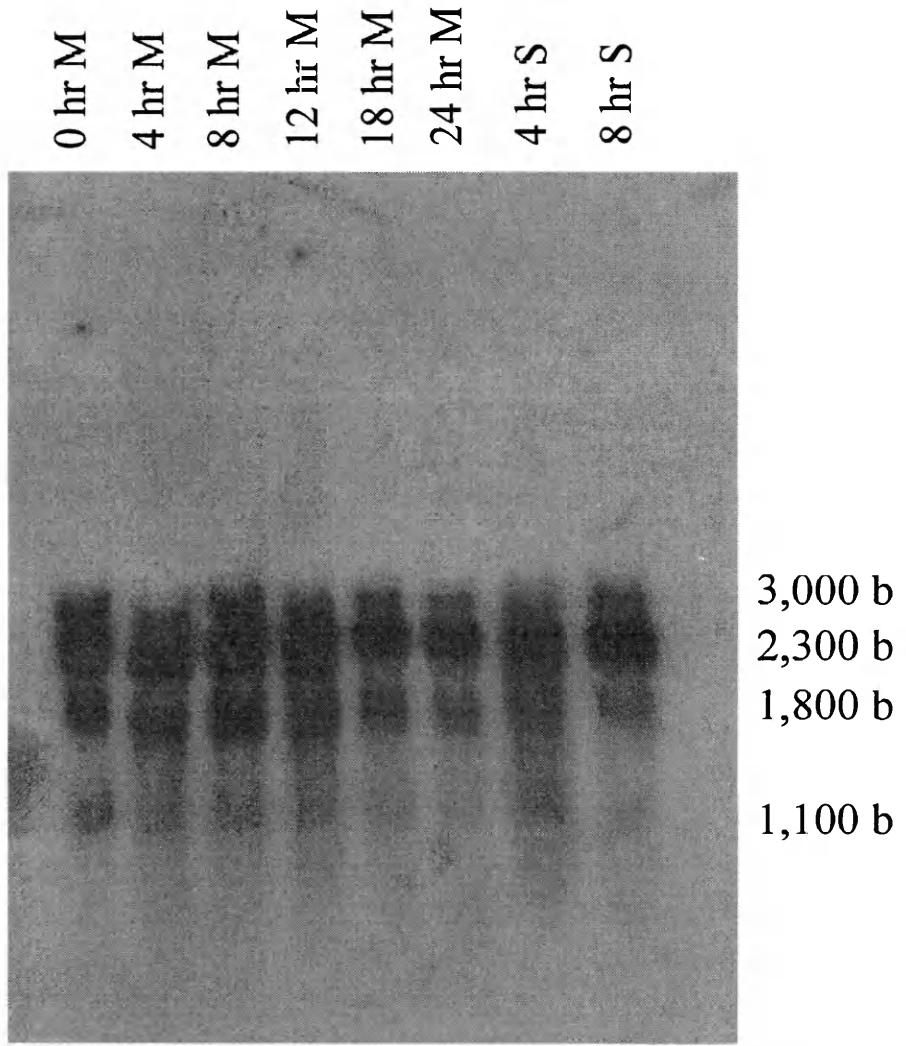
From this gel, a band which had previously hybridized with a probe of the M14 A-1 sequence, was chosen and excised. The DNA was extracted from the agarose and cloned into pUC18 vector. DH5-α cells were then transformed with the extracted DNA. Nine clones which produced white colonies on x-gal containing plates were chosen. A small scale plasmid prep was carried out for each clone and each plasmid was then incubated with PstI to check the results of the ligation.

The cut plasmids showed a large piece (3100bp) corresponding to the vector, and a smaller piece of 2700bp which corresponded to the insert (fig. 11). The gel was then blotted using the Southern blot procedure, and hybridized with a riboprobe of the M14 A-1 ubiquitin sequence. The PstI digested plasmids showed a single band of 2700bp, which corresponded to the insert (figure 12). The plasmid DNA from Clone number 5 was selected because it showed the greatest DNA concentration and sequenced at the University of Nebraska Medical Center DNA Sequencing Core Facility. The sequencing results were then used to compare the homology of our insert to that of *D. discoideum* ubiquitin using the BLAST search program. The results of the search are presented in appendix 1.

