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# Determination of the transcriptional start site and in vitro translation of the A-11 mRNA in Dictyostelium mucoroides.

Weilie Ma

# DETERMINATION OF THE TRANSCRIPTIONAL START SITE AND *IN VITRO* TRANSLATION OF THE A-11 mRNA IN *DICTYOSTELIUM MUCOROIDES*

A Thesis

Presented to the Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

by

Weilie Ma

August, 1994




# THESIS ACCEPTANCE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree Master of Arts, University of Nebraska at Omaha.

Committee

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A. F. Chase 2nd
Chair

Aug 15, 1994
Date

# ABSTRACT

Previous studies in *Dictyostelium mucoroides* have sought to identify genes specific for macrocyst development. A gene named A-11, previously isolated as a cDNA, appears to be one of these genes. The cDNA of A-11 was made from macrocyst (8h) mRNA, and Northern analysis did not detect any A-11 transcript during sorocarp formation. The cDNA sequence for A-11 has been obtained.

To determine if the cDNA of A-11 is complete, primer extension and RNA sequencing were performed using an end-labeled oligonucleotide complementary to positions 1221 to 1239 of A-11 cDNA. The two longest extension products were 102 bases and 101 bases. As there were only 84 bases from the 19-mer oligonucleotide primer to the 5'-end of A-11 cDNA, this indicated that the A-11 cDNA was not a complete reverse transcript of A-11 mRNA. It was short of full length by 18 nucleotides. RNA sequencing with dideoxynucleoside triphosphates showed the nucleotides missing from the cDNA were 5'NNTNNAACAAATAAATAA3'.

There are several AUG potential translation start sites in the 5'-end of A-11. To determine which AUG was the initiation codon, hybrid-selection of A-11 mRNA from macrocyst (4h) total mRNA and *in vitro* translation of A-11 mRNA were performed. The resulting translation product had a molecular weight of 25 kDa which approximated the size of the predicted protein initiated at the first AUG codon of A-11 mRNA.

# ACKNOWLEDGEMENTS

I'm extremely grateful to my major professor, Dr. Tom Weber. He's the best educator and adviser anyone could have hoped for. Because of his invaluable academic and technical advice, patient guidance, inexhaustible enthusiasm, and great sense of humor, this project was the most fruitful, challenging, and fun learning experience so far.

Special thanks go to my committee members, Dr. Bruce Chase and Dr. Stanley Cox, who gave generously of their time for invaluable consulting, and kindly provided access to their equipments.

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Thanks are also extended to my lab colleagues, William Arnold, Kevin Brewer, Marg Esser, and Mary Kosinski, for their brother and sister's type of generous assistance and enjoyable friendship.

Finally, I thank my parents and sister in China, for their unfailing spiritual support and love.

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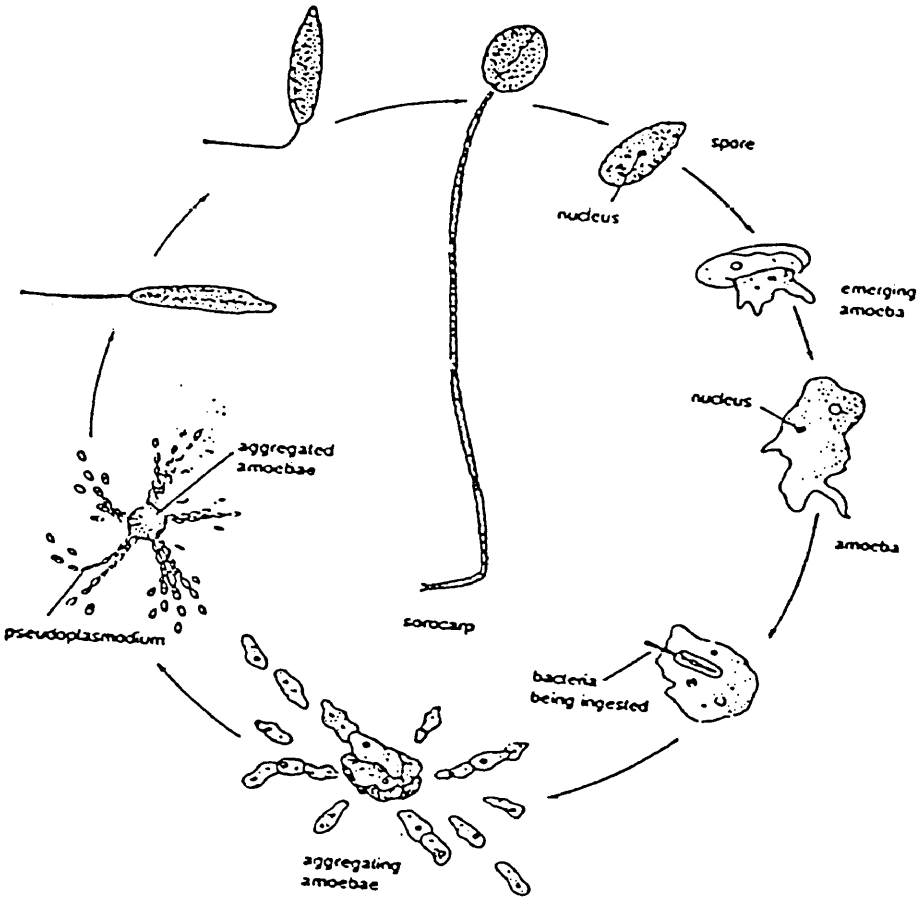
# INTRODUCTION

The cellular slime mold *Dictyostelium mucoroides*, a eukaryotic organism, provides an excellent model system with which to examine developmental processes. Unlike most other eukaryotic organisms, growth and development are two separate phases in *Dictyostelium*. Thus, developmental processes can be studied in the absence of cell division. In addition, the brevity and simplicity of the slime mold's life cycle also makes it experimentally useful. The time from initiation of multicellular development to completion of morphogenesis is about 12 hours. In a nutritive environment, *D. mucoroides* grows as separate, single-celled, haploid amoebae, doubling about every 3 hours. Bacteria, such as *E. coli*, serve as a food source. This stage of the life cycle is called the vegetative stage. Upon starvation, the individual amoebae aggregate together and develop as multicellular entities through either an asexual cycle or a sexual cycle (Fig. 1).

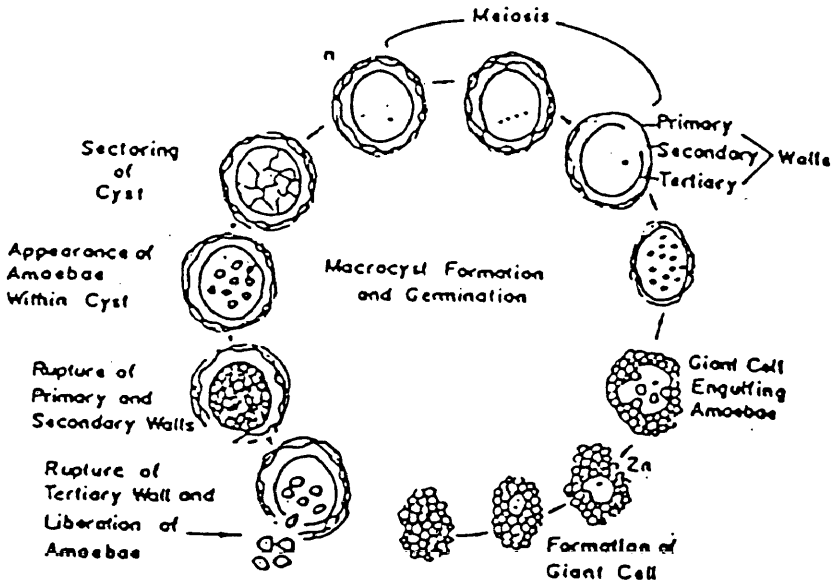
The choice of developmental pathway is influenced by environmental conditions and this can be exploited in the laboratory to direct development. Sorocarp formation, the asexual pathway, is favored when the starved amoebae are exposed to light, low humidity, low calcium, and high phosphate concentrations (Sussman, 1966); while the sexual pathway, macrocyst formation, is induced by darkness, high humidity, high population density, high calcium, and low phosphate concentrations (Nickerson and Raper, 1973). The ease of directing the choice of developmental pathway for this organism is a factor in making *D. mucoroides* a favorable experimental model to study the development of eukaryotes.

Fig. 1. Life cycle of *D. mucoroides*. (A) The asexual cycle - sorocarp formation (Kendrick, 1992). (B) The sexual cycle - macrocyst formation (Jacobson and Lodish, 1975).

A


spore
nucleus
emerging amoeba
nucleus
amoeba
bacteria being ingested
aggregating amoebae
aggregated amoebae
pseudoplasmodium
sorocarp

B


Meiosis
n
Primary
Secondary
Tertiary
Walls
Sectoring of Cyst
Appearance of Amoebae Within Cyst
Macrocyst Formation and Germination
Rupture of Primary and Secondary Walls
Giant Cell Engulfing Amoebae
Rupture of Tertiary Wall and Liberation of Amoebae
2n
Formation of Giant Cell

In the asexual cycle, the amoebae are induced to aggregate together by pulsatile signaling of cyclic AMP. The aggregation center is actually a single cell that has started sending out the distress signal, in the form of cAMP. A cell perceiving a sudden rise in cAMP does two things. First, it moves toward the source of the cAMP for about 60 seconds. In addition, it secretes more cAMP and entrains cells farther from the center of the aggregation territory. The process creates concentric waves of cells migrating in toward the aggregation center. As cells start to come closer together, they acquire surface molecules that allow them stick to one another and form lines of cells (Kessin and Campagne, 1992). The aggregation center is at first rounded but later forms a small tip and rises up as an elongate cylinder. The cylinder of cells, called a slug, soon begins to migrate in a horizontal orientation along the substratum. This migrating slug contains two zones of cells (Raper, 1940). The anterior of the slug contains cells predestined to become stalk cells (prestalk cells), and the posterior of the slug contains prespore cells. *D. mucoroides* "migrates on a stalk," that is formation of the stalk, or sorophore, begins at the site of aggregation and the stalk is produced continuously as the slug migrates across the substrate. At the beginning of culmination, the elongation of the stalk cells lifts the prespore mass from the subatrate and gives the slime mold fruiting body its final appearance. Asexual development is completed with the formation of this fruiting body, called a sorocarp. The fruiting body has two major anatomical features: a cellular stalk and a sorus, which consists of a mass of spores on top of the stalk (Kessin, 1992). Under favorable conditions, one spore is sufficient to start a new vegetative cycle.

In the sexual cycle, zygotes are founded in macrocysts which are formed to resist harsh environmental conditions. These zygotes are formed by the fusion of two haploid amoebae. The zygotes form in the center of amoeba aggregates and become "giant cells". The giant cell then phagocytizes the other cells in the aggregate so that it becomes the only cell within the cyst. A triple layered wall surrounds this cell in the mature cyst. The nucleus in this cell subsequently undergoes a series of divisions, the first of which is meiotic. After additional mitotic divisions, a macrocyst forms which is made up of many cells derived from the original zygote nucleus (Blaskovicks and Raper, 1957; Filosa, 1972; O'Day, 1981). After a period of dormancy, the macrocysts can germinate to liberate hundreds of haploid amoebae (Erdos et al. , 1973; Nickerson and Raper, 1973).

