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The effects of hypoxic conditions on Dictyostelium mucoroides.

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The Effects of Hypoxic Conditions on *Dictyostelium mucoroides*

A Thesis

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by

Mary E. Kosinski

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Thesis Acceptance

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Committee

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Date May 15, 1996

Abstract

Previously a transcript corresponding to a gene in *Dictyostelium mucoroides* designated A-11 was found in amoebae during vegetative growth and macrocyst formation. The derived amino acid sequence of A-11 was used to search Genbank to find homologous proteins. The highest degree of homology was found with a hemoglobin from the bacterium *Vitreoscilla*. The *Vitreoscilla* hemoglobin protein showed greater than 65% similarity and 50% identity with the A-11 amino acid sequence. It was reported that hypoxic conditions could stimulate the expression of the gene corresponding to the *Vitreoscilla* hemoglobin when cloned in *E. coli* cells.

The purpose of this study was to examine the effects of hypoxic conditions on *Dictyostelium mucoroides* at a morphogenetic and a molecular level. Cells developing as sorocarps were exposed to hypoxic conditions to determine if development was retarded or obstructed. It was shown that low oxygen conditions impeded development, however normal sorocarps did develop. Spores formed by cells exposed to hypoxic conditions appeared normal in morphology but were less viable than spores from normal and control sorocarps. Southern blot analysis indicated the presence of a single copy of the A-11 gene in the genome of *D. mucoroides*. RNA was isolated from cells exposed to hypoxic conditions and primer extension analysis was used to determine if the A-11 transcript was regulated by oxygen. Low oxygen did not stimulate production of the A-11 transcript. Comparison of RNA from samples taken at different times during vegetative growth and development identified A-11 as a macrocyst specific developmental gene.

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There are a number of people I wish to thank for all of their technical advice and help. Dr. Chase not only offered unlimited help but he also let me use any equipment or supplies I needed. Dr. Hanson, Dr. Mullican, and Marilynn Larson all provided excellent technical advice. These are all people I admire and are grateful to. I would also like to thank Dr. Degraw for the use of his bladder bags and Dr. Antlefinger for the use of her valve. Willy Arnold, Marg Esser, Kevin Brewer, Steve Schierner and Vince Roth all of whom provided friendship and valuable

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Introduction

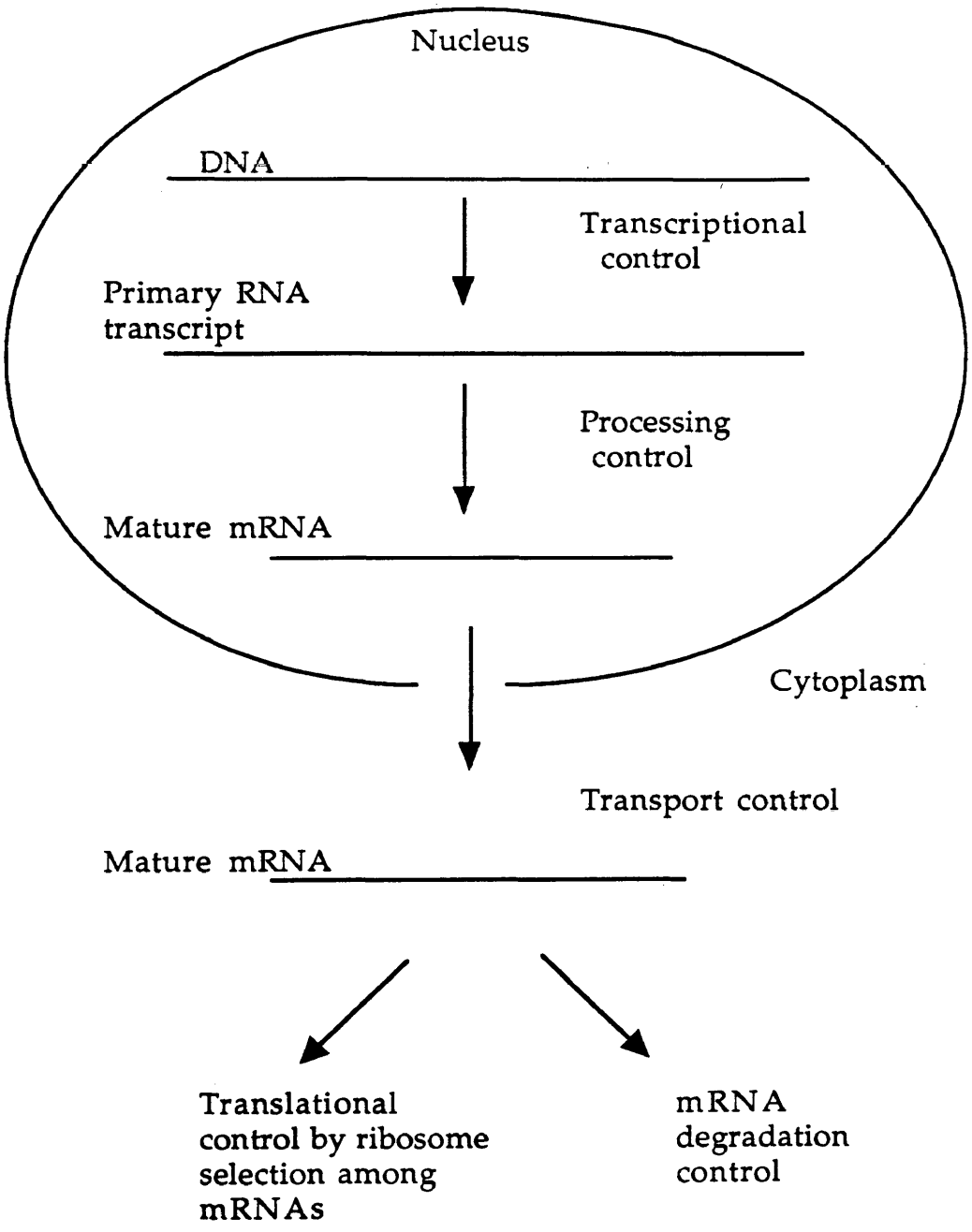
Gene regulation is an area in biology that is being studied extensively. The regulation of genes is crucial in the normal development of multicellular eukaryotic organisms. Understanding how genes are regulated is key to understanding this developmental process. Most eukaryotes have different cells, tissues and organs with specialized functions, yet all cells of the same organism have the same genotype.

Gene regulation is required to determine which genes will be expressed in specific cells and when these genes will be expressed. The central dogma of gene expression is that the genetic information or DNA is transcribed into an RNA copy which is then translated into a protein. The actions and properties of each cell type are determined by the proteins that cell contains. The amounts and types of proteins present in a particular cell is determined by each protein's corresponding mRNA concentration. Therefore the amounts and types of mRNA translated are ultimately responsible for phenotypic differences among cells.

The primary control of gene expression in higher eukaryotes occurs at the transcriptional level (Latchman, 1990). In eukaryotic organisms there are several levels at which control of gene expression can occur (Fig. 1). The decision to initiate transcription, the first step in gene regulation, is the most important point in determining whether or not genes are expressed and how much of the mRNA is produced (Lodish, 1995). Regulatory regions around the coding region, known as regulatory elements, control the initiation of transcription. These can either be positive regulatory elements, which

Figure 1.

Levels at which gene expression can be controlled in eukaryotes (Russell, 1996).


Nucleus
DNA
Transcriptional control
Primary RNA transcript
Processing control
Mature mRNA
Cytoplasm
Transport control
Mature mRNA
Translational control by ribosome selection among mRNAs
mRNA degradation control

activate transcription, or negative regulatory elements, which repress transcription.

Transcription initiation requires that specific transcription factors (TFs) bind to the gene to be transcribed. RNA polymerase II recognizes these factors and binds, along with other TFs, forming an initiation complex. Once transcription has been initiated, RNA polymerase begins moving down the DNA transcribing the gene, thus producing the primary RNA transcript.

After transcription occurs, the primary RNA transcript is further processed to produce the mature mRNA transcript. At the 5′ end of the initial transcript, a 7-methyl-guanylate cap is added. At the 3′ end of the initial transcript a stretch of 100 to 250 adenosine residues are added. Finally, introns are removed by RNA splicing before the mature mRNA is transported out of the nucleus and into the cytoplasm. Each of these steps can also be regulated to control the expression of a gene. Once the mature mRNA is in the cytoplasm it can be regulated further either by degradation or by translational control before it is translated into protein.

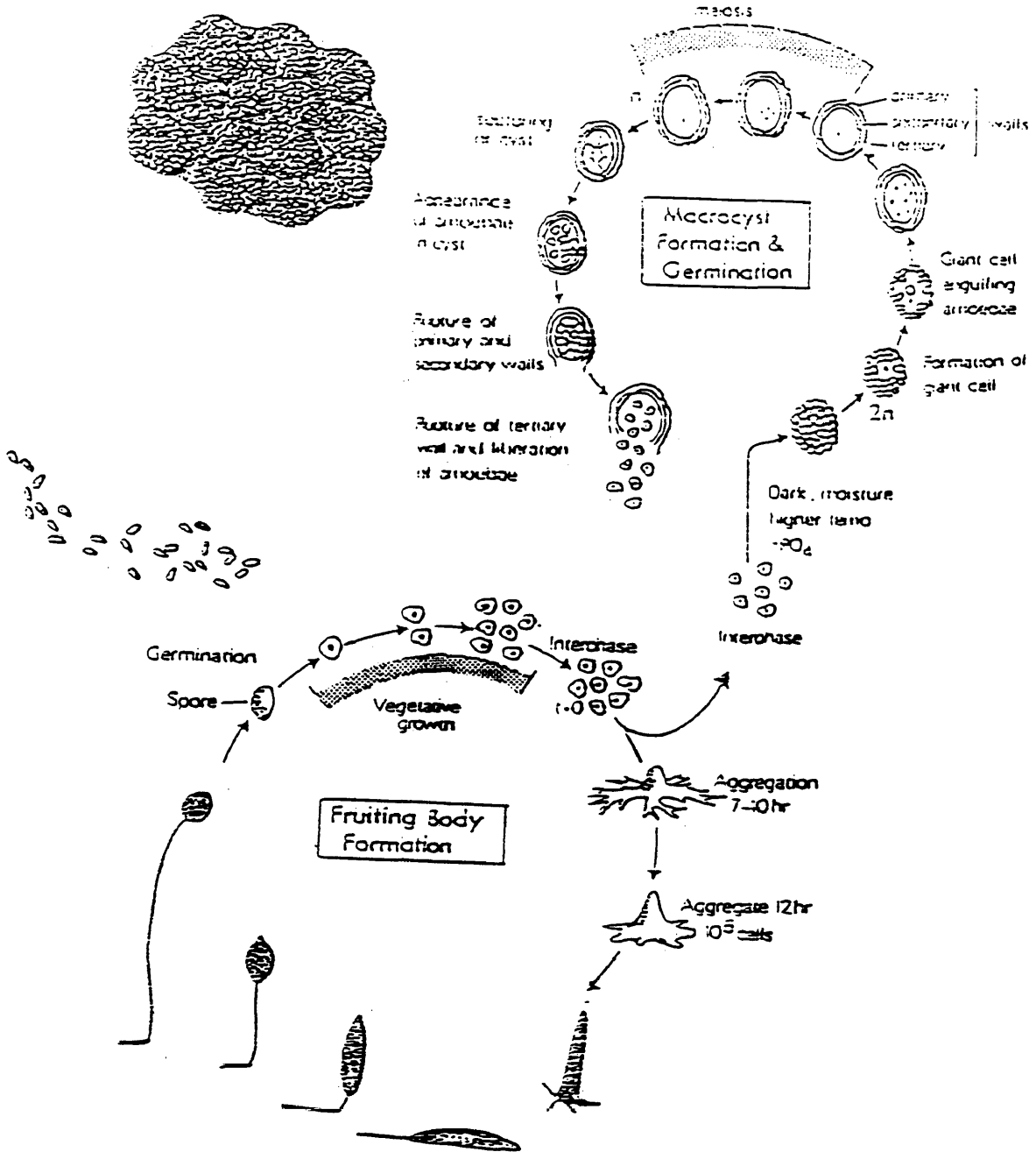
This research focused on the transcriptional regulation of a gene in *Dictyostelium mucoroides*. *Dictyostelium* was first characterized in 1869 by a German scientist, Oskar Brefeld. Since then it has gained tremendous popularity in studies of eukaryotic development and gene expression (Loomis, 1982). *Dictyostelium* has several features which make it an attractive subject. First, *Dictyostelium* is a simple eukaryote that has separate growth and developmental stages. This makes it an excellent model system for developmental studies because developmental processes can be studied without a hindrance of cell division. *Dictyostelium*'s genome is small,

containing about 50 megabases of DNA. This is only about 2% of the human genome yet it is 12 times as large as the *E. coli* genome (Loomis, 1982). *Dictyostelium* has relatively simple development, yet engages in many of the behaviors seen in embryogenesis of higher organisms. Such behaviors include migration, aggregation, differentiation, and signal transduction. Furthermore, *Dictyostelium*'s genome is maintained in a haploid condition so any mutations are seen phenotypically (Loomis 1982). Another important feature *Dictyostelium* has is that growth and development occur rapidly under laboratory conditions. Development can be complete in about 12 hours.