Figure 11. Picture of the agarose gel of the plasmid DNA extracted from the

transformed clones and digested with PstI restriction enzyme. All lanes

containing digested DNA show two bands, the larger one (3100bp) corresponds to

the vector, and the smaller one (2700bp) to the insert.
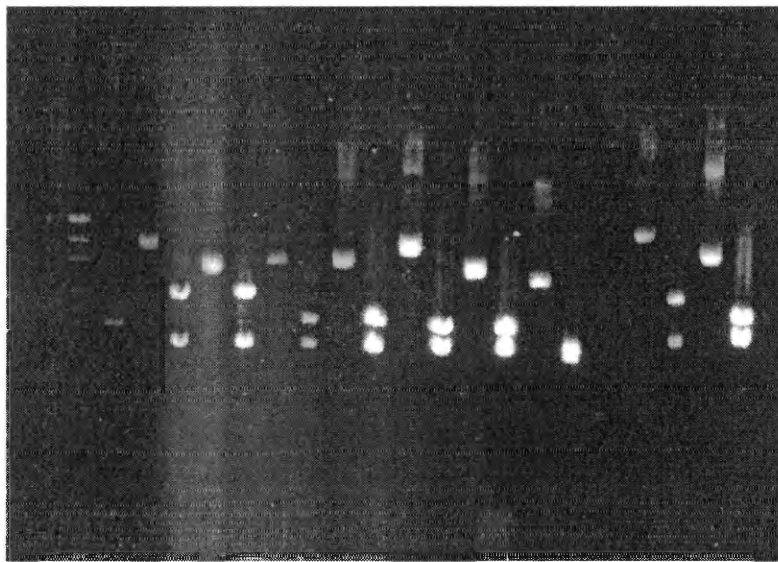
3,100 bp

2,700 bp

Figure 12. Southern blot of the plasmid DNA extracted from the transformed

clones and probed with the M14 A-1 sequence. Lane 1 is the positive control

DNA (M14 A-1). Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18, show the uncut plasmid

DNA hybridizing with the probe. Lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19, show the

insert DNA hybridizing with the probe.

3,200 bp

2,700 bp
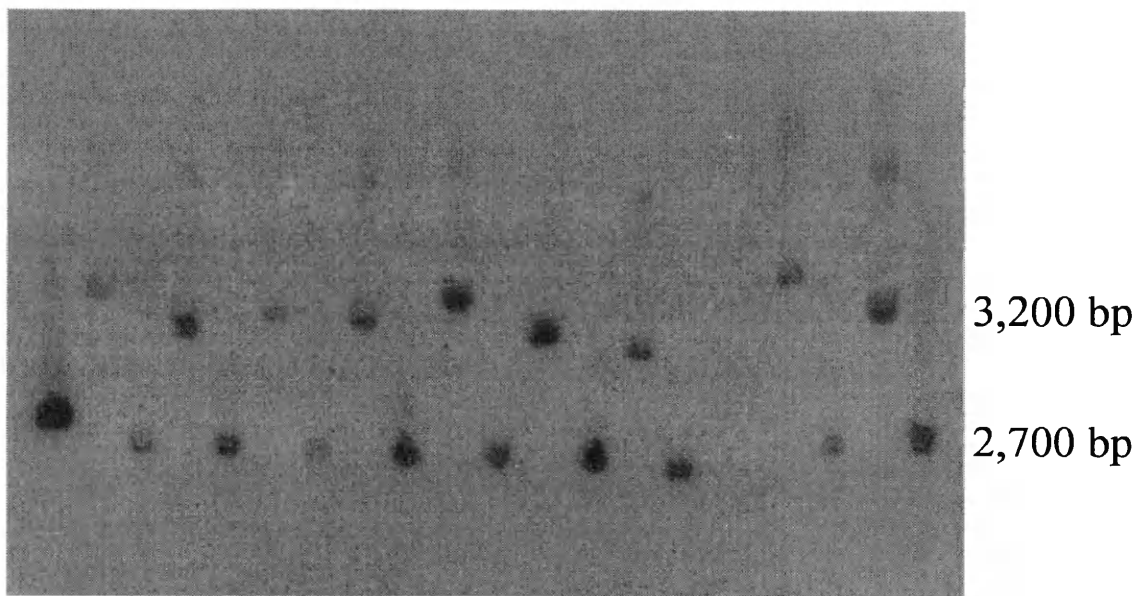
DISCUSSION

During development of an organism, a number of developmentally regulated proteins are

