

## 1. Introduction

*Dictyostelium discoideum* is a simple eucaryotic slime mold that feeds on bacteria. When nutrients are abundant, the organism exists as a unicellular amoeba. If nutrients become limited, cells start to differentiate (Darnell, 1990). During differentiation, DNA synthesis and cell division cease. The individual amoeba stream toward an aggregation center in response to cAMP secretion. Mounds of several thousand cells begin to form after 8 h of starvation, but the cells retain their individual identities and do not fuse. At about 12 h, the “first finger” stage is visible. This structure possesses a discrete tip containing prestalk cells, that will develop into stalk cells at maturation. The finger-like structure then forms a slug, which is a motile, worm-like creature that migrates toward light or warmth. At 20 h, a second finger will form. Finally, true stalk and spore cells develop. The stalk cells elongate and vacuolate, pushing down through the mass of differentiating spore cells and hoisting the mass of spore cells up along the stalk. The stalk contains cells that are destined to die, while spore cells go on to form the next generation. Each spore is surrounded by a rigid spore coat, made of glycoproteins and polysaccharides that are secreted from the spore cells. The spores are metabolically inactive and can tolerate desiccation, ultraviolet light, and other harsh treatments. When supplied with water and nutrients, the spores “germinate” to form amoeba. The life cycle is complete in about 24 h.

An “alkaline phosphatase” (AP) activity accumulates during cell differentiation in *Dictyostelium* (Krivanek *et al.*, 1958; Armant *et al.*, 1982). The enzyme is a membrane-bound glycoprotein with its catalytic domain on the extracellular surface of the membrane (Armant *et al.*, 1980). The activity of this AP was originally assayed using the artificial substrate *p*-nitrophenyl phosphate (*p*NPP). Substrate specificity studies using a partially purified preparation showed a preference for 5'AMP, and, therefore, this activity was termed 5' nucleotidase (5NU). It was not clear, however, if AP (as measured with *p*NPP as substrate) and 5NU were the same protein. Armant *et al.* (1981) showed that both AP and 5NU copurified by DEAE sephadex, phenyl sepharose and concanavalin A chromatography. A molecular weight of 120 for both activities was determined by denaturing SDS gel electrophoreses (SDS-PAGE). Both AP and 5NU activity was inhibited by inorganic phosphate and ammonia. In addition, the ratio of the two activities remained constant at all steps of the purification procedure. Thus, several lines of evidence suggested that both activities resided in similar, if not identical proteins (Armant *et al.*, 1981). Additional studies from other laboratories on the activities of vegetative cells showed identical migration of both enzyme activities on non-denaturing gels containing Triton X-100 (Bhanot *et al.*, 1985). Also, a study of a *Dictyostelium* strain with a mutationally altered AP showed the alteration in the  $K_m$  and the stability of both enzymes (MacLeod *et al.*, 1979). This also supported the concept that the two activities were the product of a single gene (Bhanot *et al.*, 1985).

The AP activity of dialyzed amoeba cell membrane preparations is inhibited by the addition of concentrated dialyzate and this inhibition can be overcome by further dialysis (Mohan Das *et al.*, 1981). The AP from culminant extracts is also inhibited by concentrated dialyzate from amoeba cells but culminant dialyzate can not inhibit the AP

from amoeba cells. This suggested that there were inhibitors produced by amoeba cells and that the AP from this stage may not function *in vivo* unless the inhibitors are removed. When differentiation occurs, the inhibitor is thought to lose its function or is degraded (Mohan Das *et al.*, 1981, and 1984).

The properties of the 5NU were studied by Armant *et al.* (1982) using a purified preparation of the enzyme. The 5NU was found to have a pH optimum of pH 9.5, a  $K_m$  for *p*NPP of 1.8 mM and for 5'-AMP is 1.2 mM, respectively, and an isoelectric point of 4.5-4.9. The enzyme was also shown to be a zinc metalloprotein. High substrate concentrations inhibited the activity as did dithiothreitol, imidazole, and phosphate. The inhibition by phosphate is irreversible under conditions of high ionic strength and alkaline pH. It is thought that these conditions reversed the inhibition by a change in the conformation of the enzyme (Bhanot *et al.*, 1989). Furthermore, the enzyme extracted from developing cells was found to bind to a concanavalin A column less efficiently than the enzyme extracted from the amoeba stage. This suggested that extracellular glycosidases that are known to be secreted after cell growth ceases may remove a sugar moiety from 5NU and, therefore, alter the ability of the 5NU to bind to the concanavalin A column (Crean *et al.*, 1977). Temperature at 50°C was reported to inhibit the function of the AP from amoeba cells. However, the activity can be recovered at this temperature by binding the enzyme to concanavalin A (Bhanot *et al.*, 1988). Alkaline phosphatase was found to be enzymatic marker of pre-stalk cells. Acid phosphatase 2 (AP2), an isozyme of acid phosphatase 1 (AP1) that is expressed in amoeba only, was also found to be expressed only in pre-stalk cells. AP1 and AP2 differ with respect to  $K_m$  and thermostability. However, the result of mutation analysis suggested that both isozymes were encoded by one gene (Loomis *et al.*, 1984).

During *Dictyostelium* development, the levels of cAMP are controlled by a balance between synthesis by adenylate cyclase and degradation by cAMP phosphodiesterase (PDE). 5'AMP, the product of cAMP degradation is then removed from the morphogen signaling pathway by 5' nucleotidase (5NU). The 5NU activity appears during the time course of cell differentiation in *Dictyostelium* and also becomes restricted to a narrow band of cells that form the interface between the prestalk/prespore zones. If 5'AMP were allowed to accumulate, feedback inhibition of cAMP degradation and a loss of potential positional information required for appropriate cellular differentiation would occur.

Gene disruption is a valuable technique to study the functional significance of a gene. Because Northern blot analysis of 5NU showed the gene is developmentally regulated and that AP was expressed at all stages of development, gene disruption for both genes would help define the role of this enzyme(s). Gene disruptions of the genes encoding glycogen phosphorylase-1 (*gp-1*) and glycogen phosphorylase-2 (*gp-2*) were performed in Dr. Rutherford's laboratory. Normally, *gp-1* is highly expressed in early development then decreases during later development. Conversely, *gp-2* is not expressed at all at the early stages, is first detected after 8 h stage then is highly expressed in the late stages of development. The results of gene disruption showed that these genes are coregulated and the loss of *gp-2* would lead to the high expression of *gp-1* (Roger *et al.*,

1993). There are many examples of the use of gene disruption in *Dictyostelium*. Restriction enzyme mediated integration (REMI) was devised for non-targeted gene disruption. REMI requires that the single-stranded DNA overhang generated by the restriction enzyme used matches the overhang on the linearized vector (Kuspa *et al.*, 1995). The surprising result showed that the DNA fragment was often integrated into the restriction sites of the host genome (Kuspa *et al.*, 1992). This method does not target a specific gene but identifies potential regulatory genes. Homologous recombination of a modified gene is used for targeted mutagenesis (Kuspa *et al.*, 1994). For example, the cDNA sequence of one developmentally regulated kinesin-related motor proteins (K7) was constructed to contain an inserted *blastidicin* resistant gene. The construct was electroporated to *Dictyostelium* cells. Western blot analysis showed that no protein (K7) was expressed in K7 “knock out” cells, although the morphology of K7 null cells formed fingers, slug, and fruiting bodies with a normal appearance (de Hostos *et al.*, 1998). Disruption of the sporulation-specific gene (*spiA*) showed normal morphology, but *spiA* strains lose viability more rapidly than a wild type strain (Richardson *et al.*, 1992). In a report in which Adenylate cyclase G (*ACG*) was disrupted, spores were not able to germinate when placed in solutions with high osmolarity (van Es *et al.*, 1996).

In another targeted disruption, a *G418* resistant gene was inserted into the *coronin* gene. After transformation, disrupted sequence was confirmed by PCR with designed primers surrounding the disrupted site. *Cor*<sup>-</sup> mutants: (1) showed multinucleate cells, (2) migrated slowly but (3) oriented normally in a cAMP gradient. Also, the mutants showed defective cytokinesis (de Hostos *et al.*, 1993). Gene disruption of a putative serine/threonine protein kinase gene (*Ddpk3*) also showed the importance of this gene in the role of aggregation even though cAMP pulse-induced expression of aggregation stage-specific genes was normal (Mann *et al.*, 1991).

In the gene targeting technique, about 35% of the transformants show homologous recombination (Rivero *et al.*, 1998). Also, electroporation was found to be most effective in introducing foreign DNA into living AX3K cells. Fluorescein-labeled dextran with molecular weights of less than  $5 \times 10^5$  were able to pass through the nuclear membrane; then the pores close within 2.5 sec after the discharge (Yumura *et al.*, 1995). Blocking the 3' ends of transforming DNA with 2'-3' dideoxynucleotides was reported to reduce the frequency of end mediated DNA insertion in *Dictyostelium* amoeba. Moreover, end modification can reduce *in vivo* ligation of the cassette. *In vivo* end ligation can occur whether the ends introduced are sticky, blunt or even compatible (Shah-Mahoney *et al.*, 1997). Not only can this technique show the importance of a gene, it also can be used to show that substitution of one gene can overcome a defective gene. Rivero *et al.*, 1996, showed that numerous actin cross-linking proteins were able to substitute for some functions of the 34-kDa protein in the 34-kDa<sup>-</sup> cells.

The only reported mutagenesis of *AP* utilized N-methyl-N'-nitro-N-nitrosoguanidine (MacLoed *et al.*, 1979). The mutation was shown to affect the activity and substrate affinity of the enzyme. Because several physical properties of the enzyme from amoeba and developed cells were indistinguishable, it was concluded that there was only one form of the enzyme (MacLoed *et al.*, 1979). Using the modern procedures of

gene disruption should provide more definitive information on the function of this enzyme.

Because the unicellular and the developmental stages are clearly separated temporally, and because the transition between these two stages is reversible until the onset of terminal differentiation, the functions of developmentally regulated genes must play very important roles. As mentioned previously, the activity of 5NU is very low at the early stage of development then increases as development proceeds. In addition the activity is localized in a narrow band of cells separating the two cell types, perhaps indicating a role for the enzyme in formation of the two cell types. Therefore, I attempted to clone the gene for 5NU and study its expression. In this dissertation I will describe: (1) the purification of the 5NU protein, (2) the determination of partial protein sequence, and (3) attempts to amplify products from genomic DNA. I will then describe a clone of 5NU obtained from the *Dictyostelium* developmentally expressed cDNA project (Morio *et al.*, 1998).

## 2. Materials and methods

### 2.1. *Dictyostelium* cell maintenance

Lyophilized spore stocks were made by plating *Dictyostelium discoideum* amoeba (strain AX3K) at the exponential growth stage on 1% agar (in double distilled H<sub>2</sub>O) plates. The cells were incubated at 20° C until spore heads of *Dictyostelium* were formed. The plates were inverted and tapped gently to cause the spores to drop to the lid of the plate. The spore heads were washed off the lid using clean milliQ H<sub>2</sub>O and transferred into an eppendorf tube (one plate/tube). The tubes were placed in a speed vacuum to dry the spores, then stored at -80°C in a desiccator containing Drierite. When fresh cells were needed, a stock tube was removed from -80°C and put on ice. A sterile pipette tip was used to pick up the dry spores and transfer them into an eppendorf tube containing 1 ml PBS (1 mM KCl, 1 mM NaCl, 0.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 3.4 mM KH<sub>2</sub>PO<sub>4</sub> pH 6.5). The cell suspension was mixed with one loop full of B/r *Escherichia coli*. The bacteria- *Dictyostelium* mixture (250 µl) was plated onto each DM agar plate (3-4 plates were prepared). One plate was spread at a time. The plates were incubated at 20°C until the spore heads formed (about 4-7 days). The plates were taken to a laminar flow hood where a sterile loop needle was used to pick up the spore heads and transfer them to 1 ml of HL5 (60 mM glucose, 1% (w/v) Oxoid peptone, 0.5% (w/v) Oxoid yeast extract, 2 mM Na<sub>2</sub>HPO<sub>4</sub> and 3 mM KH<sub>2</sub>PO<sub>4</sub>). Spore heads from 2-3 loopfuls were transferred to the same well plate. The wells were incubated at 20°C until the spores germinated. When the amoeba cell concentration was 5×10<sup>6</sup> to 1×10<sup>7</sup> cells/ml, 1 ml of the cell solution was transferred to a 125 ml Erlenmeyer flask containing 40 ml HL5 media with 100 µg/ml ampicillin and 50 µg/ml streptomycin sulfate. The flask was shaken (130 rpm) at 20°C until the cell concentration was 5×10<sup>6</sup> cells/ml. Then, the cells were transferred into larger volumes if desired, or the cells were maintained by transferring about 1 ml to a fresh 125 ml flask.