As described above, there are three properties of this organism which make it experimentally useful: (1) *D. mucoroides* has two different developmental cycles, asexual and sexual. The ease of directing the choice of developmental pathway makes the investigation of the differences between two development processes possible. (2) During asexual development, the haploid amoebae differentiate to only two different cell types, stalk or spore. With few cell types to keep track of, we can focus our attention on the biochemical and genetic changes that take places as cells move from one developmental stage to the next. (3) The sexual pathway of *D. mucoroides* is homothallic (Clark et al. , 1973). Therfore, the macrocyst formation can be examined with fewer complications than heterothallic species, such as *D. discoideum* and *D. giganteum* (Urushihara, 1992).

At the molecular level, different cell types typically result from differential gene expression. The fundamental dogma of gene expression is that DNA produces RNA which in turn produces proteins. Therefore, the genetic information in the DNA is converted into an RNA copy, which is translated into protein. Then, the action of the protein produces the specific phenotype of the cell. Thus, an understanding of how gene expression is regulated is essential for understanding the development process. In eukaryotes, gene expression is regulated basically at two levels, transcriptional and post-transcriptional.

Transcriptional control involves the determination of whether or not a gene is to be transcribed. Transcriptional activation of eukaryotic genes during development or in response to extracellular signals depends on the regulated assembly of multiprotein complexes on enhancers/silencers and promoters. Enhancers are sequences of nucleotides that potentiate the transcriptional activity of physically linked genes. An enhancer may be distant from the gene it enhances. The enhancer effect is mediated through sequence-specific DNA-binding proteins (Lewin, 1994). These observations have led to the suggestions that once the DNA-binding protein attaches to the enhancer element, it causes the intervening nucleotides to loop out to bring the enhancer into physical contact with the promoter of the gene it enhances. This loop structure then facilitates the attachment of polymerase molecules to the promoter region of the transcribed gene (Tjian and Maniatis, 1994). The complex nature of these processes provides many possibilities for controlling gene expression at the transcriptional level.

The initial transcript must be modified before becoming a mature messenger RNA. It is first modified at its 5'-end, by the addition of a cap

structure containing a modified guanosine residue, and is cleaved near its 3'-end, followed by the addition of up to 200 adenylic acid residues. Subsequently, intervening sequences, or introns, which interrupt the protein coding sequence in both DNA and the primary transcripts are removed by RNA splicing. The functional messenger RNA produced after these processes must then be transported from the nucleus to the cytoplasm where it can be translated into protein. The post-transcriptional regulation could operate at any of these stages between gene transcription and the translation of the corresponding mRNA in the cytoplasm.

Many developmentally regulated genes have been found in *Dictyostelium*. The expression of uridine diphosphoglucose pyrophosphorylase (UDPGP) of *D. discoideum* is regulated by cell-cell contact and exogenous cAMP. Unaggregated single cells do not accumulate UDPGP mRNAs. An increase in UDPGP mRNA level was observed during late development. The UDPGP mutants abort the developmental cycle, suggesting that the enzyme is essential for the completion of development (Ragheb and Dottin, 1987). Prespore EB4 gene is expressed as mRNA only after the aggregation stage of *D. discoideum* differentiation and exclusively in prespore and spore cells (Barlis, et al. , 1985). In contrast, prestalk D11 gene is expressed as mRNA exclusively in prestalk cells (Barklis and Lodish, 1983).

Dr. A. T. Weber has made a cDNA library from *D. mucoroides* macrocysts (8hr). Marilynn Larson performed Northern hybridization analysis to screen this cDNA library. She used randomly selected cDNA as probes to hybridize with total RNA isolated from macrocysts or sorocarps. She found that a cDNA probe named A-11 hybridized only with an RNA from macrocysts but

not with RNA from sorocarps. This indicates that A-11 mRNA is expressed only in macrocysts but not in sorocarps (Fig. 2). Thus, this A-11 gene might be a developmental gene regulated at the transcriptional level.

This thesis provides the answer to two questions in the study of the A-11 gene. Since cDNA is made by reverse transcription of messenger RNA catalyzed by reverse transcriptase, the cloned cDNA might be an incomplete reverse transcript of the mRNA. So, the first question is: Is this A-11 cDNA a complete transcript? This can be answered by primer extension analysis. Primer extension can map the 5'-end of the messenger RNA.The comparison of the start site of the messenger RNA and the cDNA will tell if the cDNA is complete. The start site information of the A-11 messenger RNA can also help to locate the promoter region of the A-11 gene, thus aiding in the investigation of the transcriptional regulation of A-11 gene expression. Secondly, there are several AUG potential translation start sites in the A-11 cDNA sequence (Fig. 3). Which AUG is used for initiation of translation? The A-11 mRNA was selected from the total mRNA by membrane hybridization, and translated in a rabbit reticulocyte lysate. The size determination of the translation product should help to answer this question.

Fig. 2. Northern blot analysis of the mRNA corresponding to the A-11 cDNA insert. Total RNA was isolated from stationary phase amoebae and amoebae developing along the macrocyst or sorocarp pathway at the indicated times following initiation of development. Five μg of RNA was size fractioned on a denaturing agarose gel, blotted to nylon, and probed with an SP6 generated transcript (Larson, 1991).

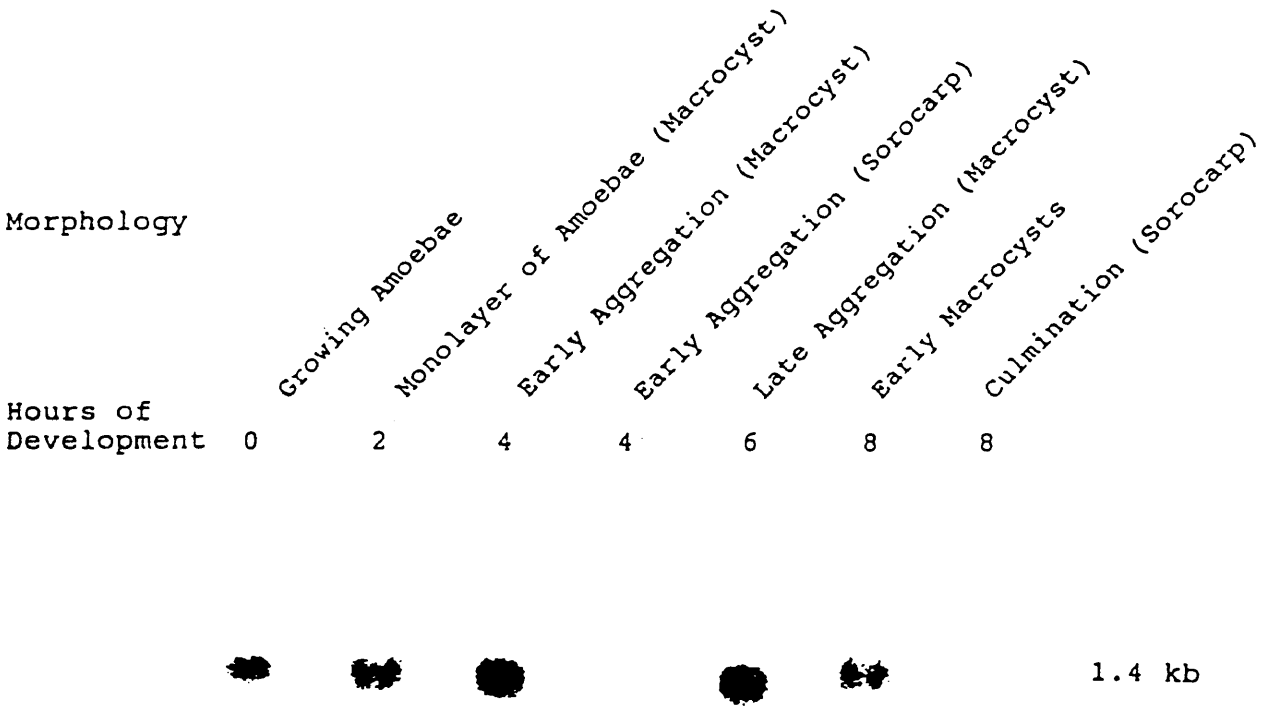

Morphology
Growing Amoebae
Monolayer of Amoebae (Macrocyst)
Early Aggregation (Macrocyst)
Early Aggregation (Sorocarp)
Late Aggregation (Macrocyst)
Early Macrocysts
Culmination (Sorocarp)
Hours of Development
0
2
4
4
6
8
8
1.4 kb

Fig. 3. The sequence of the A-11 cDNA. The nucleotides were numbered from 3' to 5'. The "N" refers to undiscernible nucleotides (Brewer, personal communication). Italicized bases indicate region to which the primer hybridizes.

```
1304 AAAACATATA AACATATAAA ATGTTAAGTC AAGAAAATAT TGATATTATT AAATCAACTG
1244 TACCAGTATT AGAAGTTCAT GGAGTTACAA TAACAAGCAC ATTTTATAAA AATATGTTTG
1184 AAGAAAATCC CCAATTATTA AATATTTTTA ACCATTCAAA TCAAAGACAA GGTAAACAAC
1124 AAACTGCATT AGCCAATACT GTATTAGCAG CTGCAGTTAA TATTGAAAAT TAAATGAATT
1064 AAATCTTGCA GGTATTGTTA ATAAACATGT TGCAACGTTT GGTGTATTAC CAGAACATTA
1004 TCCAATTGTT GGTAGAAATT ATTGGTGCAA TTAAACAAGT TTTAGGAGAA GCAGCAACTC
 944 CAGCAATTCT AAATGCATGG ACTGAGGCAT ATGGTATTAT TGCACAAGCA TTTATTGATG
 884 CAGAAGCTGC TTTATATAAA GTTACAGAAG AACAAATGGT GGTTGGAGAG ATAGTAGAGA
 824 ATTCATTGTC GAACAAAAGA TTGAAGAATC TTCAAATATT ACTTCATTTT ATTTTAAACC
 764 TGTTGATGGA AAAGCAATTG CGTCATATAT TCCAGGTCAA TATATAACAG TAAAAATTAC
 704 ATTACCATTA TCACCAGAAT CATTAGATGG AGCTACTGAT AAATGAATTA GAACTCATAT
 644 TAGACATTAC AGTTTATCAG ATGCACCTTC TGAAACTTAT TATCGTGTAC AGTAAAAGAG
 584 AAAATGCATT AAATACATCA GATCCAAATG GTGTAGTTTC AAATCATTTA CATAATAATG
 524 TTAAAGTTGG TGATATTGTA TTATTATCAC CACCAGCTGG TGATTTTGTA ATTGACAAAT
 464 CCAAATCAAA TCCAATTCTT TTGATTTCAG GTGGTGTTGG TATTACACCA TTATTCAGTA
 404 TGGTTAAAGA AACATTAGTT AAACAACCAA ATAGAGATAT TAGTTTTGTT CATTCTAGTA
 344 AATCTGAATC TGCTCAACCA TTTAAAAAAG AACTTAGAAC AATTAAAATC AAATAATAAT
 284 GTAAAATTAA ATATCATTCA CTCTGATTCT CATGGTCATA TTACTAAAGA ATCAATTACT
 224 TCATCACTAT TTGATGGCCA AGATATTAAA GATACTCAAG TCTTTATTTG TGGTCCAGTT
 164 TCTTTTATGT CAACAGNAAA CAAATTAGTT TTAGAATTAG GTTGCCNAAA ATCAAATATT
 104 TCTTATGAAG TTTTTGGTCC ATTAACTTCA GTTTAAACAA AAATATTAAT ACTAATTATA
  44 ATAAATTATT TAAATATATT TTAAACATTT AAAAAAAAAA AAAA
```

# MATERIALS AND METHODS

## ORGANISMS AND CULTURAL CONDITIONS

*D. mucoroides* strain Dm-7 was grown in association with *E. coli* B/r as a nutrient source. Stock cultures were maintained on 0.2% lactose-peptone agar plates (0.2% lactose, 0.2% peptone, 1.5% agar). To initiate vegetative growth, $1.0x10^5$ spores and 0.1 ml of a turbid suspension of *E. coli* B/r were inoculated into 250 ml flasks containing 40 ml glucose-yeast-peptone broth (GYP) (Weber and Raper, 1971). Flasks were incubated at 23°C in covered gyrotory shaking water baths. Amoebae in early stationary growth phase were harvested after 56 hr by centrifugation at 500xg for 10 min at 4°C. The amoebae were washed three times with Bonner's Salt Solution (BSS) (Bonner and Frascella, 1953) to remove bacteria, centrifuging as above.