generated and degraded. Through differential display, Esser (MA Thesis, 1995) found a

cDNA that appeared to be specific to *D. mucoroides* macrocyst development. This

cDNA showed 70% amino acid homology to ubiquitin from *D. discoideum*. Ubiquitin

has been shown to function as a signaling molecule for protein degradation in many

organisms. The purpose of this investigation was to determine the number of genes

encoding ubiquitin in *D. mucoroides* and to investigate transcription of the ubiquitin gene

at selected stages of development.

One method for detecting gene copy number is to digest the genomic DNA with

a restriction enzyme that cuts outside of the gene of interest, with an enzyme which cuts

within the gene, and then with a combination of two of these enzymes. Using the

restriction enzyme map for the ubiquitin gene sequence submitted by Muller-

Taubenberger et al. (1988), which showed high homology with the gene cloned by Esser

(MA Thesis, 1995), it was determined that the restriction enzymes PstI and PvuII cut

outside the ubiquitin gene sequence and HincII cuts within the sequence. The results of

this investigation appear to show that in *D. mucoroides* there are two genes which have

sequences similar to the putative ubiquitin sequence used for making the probe. Based

on results from restriction enzyme digest, these two sequences are 800bp from each

other. Figure 8 shows the proposed arrangement for the ubiquitin genes in *D.*

mucoroides.

From figure 7, since only one large band of 13,000bp is seen after digesting

genomic DNA with PstI restriction enzyme, the cutting sites for this enzyme must be

outside both ubiquitin sequences and quite a distance from each other. The two bands

produced by the PvuII single digest are due to cutting between as well as outside the

ubiquitin sequences, which would suggest that there is a spacing segment between the

ubiquitin sequences. Two of the Pvu II sites would be close to each other, producing the

2700bp band, and a third would be some distance away producing the 5100bp band. The

bands produced by the HincII single digest are due to three cutting sites within a short

segment of sequence containing both genes. The 3200bp fragment is produced by cutting

downstream from the gene, and also cutting very close to the 3' end of the first ubiquitin

gene. Because the gene is small, the hybridizing portion would need to be intact to

produce a detectable signal. Splitting the gene may not allow for hybridization of the

gene with the probe. For the enzymes used, this means that the HincII sites, which are

the only sites within the gene, would have to be close to either end of the gene. The

Southern blot results point to the location of these sites near the 3' end of the 200bp

ubiquitin sequence. The 1000bp band is therefore produced by having one digestion site

very near the end of the first gene and another very near the end of the second ubiquitin

gene. The portion remaining from the second gene is so small that it very likely does not

hybridize with the probe and therefore does not produce a signal. In addition, the

sequence submitted by Muller-Taubenberger (1989) and the restriction enzyme analysis

on that sequence also support the hypothesis that the HincII site is very close to the 3'

end of the gene. The predicted endonuclease site for HincII in ubiquitin is 10 to 15bp from the 3' end of the gene.

The PstI and HincII double digest produced two bands which were identical to the HincII single digest and can be explained by having the PstI cutting sites outside the HincII restriction sites. The PvuII and HincII double digest produced only one band of about 3300bp. This band is likely produced by the HincII restriction close to the 5' end but outside the gene and the HincII restriction site within the first gene. A second band should have been expected for the second gene, however, if the PvuII cutting site within the intervening sequence is located close to the beginning of the second gene, the fragment produced between the PvuII and HincII enzymes would have been very small (about 250bp). This fragment may have migrated off the gel or not have been intense enough to show on the film.

To summarize, it appears that in *D. mucoroides* there are two ubiquitin genes on the same chromosome with about 800bp between them. Giorda and Ennis (1987) reported that in *D. discoideum* there are at least six genes for ubiquitin (Giorda and Ennis, 1987). Since then, the ubiquitin genes in *D. discoideum* were reported by Kuspa and Loomis (1996), who used yeast artificial chromosome clones, to exist on four chromosomes. Using restriction enzyme mediated insertion - restriction fragment length polymorphism, Loomis et al. (1995) previously assigned the ubiquitin gene to only one chromosome. It is possible that the ubiquitin gene exists in different numbers even between very similar strains of an organism. Another explanation may be that

differences in the procedures used to determine the gene copy number produced differing results.