The B/r *E. coli* were maintained by growing a single colony from a DM agar plate (1% (w/v) oxoid peptone, 1.4% (w/v) Difco bactoagar, 1.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 11 mM KH<sub>2</sub>PO<sub>4</sub> and 11 mM glucose) at 37°C for about 16 h in 3 ml LB liquid media ( 1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.17 M NaCl, adjusted to pH 7.5 with 1 mM NaOH) and shaking at 200 rpm. The plates were inverted and incubated at 37°C for 16 h. Once the bacteria had formed a lawn, the plates were stored at 4°C. New plates of a bacteria lawn from single colonies were made every 6-8 weeks.

### 2.2. Large scale cell culture and harvesting cells at the culmination stage

Log phase amoeba were inoculated into 12, 2-L flasks containing 500 ml HL5 media with 100 µg/ml ampicillin and 50 µg/ml streptomycin sulfate. Stock cultures of amoebae were diluted to a density of 10<sup>5</sup> cells/ml in the 2-L flasks. The flasks were shaken at 20°C at 130 rpm until the density again reached log phase (5×10<sup>6</sup> cells/ml). The cells were then transferred to 1-L plastic centrifuge bottles, pelleted by centrifugation at 1,500×g, 4 min and the supernatant removed. The pellet was washed twice with cold (4° C) milliQ H<sub>2</sub>O (approximately 700 ml H<sub>2</sub>O/bottle) to remove the HL5 media. The final pellet was resuspended in small volume of H<sub>2</sub>O and transferred to preweighed 50 ml Falcon tubes. After centrifugation, the supernatant was removed and the cell pellet was

weighed. Two volumes of MES LPS (7 mM MES buffer (pH 6.5), 20 mM KCl, 5 mM MgSO<sub>4</sub>, and 50 µg/ml streptomycin sulfate) was added to the pellet. A 3.5 ml sample of the cell suspension was then pipetted onto the surface of large 2% agar plates (150×15 mm, 100 ml agar in double distilled H<sub>2</sub>O). The cells were spread evenly by touching/tapping gently with a spreader. One plate was spread at a time so that the plates would not dry out. All plates were incubated upright at 20°C until the cells reached the culmination stage. Culminants from plates were removed by adding H<sub>2</sub>O to the surface of the agar then gently scraping the cells off. About 0.5 g wet weight of cells was recovered from each plate. Cells were transferred to a centrifuge tube and centrifuged at 1,500×g, 4 min. The cell pellets were transferred into a lyophilization jar. The jar was frozen and lyophilized until the cells were dry, then stored at -80°C in a dessicator until use.

### 2.3. Equilibrating resins to be used in protein purification

Ten milliliters packed volume of concanavalin A resin was added to a 2 cm ×20 cm glass column (Pharmacia Fine Chemicals). Buffer A (25 mM Tris HCl, pH 8.5) with 0.1% Triton X-100 and 50 mM α-methyl mannoside was pumped through the column at 1 ml/min for 30 min. The resin was then washed with buffer A containing 0.1% Triton X-100 at 1 ml/min for 1 h. In order to regenerate the resin, buffer A with 0.1% Triton X-100 and 1M NaCl was pumped over the column at 1 ml/min for 30 min, then the column was pumped with buffer A containing 1% Triton X-100 at 1ml/min for 1 h.

A Diethyl Aminoethyl (DEAE) HPLC column (0.8 cm ×10 cm, 5PW, Waters) was equilibrated with buffer A containing 2M NaCl at 1 ml/min for 30 min. The column was then washed with buffer A containing 1% Triton X-100 at 1 ml/min for 1 h. The conductivity of the solution eluting from the column was tested until no salt remained.

Before use, a 300SW gel filtration column (8.0 mm ×300 mm, 300 SW, Waters) was pumped with buffer A containing 0.1% Triton X-100 and 0.3 M NaCl until the conductivity of the eluant was the same as the solution being applied to the column (about 1 h). After use, the column was pumped with milli Q H<sub>2</sub>O at 0.3 ml/min for 3 h. The flow rate of the column was controlled so that the pressure was less than 150 psi.

### 2.4. Enzyme activity assay

A visual assay for the presence of enzyme activity was performed by mixing the protein extract to be assayed (50 µl) with 50 µl of reaction mix (10 mM *p*-nitrophenyl phosphate, 20 mM MgCl<sub>2</sub> and 100 mM diethanolamine pH 9.5) in the well of microtiter plate. A sample with active enzyme produced a yellow product. Quantitative assays were performed by Spectrometry at 420 nm. A protein sample of 50 µl was mixed with 500 µl of reaction mixture and transferred to a cuvette for measurement of absorbance. The volume of the fractions from the 300SW column were small (60 µl), so the activity of each fraction was assayed by adding 1 µl of the sample to 300 µl reaction mixture and then assayed spectrophotometrically as above.

$$\text{Total units of purified protein} = \frac{(\text{the rate of dA/min})(\text{total volume of sample})}{\text{the volume assayed}}$$

## **2.5. Alkaline phosphatase (AP) purification**

Culminant cells (2 g dry weight) were used as the starting material. This is about 30 plates (150× 15 mm) or 7, 2-L flasks containing 500 ml HL5 each. The dry cells were added to 100 ml 5 mM Tris HCl (pH 7.4) containing 8.6% sucrose and homogenized by a motor-driven Teflon pestle. The homogenate was centrifuged at 700×g for 30 sec to pellet debris. The supernatant was removed to a clean tube and centrifuged at 20,000×g, for 15 min. The resulting pellet was resuspended in 100 ml of the same buffer and centrifuged 20,000×g for 15 min. The pellet was resuspended in 50 ml buffer A containing 1% Triton X-100, homogenized, and centrifuged again at 20,000×g for 20 min. The supernatant was transferred to a clean tube and used immediately in the purification.

The sample was loaded to an equilibrated ConA column (as described before) at 1 ml/min. The flow-through material was collected in a beaker. After loading the entire sample, the column was pumped with buffer A containing 1% Triton X-100 until the absorbance reached baseline. Then, buffer A containing 0.1% Triton X-100 and 0.3 M galactose was pumped onto the column until a second absorbance peak reached baseline. This fraction was collected in another beaker. Buffer A containing 0.1% Triton X-100 and 50 mM  $\alpha$ -methyl mannoside was then pumped onto the column, and about 25, 2 min fractions were collected. Pooled active fractions from the Con A column (usually about 10 fractions, 20 ml total volume), were loaded on the DEAE column (Waters) at 1 ml/min, collecting 2 min fractions. After all of the sample was loaded, the column was pumped with buffer A containing 0.1% Triton X-100 at 1 ml/min for 45 min. The column was then eluted with a 45 min gradient to buffer A containing 0.1% Triton X-100 and 0.23 M NaCl. The flow-through and gradient fractions were then assayed. Active fractions were pooled, (usually about 5 fractions) concentrated with YM100 spin columns, at 1,000×g for about 30 min or until approximately 500  $\mu$ l remained. Concentrated samples from several YM100 columns were transferred into one YM100 column, then concentrated again. Buffer A containing 0.1% Triton X-100 and 0.3 M NaCl was added to bring the volume to 550  $\mu$ l. The sample was centrifuged in a microfuge at 14,000×g for 10 min and 500  $\mu$ l was removed and loaded on the 300SW column. The column was pumped with buffer A containing 0.1% Triton X-100 and 0.3 M NaCl at 0.3 ml/min, collecting 1 min 40 sec fractions (500  $\mu$ l). About 40 fractions were collected, then assayed spectrophotometrically. Normally, fraction # 15 through # 24 were assayed. Samples from the active fractions (20  $\mu$ l) were removed and mixed with 4.5  $\mu$ l of 5×SDS loading dye (0.3125 M Tris HCl, pH 6.8, 50%, (v/v), glycerol, 10% (w/v) SDS, 25% (v/v) 2-mercaptoethanol and 0.25% (w/v) bromophenol blue) and boiled for 5 min. The samples were then loaded on 5% or 7.5% polyacrylamide gels.

## **2.6. Non-denaturing protein gels**

The protein sample was mixed with 4.5  $\mu$ l of 5× Triton X-100 loading dye (0.3125 M Tris HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) Triton X-100, and 0.25% (w/v) bromophenol blue)(without boiling) and loaded onto the non-denaturing gel. The separating gel contained 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) Triton X-100, 5% Acrylamide/Bis (30% stock), 0.05% ammonium persulfate and 0.05% TEMED. The stacking gel (4% gel) contained 0.125 M Tris, pH 6.8, 0.1% (w/v) Triton X-100, 4%

Acrylamide/Bis, 0.05% ammonium persulfate and 0.1% TEMED. The electrode running buffer contained 0.2 M Glycine pH 8.3, 25 mM Tris base, and 0.1% (w/v) Triton X-100. No standard marker was used for a non-denaturing gel.

### **2.7. Activity stain after non-denaturing gel electrophoresis**

After electrophoresis, the gel was fixed with 10% acetic acid and shaken gently at RT a few seconds. The gel was washed with buffer A at RT for 30 min then incubated overnight at RT in a reaction mixture containing 0.1 M Tris-borate, pH 9.0, 2 mM MgCl<sub>2</sub>, 6%N-N'-dimethylformamide, 0.14 mg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP).

### **2.8. Coomassie blue staining**

Coomassie stain (50% methanol, 10% acetic acid and 1.25 mg/ml Coomassie brilliant blue R-250) was added to cover the gel. The gel was shaken gently at RT for at least 30 min, washed twice with double distilled H<sub>2</sub>O, then destained in 10% methanol containing 10% acetic acid at RT for 10-15 min. The destaining solution was changed until the bands on the gel were visible and the background was minimal. The gel was shaken gently with 10% glycerol for at least 10 min then transferred to a sealing bag.

### **2.9. Silver staining**

The gel was placed in solution 1 (50% methanol and 12% acetic acid) and shaken gently at RT for 15 min, then washed twice in solution 2 (10% ethanol and 5% acetic acid) for 4 min each. Solution 3 (3.4 mM potassium dichromate and 3.2 mM nitric acid) was added to the gel and shaken gently at RT in the dark for 5 min. The gel was washed twice for 1 min with double distilled H<sub>2</sub>O. Solution 4 (12 mM silver nitrate) was added, then shaken under light at RT for 15 min. The gel was rinsed twice with cold (4° C) solution 5 (0.28 M sodium carbonate with 0.0185 % formaldehyde), then soaked with solution 5 and shaken gently until the bands of protein appeared on a light background. Solution 6 (1% acetic acid) was added to the gel to stop development. Finally, the gel was stored in a sealed seal-a-meal bag and kept at RT.

### **2.10. AP activity assay in gel slices**

A polyacrylamide gel was divided into two halves after electrophoresis. The stacking gel was removed, one-half of the gel was stained with Coomassie blue. The remainder was tested for enzyme activity. Starting from the top, the gel was cut into thin horizontal slices by a razor blade. Each slice was placed in 100 µl milliQ H<sub>2</sub>O contained in a well of microtiter plate and incubated at 4° C for 30 min. The H<sub>2</sub>O was removed and 100 µl of reaction mixture containing pNPP was added to the well and incubated until a positive reaction was obtained. The position of the slice containing enzyme activity was compared to the location of protein bands from the Coomassie blue staining.

### **2.11. Trichloro acetic acid (TCA) precipitation of protein**

Trichloro acetic acid was added to the sample to a final concentration of 10% (v/v), vortexed, and incubated on ice for 30 min. The sample was then centrifuged at 10,000×g for 15 min. The pellet was resuspended in 100 µl cold (-20°C) acetone and vortexed. After centrifugation at 10,000×g for 15 min, the pellet was resuspended in cold

acetone and centrifuged again. The final pellet was air dried and resuspended in 50  $\mu$ l stacking buffer.

### **2.12. Electroblothing of purified protein**

After electrophoresis on 5% or 7.5% SDS gel, the gel was cut into a small area containing the protein. The gel was shaken in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer (pH 11) at 4° C for at least 5 min. A polyvinylidene difluoride (PVDF) membrane, the same size as the gel, was wetted with MeOH for 1 min. Then, the membrane was placed in a tray containing electroblotting buffer (1 $\times$ CAPS containing 10% MeOH) for at least 5 min. Filter paper was cut at the same size as the gel and soaked in electroblotting buffer. A transblotting sandwich was assembled according to BioRad catalog # 170-3930-3935 and electroblotted at a constant voltage of 50 volts (170 mA-100 mA) at 4°C for 1 h. After electroblotting, the PVDF membrane was removed from the transblotting sandwich and rinsed with double distilled H<sub>2</sub>O prior to the Amido Black Staining. The transferred gel was stained with Coomassie blue to determine the efficiency of transfer to the PVDF membrane.