During vegetative growth, *D. mucoroides* exists as independent, free living amoebae. These amoebae feed phagocytically on bacteria and multiply by binary fission. Once food sources become scarce, the amoebae begin to aggregate and enter one of two different developmental pathways, sorocarp or macrocyst (Fig. 2). Aggregation is a chemotactic response to extracellular cyclic adenosine mono-phosphate (cAMP). Between 3 and 5 hours after food removal a cell begins to emit pulses of cAMP. The surrounding cells respond by not only moving chemotactically toward the cAMP but also relaying the signal further by releasing cAMP into the medium to attract more distal cells. This results in migration of aggregating amoebae up a pulsated cAMP gradient toward the aggregation center (Firtel, 1991; Shaffer, 1962, 1975, and Roos *et al.*, 1975). The level of cAMP rises significantly within the mounds and is no longer pulsatile (Loomis, 1996). Once aggregated, amoebae enter either an asexual pathway or a sexual pathway. The developmental pathway

Figure 2.

Life cycle of *Dictyostelium mucoroides*.


Macrocyst Formation & Germination
Fruiting Body Formation
Germination
Spore
Vegetative growth
Interphase
Aggregation 7–10 hr
Aggregate 12hr
Dark, moisture
2n

taken is influenced by environmental conditions (Nickerson and Raper, 1973a; Hanson and Weber, 1987) but ultimately the expression of specific genes is responsible for the orderly progression of development along the chosen pathway.

The induction of the asexual cycle or sorocarp formation, occurs when starved amoebae encounter light, low humidity, low calcium and high phosphate conditions (Nickerson and Raper, 1973a). The aggregate begins to elongate forming a migrating multicellular pseudoplasmodium. Eventually this multicellular pseudoplasmodium stops and begins to rise, producing a fruiting body. The fruiting body, known as a sorocarp, consists of a rounded sorus that is supported by a linear array of dead, vacuolated, stalk cells (Bonner 1952; Gregg and Badman 1970). The sorus contains dormant spores. Upon germination each spore yields a single amoeba (Cotter and Raper, 1966).

Induction of the sexual pathway, macrocyst formation, is prompted by darkness, high humidity, high calcium, and low phosphate conditions (Nickerson and Raper 1973a). Again starved amoebae begin to aggregate by secreting cAMP. Macrocysts formation begins by fusion of two aggregating amoebae, then their nuclei fuse (Erdos *et al.*, 1972; Raper 1984). The result of this fusion is a large, diploid, phagocytic cell. Amoebae at the periphery of the aggregate secrete a primary wall around all amoebae in the aggregate. The giant cell then begins engulfing the surrounding amoebae. Once phagocytosis is complete, a secondary and eventually a tertiary wall, rich in cellulose, are synthesized by the giant cell around the entire structure but inside the primary wall (Nickerson and Raper 1973b; Loomis 1982; Raper 1984; Erdos *et al.*, 1972). Before germination can occur, the nuclei in the macrocyst divide

meiotically followed by several mitotic divisions (Erdos *et al.*, 1972; MacInnes, and Francis, 1974). The cytoplasm then divides and forms uninucleate amoebae. Upon rupture of the cyst wall, amoebae are released.

Signals which can stimulate the expression of a particular gene can be internal, external or a combination of the two. Sorocarp formation has been predominately studied in *D. discoideum* and several genes have been found which are required for sorocarp development, although less than one fifth of the developmental genes have been well characterized (Loomis, 1996; Firtel, 1991).

One of the first signals that affects *Dictyostelium* development is environmental. It is a protein referred to as prestarvation factor which is secreted by growing cells (Rathi *et al.*, 1991). Once a certain concentration of the factor is reached, indicating cell density is high, cells respond by expressing a subset of developmental genes. One of these genes is known to be a small surface protein that mediates cell to cell adhesion, creating mounds of cells (Loomis, 1996; Rathi *et al.*, 1991; Knecht *et al.*, 1987).

Another developmental signal in *Dictyostelium* is cAMP. Increased levels of cAMP cause the repression of some genes (Ross *et al.*, 1975; Firtel, 1991) and the induction of other genes known as pulse induced genes (Mann and Firtel, 1991; Harwood *et al.*, 1992; Loomis, 1996).

After aggregation, the cell type specific genes are expressed. There are three well characterized prestalk genes, tagB, ecmA, and ecmB, and six prespore genes (Loomis, 1996., Firtel, 1991). One example of a cell type specific gene that has been characterized is tagB. This gene encodes a protein which shows similarities to known peptide transporters. Null tagB strains never form spore or stalk cells. It is believed that tagB is essential in the process of

differentiation from PST-I cells, initial prestalk cells, to PST-A cells. PST-A cells are the most anterior cells in the slug, and express high levels of ecmA, which plays a role in determination of slug shape (Early *et al.*, 1995; Jermyn and Williams, 1989, 1991). It is believed that PST-A cells produce a signal inducing the remaining PST-I cells to differentiate into PST-O cells (Loomis, 1996; Shaulsky *et al.*, 1995). PST-O cells are found at the posterior end of the slug and also play a role in limiting the expression of prespore genes. Research is currently underway to characterize this signal.

Although several developmental genes have been found in sorocarp development, little is known about the genes required for macrocyst formation. Previous research indicates that many of the developmental genes regulated in sorocarp formation are regulated at the transcriptional level (Williams *et al.*, 1979, 1980; Blumberg and Lodish, 1981; Chung *et al.*, 1981; Landfear *et al.*, 1982; Singleton *et al.*, 1987, 1988). This research focused on the transcriptional regulation of a particular gene in *D. mucoroides* macrocyst formation.

Previously a cDNA clone, A-11, was picked from a cDNA library made by A. T. Weber from 8 hour transcripts in macrocyst development. The transcript that the A-11 cDNA identified was present during vegetative growth and macrocyst formation, but not during sorocarp formation (Larson, 1991). These results indicated that A-11 could be a developmental gene regulated by transcription. Further study of this gene yielded the nucleotide sequence and from that the derived amino acid sequence was used to search the Genbank data base. A-11 showed the highest degree of homology with a hemoglobin protein found in the bacterium *Vitreoscilla*. The translated A-11 cDNA showed 65% similarity, and 50% identity. Hemogolobin-like proteins

Figure 3.

Amino acid homology of the *Vitreoscilla* hemoglobin (VtHb) aligned with the A-11 translated cDNA.

```
(A-11) 1     MLSQENIDIIKSTVPVLEVHGVTITSTFYKNMFEENPQLLNIFNHSNQRQ
             ||.|:.|:|||.|||||. ||||||.|||||:|..:|::  :|: :.|
(VtHb) 1     MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQES

(A-11) 51    GKQQTALANTVLAAAVNIEN-MN-ILQVLLINML..QRLVYYQNIIQLLV
              .|..||| |||||| |||| . : .| |.:  | | .:. :
(VtHb) 51    LEQPKALAMTVLAAAQNIENLPAILPAVKKIAVKHCQAGVAAAHYPIVGQ

(A-11) 99    EIIGAIKQVLGEAATPAILNAWTEAYGIIAQAFIDAEAALYKVTEEQMVV
             |::||||:|||:|||..||:||..|||:||:.||:.||.||  . |
(VtHb) 101   ELLGAIKEVLGDAATDDILDAWGKAYGVIADVFIQVEADLYAQAVE....
```

from five other organisms also showed 50% or higher similarities and 30% or higher identities (Brewer, 1995).

The hemoglobin protein in *Vitreoscilla* is a structural homolog of eukaryotic hemoglobins and may function as an oxygen storage trap to facilitate oxygen diffusion (Wakabayashi *et al.*, 1986). It has been shown that *Vitreoscilla* hemoglobin mRNA increases in concentration when cells are exposed to hypoxic conditions (Dikshit *et al.*, 1989).

Vitreoscilla is found in oxygen poor environmental conditions, conditions similar to those in which macrocysts are found. Macrocyst formation is stimulated in the laboratory by submerging them under a filter and buffer. It is possible that the function of the A-11 gene product is to facilitate oxygen diffusion in *D. mucoroides* as it does in *Vitreoscilla*. If this hypothesis is true, then hypoxic conditions should stimulate transcription of the A-11 gene.

Examples of genes regulated by oxygen exist in eukaryotes as well as prokaryotes. The yeast *Saccharomyces cerevisiae*, provides examples of genes whose expression is sensitive to oxygen. The CYC1 gene is paired with another gene CYC7, together they encode the cytochrome c protein, which is involved in respiratory functions. The product of this gene is induced during exponential growth when oxygen becomes limited (Zitomer and Lowery, 1992). In *D. discoideum* it has been shown that low oxygen causes changes in subunit structure of the cytochrome c oxidase. When cells enter the stationary growth phase, where it was shown that oxygen levels were very low, the smallest of the six subunits is substituted by a large polypeptide. These results show a correlation between the decrease in oxygen

concentration and expression of the alternative form of oxidase (Schiavo and Bission, 1989).

The purpose of this project was 1) assess the effects of hypoxic conditions on morphogenesis and 2) determine if the A-11 gene was a developmental gene or a gene which was regulated by oxygen. More specifically, would hypoxic conditions stimulate transcription of the A-11 gene in sorocarp cells, where it is normally not expressed?

Materials and Methods

Organisms and Cultural Conditions

Dictyostelium mucoroides Dm-7 was used in this investigation. Stock cultures were maintained on 0.2% lactose peptone plates (0.2% lactose, 0.2%peptone, 1.5% agar, 1L H_2O). Cultures of amoebae were grown in glucose yeast extract peptone broth (GYP) (2g peptone, 1g glucose, 0.5g yeast extract, 0.84g KH_2PO_4, 0.54g Na_2HPO_4, 1 L H_2O) in association with *Escherichia coli* B/r as the nutrient source. Vegetative growth was initiated by inoculating 40 mL of GYP, with 0.2 mL of 5 x 10^5 spores/mL and 0.1 mL of a turbid *E. coli* B/r suspension (Weber and Raper, 1971). Flasks were then incubated in a gyrotory shaking water bath at 23°C. Amoebae intended for vegetative studies were harvested at 48 hrs, before any cell clumping occurred. Amoebae intended for developmental studies were harvested after approximately 56 hrs of growth, at a concentration of 1 to 6 x 10^6 cells/mL. All cells were harvested by centrifugation at 500 x g (2000 rpm) in a Sorvall GSA rotor for 20 minutes at 4°C. Amoebae were washed several times with Bonner's Salt Solution (BSS)(0.60g NaCl, 0.75g KCl, 0.30g $CaCl_2$, 1L H_20) (Bonner and Frascella, 1953) and centrifuged as above to remove bacteria.

Macrocyst development was initiated in small petri dishes (48 x8.5 mm) containing an absorbent pad soaked with 1.5 mL of BSS with streptomycin (0.5 mg/mL) and a filter (Gelman Supor-450, 0.45 μM) was placed on the pad. The filter was then inoculated in a circle with 0.9 mL of a suspension containing 3.5 x 10^8 amoebae/mL. A second filter was placed

upon each inoculation and an additional 1.5 mL of BSS with streptomycin was added (Hanson and Weber, 1987). The plates were then wrapped in aluminum foil to exclude light and incubated at 23°C for 3, 6, 9, and 12 hr.

