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Exploring the Structure and Function of the SNF1 Kinase Gene in the Slime Mold *Dictyostelium Discoideum*

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EXPLORING THE STRUCTURE AND FUNCTION OF THE SNF1 KINASE GENE IN THE SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

by

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INTRODUCTION

All organisms must respond to different environmental stresses. For unicellular organisms, the most important source of stress is starvation or nutrient depletion. These microscopic life forms must respond to a constantly changing food supply in which short periods of nutrient availability are interspersed with extended periods of famine. In addition to complete starvation, these organisms are affected by other chemical changes, such as forced switching from a desirable to a less desirable carbon source, and physical changes, such as heat shock. Many microorganisms respond to such stresses by entering a different phase of their life cycle. For example, the budding yeast *Saccharomyces cerevisiae* responds by forming ascospores (1). Another unicellular organism, the slime mold *Dictyostelium discoideum* responds to stress by fruiting body formation (2). Although multicellular organisms are relatively less susceptible to drastic changes in nutrient availability, they still must respond to stresses including nutrient deprivation, heat shock, and toxins. Many multicellular organisms, including mammals, respond to stress by altering their lipid metabolism (3, 4).

In 1981, a gene encoding a protein kinase was found in *S. cerevisiae* that appeared to play a role in the yeast starvation response (5). This gene was called *SNF1* (for sucrose nonfermenting) because defects in it led to the yeast's inability to utilize sucrose as an alternative to its preferred carbon source, glucose. Subsequent studies have shown that the *SNF1* gene product is required for derepression of glucose-repressed genes (6-10) possibly acting as a transcriptional activator (11).

Several homologs to the yeast *SNF1* gene have since been identified. Examples include WPK4 in wheat (12) and RKIN1 in rye (13). Several higher plants have also been shown to contain SNF1 homologs, including NPK5 from tobacco (14), AKin10 in *Arabidopsis thaliana* (15, 16) and others in various monocotyledonous and dicotyledonous plants (17). As evidence of the conserved

nature of these homologs, cDNA from the rye kinase was recently found to rescue yeast *snf1* mutants (13) and a similar experiment using the tobacco kinase resulted in expression of the glucose-repressible SUC2 in yeast, a gene normally left inactivated in *snf1* mutants (14).

In addition to the plant homologs, SNF1 has a mammalian homolog, AMP-activated protein kinase (AMPK), that shares 46% amino acid homology with SNF1 (18). AMPK has been isolated from several mammals, including rats, pigs, and humans (19-23), and regulates both the synthesis and the breakdown of fatty acids and isoprenoids (4).

An SNF1 homolog has also been identified in the slime mold *Dictyostelium discoideum*. This gene, referred to in this paper as *Dd snf1*, shares high sequence identity with known SNF1/AMPK genes. By analogy to the yeast SNF1, *Dd snf1* may play a role in signaling *D. discoideum* to aggregate and differentiate into fruiting bodies, which is a starvation response.

In nature, *D. discoideum* exists as a soil amoeba that feeds on bacteria. In the laboratory, it can be grown on axenic medium or bacteria. It is an organism that blurs the boundaries between unicellular and multicellular organisms. When conditions are favorable, it maintains its free-living soil amoeboid growth form. However, when subjected to starvation, *D. discoideum* undergoes a remarkable transformation. Figure 1 shows the development of *D. discoideum*. Several hours after starvation, the free amoebae aggregate to form a mound. A tip forms that appears to play a role in the signaling of the cells in the mound to differentiate into one of two types of cells, spore cells and stem cells. The aggregate then extends vertically to form a finger-like structure, which can fall over to form a migrating slug. The slug moves to a more favorable location before it forms the mature fruiting body ready to disperse the spore cells, completing the developmental cycle.