### **2.13. Amido Black Staining**

A PVDF membrane was saturated with 100% MeOH for a few seconds, then stained with 0.1% amido black in 40% MeOH and 1% acetic acid. Protein bands appeared within one minute. The membrane was removed from the staining solution and destained by rinsing extensively with double distilled H<sub>2</sub>O or a solution containing 25% isopropanol and 10% acetic acid. The membrane was stored in a sealed seal-a-meal bag at RT.

### **2.14. Enzyme activity of AP transferred to a membrane (nitrocellulose or PVDF)**

The sample was loaded into two wells of a 5% SDS gel. After electrophoresis, the gel was cut into two halves. The first half was silver stained, while the second half was transferred onto a membrane by electroblotting. The membrane containing the transferred proteins was rinsed with a solution containing 100 mM Tris, pH 9.5, 100 mM NaCl and 5 mM MgCl<sub>2</sub>. The membrane was then soaked with the same solution containing 0.33 mg/ml Nitro Blue Tetrazolium (NBT) and 0.165 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). The membrane was incubated in a sealed seal-a-meal bag in the dark.

### **2.15. Preparation of protein samples for sequencing**

Samples to be prepared for N-terminal sequencing were electrophoresed on a 5% SDS gel, electroblotted to a PVDF membrane and amido black stained. The membrane was destained until the background was low enough to visualize stained protein bands, then rinsed 3 $\times$  with double distilled H<sub>2</sub>O to remove excess acetic acid. The band was excised from the membrane by a clean razor blade and transferred into a 1.5 ml eppendorf tube. Also, a blank region of membrane, approximately the same size as protein band, was cut and transferred into another 1.5 ml eppendorf tube to serve as a negative control. The PVDF-bound protein was air dried at RT and stored at -20°C. An Applied Biosystems Model 477A sequencer with on-line identification of phenylthiohydantions was used for protein sequencing.

Samples for Mass Spectrometry analysis were loaded to a 5% SDS separating gel and 4% stacking gel (20 ×22 cm). The gel was electrophored at 50 volts until the loading dye reached the boundary of the separating and stacking gel, then was changed to 200 volts for 4 h. The gel was fixed by soaking in the fixing solution (50% EtOH and 10% acetic acid) overnight. The gel was washed twice in the washing solution (50% MeOH and 5% acetic acid) for at least 30 min/wash, then, soaked in Coomassie blue stain (0.1% coomassie blue R in 45% MeOH, 10% acetic acid) at RT for 3 h to overnight. The gel was destained (50% MeOH and 5% acetic acid) until the bands were seen with minimum background. The gel was stored in 1-2 ml storage solution (5% acetic acid in milli Q H<sub>2</sub>O) in a polyester bag. The sample was sent to the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia for analysis.

### **2.16. Genomic DNA isolation for Southern Blotting**

Exponentially growing AX3K cells in 500 ml HL5 were separated into two aliquots of 250 ml each and then centrifuged at 1,500×g for 5 min. The pellet was resuspended in 30 ml TEN (10 mM Tris, pH 8.0, 10 mM EDTA and 10 mM NaCl) in a 50 ml Falcon tube and centrifuged at 1,500×g for 5 min. The pellet was once again resuspended thoroughly in 4 ml of TEN, 1 ml of 10% SDS was added then mixed gently by inversion, and incubated for 10 min at RT. The solution was recentrifuged as above and the supernatant was transferred to a fresh tube. An equal volume of Tris saturated (pH 7.6) phenol was added, and mixed by inversion. The emulsion was transferred to 30 ml borosilicate tubes (Corex) and centrifuged at 5,000×g for 10 min. The aqueous phase was transferred to a fresh borosilicate tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the aqueous solution, mixed, then centrifuged at 5,000×g for 10 min. The separation of the aqueous phase and phenol:chloroform:isoamyl alcohol extraction was repeated 2 additional times. The final aqueous phase was mixed with 3M sodium acetate (pH 5.2) to yield 0.3M final concentration. An equal volume of isopropanol was added to the solution, vortexed, and then incubated at RT for 1 h. After centrifugation at 10,000×g for 10 min, the pellet was washed with 10 ml 70% EtOH and centrifuged again at 10,000×g for 10 min. The pellet was air dried and resuspended in 1 ml TE buffer (10 mM Tris (pH8.0) and 1 mM EDTA). Ten microliters of 10 mg/ml RNase A was added and the solution was incubated at 37° C for 60 min. Phenol:chloroform:isoamyl alcohol extraction was performed, then the DNA was precipitated by adding 3M sodium acetate pH 5.2 to a 0.3M final concentration and an equal volume of isopropanol. The DNA pellet was finally resuspended in 1 ml TE buffer or milli Q H<sub>2</sub>O. Agarose gel electrophoresis or ethidium bromide fluorescent quantitation of the amount of double-stranded DNA (Sambrook *et al.*, 1989) was performed to quantitate the amount of DNA.

### **2.17. Genomic DNA isolation for PCR**

Two methods for genomic DNA isolation were used: (1) a column based method from Qiagen and (2) a procedure for a small amounts of *Dictyostelium* cells as described by Noegel *et al.* (1996). For the Qiagen method, 1× 10<sup>8</sup> amoeba were used as the starting material. The method followed is described in the QIAamp Blood kit, catalog number 29104, from Qiagen.

The method of Noegel *et al.* (1996) required AX3K amoeba (200  $\mu$ l of  $5 \times 10^6$  cells/ml). The cells were pelleted at  $2,200 \times g$  for 4 min, washed twice with 500  $\mu$ l cold ( $4^\circ\text{C}$ ) milli Q  $\text{H}_2\text{O}$ , then resuspended in 100  $\mu$ l PCR buffer (10 mM Tris (pH 9), 50 mM KCl, 0.1% Triton X-100, 0.5% NP-40 and 100  $\mu\text{g}/\text{ml}$  proteinase K). The sample was mixed by pipetting up and down twice, then incubated at  $56^\circ\text{C}$  for 45 min,  $95^\circ\text{C}$  for 10 min and chilled on ice. After centrifugation at  $14,000 \times g$  for 2 min, the supernatant was transferred into a new tube. The supernatant (15  $\mu$ l) was used for PCR analysis.

## 2.18. Assay of DNA levels:

### 2.18.1. Ethidium bromide fluorescent quantitation of the amount of DNA

Standard DNA concentrations of 1, 2, 3, 4 and 5  $\text{ng}/\mu\text{l}$  were prepared and stored at  $4^\circ\text{C}$ . The DNA standards and each sample (4  $\mu$ l) was mixed with 4  $\mu$ l of 1  $\mu\text{g}/\text{ml}$  EtBr, vortexed, then dotted on the glass surface of an Ultraviolet transilluminator. The concentration of the sample was determined by comparison to the intensity of standard DNA.

### 2.18.2. Agarose gel electrophoresis assay

Various amounts of sample were loaded onto a 1% agarose gel with 2  $\mu\text{g}$   $\lambda$ StyI as the marker. After electrophoresis, the intensity of sample band was compared to the intensity of the marker band, and the concentration of sample was estimated.

## 2.19. Southern Blot Analysis

In order to insure that the DNA was high molecular weight, 5  $\mu\text{g}$  of uncut DNA was electrophoresed on 1% (w/v) agarose gel (prepared with  $1 \times$ TBE buffer containing 50 mM Tris base (pH 8.5), 50 mM boric acid, 0.5 mM EDTA, or with  $1 \times$ TAE buffer containing 40 mM Tris acetate and 1 mM EDTA (pH 8.0)). A single band at approximately 6 kb indicated that the DNA could serve as a valid substrate for the Southern analysis. Five to ten micrograms of genomic DNA in each reaction and 8-10 different restriction enzymes were incubated overnight at  $37^\circ\text{C}$ . The digests were applied to a 0.5 cm thick 1% agarose gel with 2  $\mu\text{g}$  of standard DNA marker on the first lane of the gel (250  $\mu\text{g}$  of uncut Lambda DNA from New England Biolab was digested with 250 units of StyI in  $1 \times$  reaction buffer #3 (NEB) containing 1 mg/ml BSA at  $37^\circ\text{C}$  for at least 4 h). The reaction was terminated by heating at  $65-75^\circ\text{C}$  for 10 min. The digested DNA was mixed with  $1 \times$  agarose loading dye and aliquoted into small volumes of 200  $\text{ng}/\mu\text{l}$  and stored at  $4^\circ\text{C}$ . The gel was electrophoresed at 100-120 Volts until the bromophenol blue front was about  $3/4$  down the gel. The gel was then shaken in 0.2N HCl for 10 min. After washing the gel ten times with double distilled  $\text{H}_2\text{O}$ , denaturing solution (0.5 M NaOH and 1.5 M NaCl) was added, then shaken gently at RT for 30 min. Neutralizing solution (1 M Tris (pH 7.4) containing 3 M NaCl) was added and the gel was shaken for 30 min at RT. While the gel was shaking, hybond- $\text{N}^+$  or nitrocellulose membrane and 2 pieces of filter paper were cut to the same size as the gel. For capillary blotting, the membrane was wetted with milliQ  $\text{H}_2\text{O}$  a few seconds and then immersed in  $20 \times$ SSC (3 M NaCl and 0.3 M sodium citrate pH 7.0) for at least 5 min. For vacuum blotting, the

membrane was wetted with milliQ H<sub>2</sub>O a few seconds and then soaked with 10 ×SSC and 10 ×SSPE (1.5 M NaCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM EDTA pH 7.4).

### 2.20. Capillary Blotting

Capillary blotting was performed as described in Current Protocols in Molecular Biology. A platform was put over a tray that contained 1 l of 20 ×SSC. A long filter paper that would serve as a wick was soaked in 20 ×SSC and placed on the platform with the ends dipped into the liquid. A pipette was used to smooth out bubbles. The gel was placed on the filter paper wick with the back of the gel on top. The wet membrane was then placed on top of the gel. The cut filter papers were soaked in 20 ×SSC and placed on top of the membrane. The entire apparatus was covered with plastic wrap. The blot was set up to allow DNA to transfer about 12-16 h.

### 2.21. Vacuum Blotting

The blotting was performed by using the PosiBlot 30-30 Pressure Blotter and Pressure Control Station, as described in the instruction manual from Strategene, catalog # 400330- #400333, revision # 035001a.

After both capillary or vacuum blotting, the blot was UV crosslinked with a UV Stratalinker (model 2400 (100 V), from Strategene) for a few seconds to fix DNA to the membrane. The fixed blot was either used immediately for probe hybridization or the blot was wrapped with plastic wrap and aluminum foil and stored at -20° C until use.

### 2.22. Random Priming of a probe and Southern blotting

The DNA to be used as a probe (100 ng) (in milliQ H<sub>2</sub>O, 25 µl total volume) was boiled for 10 min, cooled in ice, then mixed with 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT, 0.2 mg/ml BSA, 6.25 A<sub>260</sub> units/ml primer “random”, pH 7.2 at 20°C, 0.5 mM deoxynucleotide mix containing no dATP, 50 µCi of α-<sup>32</sup>P dATP and 5 units of Klenow fragment. Alternatively, the reaction contained no dCTP, and 50 µCi of α-<sup>32</sup>P dCTP was used. The reaction was incubated at 37° C for at least 1 h or at RT for at least 4 h. MilliQ water was then added to bring the volume to 50 µl. A biospin 30 column was used to remove unincorporated nucleotide. After loading 50 µl of the probe to the center of the column, the column was centrifuged at 700×g for 4 min. The flow through containing the probe was transferred into a new tube. A one microliter sample was removed to scintillation fluid for determination of the level of radioactivity.

$$\text{total activity} = (\text{counts per minute (CPM)} / \mu\text{l from counting})(\text{total volume})$$

$$\text{specific activity}(\text{CPM} / \mu\text{g}) = \text{total activity} / \mu\text{g of starting DNA}$$

The probe should have a specific activity of at least 10<sup>9</sup> CPM/µg.