Sorocarp development was initiated by washing amoebae an additional time with 0.025 M phosphate buffer (2.1g KH_2PO_4, 2.6g $Na_2HPO_4 \cdot 7H_2O$, 1L H_20; pH 6.5). Small petri dishes containing an absorbent pad soaked with 1.5 mL of 0.025 M phosphate buffer and 0.5 mg/mL of streptomycin were used. A filter was placed on top of this, and again a suspension of 3.5×10^8 amoebae/mL was inoculated in a circle (Hanson and Weber, 1987). The plates were incubated in the presence of fluorescent light at 23°C for 3, 6, 9, and 12 hrs.

Amoebae used for A-11 studies of regulation were plated exactly as for sorocarp development. These plates were then placed into a vacuum desiccator, a vacuum was drawn and gas containing 5.03% O_2 in N_2 was used to fill a 2000 cc rubber bladder. The gas in the bladder was then allowed to equilibrate with the atmosphere in the desiccator. This procedure was repeated three times to ensure that the inside of the desiccator was under low (5%) O_2.

Controls were performed in a similar manner, to ensure flushing of the system had no effect on development. Plates were placed into an identical desiccator and a vacuum drawn. Atmospheric air (containing about 20% O_2) was then forced into an identical bladder and again the gas in the bladder was allowed to equilibrate with the atmosphere of the desiccator. Bladders were

checked every hour and refilled when necessary. After development for the appropriate amount of time, 3, 6, 9, or 12 hr, the cells were washed from the filters with 5 mL of cold sterile, distilled, deionized water (sterile ddH_2O).

Morphological Studies

Cells allowed to develop under low oxygen conditions were examined with a dissecting microscope and compared to normal cells to determine if development was impeded or abnormal. Photographs were taken of both control cells and low oxygen cells. Spore viability was also examined to ensure normal development had occurred. Once cells had developed completely in low oxygen a spore suspension was made. Spores were then examined and counted using phase contrast microscopy. Dilutions were made to produce spore suspensions of 3×10^3, 3×10^2, and 3×10^1 spores/mL. One tenth mL of each dilution was plated on 0.2% LP plates along with 0.1 mL of a turbid *E. coli* B/r suspension. Plates were incubated for three days at room temperature at which time plaques were counted as a measure of spore viability.

cDNA Synthesis and Cloning

Construction of a directional *D. mucoroides* strain DM-7 cDNA library utilizing the SuperScript Plasmid System (BRL Life Technologies) was performed by A.T. Weber. Poly (A)+ RNA was isolated from 8 hr macrocysts to initiate first strand synthesis. Nick translation replacement of mRNA was used to synthesize the second strand cDNA. Directionality was created by introducing two different endonuclease sites at each end of the cDNA using a

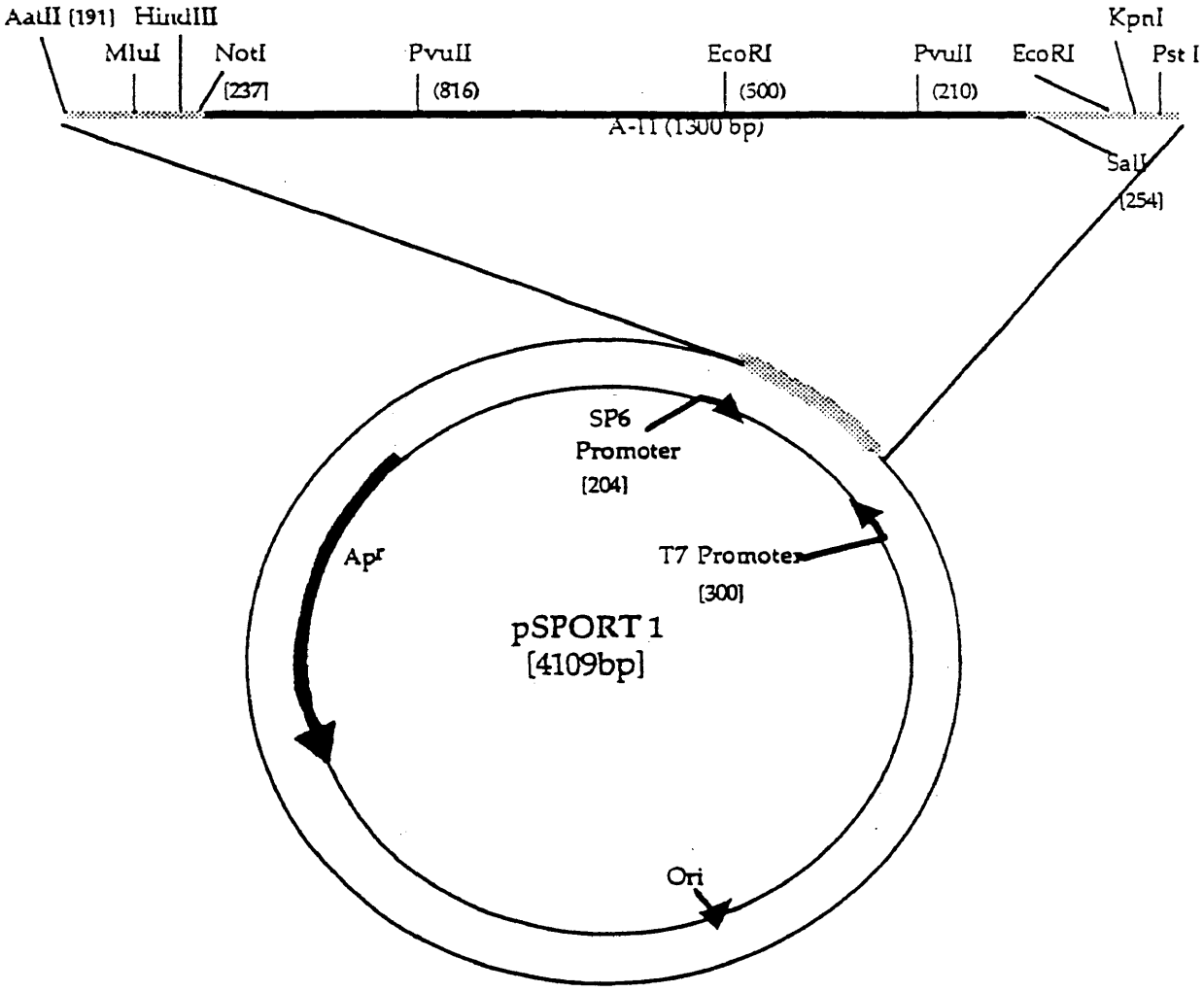
primer-adapter to initiate first strand synthesis. The primer-adapter is a primer for reverse transcriptase that also encodes a sequence for the restriction site, Not I. The resulting Not I restriction site identifies the 5′ end of the cDNA which corresponds to the 3′ end of the mRNA. The 5′ end of the mRNA is also identifiable by adding adapters to the cDNA. These are short, duplex oligomers that are blunt on one end and contain a 4 base 5′ extension, which in this system correspond to Sal I, at the other terminus (SuperScript booklet by Life Technologies). Digestion with Not I was performed to expose the 5′ end of the cDNA and it was inserted into the plasmid pSport 1 (Not I-Sal I-Cut) (Fig. 4) and maintained in *E. coli* DH5 α.

Plasmid Isolation

The A-11 clone was isolated using the alkaline lysis prep method (Promega Protocols and Applications Guide, 1991). *E. coli* containing A-11 was streaked onto Luria-Bertaini (LB) plates (10g tryptone, 5g yeast extract, 5g NaCl, 15g agar, 1L ddH_2O) with ampicillin (50 ug/mL) and incubated at 37°C overnight. A single colony of the clone was picked and used to inoculate 25 mL of LB broth. This was incubated in a shaking water bath overnight at 37°C. Cells were harvested by centrifugation at 5000 x g (6500 rpm) in a Sorvall RC-5B GSA rotor for 15 minutes at 4°C. The pellet was resuspended in 600 µL of freshly prepared cold lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) and incubated on ice for 10 minutes. After incubation, 1200 µL of freshly prepared 0.2 N NaOH and 1% sodium dodecyl sulfate (SDS) was added, mixed by careful inversion, and incubated on ice for 10 minutes.

Figure 4.

Map of the pSport plasmid vector containing the A-11 cDNA insert.


AatII (191)
HindIII
MluI
NotI
[237]
PvuII
(816)
EcoRI
(500)
PvuII
(210)
EcoRI
KpnI
Pst I
A-11 (1300 bp)
SalI
[254]
SP6
Promoter
[204]
Apr
T7 Promoter
[300]
pSPORT 1
[4109bp]
Ori

Following this, 750 µL of 3 M sodium acetate, pH 4.6 was added, gently mixed, and incubated on ice for 20 minutes. Next the sample was centrifuged at 12,000 x g for 15 minutes in a Sorval SS-34 rotor. The supernatant was transferred to another tube and DNase free RNase was added to a concentration of 1 µg/µL. This was incubated for 1 hr at 37°C. The sample was then extracted twice with Tris saturated phenol-chloroform followed by one chloroform-isoamyl alcohol (24:1) extraction. The aqueous phase was then precipitated with 2 volumes of ethanol. The pellet was collected by centrifugation at 12,000 x g for 20 minutes, washed twice with 500 µL of 70% ethanol. The pellet was air dried for about 15 to 20 minutes and then resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Quantitative and qualitative assessment of the plasmid DNA was determined by ultraviolet absorption spectrophotometry at 260 and 280 nm. Spectrophotometry was utilized to determine DNA concentration by measuring the absorbance at 260 nm with a Varian spectophotometer model 634. DNA purity was determined by the 260 nm/280 nm absorption ratio. Restriction digests were performed to linearize the plasmids. A-11 was linearized with the restriction endonuclease EcoRI or Hind III. This was performed in a 20 µL reaction containing 2 µL of 10X BSA (Bovine Serum Albumin), 2 µL of 10X buffer (buffer J and B respectively), 3 µg of DNA, 1 µl of enzyme, and brought to volume with sterile ddH_2O (Sambrook *et al.*, 1989). Two to 3 µL of the digest were combined with loading dye (0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) then loaded on a 1% agarose gel containing ethidium bromide (EtBr) (0.5 µg/mL) and run for 2 hr at 60 volts in Tris-Borate-EDTA (TBE) (5X; 0.045 M Tris-borate, 0.001 M

EDTA) using an IBI MPH electrophoresis chamber (Sambrook *et al.*, 1989). Five µL of HindIII cut lambda DNA (Sigma) was used as the molecular size standard.

Southern Blot Analysis

Probe Synthesis for Southern Blot Analysis

A-11 plasmid DNA was random prime labeled using digoxigenin (DIG)-11-dUTP. This was accomplished using the Genius DIG Kit by Boehringer Mannheim. Prior to labeling, 3 µg of A-11 plasmid was linearized with 1 µL of Eco RI at 37°C for 1 hour as previously described. The digested DNA was then purified by a phenol-chloroform extraction and ethanol precipitation. The 20 µL labeling reaction was assembled on ice in the following order; 3 µg of denatured DNA template, 2 µL of 10X hexonucleotide mixture (62.5 A_{260} units/mL of random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithioerythritol [DTE], 2 mg/mL BSA, pH 7.2), 2 µL of 10X dNTP labeling mixture(1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM Dig-dUTP, pH 6.5), to 19 µL with sterile ddH_2O, 1 µL of Klenow enzyme, labeling grade. This reaction was incubated for 1 hr at 37°C. To terminate the reaction, 2 µL of 200 mM EDTA, pH 8.0 was added and the labeled DNA was precipitated with 0.1 volume of LiCl and 2 volumes of cold ethanol for 1 hour at -80°C. Labeled DNA was collected by centrifugation in a microfuge at 13,000 x g for 15 minutes, washed with 70% ethanol, air dried and resuspended in 50 µL of TE/SDS buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0; containing 0.1% SDS).