Development of *Dictyostelium* occurs in response to amino acid starvation (24). During amino acid starvation, some cells release cyclic AMP (cAMP), which acts as a signal to other cells

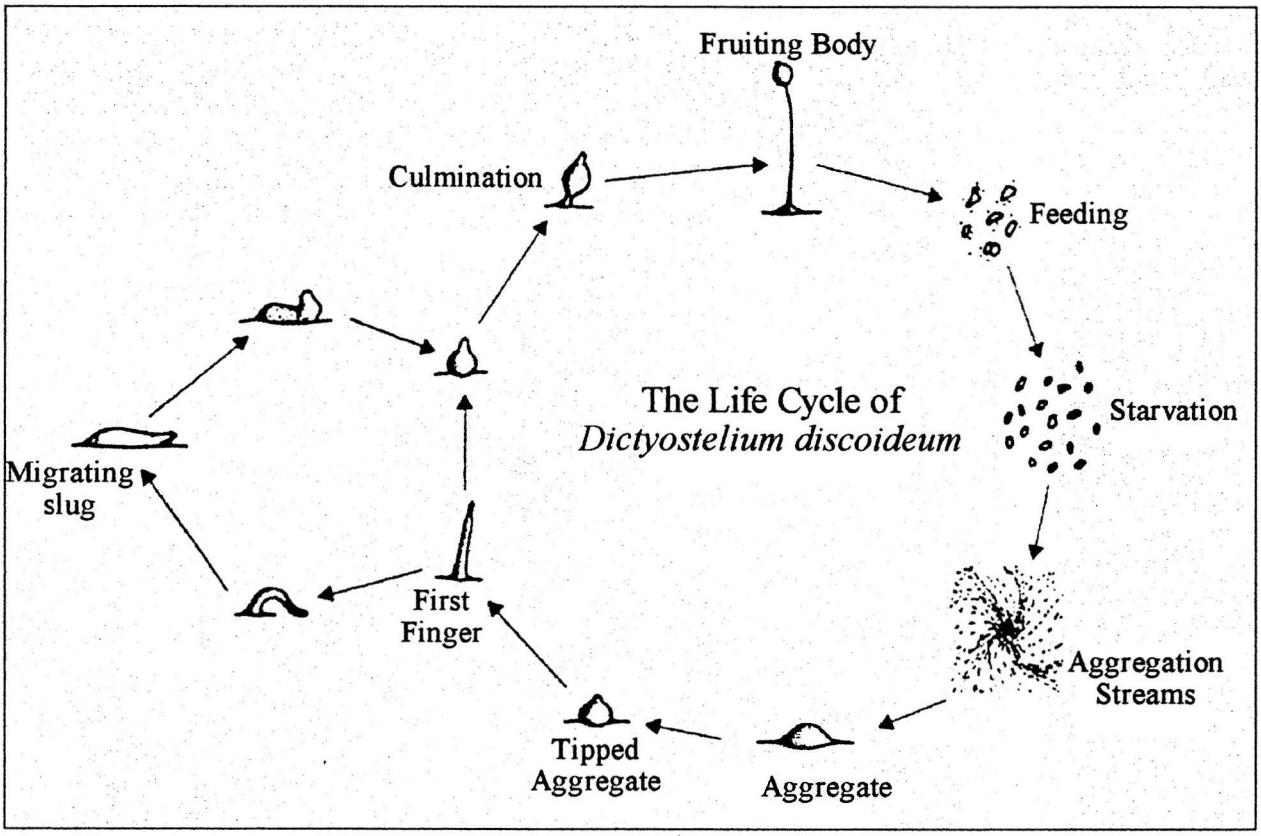

Figure 1: Development of *Dictyostelium discoideum* showing all stages of the life cycle. Diagram from Julian D. Gross (2).

that then release their own cAMP. The result is a periodic wave of cAMP, moving outward from the center of the aggregating mass. In addition to this chemical signal, cell density also plays a role in *Dictyostelium* development. Cells must be above a threshold density in order to respond to the cAMP wave (25).

Because SNF1 helps control the response of yeast to starvation and because *Dd snf1* is a highly-conserved homolog of the yeast gene, we hypothesized that *Dd snf1* also plays a role in the starvation response of *Dictyostelium*, i.e., entry into development. A better understanding of this gene's role in *D. discoideum*'s stress response may lead to a more complete understanding of the pathways involved in the stress response of more highly evolved organisms. For these reasons, we performed the following research to lay the groundwork to knowledge of the structure and function

of *Dd snf1*.