For Southern blotting the membrane containing the digested DNA was prewetted in 6×SSC. Then, 0.1 ml of prehybridization solution was added for each 1 cm<sup>2</sup> blot area. The prehybridization solution contained 6×SSC, 5×Denhart’s solution (2% Ficoll

400, 2% polyvinyl pyrrolidone and 2% BSA), 0.5% SDS, and 100 µg/ml herring/salmon sperm DNA (the stock of 5 mg/ml solution was prepared by dissolving 0.5 g herring/salmon sperm DNA in 100 ml milliQ H<sub>2</sub>O). The prehybridization/hybridization solution was prewarmed at 60°C for at least 1 h before the blot was added. The blot was incubated at 60°C for at least 1 h for a nitrocellulose membrane and at least 15 min for a Hybond-N<sup>+</sup> membrane. The labeled probe was boiled for 10 min, cooled in ice, and transferred to 0.1 ml hybridization solution (the same components as prehybridization solution, 1 ml/cm<sup>2</sup> blot area). The blot was hybridized at 60°C for about 16 h. After hybridization, the probe could be transferred to a clean tube and stored at -20°C for the next use. The blot was washed twice with an equal or double volume (compared to the blot area) of 2×SSC and 0.1%SDS (prewarmed for 5 min at 60°C). An equal (or double) volume of second wash solution (1×SSC and 0.1%SDS) was then added to the blot, washed twice at 60°C for 5 min, then rinsed briefly with 0.2×SSC and 0.2%SDS. The blot was dried briefly between 2 pieces of filter paper, then wrapped with plastic wrap and put into a cassette with Kodak X-OMAT AR film between 2 intensifying screens and stored at -80°C. The blot was not allowed to dry if it was to be reprobed.

### **2.23. 5'-end labeling of a probe with $\gamma$ -<sup>32</sup>P dATP (using synthetic oligonucleotides as probe)**

Ten picomoles of DNA was incubated with 70 mM Tris-HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 100 µCi  $\gamma$ -<sup>32</sup>P dATP, and 10 units T4 PNK at 37°C for 45 min. After incubation, the labeled probe was purified by a YM10 spin column and the specific activity was assayed with a scintillation counter.

### **2.24. Southern blotting with non-radioactive labeling probe**

The method used is described by “ECF random prime labeling and signal amplification system from Amersham Pharmacia Biotech”, catalog # RPN 5751 and RPN 5752.

### **2.25. Optimization of Mg<sup>2+</sup> concentration and temperature for PCR**

Each reaction contained 100 ng genomic DNA, 0.4 µM 5' primer, 0.4 µM 3' primer, 0.1 mM dNTP mix (pH 7.0), 50 mM KCl, 0.01% gelatin, 0.01% NP-40, 0.01% Triton X-100, 10 mM Tris-HCl (pH 8.3), and 1 unit of Taq DNA polymerase (40 µl). Various Mg<sup>2+</sup> concentrations (10 µl) of 0.50, 0.80, 1.00, 1.25, 1.50 and 2.00 mM (final concentration) were added to each reaction. A control reaction was performed containing all the ingredients except a template to test for the presence of primer dimers. The reactions were mixed well by pipetting up and down. If required, mineral oil (2 drops) was added on top of the solution. An optimum temperature for amplification was determined in a Robocycler Gradient 96 Temperature Cycler (Stratagene). The reaction was heated at 94°C for 2.5 min, followed by 34 cycles as follows: 94°C denaturing for 30 sec, various annealing temperatures (depending on the T<sub>m</sub> of each primer pair) for 30 sec, a 72°C extension for 3 min, and a final 10 min extension step at 72°C. After PCR, 20 µl of reaction was withdrawn and mixed with 3 µl of 10 × agarose loading dye and separated on a 1% agarose gel.

## 2.26. Preparation of PCR template DNA by the colony lysis procedure

A pipette tip or a sterile toothpick was used to lightly touch a bacterial colony then placed in 50  $\mu$ l colony lysis buffer (10 mM Tris at pH 8.0, 1 mM EDTA, and 0.1% Tween-20). After incubation at 95°C for 10 min, the sample was centrifuged at 14,000 $\times$ g for 2 min to pellet the cell debris. The supernatant was removed and 2-10  $\mu$ l were then immediately used for PCR analysis and the rest stored at -20°C. In addition to bacterial DNA, a small amount of *Dictyostelium* genomic DNA to be used for PCR analysis could be obtained using this method. Briefly, 10<sup>6</sup> cells of amoeba AX3K cells were centrifuged at 2,200 $\times$ g for 4 min. The cell pellet was washed twice with cold milli Q H<sub>2</sub>O and then treated as above for bacterial colonies.

## 2.27. RNA isolation

For amoeba RNA, 10<sup>9</sup> amoeba cells were transferred to a 1-L centrifuge bottle and pelleted at 2,000 $\times$ g for 3 min. The RNA isolation is described in the Rneasy Maxi Kit from Qiagen (catalog # 75161) or RNApure™ reagent, GeneHunter Corporation, (catalog # P501 or P502). In order to obtain RNA from developing cells for time course RNA, 2.5 $\times$ 10<sup>9</sup> amoebae were transferred to a 50 ml Falcon tube. The cells were pelleted at 2,000 $\times$ g for 3 min then washed twice with 10 ml MES LPS (7 mM MES (pH 6.5), 20 mM KCl, and 5 mM MgSO<sub>4</sub>). The cell pellet was then suspended in 1 $\times$ MES LPS at 400  $\mu$ l/10<sup>8</sup> cells. 400  $\mu$ l of the cell solution was plated on a 60 $\times$ 15 mm petri dish (Falcon) containing a filter patch (47 mm pure cellulose fiber filter pads, GelmanSciences) at the bottom, a filter membrane (0.45  $\mu$ m, 47 mm, grid, sterile membrane, GelmanSciences) on top, and 2 ml of MES LPS. The cells were spread evenly with a plastic spatula, then incubated at 20°C until the desired stage of development was reached. The cells were harvested by transferring a filter membrane containing the cells to the side of a plastic funnel that was applied to the side of a 50 ml Falcon tube. The cells were rinsed off the filter membrane with DEPC-treated H<sub>2</sub>O (0.002% (v/v) Diethyl Pyrocarbonate, DEPC, in double distilled H<sub>2</sub>O and autoclaved for 25 min). The cell solution was centrifuged at 2,000 $\times$ g for 3 min. The remainder of the RNA isolation was the same as described for amoeba RNA isolation. The concentration of RNA was calculated from the absorbance at 260 nm multiplied by 40 (1 absorbance unit = 40  $\mu$ g/mL RNA) and corrected for the dilution of the sample. The purity of RNA was determined from the ratio of absorbances at 260 and 280 nm; pure preparations have a ratio of 2.0. The RNA was aliquoted into small volumes and stored at -80°C. For Northern analysis, 5  $\mu$ g of RNA in 20  $\mu$ l DEPC-H<sub>2</sub>O was mixed with 3  $\mu$ l of 6 $\times$ agarose loading dye and separated on 1% agarose gels at 50-80 volts. After electrophoresis, two ribosomal bands of 17s and 26s should be visible under UV light.

## 2.28. Reverse Transcriptase-PCR (RT-PCR)

Five microliters of RNA (150 ng) in DEPC- H<sub>2</sub>O was transferred into two PCR tubes. One tube received a reaction containing reverse transcriptase while other reaction contained no reverse transcriptase. Five microliters of cocktail containing 2  $\mu$ M 3' PCR primer, 125 mM KCl, 0.025% gelatin, 0.025% NP-40, 0.025% Triton, 25 mM Tris-HCl (pH 8.3), 3.75 mM Mg<sup>2+</sup>, 0.5 mM dNTP, 20 units of RNasin, and 2.5 units of AMV RT was added to the RNA sample and mixed. The control tube contained all components

except AMV RT. The reaction was mixed by pipetting up and down, then incubated at 42°C for 1 h and 100°C for 10 min. The reaction was then cooled on ice for 5 min. PCR cocktail (40 µl) containing 31 mM KCl, 0.006% gelatin, 0.006% NP-40, 0.006% Triton X-100, 6.25 mM Mg<sup>2+</sup>, 0.5 µM 3' PCR primer, 1 µM 5' PCR primer, 2.5 units of Taq DNA polymerase was added to the template reaction. Final reaction contained 50 mM KCl, 0.01% gelatin, 0.01% NP-40, 0.01% Triton X-100, 1.5 mM Mg<sup>2+</sup>, 0.1 mM dNTP, 0.8 µM 3' PCR primer, 0.8 µM 5' PCR primer and 2.5 units of Taq DNA polymerase. The PCR program started with the denaturing temperature at 94°C for 2.5 min followed by 34 cycles of 94°C for 30 sec, various annealing temperatures for 30 sec and 72°C for 3 min followed by 72°C of extension for 10 min.

### **2.29. T-tailed pBlueScript Vector**

pBlueScript SK<sup>+</sup> (pBS) vector was purified with a Plasmid Maxi kit from Qiagen (catalog # 12163). The quantity of the plasmid was assayed by Spectrometry at 260 and 280 nm. The purity of DNA was determined from the ratio of absorbances at 260 and 280 nm (pure DNA is 1.8). Digestion of 5 µg pBS in 20 µl was performed in buffer #2 (NEB) containing 5 units of EcoRV restriction enzyme (NEB) at 37°C for at least 1 h. Then, phenol/chloroform/isoamyl alcohol and isopropanol precipitation was performed. Briefly, an equal volume of 25:24:1 phenol/ chloroform/isoamyl alcohol was added to the reaction. The sample was vortexed and centrifuged at 14,000 ×g for 15 min. The top aqueous phase was transferred into a new tube. An equal volume of chloroform was added to the solution and mixed. After centrifugation as before, the upper aqueous phase was transferred into a new tube. 1/10 Volume of 3 M sodium acetate at pH 6.3 was added to the sample and vortexed, followed by 2-3 volumes of isopropanol. After mixing, the sample was kept at -80°C for at least 5 min. The sample was centrifuged at 14,000×g for 15 min. The resulting pellet was washed with 80% EtOH and then dried by speed vacuum for about 5 min. The pellet was resuspended in 3 mM dTTP, 2 mM MgCl<sub>2</sub>, 5 units of Taq DNA polymerase, 50 mM KCl, 0.01% gelatin, 0.01% NP-40, 0.01% Triton X-100 and 10 mM Tris-HCl (pH 8.3). The reaction was incubated at 75°C overnight. The cut vector was stored at -20°C until used.

### **2.30. Test of self-ligation of cut vector**

Two ligation reactions were performed: a positive reaction containing 100 ng T-tailed pBS in 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM dATP, 25 µg/ml BSA, 50 mM Tris-HCl (pH 7.5) and 10 units of T4 DNA ligase in 10 µL; and a negative reaction contained only 100 ng T-tail pBS in 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM dATP, 25 µg/ml BSA, 50 mM Tris-HCl (pH 7.5) without ligase. The ligation reactions were incubated at 15°C overnight followed by isopropanol precipitation as above, resuspended in 10 µl milliQ H<sub>2</sub>O, and transformed into bacteria host cells as described below. The number of colonies from both reactions was compared.

### **2.31. Transformation of plasmid into bacterial host cells**

Ten microliters of the isopropanol precipitated ligation reaction was added to a gene pulser/*E. coli* pulser cuvette (0.1 cm electrode gap) from BioRad (catalog # 165-2089). Then 30 µl of electrocompetent cells (XLI blue bacteria, SURE cells, tetracycline resistant from Strategene) was transferred to the cuvette. The cuvette was tapped gently

and then electroporation was performed in a Gene Pulser (BioRad) with 960  $\mu\text{F}$  extender, 200  $\Omega$  controller, 25  $\mu\text{F}$  pulser, and at 1.8 volts. The time constant should be approximately 4.0. After electroporation, 1 ml SOC (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 9 mM NaCl, 2.5 mM KCl, 10 mM  $\text{MgCl}_2$ , and 20 mM glucose pH 7.0) was added to the electroporated cells and mixed by pipetting up and down. The solution was transferred into a small tube and shaken at 200 rpm at 37°C for 45 min. The cell solution was diluted five-fold with LB media and 100  $\mu\text{l}$  of the diluted cells were plated on LB agar plates (1.5% bacto agar in LB broth) containing 100  $\mu\text{g}/\text{ml}$  ampicillin, 10  $\mu\text{g}/\text{ml}$  tetracycline, 5 mg isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) and 1 mg 5-Bromo-4-Chloro-3-Indolyl- $\beta$ -D-galactopyranoside (X-gal). The plates were incubated at 37°C overnight and then at 4°C for about 4 h for blue-white color development. The plates were sealed with parafilm and stored at 4°C.