D. mucoroides produced the same four ubiquitin transcripts throughout development from unicellular organisms to multicellular structures. The four transcripts observed were of 3000bp, 2300bp, 1800bp and 1200bp, whether the structure was a sorocarp or a macrocyst. The band intensity did not show a significant difference between samples either. This observation supports the hypothesis that in *D. mucoroides* there are multiple transcription sites and/or multiple termination sites. If multiple polyubiquitin transcripts (more than one transcript in tandem repeats) were being synthesized, one might expect a single species to predominate over others at an early time in development. Later during development, the size of the predominating transcript may be different as evidenced by changes in the intensity of the different bands. The results of this investigation differ from those found by Giorda and Ennis (1987) and Omachi et at. (1989) in *D. discoideum*. They reported that in germinating spores, the ubiquitin gene is developmentally regulated and produces transcripts of different sizes as the spores germinate. The difference in results could be due to differences between the organisms. Both *D. discoideum* and *D. mucoroides* produce the conserved ubiquitin protein, however, they may go about this in different ways.

Ubiquitin genes occur in two forms, polyubiquitin repeats and single genes followed by a carboxyl extension protein (CEP) (Monia, 1990). In most species, mRNA transcripts encoding different polyubiquitins are expressed differentially during development but are generally shut down during the organism's growth stage, during

which the UbCEP transcript expression predominates (Monia, 1990). During times of stress and developmental changes, polyubiquitin genes are generally transcribed, allowing for the production of large quantities of ubiquitin protein. This in turn would allow the cell to selectively turn over proteins rapidly and therefore make all the changes necessary for development. The gene arrangement for the two ubiquitin genes in *D. mucoroides* does not appear to allow for this. The genomic Southern blot analysis showed two genes that are separated from each other by 800bp. This would imply that polyubiquitin transcripts in tandem repeats can not be produced. The results of the Northern blot show that polyubiquitin transcripts are not produced in *D. mucoroides*. It appears that during development of *D. mucoroides* into macrocysts, the UbCEP transcripts are the mRNA species that are produced, since the same four bands were produced during all stages of development. Sampling of the ubiquitin proteins present at each stage of development and their analysis by Western bloting, would help elucidate any changes in the number of copies of ubiquitin protein used in the ubiquitin proteasome pathway. Any changes observed in ubiquitin concentration would suggest translational control.

Another possibility which may explain the differences in results between previous research and that reported here may be the fact that Giorda and Ennis (1987) and Omachi et al. (1989) used poly(A)+ RNA as their source or RNA, whereas the source of RNA used in this investigation was total RNA. The use of poly(A)+ RNA may allow for concentration of transcripts which may have been obscured by using total RNA.

A large band produced by the digestion with PstI, which was previously shown to hybridize with the M14 A-1 sequence probe, was excised and extracted from an agarose gel. The DNA was cloned into pUC18 cloning vector and competent DH5-α cells were transformed. Southern blot analysis on the plasmid DNA of each of 9 clones produced expected results (fig 12). The control, which was the linearized pGEM-T vector with the M14 A-1 sequence, hybridized with the riboprobe synthesized from the same sequence. The uncut plasmid for each of the clones also hybridized with the riboprobe. When the insert was excised with PstI restriction enzyme, the same enzyme used during the ligation, a fragment of 2700bp hybridized with the probe for each of the clones but the larger size band, the vector, did not hybridize. All of the clones appeared to have the same insert, and therefore, the one showing the largest concentration of DNA was selected to be sequenced. The insert was sequenced in several separate runs and a total of 2600bp of sequence was obtained (Appendix 1). The BLAST computer search, however, did not match the sequence with any ubiquitin genes, even though the M14 A-1 sequence when used as a probe hybridized to the fragments of DNA carrying the putative ubiquitin gene. The sequence on the forward direction had high homology with and E. coli cell division protein (FTSY), and a signal recognition particle receptor from E. coli. On the reverse it closely matched an E. coli zinc-transporting ATPase. When the sequences were aligned with the sequence of the M14 A-1 insert, there were only approximately 100 bp that appeared to line up, and the homology between the sequences was very poor. This suggests that there is a good possibility that the clone selected for sequencing may have been a false positive, probably due to degeneracy of the code or errors during the

transcription of the probe. Performing a restriction enzyme digest on the plasmid DNA from the remaining clones may indicate which of the clones have the same insert, which would facilitate further sequencing efforts in order to determine which clone contains the ubiquitin gene.