Quantification of the probe followed the procedures recommended by Boehringer Mannheim for digoxigenin-labeled DNA. Dig-labeled control DNA was diluted to a final concentration of 1 ng/μL, 100 pg/μL, 10 pg/μL, and 1 pg/μL. The labeled DNA was diluted in the same manner. One μL spots of each dilution were placed onto Nytran membrane. DNA was immobilized on the membrane by UV crosslinking. The blots were exposed to UV light (254 nm) for 15 seconds at 160 J/m^2 (Schleicher and Schuell). Colormetric detection of the probe followed the procedures in the DIG Detection Kit No. 1 from Boehringer Mannheim Biochemicals. The membrane was wet briefly with Genius Buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). It was then incubated in Genius buffer 2 (2% [w/v]blocking reagent, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 15 minutes at room temperature. The anti-DIG-alkaline phosphatase was diluted 1:5,000 in Genius buffer 2 and the membrane was incubated in the diluted antibody for 15 minutes at room temperature. The membrane was washed twice in Genius buffer 1 at room temperature for 15 minutes and then incubated in Genius buffer 3 (100 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 2 minutes. The membrane was then incubated in Color Substrate Solution (45 μL NBT [75 mg/mL nitroblue tetrazolium salt in 70% v/v dimethylformamide], 35 μL X-phosphate solution [50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide], in 10 mL of Genius buffer 3) in the dark for approximately 30 minutes. Spot intensities of the control and experimental dilutions were compared to estimate the concentration of the probe.

Genomic DNA Isolation

DNA was isolated from *D. mucoroides* strain DM-7. To obtain amoebae for DNA isolation, cells were grown in association with *E. coli* B/r in 40 mL of GYP broth as previously described. Amoebae were harvested in early stationary phase after 56 hours of incubation. Nuclear DNA was isolated essentially as described by Richardson *et al.*, (1990). Cells were washed several times with cold ddH_2O to remove bacteria and resuspended in ice cold 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) to a volume of 1-5 x 10^8 cells/mL. After 5 minutes on ice the cells were pelleted by centrifugation at 10,000 x g in a Sorvall SS-34 rotor and the supernatant was discarded. This lysis step was repeated once and nuclei were resuspended in 0.3 volumes of proteinase K buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 0.5% SDS, 100 µg/mL proteinase K) and incubated for 1 hr at 60°C. After cooling to room temperature, DNA was extracted sequentially with phenol, phenol-chloroform, chloroform isoamyl alcohol (24:1), and then ethanol precipitated. The DNA was collected by centrifugation in a microcentrifuge for 15 minutes at 12,000 x g, washed twice with 70% ethanol, air dried and resuspended in TE, pH 8.0. Quantitative and qualitative assessment of the DNA were determined by ultraviolet absorption spectrophotometry at 260 and 280 nm as previously described.

Southern Blot Analysis

D. mucoroides DM-7 genomic DNA was digested with restriction

endonucleases Eco RI, and Pst I. Double digests for *D. mucoroides* strain were also performed with Eco RI and Pst I. In each instance 40 µg of DNA was digested with 20 units of restriction enzyme for 4 hours at 37°C (Sambrook et al., 1989). Digests were assessed for completion by examining EtBr stained gels following electrophoresis in an 0.7% mini agarose gel. Once digestion was complete samples were loaded on a 0.7% agarose gel (with no EtBr) and run for approximately 18 hours at 25 volts using electrophoresis buffer TBE. The gel was denatured for 30 minutes at room temperature in denaturing solution (0.5 N NaOH, 1.5 M NaCl). The gel was then submerged in neutralization solution (1.0 M Tris-HCl, pH 8.0, 1.5 M NaCl) for 30 minutes at room temperature (Genius system User's Guide). The DNA was transferred to Nytran (Schleicher and Schuell) by capillary transfer in 10X SSC(1.5 M NaCl, 150mM sodium citrate, pH 7.0). The DNA was then fixed to the membrane by UV crosslinking as previously described for Dig labeling. To ensure transfer was complete the gel was stained after transfer with EtBr (0.5 µg/mL) for 30 minutes and destained in ddH_2O for 30 minutes to 1 hr. The absence of stained DNA indicated complete transfer.

Prehybridization and hybridization were performed according to Boerhinger Mannheim Technical Bulletin supplied with the Dig Kit. Prehybridization was accomplished by placing the blot in a Micro-4 Hybaid hybridization oven containing 10 mLs of prehybridization solution (5X SSC, 1.0% Blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS) for 2 hrs at 42°C. After removal of the prehybridization buffer, 10 mL of hybridization buffer (5X SSC, 1.0% Blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS) was

added. The DNA probe was denatured by heating in a 65°C water bath for 10 minutes and added to the hybridization buffer to a concentration of 5 to 20 ng/mL. Hybridization occurred at 42°C for approximately 24 hrs.

Blots were washed twice in 2X wash solution(2X SSC, 0.1% SDS) for 5 minutes at room temperature, and twice in 0.5X wash solution(0.5X SSC, 0.1% SDS) for 15 minutes at room temperature (Boerhringer Mannheim Technical Bulletin). After post hybridization, colorimetric detection with NBT and X-Phosphate was performed according to the Borhringer Mannheim Technical Bulletin. This was done by first equilibrating the membrane in Genius buffer 1 (100 mM Tris-Hcl, 150 mM NaCl, pH 7.5) for 1 minute. Then the blot was gently agitated in Genius buffer 2 (2% [w/v] blocking reagent, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 30 minutes at room temperature. Toward the end of this incubation the anti-DIG-alkaline phosphatase was diluted 1:5,000 in Genius buffer 2. The blot was incubated in the anti-DIG-alkaline phosphatase buffer for 30 minutes at room temperature with gentle agitation. The blot was then washed twice in 100 mL of Genius buffer 1 for 15 minutes and equilibrated in 20 mL of Genius buffer 3 (100 mM Tris-Hcl, 1 mM EDTA, pH 8.0) for 2 minutes. Finally the blot was placed in 20 mL of color substrate solution (90 µL NBT [75 mg/mL nitroblue tetrazolium salt in 70% v/v dimethylformamide], 70 µL X-phosphate solution[50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide], in 20 mL of Genius buffer 3) wrapped in aluminum foil to exclude light and incubated in the dark for up to 20 hrs. Once the desired bands were detected the blot was washed with 50 mLs of Genius buffer 1 for 5 minutes to stop the reaction.

Primer Extension Analysis

RNA Isolation

Total RNA was isolated using the acid guanidinium thiocyanate RNA extraction method described by Chomczynski and Sacchi (1987). For RNA procedures, ribonuclease activity was reduced by using baked glassware and reagents were prepared using RNase free H_2O. RNase free H_2O was prepared by treating it with 0.1% diethyl pyrocarbonate (DEPC) (Blumberg, 1987). RNA was isolated from 3 hour, 6 hour, 9 hour, and 12 hour developmental samples , as well as from 48 hour and 56 hour vegetative samples. For each RNA preparation, 0.2 mL containing 1.8 $x10^7$ cells were vortexed in 1.8 mLs of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Next the following reagents were added; 180 µL of 2 M sodium acetate pH 4.0, 1.8 mL of phenol, and 360 µL of chloroform-isoamyl alcohol (49:1). After the addition of these reagents the sample was vortexed for 15 seconds, placed on ice for 15 minutes and centrifuged for 20 minutes at 4000 x g (5000rpm) at 4°C in a Sorvall RC-2B type SS-34 rotor. The aqueous phase was precipitated with an equal volume of cold isopropanol at -80°C overnight. The pellet was collected by centrifugation at 4°C for 20 minutes at 4000 x g (5000 rpm) in the above rotor. The pellet was then dissolved in 200 µL of denaturing solution and transferred to a microfuge tube (1.5 mL). An equal volume of cold isopropanol was then added and the sample was placed at -80°C for at least 1 hr. The pellet was again collected by centrifugation at 4°C for 15 minutes in a

microfuge at 10,000 x g (12,000 rpm) and washed twice with 75% ethanol. The pellet was air dried for about 15 minutes and redissolved in 25 μL of DEPC treated H_2O.

RNA concentration and integrity was determined by ultraviolet absorption spectophotometry and EtBr staining of 1% formaldehyde denaturing agarose gels after electrophoresis. Spectrophotometry was utilized to determine RNA concentration by measuring the absorbance at 260 nm as previously described for DNA. RNA integrity was also assessed by running samples on a 1% formaldehyde denaturing agarose gel and comparing the staining intensity of bands containing 28S and 18S ribosomal RNA molecules. Intact RNA bands in reproducible fashion, under denaturing gel electrophoresis, resulting in a two to one ratio in intensity of the ribosomal bands (Farrell, 1993). Only RNA showing this approximate ratio as assessed by visual inspection was used in further studies.

Primer Extension

A-11 PE primer, a 19-mer oligonucleotide complementary to the A-11 mRNA 5′ sequence, was prepared by Genosys Biotechnologies, Inc. Its sequence and its position on the A-11 cDNA is as follows: 5′-dCTCCATGAACTTCTAATAC-3′, complementary to positions 1221 to 1239 of the A-11 cDNA. A modified kinase reaction was used to label A-11 PE, (Sigmund et al., 1988). The following 30 μL reaction was assembled and incubated at 37°C for 40 minutes; 50 pmol of oligonucleotide, 33 pmol γ^{32}P-ATP (10 μL of 3000 Ci/mmol,10 mCi/mL), 220 pmol rATP, 10 units polynucleotide kinase, 3 μL 10X PNKB (0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50

mM DTT, 1 mM Spermidine, 1 mM EDTA), and ddH_2O to bring to a final volume of 30 µL. Following incubation the reaction was terminated by incubation at 65°C for 10 minutes. Seventy µL of TE was added and the mixture was extracted with phenol-chloroform once and precipitated with NaCl and 3 volumes of 95% ethanol at -80°C for two hours. The pellet was collected by centrifugation for 15 minutes. The pellet was then air dried and resuspended in 25 µL of TE buffer.

Primer extension was performed by first setting up a 12 µL annealing reaction which consisted of 20 µg of total RNA, 2 pmol of labeled oligonucleotide, and 1.2 µL of 10X RT buffer (0.5 M Tris-HCl pH 8.0, 0.6 M NaCl, 60 mM MgOAc, 0.1 mM DTT). The reaction was incubated at 75°C for 5 minutes and then slow cooled to less than 35°C and placed on ice. Twelve µL of 1X RT buffer containing 0.25 mM deoxynucleoside triphosphates, and 10 units of Superscript™ RNase H- Reverse Transcriptase (Life Technologies, Inc.) was added. The reaction was then incubated at 37°C for 45 minutes. The reaction was then extracted once with an equal volume of phenol-chloroform (Schumann *et al.*, 1994). Next the sample was precipitated with three volumes of ethanol and one tenth volume of NaCl at -80°C for one hour. The pellet was collected by centrifugation in a microcentrifuge at maximum speed for 15 minutes. The pellet was air dried and resuspended in 5 µL of TE buffer, 3 µL of loading dye was also added. Samples were then stored at -20°C until gel electrophoresis was performed. Samples were separated on an 8%

polyacrylamide gel containing 50% urea. Samples were run along with sequence reactions of a 1 kb DNA insert for size generation, which were generously donated by Kelley Colvin. First samples were heated at 75°C for 2 minutes and subsequently chilled on ice before being loaded onto an 8% polyacrylamide gel. Electrophoresis was carried out at a constant power of 55 watts for approximately two and a half hours or until the bromophenol blue had run off the bottom. The gel was then fixed in 10% methanol and 10% acetic acid for 30 minutes, dried at 80°C for 50 minutes and exposed to Fuji RX GCU film overnight.

In Vitro Transcripts

A positive control was prepared by synthesizing *in vitro* transcripts. These were created by linearizing the plasmid containing the A-11 cDNA with the restriction enzyme HindIII as previously described. The Promega Riboprobe kit was used to create run off transcripts according to the manufactures suggestion. To accomplish this the following 20 µL reaction was assembled at room temperature; 4 µL of 5X transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 2 µL of 100 mM DTT, 20 u of rRNasin, 4 µL of rNTP's (2.5 mM each) 1 µL of linearized DNA template, 1 µL of T7 RNA polymerase (20 u/µL), nuclease free H_2O to bring to a final volume of 20 µL. This reaction was then incubated at 37°C for 60 minutes at which time DNase was added to a concentration of 1 u/µg of template DNA, this was then incubated at 37°C for another 15 minutes. Following incubation the reaction was phenol-chloroform extracted once and

then ethanol precipitated at -80°C for one hour. RNA was collected by centrifugation in a microcentrifuge at 12,000 x g for 15 minutes. Once the pellet was dry it was resuspended in 10 µL of DEPC H_2O. The sample was then combined with an equal volume of RNA loading buffer (0.72 mL 100% formamide, 0.16 mL 10X MOPS [0.2 M 4-morpholinopropanesulfonic acid, pH 7.0, 80 mM sodium acetate, 10 mM EDTA], 0.26 mL 37% formaldehyde, 0.18 mL 0.4% bromophenol blue and 0.08 mL 0.4% xylene cyanol). The samples were denatured by heating to 65°C for 10 minutes and quick chilled on ice. Samples were run on a 1% formaldehyde agarose mini gel in 1X MOPS buffer. The gel was run at 60 volts for about 30 minutes as previously described. T7 transcripts were then gel purified using GenElute agarose spin columns from Supelco. Following elution, T7 samples were precipitated with ethanol, dried and resuspended in 5 µL of DEPC H_2O. T7 transcripts were then utilized in primer extension analysis as previously described.