To initiate macrocyst development, petri dishes (48x8.5mm) containing absorbent pads, filters (Gelman Supor450, 0.45μm) and 1.5 ml BSS with streptomycin (0.5mg/ml) were inoculated with 0.9 ml of a suspension containing $3.5x10^8$ amoebae/ml. A second filter was placed on top of each inoculated filter and an additional 1.5 ml BSS with streptomycin was added (Hanson and Weber, 1987). The plates were wrapped in aluminum foil to exclude light and were incubated at 23°C for 4 hr. Then, the cells were washed from the filters with 10 ml cold distilled deionized $H_2O$ (dd $H_2O$) and pelleted by centrifugation at 400xg for 15 min at room temperature.

To initiate sorocarp development, amoebae were washed an additional time with 0.025 M phosphate buffer (pH6.5). Petri dishes containing absorbent pads, filters and 1.5 ml 0.025 M phosphate buffer (pH6.5) with streptomycin (0.5

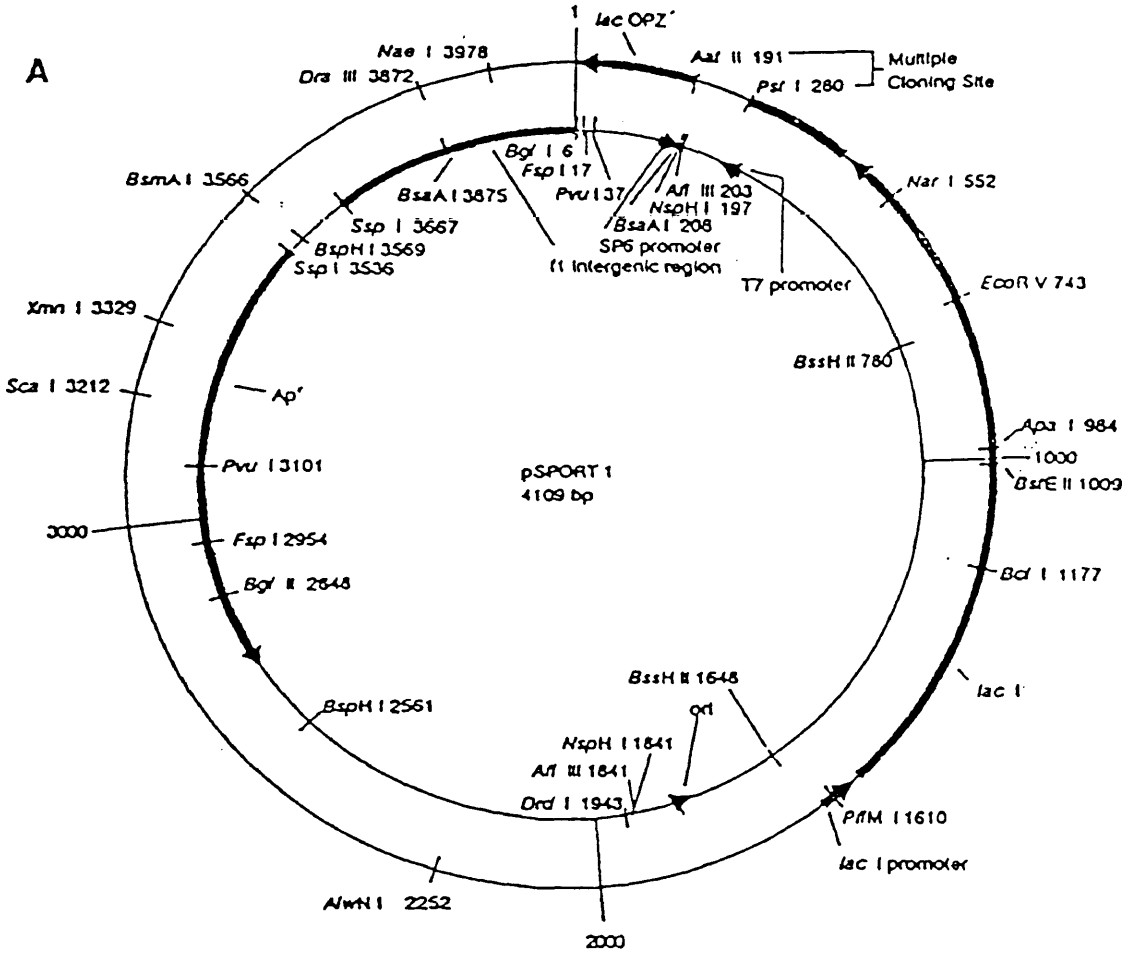
mg/ml) were inoculated with 0.5 ml of a suspension containing 3.5 x $10^8$ amoebae/ml in a ring (Hanson and Weber, 1987). The plates were incubated at 23°C with fluorescent light for 4 hr. Then the cells were washed from the filters as above.

PLASMID ISOLATION

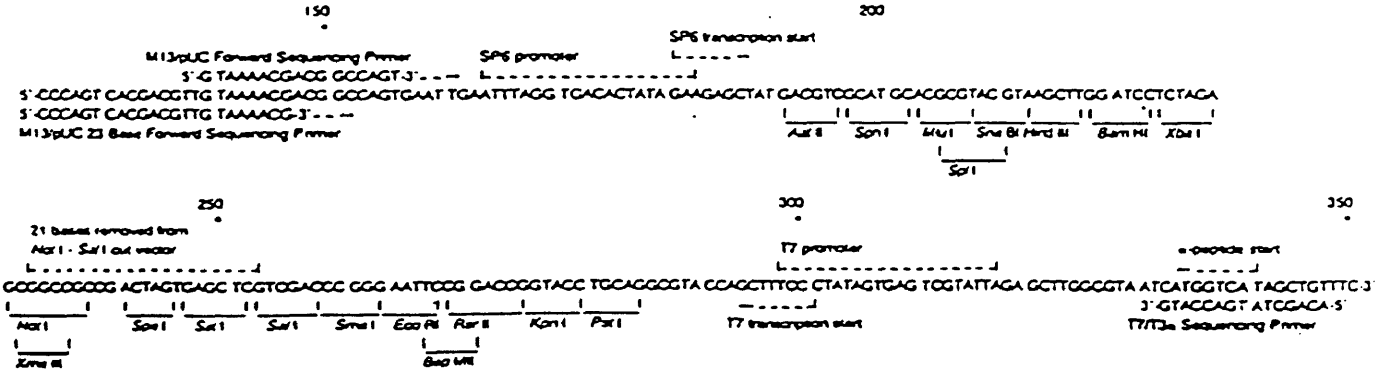
A cDNA library of *D. mucoroides* strain Dm-7 has been made by A. T. Weber using the Superscript Plasmid System (BRL). Total mRNA isolated from 8 hr macrocysts was used as the initial template. The cDNA was inserted into the plasmid pSPORT (NotI-SalI-Cut) (Fig. 4) and maintained in *E. coli* strain DH5α. The transformed DH5α has been stored at -70°C as glycerol stock. The plasmid containing A-11 cDNA was isolated using the Rapid Isolation of Plasmid DNA Procedure from Promega. The glycerol stock was picked by a sterile pipette tip and inoculated into 250 ml of terrific broth (TB) (3 grams bacto-tryptone, 6 grams bacto-yeast extract, 1 ml glycerol, 0.578 grams dibasic potassium phosphate, and 3.315 grams monobasic potassium phosphate) containing 50 μg/ml ampicillin. The cells were grown overnight in a shaker at 37°C. Then they were harvested by centrifuging at 5,000 g at 4°C for 15 minutes. The cell pellet was resuspended in 6 ml of ice-cold freshly prepared lysis buffer (25mM Tris-HCl, pH8.0, 10 mM EDTA, 50 mM glucose, and 0.8 mg/ml lysozyme) and incubated in ice water for 10 minutes. Twelve ml of freshly prepared 0.1N NaOH and 0.1% SDS was added, mixed and incubated in ice water for 10 minutes. Then, 7.5 ml of 3M sodium acetate, pH4.6, was added, mixed, and incubated in ice water for 20 minutes. After centrifuging at 12,000xg for 15 minutes, the supernatant was transferred to another tube and

Fig. 4. (A) Map of plasmid pSPORT 1. (B) Multiple cloning site of plasmid pSPORT 1. A-11 cDNA was inserted into the Not I-Sal I site with the sequence corresponding to the polyA tail at the Not I end (Gibco, BRL, catalog).

A


lac OPZ'
Aat II 191
Multiple Cloning Site
Pst I 280
Nae I 3978
Ora III 3872
Bgl I 6
Fsp I 17
Pvu I 37
Afl III 203
NspH I 197
BsaA I 208
BsaA I 3875
BsmA I 3566
Ssp I 3667
BspH I 3569
Ssp I 3536
SP6 promoter
f1 intergenic region
T7 promoter
Nar I 552
EcoR V 743
Xmn I 3329
BssH II 780
Sca I 3212
Ap'
Apa I 984
1000
BstE II 1009
Pvu I 3101
pSPORT 1
4109 bp
3000
Fsp I 2954
Bgl II 2648
Bcl I 1177
BssH II 1648
lac I'
BspH I 2561
ori
NspH I 1841
Afl III 1841
Ord I 1943
PflM I 1610
lac I promoter
AlwN I 2252
2000

B


150
200
SP6 transcription start
M13/pUC Forward Sequencing Primer
SP6 promoter
5'-G TAAAACGACG GCCAGT-3'
5'-CCCAGT CACGACGTTG TAAAACGACG GCCAGTGAAT TGAATTTAGG TGACACTATA GAAGAGCTAT GACGTCGCAT GCACGCGTAC GTAAGCTTGG ATCCTCTAGA
5'-CCCAGT CACGACGTTG TAAAACG-3'
M13/pUC 23 Base Forward Sequencing Primer
Aat II
Spe I
MluI
Sna BI Hind III
BamHI
Xba I
Sal I
250
300
350
21 bases removed from
Not I - Sal I cut vector
T7 promoter
α-peptide start
GCGGCCGCGG ACTAGTCAGC TCGTCGACCC GGG AATTCCG GACCGGTACC TGCAGGCGTA CCAGCTTTTC CTATAGTGAG TCGTATTAGA GCTTGCGCGTA ATCATGGTCA TAGCTGTTTC-3'
3'-GTACCAGT ATCGACA-5'
Not I
Spe I
Sal I
Sal I
Sma I
EcoR I
Rar II
Kpn I
Pst I
T7 transcription start
T7/T3a Sequencing Primer
Xma III
BsoBI

the precipitate was discarded. DNase-free RNase A was added to a final concentration of 20 μg/ml and the mixture was incubated at 37°C overnight. Following extractions with phenol/chloroform and chloroform:isoamyl alcohol (24:1), plasmid DNA was ethanol-NaCl precipitated and washed by 70% ethanol. The pellet was air dried and dissolved in TE buffer (10mM Tris-HCl, pH8.0, 1mM EDTA, pH8.0). The quality and quantity of plasmid DNA was determined by agarose gel electrophoresis (Berger, 1987) and ultraviolet absorption spectrophotometry, respectively.