APPENDIX 1

A. Sequence of insert DNA in the forward direction.

```
   1   CGACGGCCAG   TGCCAAGCTT   GCATGCCTGC   AGACGTCCGG   CGGTATCGGC
  51   AATCAGGACG   TCGATATTAC   GCGCTTTAGC   TGCCTGAATG   GCGTCGAAGA
 101   TAACAGAGGC   GGAATCCGCA   CCGGTATGCT   GGGCAATCAC   CGGAATATTG
 151   TTGCGCTGAC   CCCAGACCTG   AAGCTGTTCA   ACCGCTGCCG   CACGGAATGT
 201   ATCGCCCGCC   GCCAGCATCA   CCGATTTACC   CTGCTGCTCA   AACTGACGCG
 251   CCAGCTTACC   AATCGTCGTG   GTTTTACCCA   CACCGTTGAC   ACCTACCATC
 301   AGGATCACGA   ACGGCGTTTT   ACCTTCAACA   TTCAGCGGCT   CATCGACTTT
 351   CGCCAGAATC   TCGCCCATCT   CTTCTTTCAG   CAGGCCATAG   AGCGCCTCGG
 401   CATCACGAAg   CTGCTTGCGG   GATGCGCCTT   CCGTCAGATT   GGTGATTATT
 451   TTACGTGTGG   tTTCCACGCC   CACATCGGCG   ATCAACAACT   GTTCTTCCAG
 501   CTCTTCAAAC   AGATCATCGT   CGATTTTTTT   ACCGCGGAAC   AGGCTGATAA
 551   ATCCGGAACC   GAGATTTTCT   TTGGTTTTTA   ACAGGCTGCG   TTTCAGGCGC
 601   GCGAArAArC   CTTCTTTGGT   CGGkTTTTCT   TGCTCCTGGG   CrATTTCTTc
 651   AACCGGCTGC   TCTTCTTcTG   CCGGAGGAAC   TACCATcACC   GsCTcTTcTG
 701   CCGCTTCGGC   AGCCAgCGCC   TGTGCTTcCA   GCTcTTcGTC   GGtGATTTCT
 751   TcTTTAgCCG   CTTCTTcTTG   CGCCGCTTcG   ACAATcTcTA   CGGtTTCCGs
 801   TTcAgcCTGc   CACTCTTcTG   GcGAAAcCTC   TTcGGcGTTG   ACGTCTTCG
```