Results

Timing of Development

Cells were examined to determine if hypoxic conditions altered or impeded development. *D. mucoroides* vegetative amoebae were grown until a concentration of about 1 to 6 x 10^6 cells/mL were obtained. The washed amoebae were then placed on filters as described in materials and methods for sorocarp formation and examined microscopically at selected times to evaluate development. Figure 5 depicts these observations after 3, 6, and 9 hours of development for cells exposed to each of the three environmental conditions. Cells exposed to 20% oxygen in a desiccator, and the standard sorocarp cells developing in dishes on the laboratory bench, first showed signs of aggregation at 3 hours, while cells exposed to hypoxic conditions first showed signs of aggregation at 6 hours. At 6 hours of development standard sorocarp cells and cells exposed to 20% oxygen showed signs of forming mounds and ridges. At 9 hours of development these cells had formed pseudoplasmodia that were beginning to rise off the substratum, and by 12 hours these cells formed mature sorocarps, as illustrated in Figure 6. Cells exposed to hypoxic conditions did not begin to form mounds and ridges until 9 hours of development and did not form pseudoplasmodia until 12 hours of development as shown in Figure 6. Figure 6 also shows cells exposed to all three environment conditions after 24 hours of development depicting normal sorocarps from all three. Stocks produced by hypoxic cells as well as those produced by the 20% control cells appeared to be slightly shorter than those produced by the normal sorocarp cells.

Figure 5.

Developmental stages of standard sorocarp cells, cells exposed to 20% oxygen and cells exposed to hypoxic conditions. Cells were placed on filters in small petri dishes for development. Standard sorocarp cells and cells exposed to 20% oxygen are shown 3, 6, and 9 hr into development. Cells exposed to hypoxic conditions are shown at 6 and 9. At three hours cells under hypoxic conditions remained in a lawn with no sign of development and therefore were not photographed.

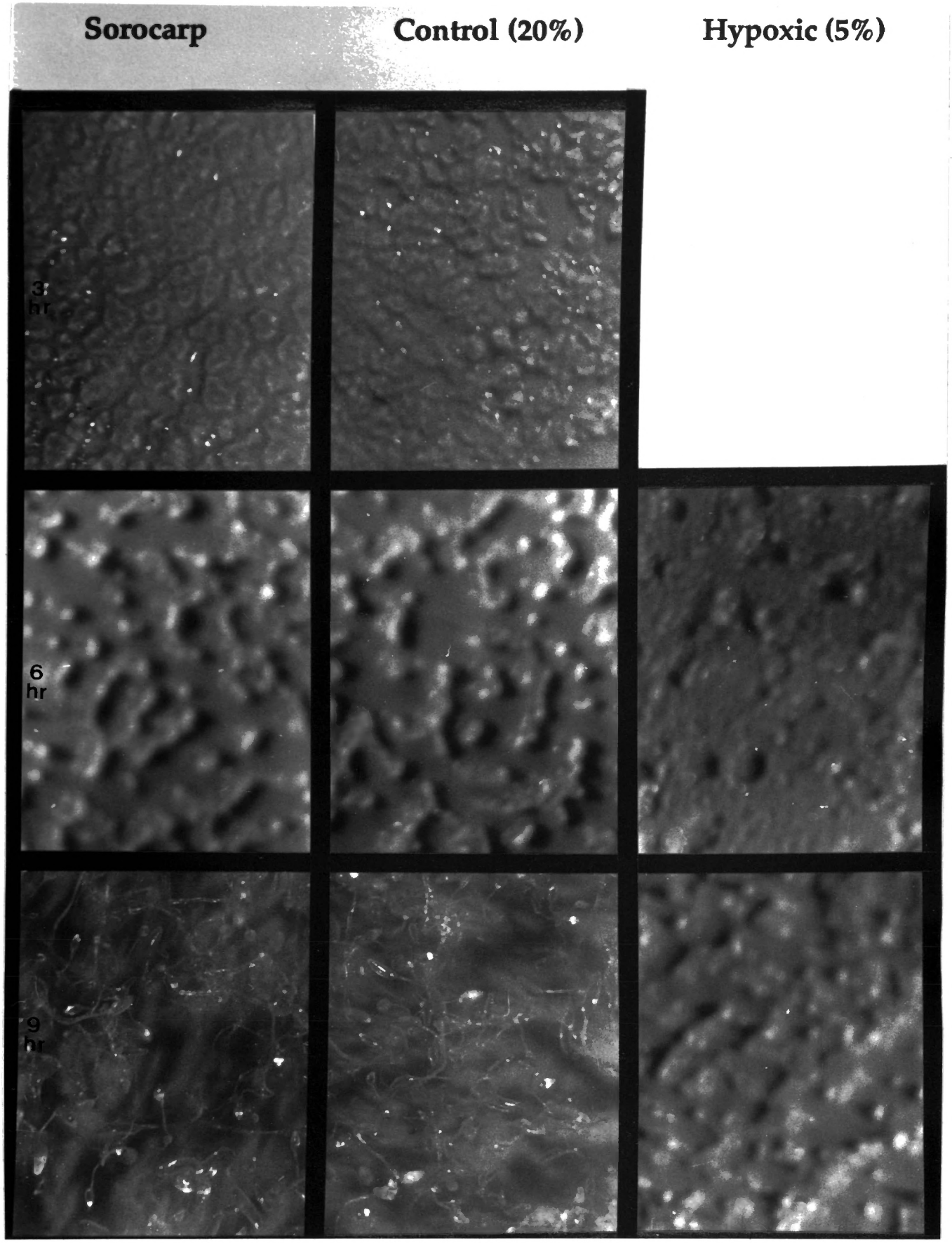
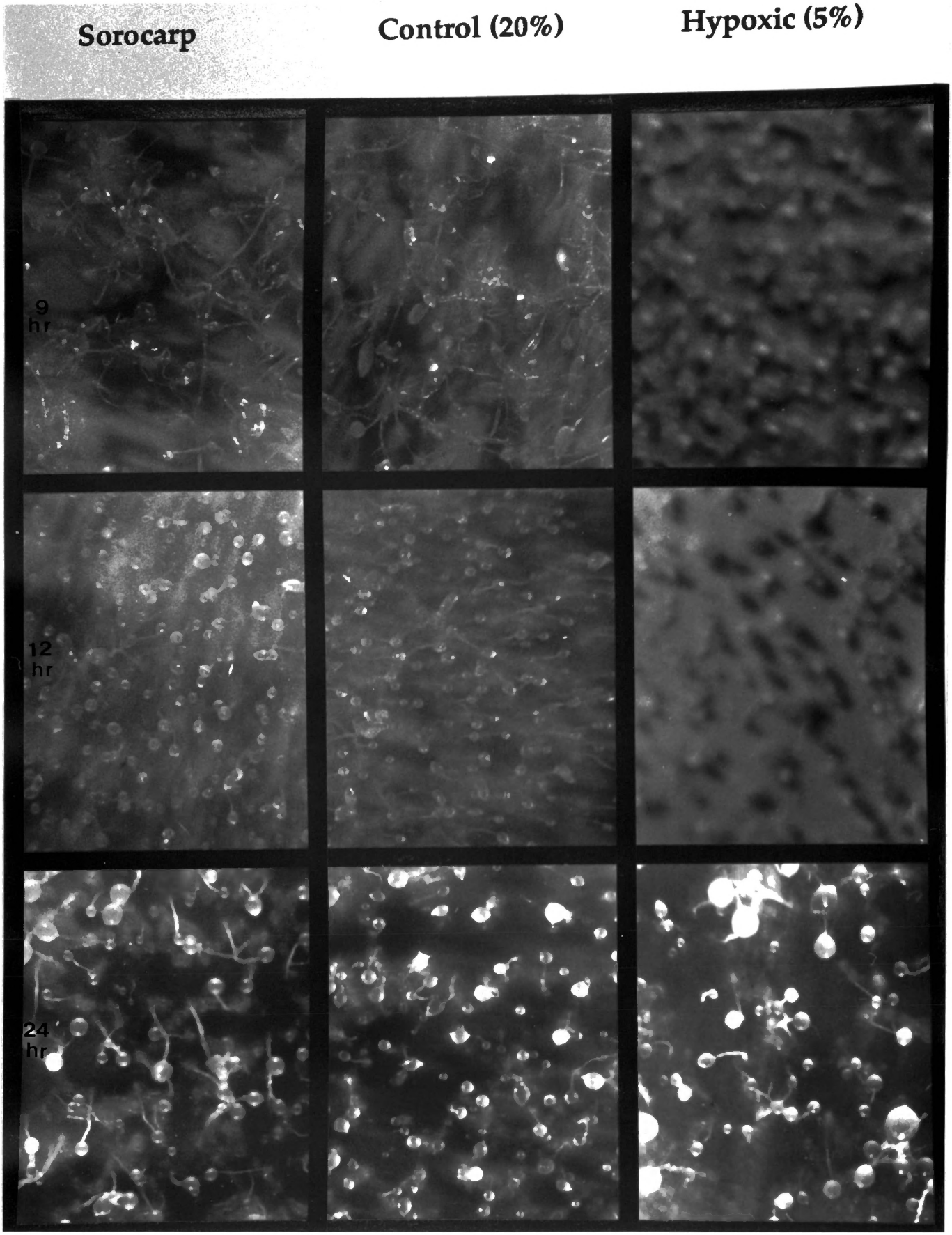

Sorocarp
Control (20%)
Hypoxic (5%)
3 hr
6 hr
9 hr

Figure 6.

Developmental stages of standard sorocarp cells, cells exposed to 20% oxygen and cells exposed to hypoxic conditions. Cells were placed on filters in small petri dishes for development. Standard sorocarp cells, cells exposed to 20% oxygen, and cells exposed to hypoxic conditions are shown 9, 12, and 24 hr into development.


Sorocarp
Control (20%)
Hypoxic (5%)
9 hr
12 hr
24 hr

Spore Analysis

Upon completion of development, spores formed by all three groups of cells were examined microscopically and tested for viability. Once development was complete, about 24 hours, wet mounts of spores were made and examined using a phase contrast microscope. Figure 7 shows photographs of spores from cells exposed to all three types of environmental conditions. Spores from hypoxic cells appeared to be more rounded than spores from the standard sorocarp cells or the 20% control cells.

Spores from all three groups of cells were then examined for viability by plating them with *E. coli* B/r on LP plates. Table 1 shows the results from one of these experiments. The percent viability for spores formed under hypoxic conditions was about half that seen for spores formed under normal oxygen concentrations.

Plasmid Analysis

The pSport plasmid containing the A-11 cDNA (pA11) was isolated to generate probes for Southern blot analysis and run off transcripts for primer extension. Isolation of pA11 was done according to the alkaline lysis method. Once isolated, confirmation of plasmid identity was made by digestion with a variety of restriction enzymes. Figure 8 illustrates restriction analysis of pA11. The A-11 cDNA is approximately 1.3 kb while the pSport vector is 4.1 kb. Enzymatic digestion confirmed the identity of the isolated plasmid as pA11. Excision of the A-11 cDNA insert was performed using MluI or KpnI and Hind III (Fig. 8). These digestions resulted in two bands, one migrating at 1.3 kb, representing the A-11 cDNA, and the other at 4.1 kb, corresponding to the vector. Digestion with EcoRI yielded two bands, a small band at

Figure 7.

Phase contrast photomicrographs of spore suspensions from all three groups of cells. Illustrated from top to bottom are spores from standard sorocarp cells, spores from cells exposed to 20% oxygen, and spores from cells exposed to hypoxic conditions.