An alternative method used was the QIAGEN Plasmid Kit (QIAGEN Inc., Chatsworth, CA). The QIAGEN plasmid purification procedure is based on the optimized alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979). The procedure has been condensed to three steps, and combined with QIAGEN resin to allow the selective purification of supercoiled plasmid DNA. For inoculation, only 100 ml TB was needed. The bacterial pellet was resuspended in 10 ml of buffer P1, and the manufacturer's protocol followed. The final pellet was redissolved in TE buffer.

## TRANSCRIPTION *IN VITRO*

Transcription of A-11 cDNA was performed essentially following the procedure of the Promega Technical Manual. The plasmid containing A-11 cDNA was isolated and linearized by HindIII. Four μl of 5X transcription buffer (200 mM Tris-HCl, pH7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 2 μl of 100 mM dithiothreitol, 0.5 μl of RNasin (40 units/μl), 1 μl of 10 mM ATP, 1 μl of 10 mM GTP, 1μl of 10 mM UTP, 1 μl of 10 mM CTP, 8.5 μl of linearized plasmid DNA (0.1μg/μl), and 1 μl of T7 RNA polymerase were added together and

incubated at 37°C for 1 hr. One µl of RQ1 RNase - free DNase (1 unit/µl) was added to remove the DNA template and the mixture was incubated at 37°C for 15 min. Two hundred and eighty µl of RNase free $H_2O$ was added to the sample, then the RNA was subsequently extracted with phenol/chloroform and chloroform:isoamyl alcohol (24:1), precipitated by ethanol, dried by lyophilization, and resuspended in RNase free $H_2O$.

DNA LABELING

A-11 plasmid DNA was digoxigenin-labeled with a standard random primed DNA labeling reaction by using the Genius™ System. Three µg (6µl) of heat-denatured A-11 plasmid DNA, 2 µl of 10X hexanucleotide mixture, 2 µl of 10X dNTP labeling mixture, 9 µl of $H_2O$, and 1 µl of Klenow enzyme (labeling grade) were added together and incubated at 37°C for 20 hrs. Then 2 µl of EDTA (200mM) was added to terminate the reaction. The labeled DNA was precipitated with ethanol/LiCl, washed by 70% ethanol, air dried, and resuspended in 50 µl of TE/SDS buffer (10mM Tris-HCl, 1 mM EDTA, pH7.0-8.0, 0.1% SDS). A DIG-labeled control DNA was used to estimate the yield of DIG-labeled DNA. A serial dilution (1:5,1:50, 1:500, 1:5000, 1:50000) of the labeled control DNA and newly labeled experimental DNA probe was made. One µl of each dilution was spotted onto a Hybond membrane (Amersham). The DNAs were fixed to the membrane by cross-linking with UV light. Then the membrane was wetted with a small amount of Genius buffer 1(100 mM Tris-HCl, 150 mM NaCl, pH7.5), incubated in Genius buffer 2 (2% (w/v) blocking reagent for nucleic acid hybridization dissolved in Genius buffer 1) for 15 minutes at room temperature, incubated in the diluted antibody (anti-DIG-alkaline

phosphatase diluted 1:5000 in Genius buffer 2) for 15 minutes at room temperature, washed twice in Genius buffer 1 at room temperature (15 minutes per wash), incubated in Genius buffer 3 (100mM Tris-HCl, 100mM NaCl, 50 mM MgCl2, pH9.5) for 2 minutes, developed in the color substrate solution (45µl NBT solution and 35 µl X-Phosphate solution in 10 ml of Genius buffer 3) in the dark for 30-60 minutes. The concentration of the experimental probe was estimated by comparing the spot intensities of dilutions of the control with those of the experimental probe.

NORTHERN BLOT ANALYSIS

RNA samples along with 2µl of loading buffer (0.72 ml 100% formamide, 0.16 ml of 10X MOPS, 0.26 ml of 37% formaldehyde, 0.18 ml of 0.4% bromophenol blue and 0.08 ml of 0.4% xylene cyanol) were loaded into wells of a 1% agarose mini-gel (0.2 g agarose, 18.9 ml 1X MOPS, 1.1 ml 37% formaldehyde). The gel was run at 75 volts for 1 hour in 1X MOPS (0.02 M mops, 0.005 M sodium acetate, 0.001 M EDTA) using an IBI QSH electrophoresis chamber. Constant recirculation of the running buffer to maintain the pH was achieved by an ISCO TRIS$^{TM}$ pump (Maniatis et al. , 1982; Davis et al. ,1986; Sambrook et al. ,1989).

The RNA was transferred to a Hybond membrane (Amersham) by overnight capillary elution in an ascending 10XSSC buffer (Schleicher and Schuell, 1987). After washing the membrane in 5X SSC for 5 minutes, the RNA was immobilized on the membrane using ultraviolet crosslinking (Church and Gilbert, 1984). The damp membrane was exposed to ultraviolet light (254nm) for 30 sec at 12 J/m$^2$.

Prehybridization and hybridization of the Hybond membrane were executed by using the Genius™ System (Boehringer Mannheim Corp. , Indianapolis, IN). The Hybond membrane with RNA was placed in a sealed hybridization bag containing 20 ml Northern prehybridization solution (5XSSC, 50% formamide, 0.02% SDS, 0.1% N-lauroylsarcosine, 2% (w/v) blocking reagent for nucleic acid hybridization, 20 mM sodium maleate, pH7.5) to prehybridize 2 hrs at 42°C. The A-11 DNA probe was heated in a boiling water bath for 10 minutes to denature the DNA, and diluted to 20 ng/ml in 10 ml Northern prehybridization solution. This became the Northern hybridization solution. The Northern prehybridization solution was discarded from the bag, and the Northern hybridization solution was added. The probe was allowed to hybridize with the membrane overnight at 42°C. The membrane was washed twice (5 minutes per wash) in 2X wash solution (2XSSC, 0.1% SDS) at room temperature, and twice (15 minutes per wash) in 0.5X wash solution ( 0.5XSSC, 0.1%SDS) at 65°C. After the membrane was washed in 50 ml maleate buffer (100 mM maleic acid, pH7.5; 150 mM NaCl) for 1 min at room temperature, it was incubated in 50 ml Northern blocking solution for 60 minutes, then in 30 ml Northern blocking solution with anti-DIG-alkaline phosphatase (1:5,000) for 30 minutes. Following 2 washes (15 minutes per wash) in 100 ml maleate buffer at room temperature, the membrane was equilibrated in 50 ml Genius buffer 3 (100 mM Tris-HCl, pH9.5; 100 mM NaCl; 50 mM $MgCl_2$) for 5 minutes and developed in the color substrate solution (45 µl NBT solution and 35 µl X-Phosphate solution in 10 ml of Genius buffer 3) in the dark for 30 minutes to 16 hrs.

## MESSENGER RNA ISOLATION

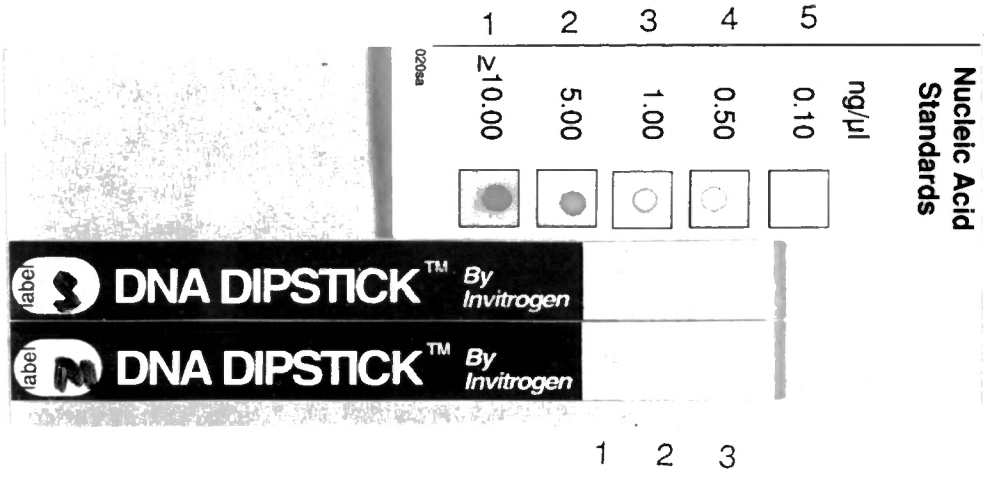
Total mRNA was isolated using the FastTrack mRNA isolation kit (Invitrogen Co. , San Diego, CA). About $9x10^8$ cells developing as macrocysts (4 hr) or sorocarps (4 hr) were harvested and lysed in a detergent-based buffer containing RNase/Protein Degrader, incubated at 45°C, and applied directly to oligo (dT) cellulose for absorption. The DNA, proteins, and cell debris were washed off the resin with a high salt buffer (Binding Buffer). Non-polyadenylated RNAs were washed off with a low salt buffer, and the mRNA was then eluted in the absence of salt (Elution Buffer) in a final volume of 400µl.

Qualitative and quantitative assessment of the total mRNA was determined by ultraviolet absorption spectrophotometry (Berger, 1987) or use of a DNA Dipstick$^{TM}$ Kit (Invitrogen Co. , San Diego). The DNA Dipstick$^{TM}$ Kit provides an accurate measurement of single or double-stranded DNA, RNA, or oligonucleotides. One microliter of each sample dilution (1:10, 1:100, 1:1000) was spotted on the Dipstick membrane, and the manufacturer's protocol followed. RNA concentration was determined by comparison of color intensity with standards. A typical result is shown in Fig. 5. The concentrations of 1:10 dilutions of both sorocarp (4 h) and macrocyst (4 h) samples were typically 70-100 ng/µl.

## ISOLATION OF cDNA INSERT

A-11 plasmid DNA was cut by HindIII and KpnI in 1X multicore buffer (Promega, Madison, WI) at 37°C overnight. The cut DNA was purified by electrophoresis on an 1% agarose gel (0.6g agarose, 60 ml 0.5XTBE, 3µl

Fig. 5. Quantification of isolated total mRNA from macrocysts (4h) or sorocarps (4h). Nucleic acid standards are shown in the first row. [Concentrations are, from left to right: (1) ≥ 10.00 ng/µl, (2) 5.00 ng/µl, (3) 1.00 ng/µl, (4) 0.50 ng/µl, (5) 0.10 ng/µl]. One microliter of each sample dilution (1:10, 1:100, 1:1000) from macrocysts (M) or sorocarps (S) was spotted on the Dipstick membrane with dilutions increasing from left to right. Nucleic acid concentration was estimated as approximately 0.7 µg/µl for both samples.


1 2 3 4 5
Nucleic Acid Standards
ng/µl
≥10.00
5.00
1.00
0.50
0.10
020aa
label DNA DIPSTICK™ By Invitrogen
label DNA DIPSTICK™ By Invitrogen
1 2 3

ethidium bromide (EtBr)) using an IBI MPH electrophoresis chamber. The gel was run at 120V for 2 hrs in 0.5X TBE
(45 mM Tris-borate, 1 mM EDTA).The desired DNA band, which was 1.3 kb, was excised under UV light and slid into dialysis tubing (Spectra, Por1, Spectrum Medical industries, INC. ).