B. Sequence of insert DNA in the reverse direction.

```
   1   TGATACGAAT   TCGAGCTCGG   TACCCGGGGA   TCCTCTAGAG   TCGACCTGCA
  51   GGAACTTTAT   CGCCCGTTGC   GCGCTCCACC   GGAATGGATT   CTCCGGTCAG
 101   GGCGCTTTCA   TCAAAACTGG   CAAACGGTGA   GAGCAGTTTA   CCGTCGGCAG
 151   GCAAACGCCC   ACCTGCGGCG   ACTTCAATCA   CATCGCCAGG   CCGCAGGCTG
 201   TTAATCGCCA   CCTCTTCCCG   CTCACCGTTA   CGCAGGCGCG   TGGCGGTTTC
 251   TGGTTTCAGC   GCCATTAAAG   CGCTAACGCC   CTGACGCGCG   CGGCTGGCGG
 301   CCCAGCCTTC   CAGTCGTTCA   CCAATCAAAA   ACAGCAGCAA   CACCATCGCA
 351   GCTTCAGCCG   TTGCGCCAAT   AAACAGTGCA   CCAATAGCGG   CTACGCTCAT
 401   TAAGGTTTCA   ATGGCGAAGT   AGCTGCCGGA   TTTGATCAGC   CGTAATGCCT
 451   GACGAGCAAT   CGGGTACAGC   CCAACCAGCG   TGGTCGCGAT   AAACGCCAGT
 501   TGCCCGAACG   GATGATTAAA   TTGCTCCAAA   CCCCAGCTGA   TTGCCATCAT
 551   CACGATAAGC   GTAATCAGCG   GCAGATTCTC   TTTCAGGCGC   GATGCTTGTG
 601   GCTCGTCGGC   GGCCTGCTCA   TCGCGCAGGG   AATAGCCCGC   TTTTTGCACC
 651   GTAGATTCAA   CCTGGGCACG   GATGTCATTG   TCGGCATCAA   CCACCAGTTT
 701   TTCGGTGGCG   AACAACACCT   GCACCTGATT   CACGCCTGCA   AGCTGGCGCA
 751   CGGCATTTTC   TACCTTGCGC   GCACAGGCGG   CGCAGTCCAT   GCCGCTGACT
 801   TTCCAGCTAT   AGCGGGAGCC   GGAGACGTTT   TCAGAGAGCG   TTGGCGAGCT
 851   GGAACATGCG   CCGTCGCAGC   AACAGTCGTT   GGCGTTCTGT   ACCGTGGTTA
 901   GCGGTTTGAA   CGCAGCAAAT   TGAGGGGCTT   TCTTGCCGTG   ATTGTCAGGA
 951   GTCGACATGG   CATCCTCCGG   TTAAGTTTTT   TCTCATTAAC   CGAAGGATAC
```

```
1001  ACTCTGGAGT   ACTCTGGAGT   CGACTCCAGA   GTCAAGTTTT   ATCAGAGATA
1051  CAGCGAGCGG   ACGATCAGGA   AGTGACCGGC   AAAGTAGCAG   GCCGCAGCAA
1101  TCGCGTTATC   CGCACGGAAG   CGTCGGCGAT   AGTGGCTCCC   CAGCCAGACA
1151  AAGTTACTGA   TAAACAGCAA   GAAGCGCCAA   CAAACGCAGA   GCGCCGGAGC
1201  GGTCGGACGG   AAGAACCACA   GTTCACCTGC   CAGCCACACC   ATCACCAGCG
1251  TCATGCCGAT   AAACGTGCAG   ATAGGCCAAC   CGGTACTCTT   CCAGCCGCGT
1301  CCAGATAATC   GCCAGTAACA   GCGCGCCCAG   AACCAGCAGC   ACCAGTGGCA
1351  GAGGCCAGAA   GAAAGAGAGC   GTCATCTGAC   TGGCGAAATA   GATGGTGTAC
1401  AGCAGGTGCG   AGAGGAAAAA   CGCGCCGATG   GCGTACATCA   GACGTTGACG
1451  TGGCAACAGG   GTTAGCGCAT   CGCCCAGCAG   TGAGGCGCAC   AGCCCTGCCA
1501  GCACCAGATA   GCTAATGGCG   TCGAACATCG   GCGCTTGCCA   GGCCAGTAAC
1551  AGCAGGAGAA   GAAGGGTTAA   CGGTTTAAAC   ACCCAGCGTT   GCCAGGTTGG
1601  CCCACGATAC   GATGCATCCA   CAGATAGCCA   TGCGGAAAGA   CAGACTGCGA
1651  TAAACGACCA   AGCATCTTAG   TCCTTGAATT   CGTTATTTTT   TCGTAAGCGA
1701  ATGCCTACGG   TCTTGCTTCC   AATTTAATGG   CGAGGGTGGA   CGGATGACAA
1751  CACCATAACT   GGCAGGGTAT   GCTTATTTCC
```

LITERATURE CITED

Barinaga, M. 1995. A new twist to the cell cycle. Science. 269: 631-632.

Blumberg, D.D. and H.F. Lodish. 1981. Changes in the complexity of nuclear RNA during development of *Dictyostelium discoideum*. Dev. Biol. 81: 74-80.

Bonner, J.T. 1982. The development of *Dictyostelium discoideum*. Academic Press Inc., New York.

Bush, J., J. Richardson and J. Cardelli. 1994. Molecular cloning and characterization of the full length cDNA encoding the developmentally regulated lysosomal enzyme β-galactosidase in *Dictyostelium discoideum*. J. Biol. Chem. 269: 1468-1476.