### 2.32. Electrocompetent Cells

XLI blue (*E coli*) SURE cells (tetracycline resistant, Strategene) were spread on LB agar plates containing 10  $\mu\text{g}/\text{ml}$  tetracycline. A sterile loop needle was used to pick up a single colony then transferred to a 250 ml Erlenmeyer flask containing 30 ml LB and 10  $\mu\text{g}/\text{ml}$  tetracycline and shaken at 200 rpm at 37°C overnight. Then, 10 ml of cell solution was transferred into 500 ml LB-tetracycline. The remainder of the solution was used to make glycerol stocks by adding 15% glycerol to the cell solution. The solution was mixed and aliquoted into a small volume and stored at -80°C. The 500 ml of the transferred cells were shaken at 37°C and 200 rpm until the absorbance at 600 nm reached 0.5-0.8 (about 4.5 h). The flask containing the cells was set on ice for 30 min and then the cells were transferred into two 1-L plastic centrifuge bottles. The cell solution was centrifuged in a GSA rotor at 5,000 $\times$ g for 10 min. The pellet was washed twice at 5000 $\times$ g for 5 min with 500 ml 10% sterile glycerol. The final pellet was resuspended in 3 ml cold GYT (10% glycerol, 0.125% w/v yeast extract, 0.25% tryptone) and mixed to resuspend. Forty-microliter aliquots were placed in eppendorf tubes, frozen by liquid nitrogen for a few seconds, and then stored at -80°C. One aliquot was used to determine the efficiency of the electrocompetent cells as follows. Intact pBS (100 ng) was transformed into the electrocompetent cells by electroporation as described above. After incubation at 37°C for 45 min, 100  $\mu\text{l}$  of undiluted,  $10^3$  and  $10^6$  diluted (in LB broth) were spread on each plate. The plates were incubated at 37°C overnight, and the efficiency of electrocompetent cells was determined by calculating colonies forming units/ $\mu\text{g}$  (CFU/ $\mu\text{g}$ ).

$$\text{CFU} = 10 (\# \text{ of colonies from one dilution})(\text{dilution factor})(\text{total sample volume in ml})$$

$$\text{CFU}/\mu\text{g} = (\text{volume of transformation in } \mu\text{l}/\mu\text{l} \text{ plated})(\text{CFU}/\mu\text{g} \text{ used for transformation})$$

The expected result should be  $10^9$ - $10^{10}$  CFU/ $\mu\text{g}$ , sometimes  $2 \times 10^{11}$  CFU/ $\mu\text{g}$ .

### 2.33. Minipreps of Plasmid DNA

PCR products were purified by the GeneClean III kit from Bio101, Inc., (catalog # 1001-600). The amount of DNA was quantitated by EtBr dot assay. The ligation with

various molar ratios of insert to vector was performed with T4 DNA ligase and followed by transformation (electroporation) as described above. A white colony was picked up with a sterile toothpick and transferred into 2 ml LB containing 100 µg/ml ampicillin (Amp) and 10 µg/ml tetracycline (Tet). About 10-15 white colonies were selected from each molar ratio ligation reaction. The cells were shaken at 37°C at 200 rpm overnight, then subjected to a boiling plasmid prep. Briefly, a small amount of the cell suspension was streaked on an LB plate containing proper ampicillin and tetracycline concentrations and incubated at 37°C until colonies were formed, then stored at 4 °C. The rest of the solution was transferred to a 2 ml eppendorf tube. A hole was made in the lid with a 25G1/2 needle. The solution was centrifuged at 14,000×g for 10 sec and the supernatant aspirated. 350 µl STET ( 8% w/v sucrose, 0.5% Triton X-100, 50 mM EDTA, pH 8.0, and 10 mM Tris-HCl, pH 8.0) was added to the sample and vortexed. Then 0.5 mg lysozyme in 10 mM Tris-HCl (pH 8.0) was added and vortexed. The mixture was boiled for 1 min and centrifuged at 14,000×g for 10 min. The bacteria pellet was removed from the supernatant by a sterile toothpick. The remaining supernatant was mixed with 25 µl equilibrated phenol, 40 µl 3 M sodium acetate (pH 6.3) and 460 µl isopropanol. The tube was vortexed after each addition. The final pellet was resuspended in 50 µl milliQ H<sub>2</sub>O, then five microliters of the sample were transferred into a new tube. Appropriate restriction enzymes were chosen to remove the insert from the vector. Digestion reaction was performed with 0.4 µg RNase A at 37°C for at least 1 h, then separated by agarose electrophoresis. After the right clone was determined, a glycerol stock was made.

### **2.34. Supercoiled sequencing**

Four micrograms of a clone from the boiling plasmid prep was resuspended in 5 µl milli Q H<sub>2</sub>O. The DNA was denatured in 2 µl denaturing solution (2N NaOH and 2 mM EDTA). The sample was vortexed, quick spun in a microfuge and incubated at RT for 5 min. Then, 7 µl of milli Q H<sub>2</sub>O and 6 µl 3M NaOAc, pH 5.2, was added to the sample and mixed. Isopropanol (75 µl) at RT was added and vortexed. The sample was incubated at -80°C for at least 10 min and spun in a microfuge at 14,000×g for 15 min. The pellet was washed with 80% EtOH and dried by inverting the tube. While inverted, a kimwipe was used to wipe the interior 2/3 of tube. Alternatively, the pellet was dried by a speed vacuum for 5 min or until EtOH had evaporated. The pellet was resuspended in 7 µl of milli Q H<sub>2</sub>O. Sequenase buffer from the Sequenase kit (USB) and 10 ng of T7 and/or M13 reverse primer was added to the DNA (final volume 10 µl). The reaction was vortexed, quick spun and incubated at 37°C for 20-30 min. A cocktail containing 17.5 mM Dithiothreitol (DTT), 0.175× dGTP labeling mix, 5 µCi <sup>35</sup>S-dATP, 2 µl of sequenase (7× dilution with glycerol dilution buffer) in 5.7 µl final volume was prepared (per one reaction). A microtiter dish (Fisher # 087728 dish, # 0877210 lid) was labeled with A,C,G and T across the top. The side of the dish was labeled with the DNA source and the type of primer. ddNTP (2.5 µl) was then added into the appropriate wells, then stored at 37°C until use. Cocktail (5.7 µl) was added to annealed DNA and primer after 20-30 min incubation. The sample was vortexed, quick spun and incubated at RT for 5 min. The prewarmed (37°C) microtiter dish was placed on a 37°C heat block. The lid

was removed, and 3.5  $\mu$ l of cocktail containing annealed DNA and primer was added to A,C,G and T wells, correctly labeled with respect to the DNA and primer source. The sample was mixed by pipetting up and down 2 $\times$  and incubated on a heat block for 5 min. Then, 4  $\mu$ l of stop reaction from USB kit was added and mixed by pipetting up and down twice. The reactions were either stored at -20°C or run on a sequencing gel immediately.

### **2.35. Preparation of DNA for sequencing**

A six percent marathon gel mix (6% 19:1 Acrylamide/Bis, 7 M urea, 1 $\times$ marathon TBE buffer, 0.045%, v/v, TEMED) was prepared. The solution was filtered and stored in a bottle wrapped with aluminum foil at 4°C. Marathon TBE buffer contained 1.35 M Tris base, 0.45 M boric acid, 25 mM EDTA disodium salt. Ninety milliliters of 6% marathon gel mix was mixed with 360  $\mu$ l 10% ammonium persulfate and poured between 2 well-scrubbed plates. The solidified gel was set up in the holding apparatus and 600 ml marathon TBE was poured into the top tank and 400 ml marathon buffer into the bottom tank. The sequencing gel was pre-run for 30 min at 1,800 V. The microtiter dish containing samples was heated to 80-90°C during the pre-run to denature the DNA and to reduce the volume. The samples were loaded into the wells and the gel was run at 1,800 V for 10 min until the sample had entered the gel. Two hundred milliliters of 3M NaOAc was added to the bottom tank, mixed by a syringe, then electrophoresis was continued at 1,800 V until bromophenol blue dye was at the bottom of the gel (approximately 2 h). The dried gel was exposed overnight at -80°C in an autorad cassette.

### **2.36. Northern Blot Analysis with radiolabelled probe (*without formamide solution*)**

Formaldehyde gels contained 1.2% agarose (in DEPC-treated H<sub>2</sub>O, 20 mM MOPS (pH 6.5-7.9), 8 mM sodium acetate, 1 mM EDTA, 2% (v/v) of 37% formaldehyde). The running buffer was 40 mM MOPS (pH 6.5-7.9), 10 mM sodium acetate, and 1 mM EDTA. Ten micrograms of RNA in 6.6  $\mu$ l DEPC-H<sub>2</sub>O was mixed with 23.6  $\mu$ l of 40 mM MOPS (pH 6.5-7.9), 10 mM sodium acetate, 1 mM EDTA, 6.7% (v/v) of 37% formaldehyde and 50% formamide. The RNA sample was heated at 55°C for 10 min, then mixed with 5  $\mu$ l formaldehyde loading dye (1 mM EDTA pH 8, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 50% (v/v) glycerol). The gel was electrophoresed at 50-60 V for 3-4 h. Half of the gel was incubated in two washes of 0.5 M ammonium acetate for 20 min each, then in 0.5 M ammonium acetate containing 0.5  $\mu$ g/ml EtBr for 45 min to overnight to stain the two ribosomal bands (17s and 26s). The other half of the gel was soaked with excess DEPC-H<sub>2</sub>O for 15 min with gentle agitation and then twice with excess 10 $\times$ SSC for 5 min. Vacuum blotting with 10 $\times$ SSC or 20 $\times$ SSC as transfer buffer and UV crosslinking were performed. After wetting with 6 $\times$ SSC briefly, the blot was prehybridized in 0.1 ml prehybridization solution/cm<sup>2</sup> blot area at 60°C for at least 15 min. The prehybridization solution contained 6 $\times$ SSC, 0.5% SDS, 5 $\times$  Denhardt's solution and 100  $\mu$ g/ml boiled herring sperm DNA. Then, the appropriate amount of probe (random priming labeling) was boiled for 5 min, chilled on ice and added to the same volume of hybridization solution. The blot was hybridized at 60°C overnight. The next day, the probe solution was transferred to a new tube and stored at -20°C for the next use. The blot was rinsed with prewarmed (60°C) 2 $\times$ SSC and

0.1% SDS briefly, then washed with the same solution for 15 min at 60°C. A wash solution of prewarmed 1×SSC containing 0.1% SDS was added to the blot and washed at 60°C for 30 min. Then the blot was rinsed briefly with prewarmed 0.2×SSC containing 0.2% SDS. The blot was dried briefly between 2 pieces of filter paper, wrapped with plastic wrap, and exposed to a film in an autoradiogram cassette.

### **2.37. Northern Blot Analysis (with formamide solution)**

The procedure was the same as the method without formamide solution except the prehybridization/hybridization solution contained 5 ×SSC, 5 ×Denhardt's solution, 50% (v/v) formamide, 1% (w/v) SDS and 100 µg/ml boiled herring sperm DNA. The prehybridization/ hybridization temperature was 42°C. The blot was washed with a double volume of wash solution/ cm<sup>2</sup> of blot area, then twice with 2 ×SSC and 0.1% SDS at RT, 5 min/wash with gentle agitation. Then, the blot was washed twice with 0.2 ×SSC and 0.1% SDS at RT, 5 min/wash with gentle agitation. If necessary, 2 additional washes using prewarmed (42°C) 0.2 ×SSC and 0.1% SDS were performed at 42°C, 15 min/wash. If further washing was required, two washes using prewarmed (68°C) 0.1 ×SSC and 0.1% SDS could be performed at 68°C, 15 min/wash. The blot was dried briefly between two pieces of filter paper and wrapped in plastic wrap and exposed to a film in a cassette.

### **2.38. Reprobing a nylon membrane (Northern blot membrane)**

A membrane that had been used previously in a Northern blot was washed three times in boiling milliQ H<sub>2</sub>O with gentle agitation for 15 min each. The membrane was scanned with a Geiger counter to determine if the first probe had been removed. If the membrane was still radioactive, it was shaken with boiled 0.1% SDS in double distilled H<sub>2</sub>O with gentle agitation for 15 min. Then the blot was dried briefly between 2 pieces of filter paper. The blot could be reprobed with the second probe immediately or wrapped with plastic wrap and stored at -20°C until use.