The dialysis tubing was prepared following the procedure of Sambrook et al. ,1989. The dialysis tubing was cut into convenient lengths (10-20 cm), boiled for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH8.0), rinsed thoroughly in distilled water, then boiled for 10 minutes in 1 mM EDTA (pH8.0). After the dialysis tubing was cooled down, it was stored at 4°C. Before use, the tubing was washed inside and out with 0.5X TBE. The agarose slice containing the DNA band was floated in 200 µl 0.5X TBE in the tubing. The tubing was sealed by a closure at each end and placed in a minigel apparatus which was filled with sufficient 0.5X TBE to cover the tubing. The DNA was electroeluted at 2 V/cm for 2 hrs. After electroelution was complete, the polarity of the electrodes was reversed for 30 sec at 100 V to dislodge DNA from the tubing wall. The eluted DNA was then collected in a 1.5 ml microfuge tube. Another 200 µl 0.5X TBE was used to rinse the tubing, and combined with eluted DNA. Following extractions with phenol/chloroform and chloroform/isoamyl alcohol, the eluted DNA was ethanol/NaCl precipitated and washed by 70% ethanol (Ausubel et al. , 1992).

HYBRID SELECTION OF mRNA

Four µg of A-11 cDNA insert was dissolved in 80 µl of diethyl pyrocarbonate (DEPC) treated water. The DNA was denatured by boiling for 3

min, then quick-chilled in cold methanol, made 10X SSC by adding an equal volume of ice-cold 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH7.0), and blotted onto a 1 cm by 1 cm piece of nitrocellulose membrane (Bio-Rad laboratories, CA) which was prewetted by DEPC treated water. The wetted nitrocellulose paper was cut to four pieces (25 $mm^2$ each), sealed in a 1.5 ml microfuge tube, and baked for 2 hour at 80°C in a vaccum oven.

Eight pieces of membrane with bound A-11 insert were placed in a 1.8 ml round bottom CRYOS$^{TM}$ vial (Sumitomo Bakelite Co. , Japan) with 800 µl of DEPC treated water and heated to 100°C for 1 minute. The water was then removed and replaced by 800 µl of hybridization mix (0.7 M NaCl, 10 mM MOPs, pH6.4, 8 mM EDTA, 0.5% SDS) and prehybridized at 40°C for 1 hour with shaking. The paper was then transferred to a fresh tube containing 10 µg of total mRNA which had been dissolved in 800 µl of hybridization mix and heated to 65°C for 10 minutes. After an 18-h incubation at 40°C with shaking, the hybridization mix was removed and the filter was successively washed three times at room temperature with 1X SSC/0.5%SDS, five times at 50°C with 0.1X SSC/0.1% SDS, two times at room temperature with 0.1X SSC, once with ice-cold 2 mM EDTA (pH7.2), and two times with ice-cold water. The hybridized mRNA was eluted from the paper by heating to 90°C for 5 minutes in 200 µl of DEPC treated water containing 10 µg yeast tRNA (Gibco BRL, MD) as carrier. Immediately after heating, the tube was snap frozen in cold methanol, and thawed slowly on wet ice. The filters were then removed, and the mRNA solution was lyophilized reducing the volume to about 10 µl (Liebhaber and Cash, 1985).

TRANSLATION AND GEL ANALYSIS

Translation of the mRNA was achieved by using the Rabbit Reticulocyte Lysate System (Promega Corp. , Madison, WI). Thirty five µl of rabbit reticulocyte lysate, 1 µl of Rnasin ribonuclease inhibitor, 1 µl of 1 mM amino acid mixture (minus methionine), 4 µl of [$^{35}$S] methionine at 10 mCi/ml, and 9 µl of RNA substrate in water that had been preheated to 65°C for 5 minutes to remove the secondary structure were added together and incubated at 30°C for 2 hrs. The translation products were analyzed by SDS polyacrylamide Gel Electrophoresis using Mini-Protean$^{TM}$ II Dual Slab Cell (Bio-Rad). The Laemmli buffer system was used in SDS gel electrophoresis (Laemmli, 1970). Discontinuous gels were cast following the procedures provided in the Bio-Rad Instruction Manual. A 12% separating gel was used along with a 4% stacking gel. Five µl of translation mixture was added to 10 µl of loading buffer (4 ml distilled water, 1 ml 0.5 M Tris-HCl, pH6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2 mercaptoethanol, 0.2 ml 0.05% Bromophenol blue) and preheated at 100°C for 2 minutes. Two µl of Rainbow$^{TM}$ colored molecular weight protein standard consisting of lysozyme, 14.3 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 46 kDa; bovine serum albumin, 69 kDa; phosphorylase b, 97.4 kDa; myosin, 200 kDa (Amersham), and 1 µl of $^{14}$C-labeled molecular weight protein standard consisting of lysozyme, 14.3 kDa; ß-lactoglobulin, 18.4 kDa; α-chymotrypsinogen, 25.7 kDa; ovalbumin, 43 kDa; bovine serum albumin, 68 kDa; phosphorylase B, 97.4 kDa; myosin (H-chain), 200 kDa (BRL) were used for molecular weight comparisons. Electrophoresis was carried out at 200 volts until the tracking dye was at the end of the gel. The gels were then removed, fixed for 3 minutes in a solution containing 10% (w/v)

trichloroacetic acid, 10% (v/v) glacial acetic acid, 30% (v/v) methanol, soaked in 15-20 volumes of water for 30 minutes, soaked in 8-10 volumes of Fluoro-Hance (Research Products International Corp. , IL) for 30 minutes, and dried at 80°C for 1 hour with Hoffer SE540-Slab Gel Dryer (Hoeffer Scientific Instruments). The dried gels were exposed to Kodak X-Omat AR film (Eastman Kodak Company) at -80°C for 1-3 days.

## TOTAL RNA ISOLATION

Total RNA isolation was performed based on Marilynn Larson's method which is a modified form of the method of Chomczynski and Sacchi (1987). For each preparation, $1.8 \times 10^7$ cells were vortexed in 1.8 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The addition of 180 µl 2 M NaOAc (pH4), 1.8 ml TE saturated phenol and 360 µl chloroform-isoamyl alcohol (49:1) was carried out with mixing after addition of each reagent. The sample was vortexed for 10 sec, chilled on ice for 15 min and centrifuged for 20 min at 4000xg at 4°C. The aqueous phase was precipitated with an equal volume of isopropanol at -70°C for 1 hour. To collect the pellet , the sample was centrifuged for 20 min at 4000xg at 4°C. The pellet was dissolved in 200 µl of denaturing solution and transferred to a 1.5 ml microfuge tube. An equal volume of isopropanol was added and the sample was incubated at -20°C for 1 hour. The pellet was collected by centrifugation at 4°C for 10 min in a microfuge at 10,000xg and washed twice with 70% ethanol. After air drying, the pellet was redissolved in DEPC treated $H_2O$.

PRIMER EXTENSION ANALYSIS

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 (Fig. 3) is as follows: 5'-dCTCCATGAACTTCTAATAC-3' (complementary to positions 1221 to 1239 of A-11 cDNA). Oligonucleotides were labeled by a modified kinase reaction (Sigmund et al., 1988). Fifty pmol of oligonucleotides, 33 pmol [$\gamma$-$^{32}$P] ATP, 220 pmol rATP, 10 units polynucleotide kinase, 3 µl 10X PNKB (0.5 M Tris-HCl, pH7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA), and deionized and distilled $H_2O$ ($ddH_2O$) to bring the final volume to 30 µl were added together and incubated at 37°C for 40 min, then at 65°C for 10 min. Another 70 µl of TE was added, and the mixture was extracted with phenol-chloroform once, and precipitated with NaCl and 3 volumes of 95% ethanol. The pellet was dried in a Speed Vac Concentrator and resuspended in 25 µl TE. Primer extension was based on the method of Schumann et al. (1994). In a 10 µl assay mixture containing 50 mM Tris-HCl (pH8.3) and 75 mM KCl, 2 pmol of labeled oligonucleotide was annealed to 2.8 µg of poly(A) RNA. The annealing mixture was heated to 68°C for 5 min, slowly cooled down to less than 35°C, and put on ice. Five µl of buffer containing 50 mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 2.5 mM deoxynucleoside triphosphates (dNTPs), and 10 units of Superscript$^{TM}$ RNase H$^-$ Reverse Transcriptase (Life Technologies, Inc. , BRL) was added. Assay mixtures were incubated at 42°C for 30 min. Then, 5 µl of stop solution (United States Biochemical) was added. The reaction mixture was heated to 75°C for 2 min and chilled on ice, and products were separated on 8% polyacrylamide gels containing 50% urea (Sequencing Support Service, USB, Ohio). The

sequencing reaction products of M13mp18 single strand control DNA (SequenaseR Version 2.0 DNA Sequencing Kit, USB) was also separated on the same gel for use as a standard size marker labeled with [$^{35}$S] dATP. The gel was prerun for 30 min at a constant power of 55 watts (approximately 1200 volts, 45 mA), and after loading the samples, run for about 2 hours at 55 watts until the bromophenol blue dye ran off the bottom. Then, the gel was fixed in 10% methanol and 10% acetic acid for 30 min, dried at 80°C for 40 min. The dried gels were exposed to Kodak X-Omat AR film (Eastman Kodak Company) for 1-3 days.

The 5' sequence of the A-11 mRNA was provided by a primer-extension reaction with dideoxyribonucleoside triphosphates. In a 12 μl assay mixture containing 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT, 4 pmol of labeled oligonucleotide was annealed to 6 μg of poly (A) RNA. The annealing mixture was heated to 68°C for 5 min, slowly cooled down to less than 35°C, and put on ice. Ten units (1 μl) of AMV Reverse Transcriptase were added to the annealing mixture. The concentrations of dNTPs and ddNTP in termination mixes were: 0.2 mM dNTPs and 0.08 mM ddATP in ddA mix; 0.2 mM dNTPs and 0.1 mM ddCTP in ddC mix; 0.2 mM dNTPs and 0.1 mM ddGTP in ddG mix; 0.2 mM dNTPs and 0.08 mM ddTTP in ddT mix. Two and a half μl of annealing mixture was aliquoted to tubes containing 2.5 μl of termination mixes. After 30 minutes' incubation at 42°C, 5 μl of stop solution was added. The products were separated and analyzed as above.

# RESULTS

## *IN VITRO* TRANSCRIPTION

The transcriptional products obtained *in vitro* from cloned A-11 cDNA were used as a positive control in Northern Blot analysis and for testing the conditions of mRNA selection (Fig. 6). They were also used in *in vitro* translation as a positive control. *In vitro* transcription of A-11 cDNA was started from the T7 promoter. The plasmid vector was linearized by HindIII to produce a complete transcript of the cDNA insert that stopped at the HindIII restriction site (Fig. 4). Undigested plasmid DNA can give rise to very long transcripts, thus, incomplete digestion during linearization of the A-11 plasmid DNA is the probable explanation for the additional products obtained from some *in vitro* transcription reactions (Fig. 6). Complete linearization of the A-11 containing plasmid vector resulted in one main transcriptional product when analyzed by electrophoresis (Fig. 7), or by Northern blot (lane 5 of Fig. 8).

## HYBRID SELECTION OF mRNA

The A-11 mRNA was isolated from total mRNA by hybrid selection. Linearized plasmid vector containing the A-11 insert, or the excised A-11 cDNA insert were denatured and blotted on nitrocellulose membrane for hybrid selection. The plasmid was linearized by HindIII. A-11 cDNA insert was cut out of the plasmid using a double restriction digestion by HindIII and KpnI. The completeness of the digestion was checked by electrophoresis (Fig. 9). The excised cDNA was purified by electrophoresis (Fig. 9), and isolated by electroelution using dialysis tubing (Fig. 10). The sizes of the A-11 containing

Fig. 6. Comparative Northern analysis of different elution conditions during hybrid selection. *In vitro* transcripts of A-11 cDNA were used to test the elution conditions during hybrid selection. The elution mixtures were then separated on a 1% agarose gel containing 0.66 M formaldehyde and analyzed by Northern blot. Lane 1, transcripts were eluted by heating to 80°C for 10 min. Lane 2, transcripts were eluted by heating to 90°C for 5 min. Lane3, transcripts were eluted by heating to 100°C for 1 min. Lane 4, transcripts undergo prehybridization and hybridization procedures with no nitrocellulose membrane addition in the hybridization mix to test for RNase contamination during hybrid selection. Lane 5, negative control, *in vitro* transcripts were not added to hybridization mix. Lane 6, positive control, 10 ng of *in vitro* transcript.