METHODOLOGY AND RESULTS

Construction of Genomic Mini-Library

I first attempted to create a *Dictyostelium* genomic mini-library. Dr. Greg Podgorski provided a 1.3 kb *Dd snf1* cDNA fragment. This fragment could be used to probe a genomic mini-library for the entire *Dd snf1* gene. The *Dd snf1* gene is approximately 4.1 kb in length and is flanked by BclI endonuclease sites. Several micrograms of BclI-cut *Dictyostelium* genomic DNA provided by Dr. Podgorski were run on a 1% agarose gel in parallel with a HindIII-cut lambda standard. After running the gel, I visualized the ethidium bromide-stained DNA on a UV light box and identified a portion of the DNA streak corresponding to sizes between 3.9 and 4.3 kb. This portion of the gel was excised. A hole twice the size of the excised block was made in another portion of the gel. The section of gel containing the DNA was then rotated horizontally 90 degrees and placed in the upper half of the hole. The lower half of the hole was then filled with low melting temperature (LMT) agarose. When the LMT agarose solidified, the gel was run for a few more minutes to move the DNA from the standard agarose into the LMT agarose. The gel was visualized again to ensure that the DNA was completely in the LMT agarose. The LMT agarose was then removed and trimmed of the borders that did not contain DNA. This procedure provided an easy method for obtaining size-selected DNA from standard agarose.

The size-selected DNA was then ligated into a BamHI-cut pBluescript II (KS)+ (pBS) vector using T4 DNA ligase. Three ligations were set up corresponding to nominal 3:1, 1:1, and 1:3 insert:vector ratios. A vector-only positive control was included along with a negative control that lacked ligase. All ligations were allowed to incubate overnight at 20°C.

Following incubation, the ligation mixtures were used to transform DH5α *E. coli* cells by

electroporation using 0.2 cm gap cuvettes according to instructions provided by BioRad, the manufacturer of the electroporator. All five ligation mixtures were transformed. A positive control using 0.1 ng of PBS and a negative control using only water were included in the transformations. After the cells were allowed to recover for one hour by shaking at 37°C in SOC medium, they were plated on petri dishes containing LB medium in agar with ampicillin added at a concentration of 100 μg/mL for selection of cells containing plasmids. X-Gal and IPTG were added for blue-white color selection of colonies containing plasmids with inserts. The plates were incubated at 37°C overnight. Following the incubation period, the plates were scored for blue and white colonies. As Table 1 indicates, the 1:3 insert:vector ratio yielded the highest proportion of white colonies to blue colonies.

Sample	DNA in Sample	Volume Plated	Colonies Blue	Colonies White
- control	0μL	200μL	--	5
+ control	1	1	--	259
- ligase	1	200	--	11
+ ligase	1	200	3	1
1:3	1	200	49	34
1:1	1	200	28	12
3:1	1	200	37	5

Table 1: Results of transformations into DH5α *E. coli* cells.

Although both the transformation efficiency and the ratio of white colonies to blue colonies were lower than desired, several colonies were examined for the presence of insert. This was accomplished by inoculating twelve tubes containing 3 ml of Terrific Broth with twelve white colonies from the 1:3 insert:vector ratio plate. In addition, three similar