### **2.39. Knock out mutagenesis**

A pBSR 19 plasmid containing a *Blasticidin* resistant gene (BSR) flanked by an actin 15 promoter and an actin 8 terminator was obtained from William Loomis (University of California at San Diego). The plasmid was transformed into XLI blue bacteria and glycerol stock was made. The BSR cassette containing BSR and flanking actin promoter and terminator was excised from the plasmid by digestion with 1 unit/µg DNA *Bam*HI at 37°C for at least 1 h. After electrophoresis on 1×TAE agarose gel, the 1.3 kb band containing the BSR cassette was cut out of the gel, gene cleaned, and the amount of DNA determined by an ethidium dot assay. Also, the SSK273 plasmid containing the *5NU* cDNA was digested with *Bam*HI, then precipitated with 3M NaOAc and isopropanol. The *Bam*HI-digested SSK273 was treated with 1 unit of CIAP/µg DNA in the corresponding buffer. Ligation of BSR cassette/*Bam*HI as insert and SSK273 plasmid/*Bam*HI/CIAP as vector was performed with molar ratio of 12.5:1. Plasmid mini preps were performed and the miniprep DNA was digested with *Bam*HI. The presence of the insert was confirmed by PCR using CC1 (5' primer, 5' ATG GTG GAT TCT TGG

TGT A 3') and NU1R (3' primer, 5' TGA GTG GTT TTA TTA TTA TTA TCT 3'). Maxi plasmid preps of the clone containing the insert were prepared and glycerol stocks obtained.

#### 2.40. AX3K transformation with the blasticidin knockout construct

Thirty micrograms of SSK273 plasmid containing the BSR cassette and flanking 5NU sequences were digested with 3 units of *DraII* in buffer L (BMB) at 37°C for at least 1 h and precipitated with 3M NaOAc (pH 6.3) and isopropanol. The pellet was resuspended in milli Q H<sub>2</sub>O and redigested with 3 units of *SacII* in buffer #4 (NEB) at 37°C for at least 1 h and precipitated as above. The final pellet was resuspended in a small amount of milli Q H<sub>2</sub>O (50-100 µl). In order to examine the digested DNA, a microgram sample was mixed with 3 µl 10×agarose loading dye and loaded on 1% agarose gel with 2 µg  $\lambda$ *SlyI* as a standard marker.

A volume of 25-50 ml of AX3K amoeba cells ( $10^6$  to  $4 \times 10^6$  cells/ml) was centrifuged at  $2,200 \times g$  for 4 min. The cell pellet was resuspended in cold (4°C) electroporation buffer to obtain  $10^7$  cells/ml. The electroporation buffer contained 10 mM NaPO<sub>4</sub> pH 6.1 (0.1 M NaH<sub>2</sub>PO<sub>4</sub> was adjusted to pH 6.1 with 0.1 M Na<sub>2</sub>HPO<sub>4</sub>), and 50 mM sucrose (filter sterilized). A 0.4 cm cuvette was chilled on ice for at least 5 min. Cell solution (0.8 ml) was aliquoted in eppendorf tubes and kept on ice until 10-25 µg of the BSR construct was added. One tube contained only cells (no DNA) to serve as a control. The DNA/cell mix was then transferred to a chilled cuvette and electroporated at 3 mF, 2.5 KV/cm. The time constant ranged from 0.5 to 1.1 msec. After electroporation, 200 µl of electroporated cells were transferred into 10 ml HL5 media containing 100 µg/ml ampicillin and 50 µg/ml streptomycin sulfate in a tissue culture plate (100 × 20 mm polystyrene, Corning, catalog # 25025) and swirled gently. The plates were then incubated at 20°C for 24 h. Next, 50 µg blasticidin was added to each plate and swirled gently. Controls containing untransformed cells were cultured with and without blasticidin. The plates were incubated at 20°C until colonies of *Dictyostelium* amoebae were visible (3-5 days or longer). Each colony was circled (15-20 colonies/plate) under an inverted microscope at 40× magnification. The plate was tilted and a sterile pipette was used to remove HL5 media. Four ml of sterile 1×PBS was transferred into the plate. Under the microscope, a colony was picked up directly with a 20 µl pipette tip. The transferred colony was transferred immediately to 100 µl B/r *E. coli*. The *E. coli* was prepared from glycerol stock by thawing on ice, then removing to 3 ml LB by a sterile loop. The tube was shaken at 37°C, 250 rpm overnight. A sterile loop needle was dipped into the solution and streaked onto a DM agar plate to get single colonies. The plate was incubated at 37°C until colonies grew. A sterile loop was used to pick up a single colony and transferred to 3 ml LB. The plate containing the rest of colonies was parafilm and stored at 4°C. The tube was shaken at 37°C, 250 rpm overnight.

The *Dictyostelium* colony was mixed with *E. coli* gently and spread on a DM agar plate. This step was repeated until all colonies were selected. The plates were incubated at 20°C until clear plaques were observed as formed by *Dictyostelium* amoeba that had eaten the surrounding lawn of bacteria. Alternatively, pooled colonies were also

performed. In this procedure, after *Dictyostelium* colonies were visible, the plate was tilted and HL5 media was removed from the plate by a sterile pipette. Four ml of sterile 1×PBS was added to the plate. All plates were scraped with a sterile spatula and then transferred to a 50 Falcon tube. The tube was centrifuged at 1,500 xg for 4 min. The cell pellet was resuspended in 300 µl sterile 1×PBS and mixed gently. Various dilutions were made with sterile 1×PBS, then 100 µl from each dilution was mixed with 100 µl B/r *E. coli*. The solution from each dilution was spread onto a DM agar plate. The plates were incubated at 20°C until *Dictyostelium* amoeba plaques formed. A sterile loop needle was used to pick up amoeba cells from a plaque and then transfer them to a culture well (well diameter 16 mm, tissue culture treated, polystyrene, Corning, catalog # 25820) containing 2 ml HL5, 100 µg/ml ampicillin, and 50 µg/ml streptomycin sulfate. The culture plates were incubated at 20°C. At intervals during the incubation, fresh HL5 media containing appropriate antibiotics was added to a well to maintain 2 ml volume. When the cell concentration reached  $2 \times 10^6$  to  $4 \times 10^6$  cells/ml, the solution was divided into 3 samples. Two samples were transferred to 1 ml HL5 media containing ampicillin and streptomycin sulfate, one of which contained 50 µg blasticidin. Transformant DNA was isolated from the remaining sample according to Noegel *et al.* (1996). PCR analysis was performed to detect the disruption of the 5NU gene.

When the cells growing in the wells reached a concentration of  $1-2 \times 10^6$  cells/ml, a sample was transferred to 50 ml HL5 containing 100 µg/ml ampicillin and 50 µg/ml streptomycin sulfate in a 250 ml Erlenmeyer flask. The flasks were shaken at 130 rpm at 20°C until the concentration reached  $4 \times 10^6$  to  $5 \times 10^6$  cells/ml. The cells were plated onto a filter membrane, saturated with MES LPS as described earlier, and incubated at 20°C until slugs were formed. Then, Northern blot analysis with random priming of 5NU probe was performed.

### 3. Results

#### 3.1. Purification of 5NU

The method used to purify alkaline phosphatase and 5NU from *Dictyostelium* was adapted from Armant and Rutherford (1981). The protein was first purified from 2 g dry weight culminant cells as described in Materials and Methods. Fig. 1 shows the elution of 5NU from Concanavalin A affinity column by buffer A containing 0.1% Triton X-100 and 50 mM  $\alpha$ -methyl mannoside. Normally, active protein was found in fraction numbers 56-74 (fraction volume is 2 ml) when assayed with *p*NPP as substrate. The pooled active fractions were then applied to a DEAE HPLC column. Fig. 2 shows the elution of activity from this column when a gradient of buffer A containing 0.1% Triton X-100 and 0.3M NaCl was applied. Fraction numbers 60-72 were usually found to contain the active fractions. The pooled fractions from the DEAE HPLC column were pooled and concentrated then applied to a 300SW gel filtration column (Fig. 3). The protein eluted at a position corresponding to ~90 kDa; this differs from the 120 kDa 5NU that Armant and Rutherford (1981) purified. A sample of each of the fractions from the 300SW column was loaded on a denaturing gel (5% or 7.5% SDS gels were compared and the result showed that there were no differences in the results - data not shown). After silver staining of the SDS gel, three bands of 90, 120, and >120 kDa bands were seen. The intensity of the bands coincided with the enzyme activity found in the fractions (Fig. 4). In addition a fourth band at 45 kDa band was sometimes detected (not shown).

I next attempted to determine which of the bands from the SDS PAGE gel shown in Fig. 4 corresponded to 5NU protein. Therefore, the gel was analyzed for phosphatase activity as described in Materials and Methods. However no activity was observed. Although no enzyme activity was measured on SDS PAGE gels, clear bands of activity were obtained with non-denaturing gels (Fig. 5). This showed that the enzyme was active after electrophoresis in polyacrylamide gels. I next tested if electroblotting the protein onto a PVDF membrane after SDS PAGE would yield active enzyme. In order to test the efficiency of the transfer procedure, glycogen phosphorylase A and bovine serum albumin were also transferred to the membrane (Fig. 6). Although the standard proteins were transferred to the membrane, no protein was visible in the lane containing the purified 5NU (Fig. 6A, lane 6). In Fig. 7, a sample of an active fraction from the 300SW column was applied to SDS PAGE then electroblotted onto a nitrocellulose membrane and stained for activity with the reaction mixture containing NBT and BCIP. Commercial alkaline phosphatase from CalBiochem (calf intestine) and Sigma (hog intestinal mucosa) with various dilutions were also included as control activities. All sources of AP showed enzyme activity, although the commercial enzymes showed smeared bands. For the purified *Dictyostelium* protein (lane 7), the 45 kDa band was visible within 5 min of incubation. About 10 min later the 90 kDa band began to show activity; and after incubation overnight, the 120 and >120 kDa activity bands were visible (Fig. 7).

### 3.2. Attempts to concentrate the active 300SW fractions and perform preparative SDS PAGE.

In order to obtain a sufficient amount of protein for sequencing, the active fractions from the 300SW column were pooled, concentrated and separated by preparative SDS PAGE. However, when this was attempted, discrete bands were not visible, but instead a “smear” was observed. I then carried out a series of tests to determine the cause of the smearing. For example the effect of 5% SDS gels with and without 4% stacking gel was tested. Glycogen phosphorylase A and ovalbumin were used as controls. The results showed that the band smearing from the gel containing a 4% stacking gel was reduced (Fig. 8A) as compared to the gel without a stacking gel (Fig. 8B) but neither yielded clear separation of the bands from the pooled 300SW column (lane 8 and 9). Because smearing was observed only after concentration of the 300SW fractions, I next tested the effect of increased concentration of protein on banding. BSA at 1, 5 and 20  $\mu\text{g}$  was suspended in bufferA (no Triton X-100 added) and subjected to SDS PAGE (Fig. 9). Sharp bands were present, even to 20  $\mu\text{g}$  protein indicating that the amount of protein does not result in the smear observed with the concentrated column fractions. A similar test was performed with BSA except in this case the protein was dissolved in bufferA containing Triton X-100 and then concentrated prior to electrophoresis. Five micrograms of BSA was suspended in bufferA containing 0.3M NaCl and 0.1% Triton X-100. This is the same buffer conditions as in the 300SW fractions. A YM10 spin column and an Amicon stirred cell (YM10) were used to concentrate the protein solution. After electrophoresis and Coomassie blue stain, the smear from both concentrated samples were visible (Fig. 10). This result indicated that the protein smear was due to some component of the buffer used in the 300SW chromatography. To test if this component was Triton X-100, samples containing 5  $\mu\text{g}$  BSA in either 6 ml buffer A containing 0.3 M NaCl and increasing amounts of Triton X-100 (to 10%) were prepared and loaded on a 5% gel (Fig. 11). After electrophoresis and Coomassie blue staining, it was apparent that increased concentration of Triton X-100 resulted in the smear (Fig. 11, lane 5). Although the Triton X-100 concentration in the pooled column fractions was only 0.1%, the subsequent concentration of these fractions prior to preparative SDS PAGE could result in increased levels of Triton X-100. Therefore, the effect of concentrating 5  $\mu\text{g}$  BSA in 0.1% Triton X-100, followed by SDS PAGE was tested. As shown in Fig. 9, 5  $\mu\text{g}$  BSA produced a sharp band when dissolved in the absence of Triton X-100. Likewise, the presence of 0.1% Triton X-100 did not result in smearing if the sample was not concentrated (Fig. 11, lane 3). However in Fig. 12, 5  $\mu\text{g}$  BSA resulted in smearing when 0.1% Triton X-100 was concentrated prior to SDS PAGE. Therefore, I concluded that the smearing of concentrated 300SW fractions was due to Triton X-100 in the buffer.