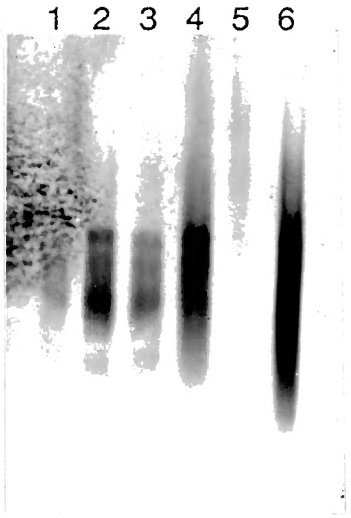

1 2 3 4 5 6

Fig. 7. Electrophoretic analysis of *in vitro* transcript of A-11 cDNA. The plasmid vector of clone A-11 was linearized by HindIII. *In vitro* transcription of A-11 cDNA was started from T7 promoter and stopped at HindIII restriction site. The transcription product was analyzed on a 1% agarose gel containing 0.66 M formaldehyde. Lane 1, HindIII cut lambda DNA. Lane 2, 0.2 μg of clone A-11. Lane 3, 0.05 μg of linearized clone A-11 by HindIII. Lane 4, 0.2 μg of excised A-11 cDNA by HindIII and KpnI. Lane 5, *in vitro* transcript of A-11 cDNA.

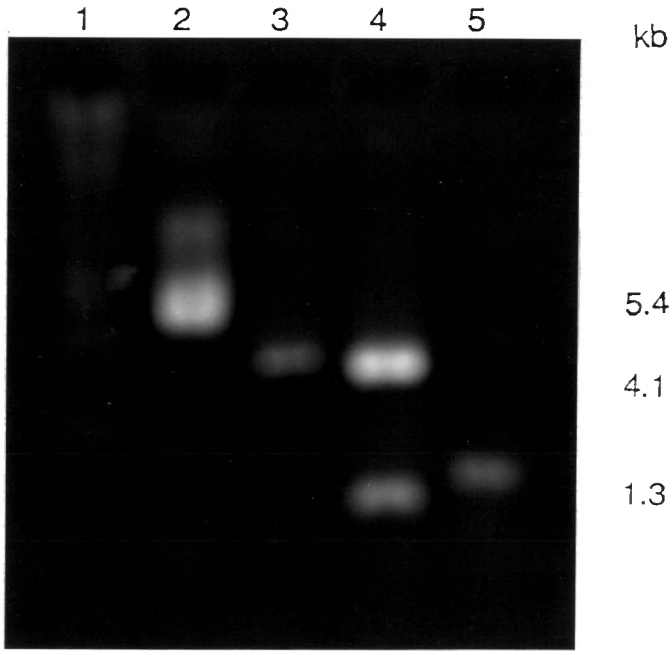

1
2
3
4
5
kb
5.4
4.1
1.3

Fig. 8. Northern analysis of RNA isolated by hybrid selection. Lane 1, 10 ng of *in vitro* transcript of A-11 cDNA. Lane 2 and 3, elution mixtures resulted from hybrid selection of *in vitro* transcript of A-11 cDNA. Lane 4, 70 ng of total mRNA from macrocyst (4h). Lane 5, elution mixture resulted from hybrid selection of total mRNA from macrocyst (4h).


1 2 3 4 5

Fig. 9. Electrophoretic analysis of cDNA clone A-11 linearized with HindIII and cDNA insert excised with HindIII and KpnI. Lane 1, HindIII cut lambda DNA. Lane 2, 0.2 µg of clone A-11. Lane 3, 0.1 µg of linearized clone A-11 by HindIII. Lane 4, 0.1 µg of excised A-11 cDNA by HindIII and KpnI.

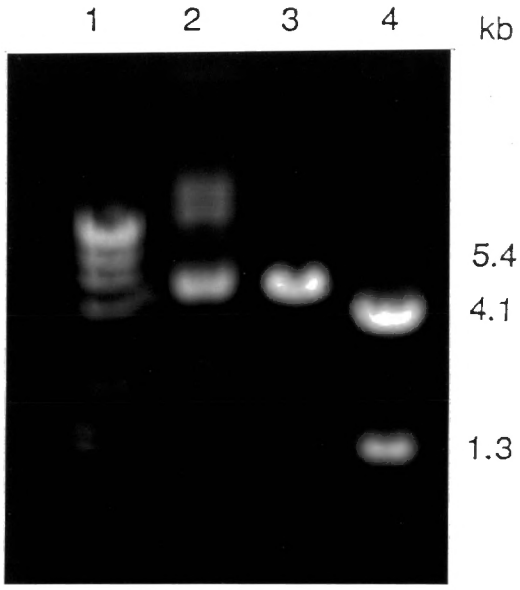
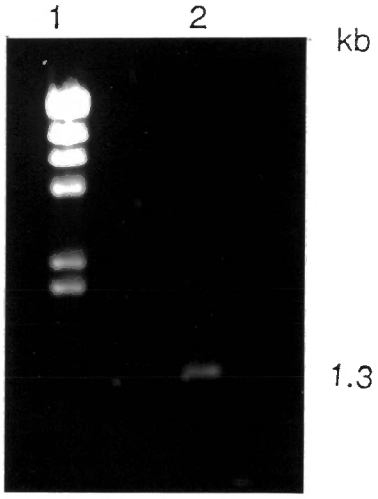

1
2
3
4
kb
5.4
4.1
1.3

Fig. 10. Electrophoretic analysis of excised A-11 cDNA insert isolated by electroelution. A-11 cDNA insert was cut out of the plasmid by HindIII and KpnI. The excised cDNA was purified by electrophoresis, and isolated by electroelution using dialysis tubing. Lane 1, HindIII cut lambda DNA. Lane 2, excised A-11 cDNA insert isolated by electroelution.


1
2
kb
1.3

plasmid DNA and A-11 cDNA insert are 5.4 kb and 1.3 kb, respectively. Nitrocellulose membranes with bound A-11 cDNA insert selected A-11 mRNA more efficiently than membranes with bound linearized A-11 plasmid DNA. So membranes with bound A-11 cDNA insert were preferred for use in hybrid selection.

Different temperatures for eluting the hybridized mRNA from nitrocellulose membrane were tested. The best result was obtained by heating to 90°C for 5 minutes (Fig. 6). The RNA was difficult to redissolve once it was completely dry. Thus, the step of precipitation by ethanol after elution was replaced by lyophilization of the elution mixture to reduce the volume to 10 μl.

To confirm that A-11 mRNA was in the elution mixture after selection, the results of hybrid selection were analyzed by Northern blots (Fig. 8). Both the *in vitro* transcript and natural A-11 mRNA produced *in vivo* were successfully selected. As shown in Fig. 8, the RNA bands of the *in vitro* transcript and the selected A-11 mRNA migrated about the same distance from the wells during electrophoresis. This result suggests that the cloned A-11 cDNA is full length or nearly so.

This Northern analysis showed that A-11 mRNA was in the final elution mixture, but it couldn't exclude the possibility of the presence of other mRNAs resulting from low stringency washing conditions during hybrid selection. These mRNAs, if present, would be translated in rabbit reticulocyte lysate and give rise to protein bands on an SDS-PAGE gel.

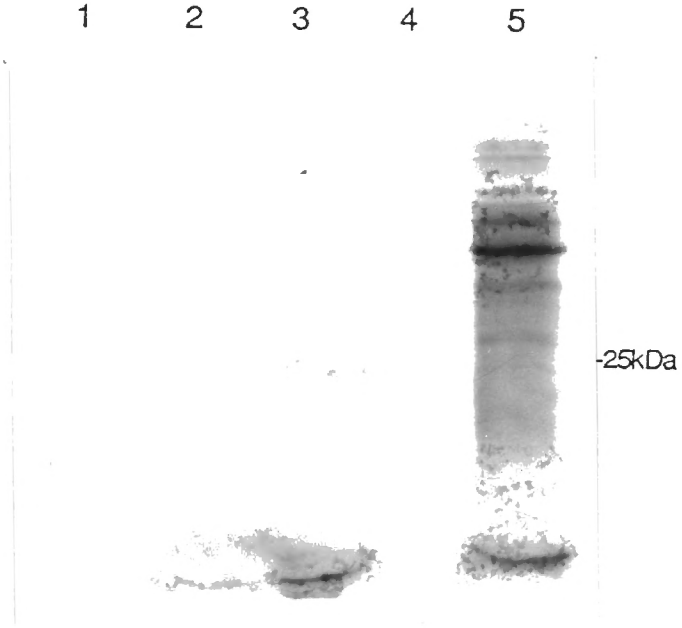
TRANSLATION ANALYSIS

Four translation reactions, each with a different template, were performed and the products obtained were analyzed on the same SDS-PAGE gel. The templates used in the translation mixtures were: (1) selected mRNA (A-11 mRNA); (2) *in vitro* transcript of A-11 cDNA; (3) total mRNA; (4) no mRNA. The autoradiogram prepared from the dried gel was developed after 1-day or 4-day exposures. After 1-day exposure the lane loaded with translation products from the total mRNA template showed several sharp bands. Three bands with labeled proteins of similar electrophoretic mobility appeared in the lanes loaded with translation products from the selected mRNA and the *in vitro* transcript. Three corresponding bands appeared in the lane with translation products from total mRNA (Fig. 11A).

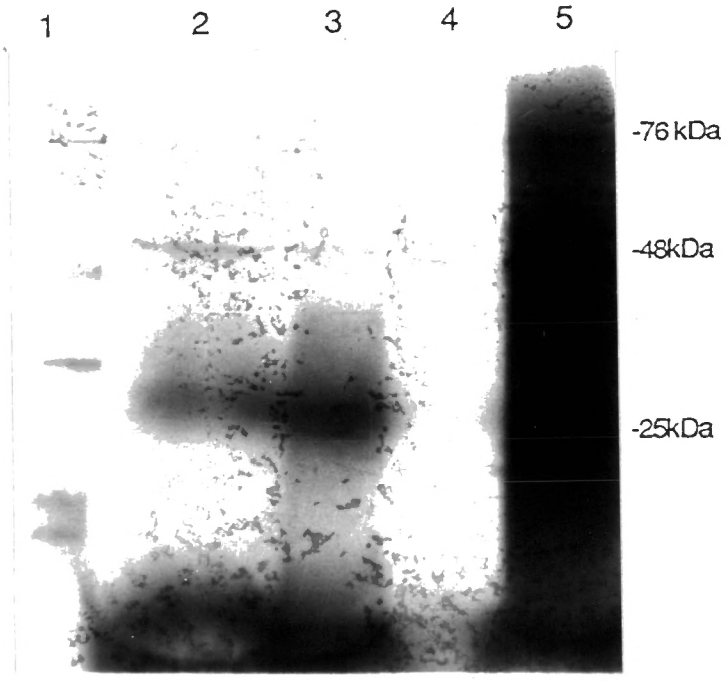
In order to get darker bands in the lane with products from the selected mRNA, a 4-day exposure was performed (Fig. 11B). The lane with translation products from total mRNA was a dark smear due to over exposure. This was expected since many different mRNAs would be translated into many different sized polypeptides and each polypeptide band became darker after longer exposure.Three polypeptides, here designated P76, P48, and P25, the sizes of which were 76 kDa, 48 kDa, and 25 kDa, respectively, were produced in the selected mRNA translation mixture and in the *in_vitro* transcript translation mixture. P76 and P48 were also generated in the control translation mixture with no mRNA addition. This indicates that P76 and P48 were translated from the nondegraded mRNA in rabbit reticulocyte lysate. Thus, P25 is the only translation product that could be attributed to the template in the selection

Fig. 11. Translation analysis of A-11 mRNA that was hybrid-selected from macrocyst (4h) total mRNA. The procedures for hybrid selction and *in vitro* translation were described in materials and methods. Translation products were separated by SDS-PAGE and analyzed by autoradiography. (A) Autoradiogram exposed for 1 day. (B) Autoradiogram exposed for 4 days. Lane 1, $^{14}C$-labeled molecular weight protein standards. Lane 2, translation products from the selected mRNA. Lane 3, positive control, translation products from the *in vitro* transcript of A-11 cDNA. Lane 4, negative control, translation mixture with no RNA addition. Lane 5, translation products from macrocyst (4h) total mRNA.