Sorocarp Spores

Control Spores (20%)

Hypoxic Spores (5%)

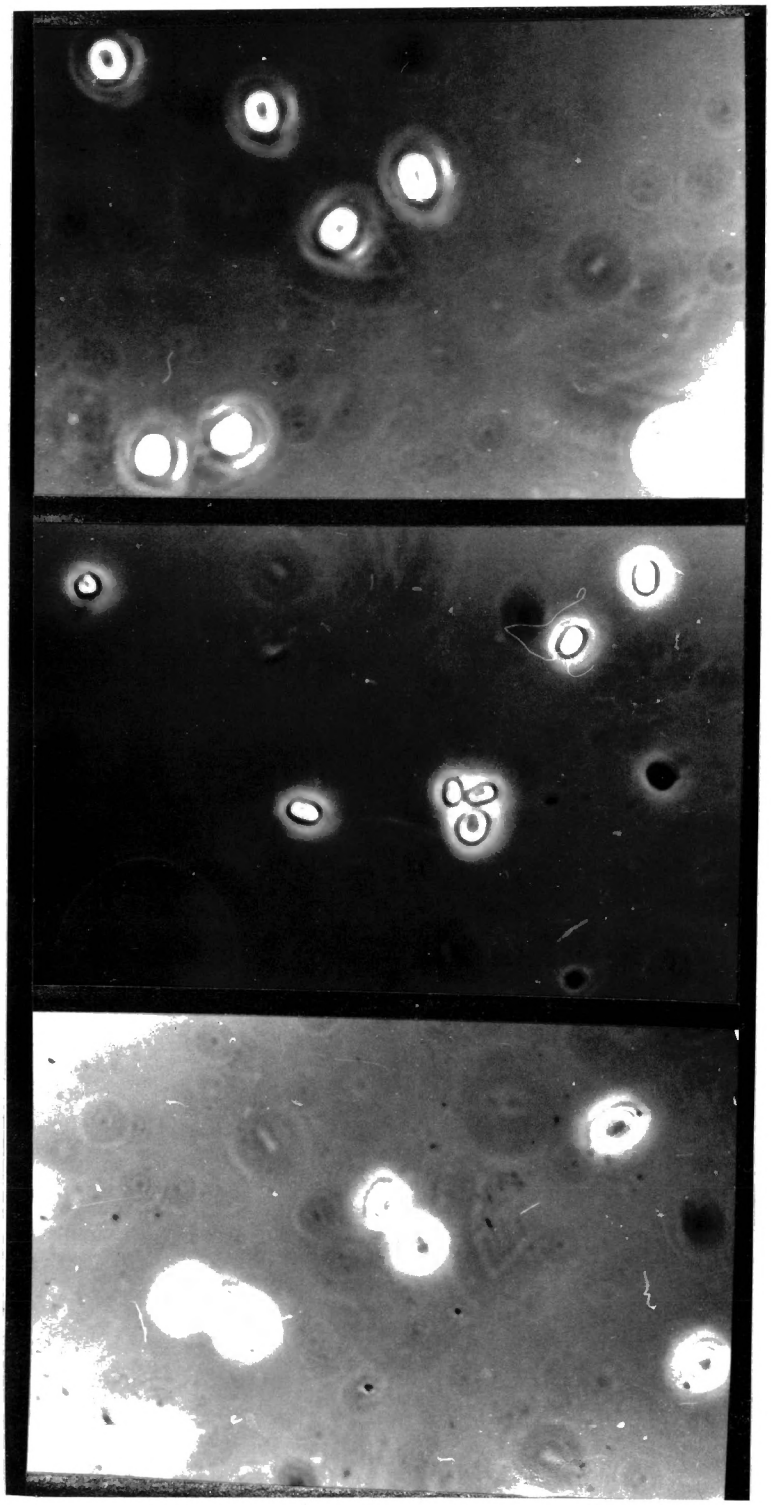


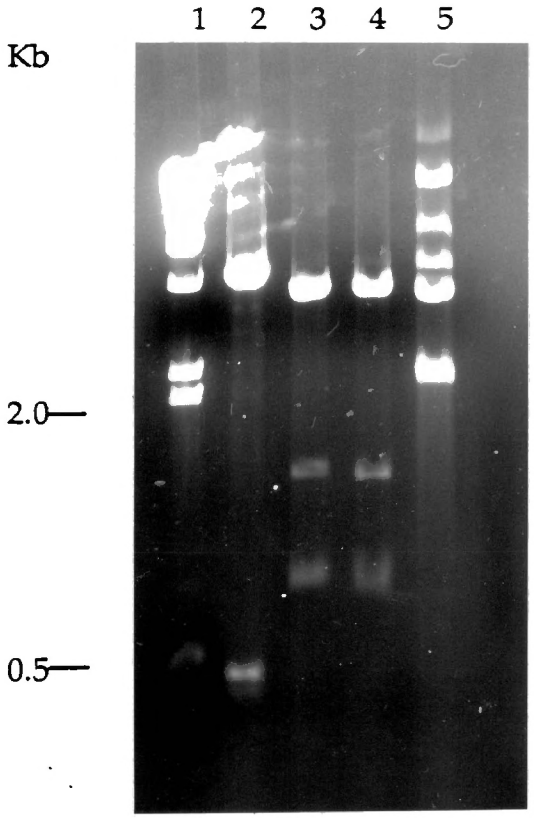
Table 1

Spore viability as determined by ability to germinate and produce a plaque in a lawn of bacteria after 72 hrs. of incubation at room temperature.

Spore Type	Percent Viable
Standard sorocarp	88%
20% Oxygen	79%
Hypoxic (5% O_2)	42%

Figure 8.

Electrophoretic analysis of restriction digests of pA11 examined on a 1% agarose gel. Lane 1, is Hind III cut lambda. Lane 2, pA11 digested with EcoRI. Lane 3, pA11 digested with KpnI & HindIII. Lane 4, pA11 digested with MluI. Lane 5, uncut pA11


1 2 3 4 5
Kb
2.0
0.5

approximately 430 bp and a larger band at 5 kb.

Genomic DNA Analysis

Genomic DNA was utilized to determine the gene copy number for A-11 in *D. mucoroides*. *D. mucoroides* genomic DNA was isolated essentially according to Richardson *et al.*, (1990). Isolated genomic DNA was digested to completion with selected restriction enzymes.

Southern Blot Analysis

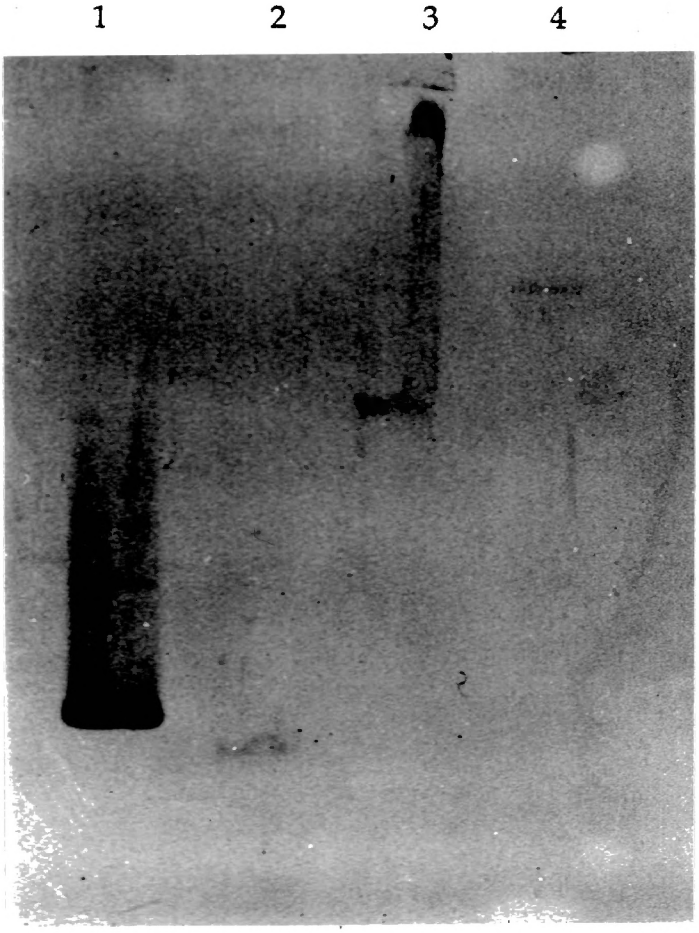
Restriction enzyme digested *D. mucoroides* genomic DNA was transferred to a membrane and then probed with labeled A-11 cDNA to determine the gene copy number for A-11. The results of these experiments are shown in Figure 9. Figure 9 is the Southern blot generated from *D. mucoroides* genomic DNA. Only one band was seen in each of the single digests. The double digest also only produced one band which was smaller than either band from the single digest. The band created by the double digest migrated close to the linearized A-11 cDNA, indicating a size of approximately 5.3 kb. Bands resulting from either of the single digests were much larger.

In Vitro Transcripts

In vitro transcripts were utilized as a positive control for primer extension reactions. *In vitro* transcripts were made by runoff transcription of the A-11 cDNA utilizing the T7 promoter. Hind III was used to linearize pA11. This produced a complete transcript of the cDNA insert starting at the T7 promoter and stopping at the Hind III restriction site. Lane 2 of Figure 10

Figure 9.

Southern blot analysis of *D. mucoroides* genomic DNA probed with A-11 cDNA. Lane 1, linearized pA11 plasmid. Lane 2, the double digest of *D. mucoroides* genomic DNA using EcoRI and PstI. Lane 3, *D. mucoroides* genomic DNA digested with PstI and lane 4, *D. mucoroides* genomic DNA digested with EcoRI.


1
2
3
4

showed two bands, the larger corresponding to the template DNA. After the addition of DNase only RNA transcripts remained (lane 3).

RNA Analysis

To assay for the native A-11 transcript, total RNA was extracted from developing cells exposed to different environmental conditions. Total RNA was isolated using a guanidinium thiocyanate extraction method. RNA samples were assessed for quantity and quality by spectophotometry and agarose gel electrophoresis. Figure 11 depicts results of an agarose gel electrophoresis of intact RNA. Intact RNA is shown here by the 2:1 ratio of intensity of the 28S to the 18S ribosomal RNA bands.

Primer Extension Analysis

To determine if the A-11 transcript was induced by hypoxic conditions, primer extension analysis was used. Using the same developmental system as previously described, total RNA from developing cells exposed to the different environmental conditions was isolated. The A-11 PE primer was then annealed to this total RNA. The A-11 PE primer anneals near the 5′ end of the transcript, 83 bases downstream of the 5′ end of the transcript. The full length labeled extended primer from the *in vivo* transcript appeared at 102 bases on an 8% polyacrylamide gel (Fig. 12). The labeled extended primer from *in vitro* transcription product appeared at 144 bp (Fig. 13), the difference can be accounted for by the 31 bp of the T7 promoter and 11 bp added by the linkers. No detectable bands were present in cells exposed to hypoxic conditions (Fig. 12 and Fig. 13).

To ensure that the above primer extension reaction was sensitive enough to detect the A-11 transcript if it was present in small amounts, a titration experiment was performed using the *in vitro* transcripts (Fig 14). Each lane represents a one half reduction in the amount of RNA transcript added. Transcription products are detectable down to 0.5 pmol of RNA transcript.

To confirm that A-11 was a developmentally regulated gene, primer extension was performed on 48 hr and 56 hr vegetative samples (Fig. 15). RNA from 56 hr vegetative cells in lane 1 produced a band at 102 bases, corresponding to the full length *in vivo* transcription product. Lane 2 represents RNA from 48 hr vegetative sample showing no band at 102 bp.

Figure 10.

Electrophoretic analysis of T7 runoff transcripts. Lane 1, Hind III cut lambda DNA. Lane 3 is the reaction after transcription before the addition of DNase. Lane 5 is the final product after DNase treatment, phenol-chloroform extraction and ethanol precipitation.