In order to overcome the effect of Triton X-100 on preparative SDS PAGE, we tested a treatment designed to remove Triton X-100 from solution. Two samples containing 5  $\mu\text{g}$  BSA were suspended in 6 ml buffer A containing 0.3 M NaCl and 0.1% Triton X-100. The first sample was directly concentrated with a YM10 spin column and speed vacuum to yield a final volume of 20  $\mu\text{l}$ . The second sample was shaken with 0.1 g Bio-Beads SM-2 Adsorbent (BioRad) for 2 h. This represented a 1:10 (v/w) ratio of Triton X-100 and Bio-beads SM-2 Adsorbent. The supernatant was then concentrated as

above. Both samples were electrophoresed on a 5% SDS gel and Coomassie blue stained (Fig. 13). A single band of the concentrated protein was visible for the sample treated with Bio-Beads SM-2 Adsorbent (Fig. 13B) while smearing appeared for the sample that was not treated (Fig. 13A). I next tested the effect of removing Triton X-100 with Bio-Beads SM-2 Adsorbent on fractions from the 300SW gel filtration chromatography. Fig. 14 shows that Bio-Beads SM-2 Adsorbent treatment considerably improved the banding pattern of the concentrated fractions. Four bands of approximately 45, 90, 120, and >120 kDa bands were seen after Coomassie blue stain. Fig. 15 confirms the result that Bio-Beads SM-2 Adsorbent eliminated smearing of the concentrated protein. Twenty microliters of each active fraction from 300SW gel filtration and the concentrate of the pooled fractions after treated with biobeads were electrophoresed on a 5% SDS gel. After silver staining, three bands (90, 120 and >120 kDa) were seen clearly from the most active fractions (Fig. 15, lane 2-4) and from the pooled and concentrated fractions (lane 7).

### 3.3. Preparative SDS PAGE and protein sequence analysis

Three separate purifications of 5NU were pooled together and treated with Bio-Beads SM-2 Adsorbent at the ratio of 1:10 Triton X-100:Bio-Beads SM-2 Adsorbent. The samples were then subjected to preparative SDS PAGE and transferred to PVDF membrane as described in Materials and Methods. The 45 and 90 kDa bands were cut out of the PVDF membrane after electroblotting. The polypeptides were sequenced using an Applied Biosystems Model 477A Sequencer with on line identification of phenylthiohydrazones. N-terminal sequence was obtained from both bands, and internal sequence was obtained from the 90 kDa band. The N-terminal sequence for 90 kDa was QYSQTNEKIIVTGE. The N-terminal sequence for the 45 kDa band was MLVVKTNVYNSPGCTGGVNK. Also three short sequences from the 90 kDa band were obtained from Mass Spectrometric analysis, GYSQTNEKIIVTGEC\*K, GXDVNHDSENEFK and FKYEXPXNESXSVDXK. C\* designates carbamidomethyl modified C and X designates either I or L (Mass Spectrometric analysis can not distinguish between the two amino acids). Inspection of the 90 kDa N terminal sequence and one of the sequences from Mass Spectrometric analysis showed (90%) identity. Using these peptide sequences, no matching protein sequences were found in the databank.

### 3.4. Southern blot analysis with phos B3 and C3 oligonucleotides

PCR primers were designed from the derived DNA sequence above as in Table 1. Phos B3 and C3 oligonucleotides were used as probes for Southern blot analysis. Genomic DNA was digested with various restriction enzymes, separating on 1% agarose gel (Fig. 16, lane 1, standard lambda StyI marker; lane 2-9 contains 5 µg genomic DNA digested with 10 units of *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I and *Xho*I, respectively) and transferred to nitrocellulose membrane by either capillary or vacuum blottings. End-labeled probes of phos B3 and phos C3 with high specificity ( $3.4 \times 10^7$  and  $2.2 \times 10^7$  CPM/µg, respectively) were used as probes. Similar patterns of hybridization were observed in some lanes, indicating the original peptides were from the same protein.

### 3.5. Temporal expression of 5NU as measured by RT-PCR

Total RNA from 20 h of development was isolated as described in Materials and Methods. Two sharp ribosomal bands of 17s and 26s were seen on a 1% agarose gel indicating the lack of RNA degradation in this preparation (Fig. 17). RT-PCR of three previously studied genes was performed to assess the condition of this slug mRNA. The control genes included (1) *glycogen phosphorylase-2 (gp-2)* (5' GCA GGT TTA GGT AAT GGT GG 3' as the 5' primer and 5' TCA CCA TGG GAA ATT GAA CG 3' as 3' primer), (2) *glycogen synthase (gs)* (5' GAA GCA TCT AAA CGT GGT ATC 3' as 5' primer and 5' CAG AGT GGA TCA AAG ATG ACA GTT G 3' as 3' primer) and (3) *cyclic AMP phosphodiesterase inhibitor (PDI)* (5'GAT AAA TGC ACT AGC CCA G 3' as 5' primer and 5' GGT GGT TGG CCA TGT ATT AA 3' as 3' primer). These same primers were also used to amplify genomic DNA (200 ng) fragments to be used as a control. The expected PCR products of 200, 400 and 529 bp (as well as other bands) were obtained with slug RNA as template for *gp-2*, *gs* and *PDI*, respectively (Fig. 18, lane 2, 4 and 6). PCR products of 300, 500 and 629 bp were obtained with genomic DNA as template for *gp-2*, *gs* and *PDI*, respectively (Fig. 18, lane 3, 5 and 7). The increased size of the PCR products from genomic DNA represented the intron that was known to occur between the PCR primers. The results of the control amplifications showed the mRNA of the slug preparation was not degraded. Then, RT-PCR of amoeba RNA and slug RNA was performed using the 5NU phos B5 as 3' primer and phos N as 5' primer. A PCR product was obtained with slug RNA as template but not from amoeba RNA (Fig. 19). This was the first evidence that the 5NU gene is developmentally regulated (later in this dissertation, additional data will be presented from Northern blotting).

### 3.6. PCR amplification of genomic DNA with the degenerate primers

Genomic DNA was then used as template for amplification by the 5NU primer pairs, phos A31 and phos B51, phos A31 and phos B5, and phos C3 and phos B5. PCR fragments of 400, 600 and 800 bp PCR products were obtained respectively (Fig. 20). The PCR conditions for phos A31 and phos B51 were 94°C for 1 min, 40°C for 1 min and 72°C for 2 min, 1.5 mM MgCl<sub>2</sub> at final concentration (Fig. 20A). A 600 bp PCR product was obtained with primers phos A31 and phos B5; with the conditions of 0.6 mM Mg<sup>2+</sup> and 94°C for 1 min, 37°C for 1 min and 72°C for 2 min (Fig. 20B). An 800 bp PCR product was obtained with primers phos C3 and phos B5 with the conditions of 1.5 mM Mg<sup>2+</sup> and 94°C for 1 min, 44°C for 2 min and 72°C for 2 min (Fig. 20C). All amplifications were performed for 40 cycles. The bands were cut out of a 1% TAE agarose gel and gene cleaned. An ethidium bromide dot assay was used to determine the quantity of the products. The PCR products from genomic DNA amplification of 400, 600 and 800 bp were cloned into T-tailed pBlueScript vector with the insert and vector molar ratio of 1:1 (phos A31 and phos B51), 3:1 (phos C3 and phos B5; phos A31 and phos B5). The blunt end ligation reactions were performed at 15°C overnight. The plasmids were transformed into XL1 blue bacteria by electroporation at time constant of about 4.0. White colonies of transformants were selected, and after overnight growth subjected to boiling plasmid preps. The clones were cut with *Bam*HI and *Hind*III to confirm the insert sizes. Insert of 400, 600, and 800 bp were obtained (Fig. 21A, B and C).

### 3.7. Clones of 5NU and AP

A database search from the *Dictyostelium* developmental cDNA project at the institute of Biological Science, University of Tsukuba, Japan revealed a cDNA that matched the peptide sequences that I had obtained for 5NU, as well as a cDNA with high identity to alkaline phosphatases from other organisms (Fig. 22). An additional search of all known DNA sequence databases confirmed the previous data that the 5NU protein is unique. Five clones were obtained from the cDNA project. SLA872, and SSA425 contained the full and partial sequences of 1.8 and 0.8 kb of *AP*, respectively. SSK273, SSG581 and SSM754 contained the full and partial sequences of 1.9, 0.7 and 1.0 kb of 5NU, respectively. The plasmids were electroporated into XLI Blue bacteria at a time constant of about 4.0. White colonies of each clone were selected and boiling plasmid preps were performed after overnight growth. The clones were cut with *SalI* and *NotI* to release the inserts. After electrophoresis on 1% TAE agarose gel, the expected sizes of each cDNA were seen (Fig. 23). The 1.8 kb band of the SLA872 (*AP*) cDNA and the 1.9 kb band of the SSK273 (5NU) cDNA were cut out of the TAE gel and gene cleaned. The cDNA sequences were quantitated by Ethidium dot assay and 100 ng was fluorescein labeled and used as probes. For Southern analysis of the *AP* probe, two  $\mu$ g of genomic DNA was digested with 10 units of *ClaI* (lane 2 and 5, Fig. 24A), *EcoRI* (lane 3 and 6, Fig. 24A), and *HindIII* (lane 4 and 7, Fig. 24A). In the case of the 5NU probe, 2  $\mu$ g of genomic DNA was digested with 10 units of *RsaI* (lane 5, Fig. 25A), *Sau3A* (lane 6, Fig. 25A) and *SspI* (lane 7, Fig. 25A) and electrophoresed on 1% TBE agarose gel. After vacuum blotting onto nylon membrane, hybridization was performed as described in the Materials and Methods. After overnight exposure of both blots in a phosphoscreen, the pattern of hybridized bands from the *AP* and 5NU were seen (Fig. 24B and 25B, respectively). The results suggest that only one form of the gene is present for both *AP* and 5NU. Both the 5NU and *AP* clones were sequenced as described in Materials and Methods, and shown in Fig. 26 and 27.

The derived amino acid sequence of 5NU is shown in Fig. 28. An open reading frame exists starting at the ATG (+112) through position +1845. The ATG at position +112 is preceded by a stretch of 28 A's, a result that is characteristic of many of the start site of translation in *Dictyostelium* genes. However, it is curious that the N-terminal amino, according to the results of Edmund degradation analysis, occurs at the Glycine residue at position +239. It is possible that the real N-terminal amino acid was blocked and that the 239 glycine represents the start of a degradation product, although no evidence for this is available. Three of the peptide sequences from Mass Spectrometric analysis and one from Edmund degradation were found in the derived protein sequence, thus confirming the identity of this cDNA clone as the 5NU protein. These sequences are underlined in Fig. 28. The sequence GYSQTNEKIIVTGE was found by both Mass Spectrometric and Edmund degradation. The derived amino acid sequence and this peptide sequence is identical over the 14 amino acids. The sequence FKYELPINESLSVVDIK was identical to the derived sequence except that the position of the I and N were reversed. The sequence GXDVNHDSVEFK was identical in 10 of the 12 amino acids.

The derived protein has a molecular weight of 63,423 Daltons, with 39 strongly basic amino acids, 49 strongly acidic amino acids, 165 hydrophobic amino acids, and 262 polar amino acids, and an isoelectric point of 4.869. It should be noted that the estimated size of the 5NU protein from SDS PAGE analysis was estimatedly 90,000 Daltons, a result considerably different from that estimated by translation of the cDNA clone.

In addition to cDNA clones for 5NU and AP, the cDNA library also contained a clone that matched the N-terminal sequence of the 45 kDa polypeptide that was purified from the Con A affinity column. The protein derived from this cDNA has a molecular weight of 26,152 Da. As in the case of 5NU, the size of the polypeptide as estimated from SDS PAGE is considerably larger than that derived from the cDNA. The reasons for this difference in apparent size is not obvious. However, I have observed that the presence of concentrated solutions of Triton X-100 caused severe smearing of the bands on SDS gels, and it is possible that these preparations do not migrate in the gel according to their true size. It is clear however, that the 45 kDa peptide is not a degradation product of the 90 kDa peptide, because the sequence of their cDNAs is not similar. Searches of Gene Bank did not reveal any proteins with high sequence similarity to the 45 kDa sequence. I conclude that the 45 kDa peptide is a unique *Dictyostelium* protein with “alkaline phosphatase” activity.