A




B



mixture. Further, A-11 mRNA is the only mRNA left in the elution mixture after hybrid selection.

## PRIMER EXTENSION ANALYSIS

Primer extension analysis was performed to map the 5' end of the A-11 mRNA and determine whether the A-11 cDNA is full length. The products of primer extension, using an end-labeled oligonucleotide complementary to positions 1221 to 1239 of A-11 cDNA (Fig. 3), were run on a 8% polyacrylamide sequencing gel in parallel with the products of a sequencing reaction of M13mp18 to provide a size standard. The two bands representing the longest extension products, which were also the major bands, were 102 bases and 101 bases (Fig. 12). The presence of multiple bands could represent multiple start sites of transcription or slippage of reverse transcriptase due to the high A-U content of the 5'-untranslated region of the mRNA. There were only 84 bases from the 19-mer oligonucleotide to the 5' end of A-11 cDNA. This indicates that the A-11 cDNA is not a complete reverse transcript of A-11 mRNA. It is short of full length by about 18 nucleotides.

Primer extension with dideoxynucleoside triphosphates gave additional information about the sequence of the A-11 mRNA start site (Fig. 13 and Fig. 14). The 5' end mRNA sequence matches the 5' end A-11 cDNA sequence, except that the nucleotides 5'NNTNNAACAAATAAATAA3' (positions -21 to -38 of Fig. 13) were missing in the A-11 cDNA. However, the first AUG (translation start site) was in A-11 cDNA.

In Fig. 14, the identical primer extension products obtained in lane 1 and lane 2 using independently isolated total mRNA template showed the

Fig. 12. Analysis of the start site of A-11 mRNA by primer extension. Primer extension was performed as described in materials and methods. The reaction products were separated on an 8% polyacrylamide gel containig 50% urea and analyzed by autoradiography. The sequencing reaction products of M13mp18 single strand control DNA were also separated on the same gel for use as a standard size marker. The lanes labeled A, C, T, G represent the sequencing reaction with M13mp18. Lane 1, primer extension using 2.8 μg of macrocyst (4h) total mRNA. Lane 2, primer extension using 10 μg of macrocyst (4h) total RNA.

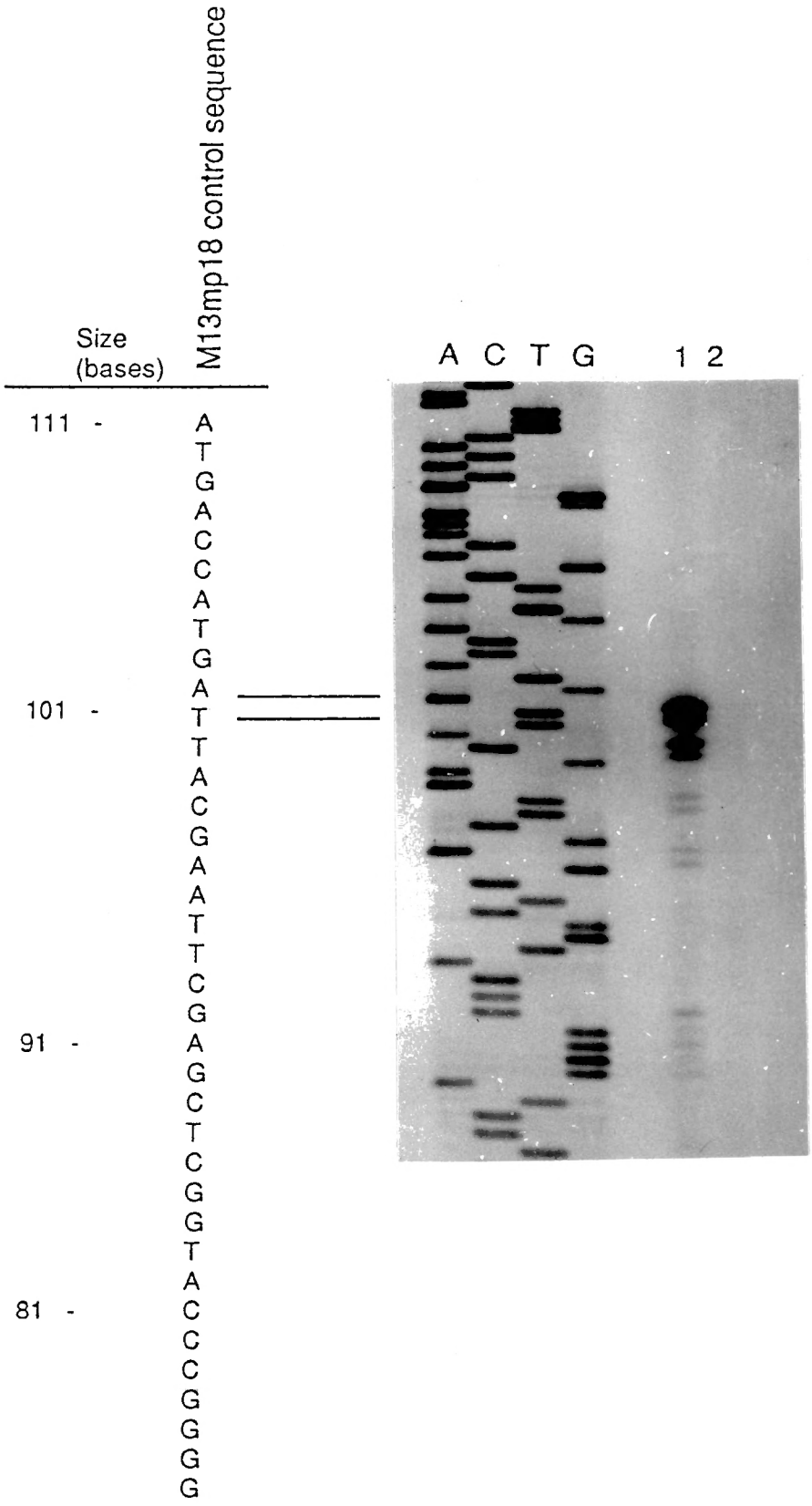
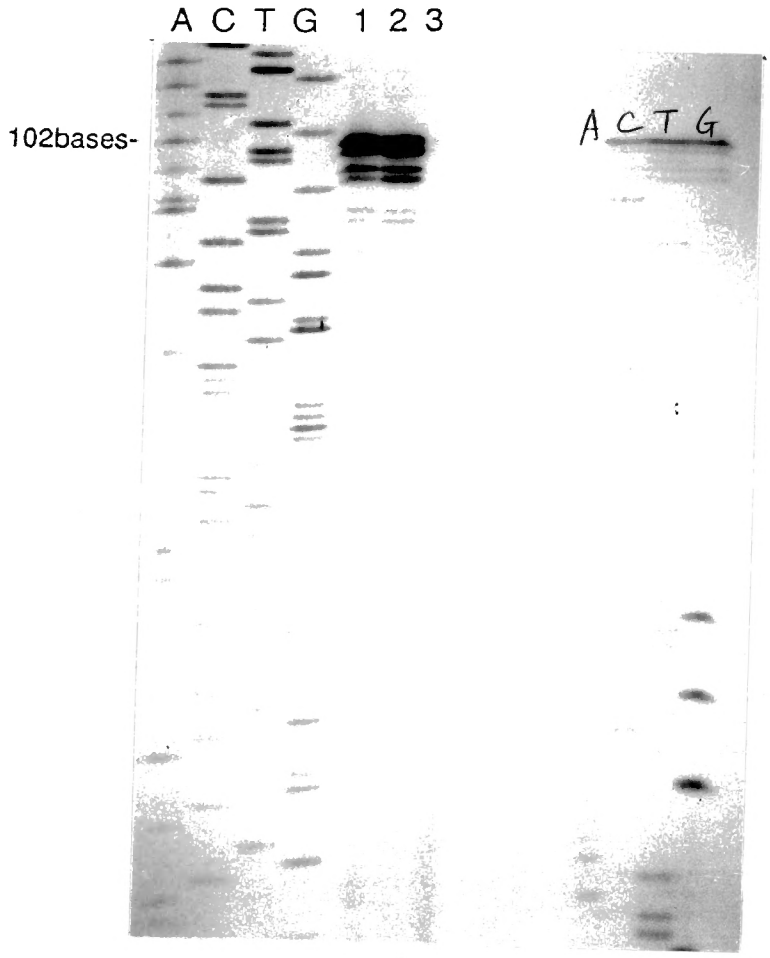

Size
(bases)
M13mp18 control sequence
111 -
101 -
91 -
81 -
A T G A C C C A T G A T T A C G A A T T C G A G C T C G G T A C C C G G G G
A C T G
1 2

Fig. 13. The sequence of A-11 mRNA. The sequence of the 5'-end of the A-11 mRNA was obtained by primer extension analysis as described in materials and methods. The numbering begins at the first ATG. The 5'-end nucleotides missing from the A-11 cDNA are shown in italics. The ATGs in the first 300 nucleotides are underlined.