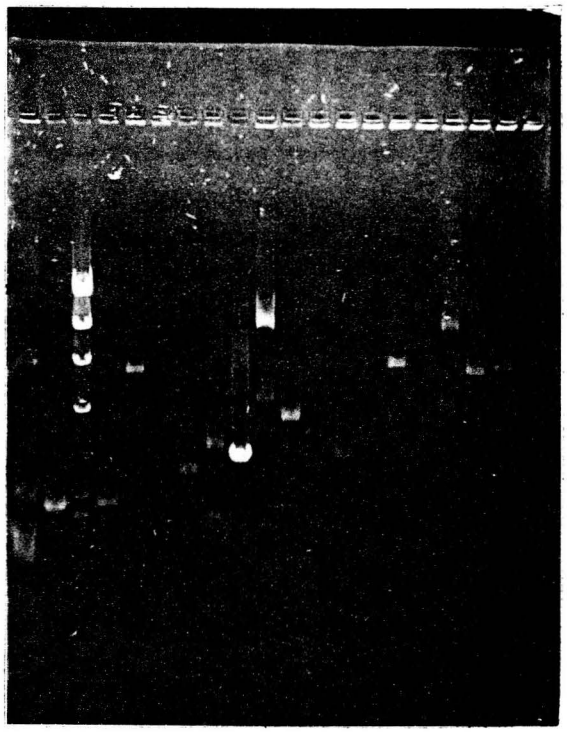

Figure 2: Results of gel electrophoresis.
Lane 1: Uncut 1:3 insert:vector ratio DNA
Lane 2: PstI-cut 1:3 ratio DNA
Lane 3: HindIII-cut λ standard
Lanes 4-8: PstI-cut 1:3 ratio DNA
Lane 9: Uncut pBSII vector
Lane 10: Uncut 1:3 ratio DNA
Lanes 11-16: PstI-cut 1:3 ratio DNA
Lane 17: Uncut 1:1 ratio DNA
Lanes 18-20: PstI-cut 1:1 ratio DNA

cultures were inoculated with three white colonies from the 1:1 insert:vector ratio plate. Cells were grown overnight with shaking at 37°C. Following incubation, the DNA was extracted from the cells using the rapid miniprep protocol provided by Dr. Greg Podgorski. After extraction, the DNA was cut with Pst1 to linearize the plasmid DNA and run on a 1% agarose gel. As Figure 2 indicates, only 3 out of nine lanes containing the 1:3 insert:vector ratio DNA contained insert. However, all three of the lanes containing the 1:1 insert:vector ratio DNA contained insert. To confirm this high percentage of plasmids with insert in the 1:1 ratio, twelve more white colonies from this ligation were inoculated into Terrific Broth and the DNA extraction procedure was repeated. All ten cultures that produced visible amounts of DNA contained insert.

Although all of the 1:1 insert:vector ratio colonies tested appeared to contain insert, the colonies on the plate were at such low density that the plates were unsuitable for use in probing for the *Dd snf1* gene. To solve this problem, another transformation was performed using the 1:1 ratio DNA. This time, however, the cells were plated at higher densities. One plate was inoculated with 200 μL of cells, one with 500 μL, and one with 1000 μL. After incubation the 500 μL plate gave an optimal number of colonies (about 2000) with acceptably low background of blue colonies.

The 1:1 ratio ligation was then scaled up 30-fold to produce the required amount of DNA and transformations were performed using this DNA. Instead of obtaining results similar to the previous transformations, however, I found extremely high colony density on each plate. In addition, no blue colonies were present and the colony morphology was different than on the previous plates. I therefore suspected a contaminating plasmid DNA was present in one of the reagents used in the transformation. To test this, several additional ligations and transformations were performed over the course of one month. The cuvettes, which are reused for electroporation, were found to be contaminated. This problem was corrected and another ligation was performed using the 1:1

insert:vector ratio.

Transformations using this ligation mixture did not produce colonies. I therefore hypothesized that the low melting temperature agarose containing the DNA was inhibiting the transformations. Therefore, I attempted several different methods of removing the DNA from the agarose. These procedures included a modification of the freeze-squeeze method, removal of the agarose by centrifuging through sterile fiberglass (a protocol borrowed from Dr. Ann Anderson's laboratory), and use of National Scientific's QuicKit. The QuicKit method yielded the best results.

Although the QuicKit method of removing the DNA from the gel gave the highest recovery of DNA, the transformation efficiency remained low. I prepared a new solution of ligase buffer, but this did not solve the problem. Dr. Podgorski speculated that there was a problem with the $MgCl_2$, the PEG, or both of these solutions used in the transformation. I therefore set up several ligations and transformations to pinpoint the problem. After repeating the procedure several times, I determined that our ligation buffer was faulty. I used the T4 ligase buffer from New England Biolabs and this buffer appeared to be superior.

In spite of using the new buffer, I was still unable to approach my initial favorable results. After many attempts to create the mini-library in this manner, I switched to a different method to obtain the *Dd snf1* genomic clone.

Attempt to clone *Dd snf1* using inverse PCR

As a result of my inability to create a size-selected genomic mini library, I turned to another technique for cloning the *Dd snf1*. This technique, called reverse PCR, has been used with some success in other labs (Dr. Greg Podgorski, personal communication). This technique involves the synthesis of oligonucleotides that prime DNA replication in the opposite direction of normal PCR.