Carlson, B. 1996. Patten's Foundations of Embryology. 6th ed. McGraw Hill, Inc., New York.

Chin, D.T. , N. Kuehl, L., Rechsteiner, M. 1986. The degradation of guanidated lysozyme in reticulocyte lysate. J. Biol. Chem. 261: 3883-3890.

Chomczynski, P., and N. , Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thyocianate-phenol-chloroform extraction. Analytical Biochemistry 162: 156-159.

Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. Cell. 79: 13-21.

Ciechanover, A. and A.L. Schwartz. 1994. The ubiquitin-mediated proteolitic pathway: mechanisms of recognition of the proteolitic substrate and involvement in the degradation of native cellular proteins. The FASEB journal. 8:182-191.

Clark, A., N. Anson, M. Sudharasi, R.A. Firtel. 1997. A ubiquitin-conjugated enzyme is essential for developmental transitions in *Dictyostelium*. Mol. Biol of the Cell. 8: 1989-2002.

De Lozanne, A., M. Lewis, J.A. Spudich, L. Leinwald. 1985. Cloning and characterization of non-muscle myosin heavy chain cDNA. Proc. Natl. Acad. Sci. 81: 6807-6810.

Deverotes, P. 1989. *Dictyostelium discoideum*: A model system for cell-cell interactions in development. Science. 245: 1054-1058.

Erdos, G. , A.W. Nickerson, K.B. Raper. Fine structure of macrocysts in *Polysphondylium violaceum*. Cytobiologie. 6: 351-366.

Gaskins, C., A.M. Clark, L. Aubury, J.E. Segall, and R.A. Firtel. 1996. The *Dictyostelium* MAP kinase ERK2 regulates multiple independent developmental pathways. Genes Dev. 10: 118-128.

Gaskins, C., M. Maeda, and R.A. Firtel. 1994. Identification and functional analysis of a developmentally regulated extracellular signal-regulated kinase gene in *Dictyostelium discoideum*. Mol. And Cell. Biol. 14: 6996-7012.

Giorda, R. , H. Ennis. 1987. Structure of two developmentally regulated *Dictyostelium discoideum* genes. Molecular and Cellular Biology. 6: 2097-2103.

Glick, B.R. and J.J. Pasternack. 1994. Molecular Biology and Biotechnology: principles and applications of recombinant DNA. American Society for Microbiology. Washington, D.C.

Gregori, L. , D. Marriott, C.M. West, V. Chau. 1985. Specific recognition of Calmodulin from *Dictyostelium discoideum* by the ATP, ubiquitin-dependent degradative pathway. J. Biol. Chem. 260: 5232-5235.

Hershko, A. 1983. Ubiquitin: Roles in protein modification and breakdown. Cell. 34: 11-12.

Hershko, A., E. Eytan, A., Chiechanover, and A.L , Haas,. 1982. J. Biol. Chem. 257: 13964-13970.

Jaqiet, M., D. Part, and B. Felenbok. 1981. Changes in the polyadenylated messenger RNA population during development of *Dictyotelium discoideum*. Dev. Biol. 81: 155-166.

Kikkawa, U. , S. Mann, R.A. Firtel and T. Hunter. 1992. Molecular cloning of Casein Kinase II α subunit from *Dictyostelium discoideum* and its expression in the life cycle. Molecular and Cellular Biology. 12: 5711-5723.

Kindle, K. and R.A Firtel. 1978. Identification and analysis of *Dictyostelium* actin genes, a family of moderately repeated genes. Cell. 15: 763-778.

Kuspa, A. and W.F. Loomis. 1996. Ordered yeast artificial chromosome clones representing the *Dictyostelium discoideum* genome. Proc. Natl. Acad. Sci. 93: 5562-5566.

Latchman, D. 1990. Gene regulation: A eukaryotic perspective. Unwin Hyman Inc., London, England.

Lodish, H., D. Baltimore, A. Berk, S.L. Zipurski, P. Matsudaira, and J. Darnell. 1995. Molecular Cell Biology. 3rd ed. Scientific American Books. New York.