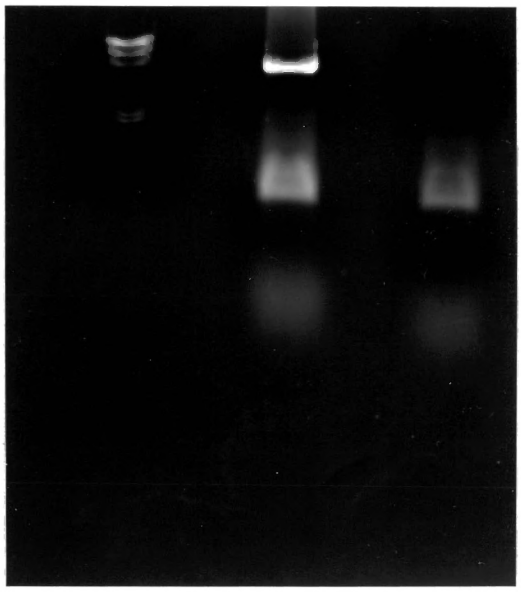

1
2
3

Figure 11.

Electrophoretic analysis of total RNA. Lane 1, RNA isolated 2 hr into development from cells exposed to 5% oxygen conditions. Lanes 2 & 3, RNA isolated at 4 hr into development from cells exposed to 5% oxygen conditions. Lane 4, RNA isolated at 6 hr into development isolated from cells exposed to 5% oxygen conditions. Lane 6, RNA isolated at 8 hr into development from cells exposed to 5% oxygen conditions, and lane 7, vegetative RNA isolated at 56 hr of vegetative growth.

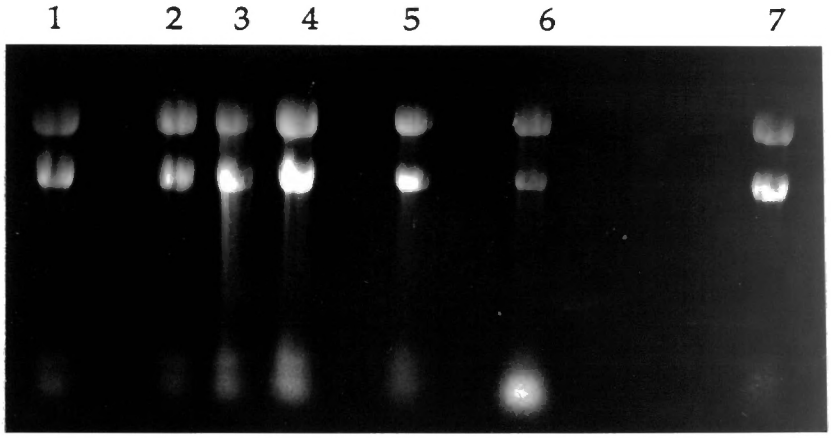

1
2
3
4
5
6
7

Figure 12.

Primer extension analysis of RNA extracted from cells exposed to different environmental conditions. Lanes 1 through 4 are sequence derived from a 1.0 kb cloned fragment (kindly provided by Kelley Colvin) using the m13 forward primer, loaded G, A, T and C respectively. Lane 5, RNA isolated from 9 hr macrocyst cells. Lane 6, RNA isolated from 9 hr sorocarp cells. Lane 7, RNA isolated from 6 hr macrocyst cells. Lanes 8 through 11 contain RNA isolated from cells exposed to hypoxic conditions at 3, 6, 9, and 12 hr respectively. Lane 12, RNA extracted from 12 hr sorocarp cells. Lane 13, RNA isolated from 12 hr cells exposed to 20% oxygen.

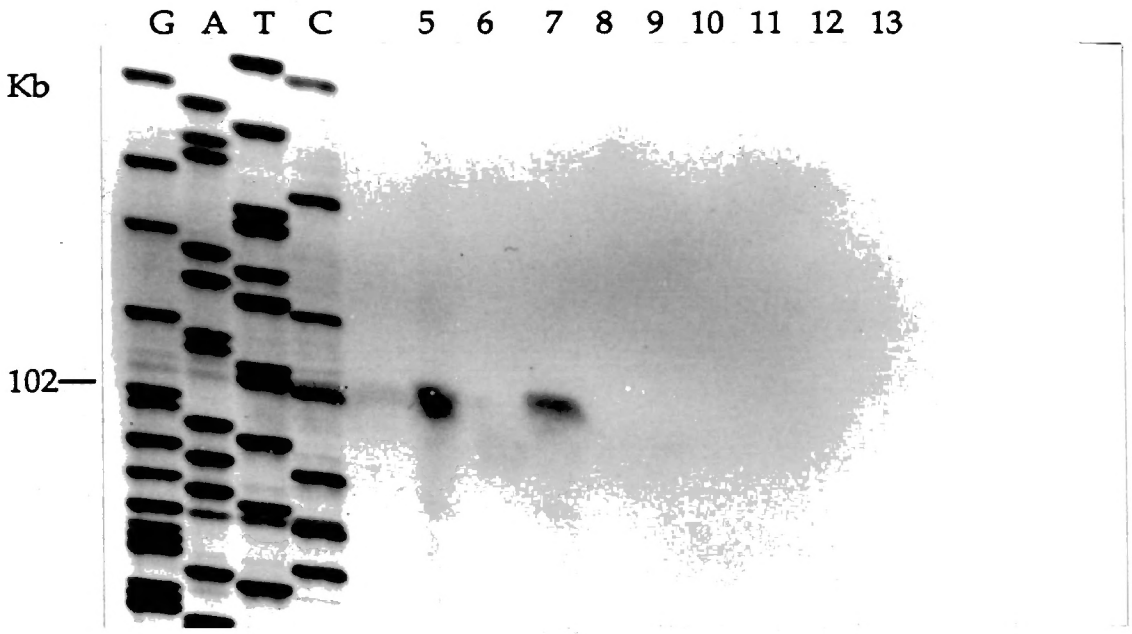

G A T C
5 6 7 8 9 10 11 12 13
Kb
102—

Figure 13.

Primer extension analysis of RNA extracted from cells exposed to different environmental conditions, run independently from the previous primer extension procedure. Lanes 1 through 4 is sequence derived from Kelley Colvins 1.4 kb insert using the forward m13 primer. Lanes 1 through 4 were loaded G, A, T, and C respectively. Lane 5, RNA isolated from 9 hr macrocyst cells. Lane 6, RNA synthesized by *in vitro* runoff transcripts. Lanes 7 through 9 contains RNA extracted from 6, 9, and 12 hr cells that were exposed to hypoxic conditions. Lane 10, RNA isolated from 12 hr cells exposed to 20% oxygen. Lane 11, RNA extracted from 12 hr sorocarp cells.

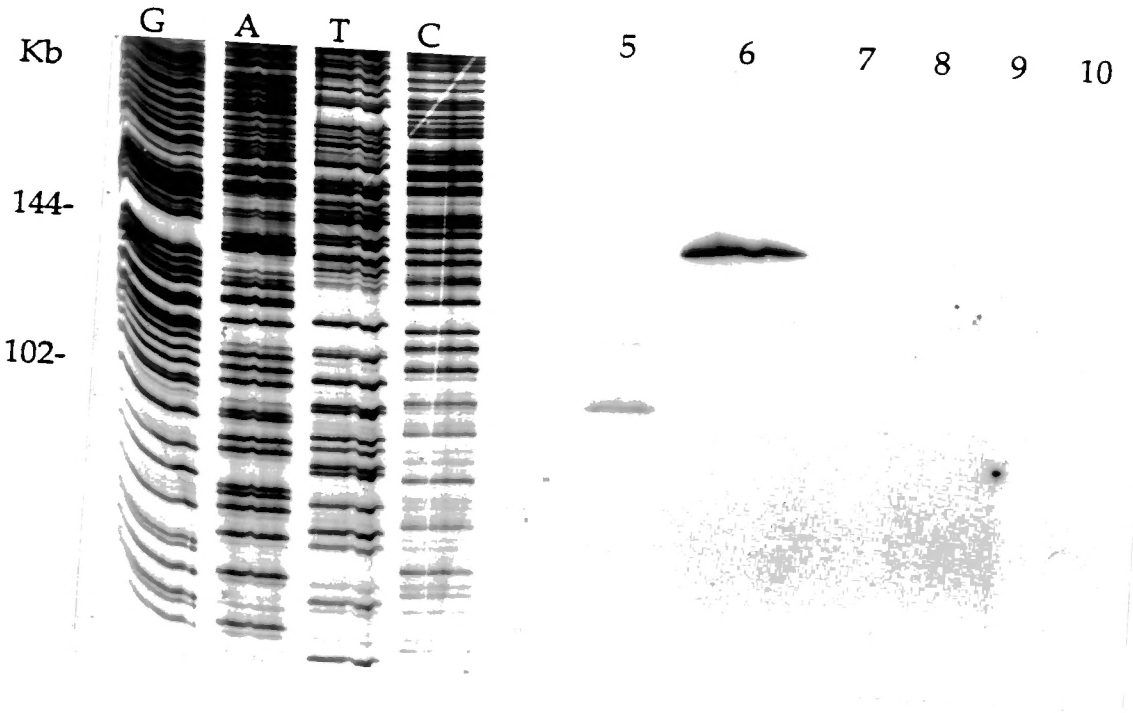

G
A
T
C
Kb
144-
102-
5
6
7
8
9
10

Figure 14.

Primer extension titration analysis utilizing *in vitro* transcripts. All lanes contained the same amount of; labled primer, 2 pmol, reverse transcriptase, 10 units, and nucleotides, 0.25 mM. Lane 1, contains 4 pmol of T7 transcript. Lane 2, contains 2 pmol of T7 transcript. Lane 3, contains 1 pmol of T7 transcript. Lane 4 contains 0.5 pmol of T7 transcript. Lane 5, contains 0.25 pmol of T7 transcript.

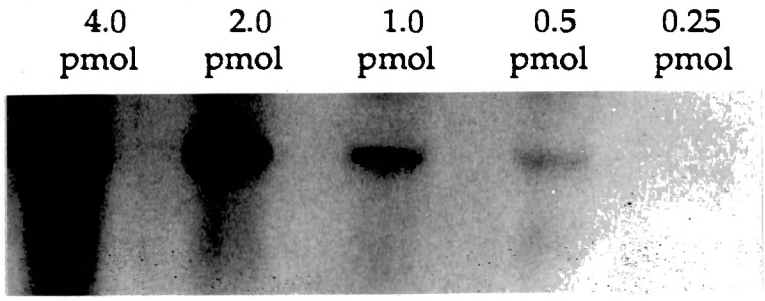
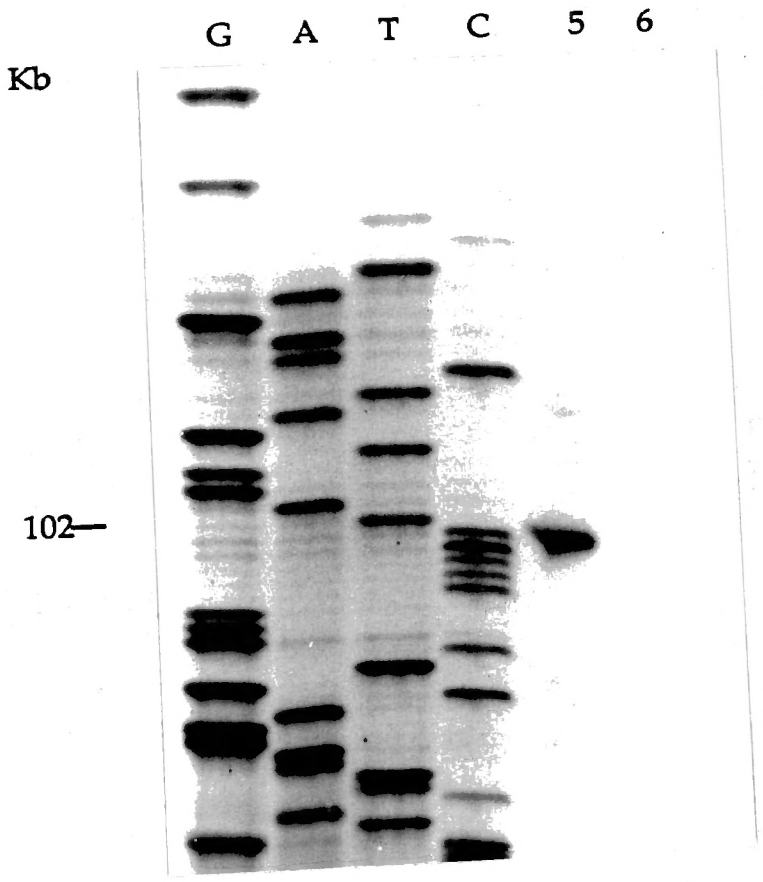

4.0 pmol
2.0 pmol
1.0 pmol
0.5 pmol
0.25 pmol

Figure 15.