| | | -38 | *NNTNNAAC* | *AAATAAATAA* | AAAACATATA | AACATATAAA |
|---|---|---|---|---|---|---|
| 1 | ATGTTAAGTC | AAGAAAATAT | TGATATTATT | AAATCAACTG | TACCAGTATT | AGAAGTTCAT |
| 61 | GGAGTTACAA | TAACAAGCAC | ATTTTATAAA | AATATGTTTG | AAGAAAATCC | CCAATTATTA |
| 121 | AATATTTTTA | ACCATTCAAA | TCAAAGACAA | GGTAAACAAC | AAACTGCATT | AGCCAATACT |
| 181 | GTATTAGCAG | CTGCAGTTAA | TATTGAAAAT | TAAATGAATT | AAATCTTGCA | GGTATTGTTA |
| 241 | ATAAACATGT | TGCAACGTTT | GGTGTATTAC | CAGAACATTA | TCCAATTGTT | GGTAGAAATT |
| 301 | ATTGGTGCAA | TTAAACAAGT | TTTAGGAGAA | GCAGCAACTC | CAGCAATTCT | AAATGCATGG |
| 361 | ACTGAGGCAT | ATGGTATTAT | TGCACAAGCA | TTTATTGATG | CAGAAGCTGC | TTTATATAAA |
| 421 | GTTACAGAAG | AACAAATGGT | GGTTGGAGAG | ATAGTAGAGA | ATTCATTGTC | GAACAAAAGA |
| 481 | TTGAAGAATC | TTCAAATATT | ACTTCATTTT | ATTTTAAACC | TGTTGATGGA | AAAGCAATTG |
| 541 | CGTCATATAT | TCCAGGTCAA | TATATAACAG | TAAAAATTAC | ATTACCATTA | TCACCAGAAT |
| 601 | CATTAGATGG | AGCTACTGAT | AAATGAATTA | GAACTCATAT | TAGACATTAC | AGTTTATCAG |
| 661 | ATGCACCTTC | TAGAACTTAT | TATCGTGTAC | AGTAAAAGAG | AAAATGCATT | AAATACATCA |
| 721 | GATCCAAATG | GTGTAGTTTC | AAATCATTTA | CATAATAATG | TTAAAGTTGG | TGATATTGTA |
| 781 | TTATTATCAC | CACCAGCTGG | TGATTTTGTA | ATTGACAAAT | CCAAATCAAA | TCCAATTCTT |
| 841 | TTGATTTCAG | GTGGTGTTGG | TATTACACCA | TTATTCAGTA | TGGTTAAAGA | AACATTAGGT |
| 901 | AAACAACCAA | ATAGAGATAT | TAGTTTTGTT | CATTCTAGTA | AATCTGAATC | TGCTCAACCA |
| 961 | TTTAAAAAAG | AACTTATAAC | AATTAAAATC | AAATAATAAT | GTAAAATTAA | ATATCATTCA |
| 1021 | CTCTGATTCT | CATGGTCATA | TTACTAAAGA | ATCAATTACT | TCATCACTAT | TTGATGGCCA |
| 1081 | AGATATTAAA | GATACTCAAG | TCTTTATTTG | TGGTCCAGTT | TCTTTTATGT | CAACAGNAAA |
| 1141 | CAAATTAGTT | TTAGAATTAG | GTTGCCNAAA | ATCAAATATT | TCTTATGAAG | TTTTTGGTCC |
| 1201 | ATTAACTTCA | GTTTAAACAA | AAATATTAAT | ACTAATTATA | ATAAATTATT | TAAATATATT |
| 1261 | TTAAACATTT | AAAAAAAAAA | AAAA | | | |

Fig. 14. RNA sequencing by primer extension. Primer extension reactions with or without dideoxyribonucleoside triphosphates were performed as described in materials and methods. The reaction products were separated on an 8% polyacrylamide gel containing 50% urea and analyzed by autoradiography. The lanes labeled A, C, T, G represent the sequencing reaction with macrocyst (4h) total mRNA. Lane 1, primer extension using 2.8 μg of macrocyst (4h) total mRNA isolated. Lane 2, primer extension using 2.8 μg of macrocyst (4h) total mRNA isolated during a second experiment carried out independently of that in lane 1. Lane 3, primer extension using 2.8 μg of sorocarp (4h) total mRNA isolated.


A C T G 1 2 3
102bases-
A C T G

reproducibility of the results. Primer extension products from same amount of macrocyst (4h) mRNA and sorocarp (4h) mRNA were separated in lane 2 and lane 3, respectively. The specificity of the primer extension reactions was obtained by comparing the bands of lane 2 and lane 3. The absence of bands in lane 3 indicated that no template was primed for reverse transcription from sorocarp (4h) total mRNA. This suggested that the primer specifically recognized A-11 mRNA.

# DISCUSSION

A cDNA library of *D. mucoroides* strain Dm-7 was made from macrocyst (8h) messenger RNA by A. T. Weber. Using Northern hybridization analysis to screen this cDNA library, Marilynn Larson found that a cDNA named A-11 only hybridized with an RNA from macrocysts but not with RNA from sorocarps. This indicated that A-11 messenger RNA was expressed only in macrocysts but not in sorocarps.

Primer extension and RNA sequencing was performed to map the 5'-end of the A-11 mRNA (Fig. 12 and Fig. 14). Extension from the primer with macrocyst (4h) total mRNA produced multiple bands (Fig. 12). The longest two products, which were also the major ones, were 102 bases and 101 bases. The smaller extension products might result from multiple transcriptional start sites or from the premature termination of reverse transcription due to the high A-U content of the 5'-untranslated region of the mRNA. Since the position of the TATA box presumably determines the site of transcriptional initiation in eukaryotes having a TATA box containing promoter (Mathis and Chambon, 1981), multiple TATA boxes upstream could account for the heterogeneity seen at the 5'-end of the A-11 mRNA. The upstream sequence should be obtained to answer this question.

The location on a DNA strand to which RNA polymerase binds to initiate transcription is the transcription controlling sequence adjacent to the start of the gene. This transcription-controlling sequence is called the promoter. The highly conserved sequence in most promoters recognized by RNA polymerase II is the TATA box. The TATA box is located at a fixed position about 30 bases

upstream of the start site (Breathnach and Chambon, 1981; Bucher and Trifonov, 1986). More recently, several genes have been studied in which no TATA box is evident by sequence analysis. Some of these genes contain a GC-rich stretch of 20-50 nucleotides that lies within the first 100-200 bases upstream of the start site (Sehgal, et al. , 1988). Thus the 5'-end mRNA sequence as determined in this study will help to locate the TATA box or the GC-rich stretch in the promoter region.

In the absence of A-11 mRNA templates as seen in Northern blot analysis of RNAs from the sorocarp pathway, extension from the primer with sorocarp (4h) total mRNA served as a good negative control. It confirmed the specificity of the primer to the A-11 mRNA.

The 5'-end mRNA sequence obtained from primer extension matches the sequence at the 5'-end of A-11 cDNA, except that the nucleotides 5'NNTNNAACAAATAAATAA3' (positions -21 to -38 of Fig. 13) were missing in the A-11 cDNA. The first ATG (putative translation start site) was in the A-11 cDNA. If the first ATG is assumed to be the initiation codon, the leader sequence, from transcriptional start site to the first AUG, is 38 nucleotides long with an A+T content of 92%. It falls in the 20 to 100 nucleotides which is the range of leader sequences on most eukaryotic mRNA (Kozak, 1987).

The *in vitro* translation from the hybrid-selected A-11 mRNA resulted in only one translation product (Fig. 11B). The molecular weight of this translation product was estimated to be 25 kDa by comparing its migration with the migration of protein standards on SDS-PAGE. This size matches the size of the longest predicted protein product which initiates at the first ATG site and has

an expected molecular weight of about 23 kDa according to the cDNA sequence (Fig. 15).

There are several ATG sites in the 5'-end of A-11. The first ATG of A-11 is very likely to be the initiation codon, because:

(1) Although initiation is not restricted to the first ATG codon in all cases, initiation is at least limited to ATG codons in the vicinity of the 5'-end. In no case do eukaryotic ribosomes initiate *de novo* in the middle of an mRNA (Kozak, 1989). There are five ATG sites underlined in Fig. 13 in the first 300 nucleotides of A-11 mRNA. One of them should be the translation start site.

(2) According to the scanning mechanism for initiation of translation in eukaryotes, the 40 S ribosomal subunit binds initially at the 5'-end of mRNA and then migrates, stopping at the first AUG codon in a favorable context for initiating translation. The "first-AUG rule" holds for some 90-95% of the hundreds of vertebrate mRNA sequences that have been analyzed (Kozak, 1987).

(3) The consensus sequence for initiation in higher eukaryotes is A/GCCATGG (Kozak, 1989). The consensus sequence for initiation in *D. discoideum* is AXAATGG (Schatzle, et al. , 1991). Further, as long as there is a purine in position -3 (The A of the ATG codon is designated +1, with positive and negative integers proceeding 3' and 5', respectively. ), deviations from the rest of the consensus sequence only marginally impair initiation (Kozak, 1989). The position -3 of the first ATG of A-11 mRNA is an A. Compared with the other five ATG contexts at 5'-end of A-11 mRNA, the first ATG context shows the most similarity to other translation initiation context in *Dictyostelium* (Fig. 16), and thus appears to be the most favorable.

Fig. 15. Predicted amino acid sequence of the A-11 gene. Translation was assumed to initiate at the first ATG codon.

```
1                                                   11
met leu ser gln glu asn ile asp ile ile lys ser thr val pro

                        21
val leu glu val his gly val thr ile thr ser thr phe tyr lys

31                                                  41
asn met phe glu glu asn pro gln leu leu asn ile phe asn his

                        51
ser asn gln arg gln gly lys gln gln thr ala leu ala asn thr

61                                                  71
val leu ala ala ala val asn ile glu asn ... met asn ... ile

                        81
leu gln val leu leu ile asn met leu gln arg leu val tyr tyr

91                                                  101
gln asn ile ile gln leu leu val glu ile ile gly ala ile lys

                        111
gln val leu gly glu ala ala thr pro ala ile leu asn ala trp

121                                                 131
thr glu ala tyr gly ile ile ala gln ala phe ile asp ala glu

                        141
ala ala leu tyr lys val thr glu glu gln met val val gly glu

151                                                 161
ile val glu asn ser leu ser asn lys arg leu lys asn leu gln

                        171
ile leu leu his phe ile leu asn leu leu met glu lys gln leu

181
arg his ile phe gln val asn ile ***
```

Fig. 16. Comparison of *Dictyostelium* translation initiation context. The mRNA sequences of the translation initiation context are shown. The ATG codons are underlined. The number after A-11 indicates the sequential appearance of ATG in A-11 mRNA.

| | | |
|---|---|---|
| EB-4 | AAAATGC | (Barklis, et al. ,1985a) |
| Phosphodiesterase | AAAATGG | (Podgorski, et al. , 1989) |
| Dd31 | AAAATGG | (Richardson, et al. , 1991) |
| DG17 | AAAATGT | (Driscoll and Williams, 1987) |
| D19 | AAAATGA | (Early, et al. , 1988) |
| D11 | AAAATGT | (Barklis, et al. , 1985b) |
| UDPGP | AAAATGA | (Ragheb and Dottin, 1987) |
| A-11(1) | AAAATGT | |
| A-11(2) | TTCATGG | |
| A-11(3) | AATATGT | |
| A-11(4) | TAAATGA | |
| A-11(5) | AACATGT | |

(4) When there are fewer than 10 nucleotides between the cap and the first ATG codons, ribosomes may initiate at the first and second codons (Kelley, et al. , 1982; Peterson and Piatigorsky, 1986; Strubin, 1986). The leader sequence of A-11 mRNA is 38 nucleotides long which is long enough for the 40 S ribosome subunit to recognize the first ATG codon.

(5) The translation product from the hybrid-selected A-11 mRNA has a similar molecular weight to the predicted protein initiated from the first ATG codon. In the first 300 nucleotides, the only ATG in a different reading frame would produce a peptide only 4 amino acids in length.

To further confirm that translation of the A-11 gene initiates at the first ATG codon, the A-11 protein needs to be purified. Antibody to the A-11 protein could be produced by using the *in vitro* translation product as the antigen. The A-11 protein could be purified by precipitation with antibody. The sequence of the A-11 protein then could unequivically demonstrate which ATG is the true translation initiation site. Also, the structure and the properties of the A-11 protein should reveal the function and the developmental importance of this protein.

In conclusion, the start site of A-11 mRNA was obtained by primer extension. This would help to locate the promoter region of the A-11 gene, thus aiding in the investigation of transcriptional regulation of A-11 gene expression. A-11 mRNA was hybrid-selected and translated *in vitro*. The translation product had a molecular weight of 25 kDa which approximated the size of the predicted protein initiated at the first ATG codon of A-11 mRNA. The resulting translation product could be used in future experiments to produce antibody for the purification and quantitation of the A-11 protein produced *in vivo*.

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