Loomis, W.F., D. Welker, J. Hughes, D. Maghakian, A. Kuspa. 1995. Integrated maps of the chromosomes in Dictyostelium discoideum. Genetics. 141: 147-157.

Loomis, W.F. 1975. Dictyostelium discoideum a developmental system. Academic Press Inc., New York.

Mann, S.K.O. and R.A. Firtel. 1993. CAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during Dictyostelium development. Development. 119: 135-146.

Marx, J. 1992. Dictyostelium researchers expect a gene bonanza. Science. 258: 402-403.

Meyers, R. 1995. Molecular Biology and Biotechnology. VCH Publishers Inc., New York

Monia, B., D.J. Ecker, S.T. Crook. 1990. New perspectives on the structure and function of ubiquitin. Biotechnology. 8: 209-215.

Moriyama, R. and K. Yanagisawa. 1987. Synthesis of specific proteins for sexual development in Dictyostelium discoideum. Cell Differentiation. 23 :143-152.

Muller-Taubenberger, A., J. Hagmann, A., Noegel and G. Gerish. 1988a. Ubiquitin gene expression in Dyctiostelium is induced by heat and cold shock, and inhibitor of protein synthesis. Journal of Cell Sci. 90: 51-58.

Muller-Taubenberger, A., M. Westphal, E. Jaeger, A. Nogel, and G. Gerish. 1988b. Complete cDNA sequence of a Dictyostelium ubiquitin with a carboxy-terminal tail and identification of the protein using and antipeptide antibody. FEBS lett. 229: 273-278.

Muller-Taubenberger, A. , H. R. Graack, L. Grohmann, M. Schleicher and G. Gerish. 1989. An extended ubiquitin of Dictyostelium is located in the small ribosomal subunit. J. Biol. Chem. 264: 5319-5322.

Mutzel, R. 1995. Molecular biology, growth and development of the cellular slime mold Dictyostelium discoideum. Experientia. 51: 1103-1115.

Nevins, J.R. 1983. The pathway of eukaryotic mRNA transcription. Ann. Rev. in Biochem. 52: 441-446.

Nickerson, A.W. and K. B. Raper. 1973. Macrocysts in the life cycle of the dictyostelaceae. 1. Formation of the macrocysts. Amer. J. Bot. 60: 190-197.

Nickerson, A.W. and K. B. Raper. 1973. Macrocysts in the life cycle of the dictyostelaceae. II.. Germination of the macrocysts. Amer. J. Bot. 60: 247-254.

North M. and D. Cotter. 1991. Analysis of *Dictyostelium mucoroides* macrocysts by using a simple breakage and fractionation procedure. Amer. Soc. Microbiol. 57: 307-310.

O'Day, D. H. and M. Lydan. 1989. The regulation of membrane fusion during sexual development in *Dictyostelium discoideum.* Biochem. Cell. Biol. 67: 321-326.

O' Day, D. H. and K. E. Lewis. 1981. Pheromonal interactions during mating in *Dictyostelium.* Academic Press, New York.

O'Day, D. H. 1979. Aggregation during sexual development in *Dictyostelium discoideum.* Can. J. Microbiol. 25:1416-1426.

Omachi, T., R. Giorda, D. Shaw and H. L. Ennis. 1989. Molecular organization of developmentally regulated *Dictyostelium discoideum* ubiquitin cDNAs. Biochemistry. 28: 5226-5231.

Rechsteiner, M. 1991. Natural substrates of the ubiquitin proteolytic pathway. Cell. 66: 615-618.

Richardson, D.L., C.B. Hung, W.F. Loomis. 1991. A prespore gene, Dd31, expressed during culmination of *Dictyostelium discoideum.* Dev. Biol. 144 (2): 269-80.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor, New York.

Vijay-Kumar, S., C.E. Bugg, W.J. Cook. 1987. Structure of ubiquitin redifined at 1.8 A resolution. J. Mol. Biol. 194: 531-544.

Waterfall, W. 1983. Slime mold: singular and plural. From NSF Mosaic Reader.

Williams, J.G., M.M. Lloyd, and J.M. Devine. 1979. Characterization and transcription analysis of a cloned sequence derived from a major developmentally regulated mRNA of *Dictyostelium discoideum.* Cell. 17: 903-913.