Primer extension analysis of vegetative samples. Lanes 1 through 4 contain sequence from Kelley Colvin's 1.0 kb fragment, derived by the M13 reverse primer. Lanes 1 through 4 contain G, A, T, and C respectively. Lane 5 represents RNA extracted from 56 hr vegetative cells, in which clumping cells were visable. Lane 6 corresponds to RNA isolated from 48 hr vegetaive cells, in which no clumping had occurred.


G
A
T
C
5
6
Kb
102

Discussion

A cDNA library of *D. mucoroides* strain Dm-7 was constructed by A. T. Weber using eight hour transcripts in macrocyst development. Using Northern blot analysis to screen this cDNA library, Marilyn Larson found the cDNA clone A-11 to hybridize to RNA from vegetative amoebae and RNA from macrocysts, but not to RNA from sorocarps. The nucleotide sequence was obtained by Kevin Brewer, who also derived the amino acid sequence and used that sequence to search the Genbank data base. The protein found with the highest degree of homology to the A-11 product was a bacterial hemoglobin from *Vitreoscilla*. It has been shown that under low oxygen conditions this hemoglobin in *Vitreoscilla* can function as a terminal cytochrome oxidase when the gene is transformed into *E. coli* mutants (Dikshit, *et al.*, 1990). An increase in concentration of *Vitreoscilla* hemoglobin mRNA concentration occurs when cells are exposed to hypoxic conditions.

The A-11 gene product of *D. mucoroides* may have a function similar to the *Vitreoscilla* hemoglobin. In laboratory conditions macrocyst formation is stimulated by submersion under a filter and buffer. Vegetative amoebae are grown in association with bacteria as a nutrient source, in flasks containing 40 mL of medium. This medium might be low in oxygen as both amoebae and bacteria are respiring. Sorocarp formation occurs on the surface of filters where oxygen is abundant. Considering the above, the A-11 gene might not be a developmentally regulated gene but a gene regulated by oxygen concentration. If the product of the A-11 transcript serves a function in vegetative cells and macrocysts similar to the hemoglobin in *Vitreoscilla*,

cells forming sorocarps cells might also require this product if faced with hypoxic conditions.

The first parameter in determining if the A-11 transcript was regulated by hypoxic conditions was to determine if a low oxygen environment affected development. It was reported that *Dictyostelium discoideum* cells were able to tolerate 5% oxygen, while oxygen concentrations falling below this level dramatically affected growth (Schiavo and Bission, 1989). Therefore, sorocarp cells were allowed to develop in an environment containing 5% oxygen and examined for developmental defects (Fig. 5 and Fig. 6). Examination of cells exposed to all three environmental conditions revealed that cells exposed to hypoxic conditions lagged behind the development of normal sorocarp cells and cells exposed to 20% oxygen. Control cells which were placed into a desiccator and allowed to develop with atmospheric air showed no difference in timing of development from the normal sorocarp cells, which were allowed to develop on the laboratory bench. Therefore the cells exposed to 20% oxygen served as a good control for the remaining experiments.

Development of cells exposed to hypoxic conditions seemed to be retarded by approximately three hours. Cells exposed to hypoxic conditions did not start aggregation until six hours of development, however, control cells began to aggregate at only three hours of development (Fig. 5). After 24 hours of development, cells exposed to all three environmental conditions developed into normal sorocarps, indicating hypoxic conditions did not terminate development.

Cells exposed to hypoxic conditions as well as control cells exposed to 20% oxygen appeared to have shorter stalks than normal sorocarps. Since this abnormality is seen in the control cells (20% cells) it could be explained by the

dry environment that flushing the system might have created. The sheath that envelops the actively migrating pseudoplasmodium in *D. discoideum* is essentially fluid in character containing a substantial amount of cellulose (Loomis, 1975, and Raper 1984). Exposure of the pseudoplasmodium to just a few minutes of dry air results in a tougher sheath. With prolonged exposure to dry air, the sheath becomes progressively tougher. The sheath becomes so tough that if a thin glass rod is inserted through it, the sheath will collapse causing it to be completely destroyed. This does not occur when the sheath is exposed to air; a thin glass rod can be passed through the sheath without causing any damage (Raper, 1984). The sheath has been proposed to provide traction for amoeboid motion by the cells inside (Inouye and Takeuchi, 1979., Shaffer, 1962 and Loomis, 1982). The young sorogen, a structure in the process of developing into a fruiting body, of *D. mucoroides* is structurally comparable to the migrating pseudoplasmodium of *D. discoideum*, except for the presence of the developing sorus (Raper, 1984). It is possible that repeated flushing of air through the developmental system created a dry environment inside the desiccator. This dryness could have affected the sheath around the stalk of the developing sorocarp causing it to become tough and even brittle. This might affect the stalk height by not allowing the movement of cells as easily through the tube, thus creating a shorter stalk.

Once it was determined that normal sorocarps did develop under low oxygen conditions an analysis of the spores from cells exposed to hypoxic conditions was performed. In comparison with the control cells, spores from hypoxic cells looked morphologically normal under a phase contrast microscope (Fig. 7). However, hypoxic cells appeared to produce spores that

were slightly rounder than those produced by the control cells. Viability tests showed that spores from hypoxic cells were significantly less viable than spores from either of the control cells. Spores obtained from the cells exposed to the 20% oxygen were slightly less viable than spores from the normal sorocarp control. This difference in viability might again be attributed to the dry environment of the developmental system.

Exposure to dry air may slightly affect the spore by altering the cellulose structure within the spore wall, decreasing spore viability and resistance. This might explain why spores exposed to the developmental system designed in this experiment are slightly less viable than normal sorocarp spores. The normal spore wall is a multilayered structure containing both protein and cellulose. It appears that spore formation occurs in part from prespore vesicles fusing with the plasma membrane of spore cells and contributing their contents to the outer wall (Loomis, 1975). Several mutant strains have been found in *D. discoideum* which illustrate that fruiting body formation does not ensure the ability of prespore cells to undergo normal encapsulation. Spores from these mutant strains are less viable and less resistant than wild type spores. (Loomis, 1975., Katz and Sussman, 1972).

The significant difference in spore viability observed in spores from hypoxic cells must be attributed to hypoxic conditions, since control cells did not show such a dramatic loss in viability. Hypoxic conditions may affect spore viability directly or indirectly through a process such as modifying encapsulation. An examination of spore ultrastructure might reveal greater information of the effects hypoxia has on encapsulation.

Before initiating studies of A-11 regulation, the copy number of the A-11 gene was determined. If multiple copies of the A-11 gene existed in the

genomic DNA of *D. mucoroides* then synthesis of the transcripts could be regulated differently. If one transcript were expressed for a particular environmental condition, such as hypoxia, but another transcript were produced for a different condition, perhaps macrocyst development, it would be difficult, by monitoring the concentration of A-11 transcript to determine what condition was responsible for A-11 regulation. To clarify this question Southern blot analysis was used. The Southern blot generated from *D. mucoroides* (Fig. 9) showed one band, indicating only one copy of A-11 in the genome of *D. mucoroides*. Since a control of the plasmid containing the A-11 cDNA (pA11) was also probed on this membrane, some information could be generated about the size of these bands on the Southern blot. The band generated from the double digest runs slightly faster than the pA11 plasmid. Therefore, since the pA11 plasmid has a known size of 5.3 kilobases, the band from the double digest is slightly smaller than 5.3 kilobases. The size of the DNA fragments in the other bands were much larger in size than the pA11 plasmid. About 25 to 30% of *Dictyostelium's* genome has been characterized, through DNA reannealing reactions, to be repetitive sequences. A substantial portion of these repetitive sequences, approximately 70%, have been shown to encode ribosomal RNA. The single copy portion of *Dictyostelium's* DNA makes up between 70 to 75% of the genome (Loomis, 1975, 1982). Relatively few genes, the actin genes being an example, are maintained in multiple copies (Loomis, 1982).

To determine if the A-11 transcript was regulated by oxygen concentration, primer extension analysis was used. If the A-11 transcript were present in sorocarp cells exposed to hypoxic conditions then the primer would bind to the complementary sequence at the 5′ end of the RNA

transcript. Upon extension of the annealed primer a band of 102 bases would be generated. This is 18 nucleotides longer than the A-11 cDNA. Weilie Ma had determined that the A-11 cDNA was short 18 nucleotides (Ma, 1994). At 102 bases no bands were detected in any of the samples except the lane containing macrocyst RNA, which served as a positive control. RNA samples were taken from cells exposed to hypoxic conditions up to twelve hours of sorocarp development and the transcript could not be found at any time (Fig. 12)

Before ruling out oxygen regulation, the sensitivity of primer extension analysis was examined. Sensitivity was tested by creating *in vitro* transcripts and diluting them to determine the minimal amount of RNA necessary for detection. The *in vitro* transcripts yielded a band at 144 bases. This length is due to the addition of the plasmid sequence caused by the linearization of the plasmid and the addition of sequence by the polylinkers during cloning, as explained in the results. A titration experiment showed that transcript could be detected when only 0.5 pmol of *in vitro* transcript was present (Fig. 14). Therefore, the A-11 transcript did not appear to be regulated by oxygen. If oxygen did regulate this transcript then low oxygen concentrations would stimulate transcription of this gene, causing a detectable level of transcript to be present.

If the A-11 transcript was not regulated by oxygen then perhaps it was under developmental control. However, A-11 was reported to be present in both macrocysts and vegetative cells (Larson, 1991). After 48 to 56 hours of growth, vegetative amoebae began to aggregate together, forming clumps which resembled the beginnings of macrocyst formation. These cells have already chosen the developmental pathway of macrocyst formation, thus it

can be assumed early genes required for macrocyst formation are being expressed. Vegetative cells isolated after 48 hour of growth might be considered zero hour macrocysts and thus contain transcripts required for macrocyst formation. Only cells isolated before clumping should be considered as vegetative.

To confirm that the A-11 gene was a developmental gene, required in macrocyst formation, primer extension analysis was performed on 48 and 56 hour vegetative amoebae (Fig. 15). These results showed one band at 102 bases in the RNA sample from the 56 hour amoebae, indicating the A-11 transcript was present. No band was detected in samples of RNA from the 48 hour vegetative amoebae. This experiment confirms A-11 as a developmental gene present only during macrocyst formation.

What kind of function does a developmental gene, containing homology with the *Vitreoscilla* hemoglobin, have in macrocyst cells? Macrocyst formation occurs when amoebae aggregate together forming a large mass of cells. Conditions inside this mass are likely to be low in oxygen. If such conditions existed, amoebae within the middle regions of the cyst would be under hypoxic conditions. Considering this, it would be advantageous for amoebae to encode a protein which would help facilitate oxygen diffusion. If the function of the A-11 product serves a function similar to the *Vitreoscilla* hemoglobin, it may be essential for viability of amoebae within the forming macrocyst.

Recently, it has been reported that hypoxia can induce apoptosis in oncogenically transformed cells. More importantly, it has also been shown that hypoxia selects for cells which are mutated in the tumor suppressor gene, p53. Therefore, hypoxia provides a physiological selective pressure in tumor

cells for the growth of cells that have lost their apoptotic potential through p53 mutations. Since hypoxia is a commonly found physiological stress in developing tumor cells, this may help explain why p53 is one of the most commonly found mutated genes in tumors (Graeber, *et al.*, 1996).

Macrocysts may contain hypoxic regions, similar to regions in masses of tumor cells. If tumor cells had the ability to stimulate expression of a gene which helped oxygen diffusion, then hypoxic conditions might not be as prevalent. If hypoxic conditions were relieved in developing tumor cells, mutations in p53 might not be selected. Therefore, active p53 would be expressed leading to cell apoptosis and tumor growth would be inhibited.

Future research on the *D. mucoroides* developmental gene A-11 should include locating the promoter element controlling this gene and then assaying for transacting factors which may regulate this transcript. Another interesting experiment which could be performed would be to clone in a reporter gene and assay for activity *in situ*. Other possible future experiments could include isolating the protein corresponding to this transcript, and gene knockout procedures.

In summary hypoxic conditions proved to retard development and affect spore viability, however, low oxygen did not appear to be the signal regulating the transcription of A-11. A-11 was determined to be developmentally regulated and specific to macrocyst development.

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