Dr. Podgorski and I ordered primers from the Utah State University Biotechnology Center

based on the known portion of the *Dd snf1* gene. We then attempted the reverse PCR technique several times with various concentrations of DNA and reagents. The results of the procedure in every case were disappointing. Again, no significant results were obtained. After several attempts, this procedure was also abandoned.

The *Dd snf1* was isolated from a new genomic mini-library

Dr. Podgorski created a new size-selected genomic mini library and screened it for the *Dd snf1* gene using a ^{32}P-labeled *Dd snf1* cDNA fragment as a probe. In this manner, the *Dd snf1* gene was isolated. Nucleotide sequencing of the gene is currently underway and the available results confirm that a *Dictyostelium discoideum* homolog of the yeast SNF1 gene has been isolated.

Antisense as a test of gene function

Generating an antisense vector

In an attempt to assess the function of the *Dd snf1* gene product, I began to work in collaboration with Sandy Sung, a graduate student in Dr. Podgorski's lab, to create a regulatable antisense RNA. At first, we attempted to use a 1.3 kb cDNA fragment of the gene in a PVEII-MCS vector (provided by Dr. Podgorski). The PVEII-MCS vector contains the discoidin promoter, which is regulated by the concentration of folic acid in the medium. When folate is present in a growing culture, the promoter is off. When cells are starved and folate is removed from the medium, however, the promoter permits high levels of transcription of the genes under its control.

To assess the role of the *Dd snf1* gene product in *D. discoideum*, we attempted to insert the 1.3 kb cDNA fragment into PVEII-MCS in the antisense orientation. In principle, transforming this construct into *D. discoideum* cells should allow regulation of the *Dd snf1* mRNA to study gene function. By starving the cells and depleting the medium of folate, antisense RNA could be produced. This antisense RNA would then bind to any *Dd snf1* mRNA, inhibiting translation of the gene.

We attempted to place the 1.3 kb *Dd snf1* cDNA fragment in the antisense orientation directly downstream from the discoidin promoter of PVEII-MCS. We were unsuccessful.

Sandy Sung then used the same technique to insert a smaller 0.7 kb EcoRI-HincII fragment from the 5' end of the cDNA into the PVEII-MCS vector. Ms. Sung was able to accomplish this. She then transformed DH5α *E. coli* cells with the antisense construct, generating DH5α colonies containing the DNA in the antisense orientation. This was verified by extraction of the DNA from the cells, cutting the DNA with PstI, and running it on an agarose gel (Figure 3).

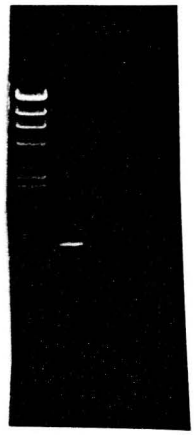

Figure 3: Results of gel electrophoresis showing a ~0.7 kb DNA band corresponding to the antisense cDNA

Transformation of *D. discoideum* with the antisense construct

I used the antisense construct created by Ms. Sung to transform *D. discoideum*. This necessitated the isolation of a large amount of DNA. This was done by growing a 1 L culture of the desired transformant in LB medium with 50 μg/mL ampicillin. I then extracted the DNA from the cells using a medium scale isolation procedure provided by Dr. Podgorski. I next grew *D. discoideum* cells to a density of ~$2x10^6$ cells/mL in HL5 medium. These cells were then transformed with the antisense construct using electroporation according to a protocol modified from procedures of the Devreotes and Kessin Laboratories. I am currently selecting these transformants based on resistance to the antibiotic G418.

DISCUSSION

The *Dd snf1* gene shares a high level of sequence homology with other SNF1/AMPK genes. This indicates that it is a member of this highly conserved family of genes that play an important role

in responses to stress. *Dictyostelium discoideum* responds to a major stress, starvation, by development to form a terminally differentiated fruiting body. We hypothesized that the *Dd snf1* gene product played a role in this process.

Although the results of this research are incomplete, this lab will continue to study the function of this gene. Knowledge of this gene's role in the stress response of *D. discoideum* may lead to a better understanding of the role of related genes in the stress responses of other organisms.

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