### **3.8. Expression of 5NU and AP during *Dictyostelium* development**

In order to determine if the RNA preparations used to measure the levels of 5NU and AP were not degraded, the *Glycogen phosphorylase-2 (gp-2)* gene was used as a control. This gene has been studied in Dr. Rutherford's lab and is known to be developmentally regulated (Brickey *et al.*, 1990). Genomic DNA was amplified by two *gp-2* primers; 5' GCA GGT TTA GGT AAT GGT GG 3' as 5' primer and 5' TCA CCA TGG GAA AAT GAA CG 3' as 3' primer. The 300 bp *gp-2* product was separated on 1% TAE agarose gel (Fig. 29). The band was cut out of the gel, gene cleaned and quantitated by EtBr dot assay. For Northern analysis, total RNA was isolated by the method described by GenHunter (Nashville, TN) at 4 h intervals during the time course of development. Ten micrograms of RNA from each time point was separated on a 1.2% formaldehyde gel, then stained with EtBr. Two bands of 17s and 26s rRNA were observed, indicating the lack of RNA degradation (Fig. 30A). After vacuum blotting of the RNA from each time point onto a nylon membrane, the blot was hybridized with radiolabeled *gp-2* probe as described in Materials and Methods. As expected, a hybridized band was seen from RNA of 12, 16 and 20 h of development (Fig. 30B) (Rutherford *et al.*, 1992). The result confirmed that the *gp-2* gene is developmentally regulated, and that the RNA preparation could be used in a valid Northern analysis.

The fragment encoding 5NU was then radiolabelled by random priming and used as a probe for Northern analysis. RNA was extracted at four hour intervals over the time course of development as described above. Again, the extent of RNA degradation from each stage was determined by the visible bands of 17s and 26s rRNA. Equal amounts of RNA from each stage (as determined spectrometrically) was loaded on the gel and verified by the intensity of the bands on 1.2% formaldehyde gel after staining with EtBr (Fig. 31A). Fig. 31B shows 5NU mRNA was present after 8 h of development. No

expression of this gene is apparent at the early stages of development or in undifferentiated cells. Thus the *5NU* is developmentally regulated.

The same blot that was first hybridized with the *5NU* gene was rehybridized with an *actin* probe after the first probe was removed. *Actin* is known to be an abundant gene that is expressed at all stages of development of *Dictyostelium*. In order to obtain the *actin* probe, genomic DNA was amplified by using 5' GGA CGG TGA AGA TGT TCA AG 3' as the 5' primer and 5' CTC CCA CAC TGT ACC AAT CT 3' as the 3' primer. The 500 bp PCR product was seen on a 1%TAE agarose gel (Fig. 32). The band was cut out of the gel, gene cleaned and quantitated by EtBr dot assay. The *actin* PCR product was random primed and hybridized to the blot. After overnight exposure of the blot, the hybridized band of *actin* was shown in RNA from all stages of development confirming that mRNA was present at all stages on the blot (Fig. 33). In order to determine the exact timing of the *5NU* expression, RNA from 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 h of development was isolated by GenHunter method. Five micrograms and 10 µg of RNA from each stage, as determined by absorbance at 260 and 280 nm, were electrophoresed on a 1% agarose gel and 1.2% formaldehyde gel. Two bands of 17s and 26s rRNA were seen from both gels (Fig. 34A and 34B, respectively). After vacuum blotting, the membrane was hybridized with the random primed *5NU* probe. After overnight exposure, a band was visible beginning at 5 h of development and increasing in intensity with time of development (Fig. 34C). The *actin* probe was used again to confirm that mRNA was present in each lane on the blot. The hybridized band of the *actin* was seen from RNA from all stages (Fig. 35).

The expression of an additional developmentally regulated gene was determined, *Transcription Factor II (TFII)*, in order to insure the quality of the RNA preparation. Fig. 36 shows that the expression of *TFII* is developmentally regulated (as previously described, Warty *et al.* unpublished results). Expression of *TFII* could be observed only after 8 h of development.

A Northern analysis of *AP* expression was performed with this same RNA preparation from 4 h time points of development. The 17s and 26s rRNA bands were seen on 1.2% formaldehyde gel (Fig. 37A). After vacuum blotting of RNA onto a nylon membrane and hybridizing with *AP* probe, 4 hybridized bands were present at all stages of development of *Dictyostelium* (Fig. 37B). The two top bands are at the same Rf as the two rRNA bands of 17s and 26s and may indicate the probe binds to rRNA under these hybridization conditions. The two lower bands may represent two forms of *AP* mRNA. More experiments will be performed in the future to study the expression of the *AP* gene. The result indicated that the expression of *AP* is found at all stages of development of *Dictyostelium*. After the *AP* probe was removed, the same blot was hybridized again with *actin* probe to determine the condition of RNA. The expression of this gene was observed at all stages of development of *Dictyostelium* as expected (Fig. 38).

### 3.9. Gene disruption of the *5NU* with *blasticidin resistant gene*

In order to determine the functional significance of the *5NU* to *Dictyostelium* development, I attempted to disrupt the endogenous gene by targeted homologous recombination. A *blasticidin* resistant gene (BSR) was inserted into the internal sequence of the *5NU* gene. pBSR19 is a plasmid containing a cassette of BSR flanked with an actin 15 promoter and an actin 8 terminator in pGEM3 vector (Fig. 39). In order to obtain a high yield of pBSR19, a maxiprep was performed by using a Qiagen plasmid maxi kit. One microgram of pBSR19 (as determined by absorbance at 260 and 280 nm) was digested with 10 units of *Bam*HI at 37°C for at least 1 h. After electrophoresis on an 1% TAE agarose gel, the 1.4 kb band of the BSR cassette was seen (Fig. 40). The pBSR19 without addition of a restriction enzyme was used as a control. The 1.4 kb band of BSR cassette was cut out of the gel, gene cleaned and quantitated by EtBr dot assay. Also SSK273, which is the plasmid containing *5NU* cDNA in pBS vector, was digested with *Bam*HI to provide compatible ends with BSR cassette. Fig. 41, lane 2 shows the 1.9 kb band of undigested *5NU* and the 414 and 1,507 bp bands of *Bam*HI digested *5NU* cDNA in lane 3 and 4. The result showed that *Bam*HI cuts the *5NU* cDNA only once. The digested SSK273 plasmid and the BSR cassette were mixed and ligated with a molar ratio of 12.5:1, insert:vector overnight at 15°C. The ligation reaction was then electroporated into XLI blue bacteria at a time constant of 4.0. White colonies were selected and boiling plasmid prep was performed. Each miniprep was digested with *Bam*HI to release the BSR cassette. The 1.4 kb band was present in one of the clones (Fig. 42, lane 5). Maxipreps and glycerol stocks of this clone were obtained.

In order to screen the *Dictyostelium* transformants for insertion of the BSR within the *5NU* gene, I used a PCR based approach. Several primers were designed on either side of the BSR insertion site. 5' primers are CC1 (5' ATG GTG GAT TCT TGG TGT A 3'), CC3 (5' CAA AAT GTG GTT ATA GTC AAA 3') and R1 (5' TCA AAT CCA GAA ACA AAC AAA ACA TCA A 3'). 3' primers are CC2 (5' TTC TAT CCA CCA AGT TTT T 3'), NU1R (5' AGA TAA TAA TAA AAC CAC TCA 3') and K1 (5' TGT TAC TAT GAA GCT GGT GTT GAA ATT T 3'). Fig. 43 shows the position of each primer and the *Bam*HI restriction site in the *5NU* cDNA sequence. Optimum conditions for the PCR reaction of each pair of primers was determined. Genomic DNA (100 ng) was used as template. Fig. 44 shows the determination of optimum conditions for primer pair CC1 and CC2. A 270 bp product was amplified. Conditions for optimum amplification of the 1,187 bp of PCR product from CC1 and K1 primers are shown in Fig. 45. Conditions were also determined for CC1 and NU1R primers (Fig. 46, top, 409 bp) and R1 and NU1R primers (Fig. 46, bottom, 209 bp).

I next tested if the primers could be used to detect the presence of the BSR cassette within the *5NU* cDNA. The SSK273 plasmid containing the BSR cassette (100 ng) was used as a template and the optimum condition for PCR amplification using CC1 and CC2 as primers was determined. The correct product of 1,646 bp was obtained in reaction mixtures containing 2.0 mM Mg<sup>2+</sup> at final concentration with various temperature from 47-58°C (Fig. 47). Also CC3 and NU1R primers were used to amplify the SSK273 plasmid containing the BSR cassette (200 ng). The conditions for the PCR reaction were in 0.5, 0.8, 1, 1.25 and 1.5 mM Mg<sup>2+</sup> and the reaction was amplified by

94°C for 2.5 min, the 34 cycles of 94°C for 30 sec, 42.4°C for 30 sec and 72°C for 3 min and followed by the extension at 72°C for 10 min. After electrophoresis, a 1,748 bp product was obtained with maximum amplification at 0.5 mM Mg<sup>2+</sup> (Fig. 48). These results confirmed the restriction enzyme digestion, that the BSR cassette was inserted correctly into the *5NU* cDNA.

In order to screen potential knockout clones, I used a method described by Noegel *et al.* (1996) to isolate small amounts of DNA for PCR amplifications. Genomic DNA from non-transformed AX3K amoeba cells of *Dictyostelium* was isolated by this method and used as a control. CC1: K1, and R1: K1 primers were used to determine the condition of the genomic DNA. A PCR product of 1,187 bp was obtained with CC1 and K1 primers, using 100 ng genomic DNA as a template. The PCR reaction was performed in 2 mM Mg<sup>2+</sup> with 94°C for 2.5 min, followed by 34 cycles of 94°C for 30 sec, 48°C for 30 sec and 72°C for 3 min with the extension of 72°C for 10 min (Fig. 49, lane 2). A PCR product of 1,032 bp was obtained from the R1 and K1 primers as expected. The PCR reaction was performed in 1.25 mM Mg<sup>2+</sup> with 94°C for 2.5 min, followed by 34 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 3 min with the extension of 72°C for 10 min (Fig. 49, lane 3). Also CC1 and K1 primers were used to detect the BSR cassette inserted into the SSK273 plasmid (Fig. 50). The PCR conditions were 2mM Mg<sup>2+</sup>, amplified at 94°C for 2.5 min, 34 cycles of 94°C for 30 sec, 48°C for 30 sec and 72°C for 3 min and followed by extension at 72°C for 10 min. After electrophoresis, a product of the correct size of 2,563 bp was obtained (Fig. 50). These results showed that this method could be used to screen genomic DNA of clones for insertion of the knockout cassette.

### **3.10. Transformation of disrupted *5NU* into the *Dictyostelium* genome**

The SSK273 plasmid containing the BSR cassette was digested with *Dra*II and *Sac*II to release the BSR cassette flanking *5NU* sequence. The digested plasmid was treated with calf intestinal alkaline phosphatase to prevent self-ligation. The sample was separated on a 1% TAE agarose gel yielding the 2.9 kb pBS vector, and the 3.3 kb BSR cassette flanking the *5NU* cDNA (Fig. 51). The upper band was removed, gene cleaned, and transformed into *Dictyostelium* amoeba. The cells were subjected to blasticidin selection as described in Materials and Methods. Resistant colonies were present after 5-6 days. Genomic DNA of transformants that were resistant to blasticidin was isolated by the method described by Noegel *et al.* (1996), then subjected to PCR amplification with various primers (Fig. 52). The results show that the PCR fragments obtained were of a size expected for the endogenous *5NU* gene. Therefore, the *5NU* gene was not disrupted, and the BSR cassette had been inserted in a random site. To test further for “knockout” of the endogenous gene by the BSR insertion, I performed Northern analysis of the transformants. RNA from transformants and non-transformed cells at 20 h of development was prepared and hybridized using *5NU* cDNA as the probe (Fig. 53). The results show that the size of the mRNA from blasticidin resistant clones, and that from untransformed cells was identical. This result confirms the PCR analysis, and again shows the random integration of the BSR cassette into the *Dictyostelium* genome.

### 3.11. Construction of the *AP* knockout vector with the *blasticidin resistant gene*

In order to disrupt the *AP* gene, the *blasticidin* resistant gene (BSR) was inserted into the internal sequence of the *AP* gene. The SLA872 which is the plasmid containing *AP* cDNA in pSPORT1 vector was double digested with *Nsi* and *MunI* restriction enzymes. Then, treated with calf intestinal alkaline phosphatase to prevent self-ligation. After EtOH precipitation, the sample was applied to agarose gel electrophoresis, and the cut form of the SLA872 plasmid was obtained (Fig. 54). In order to provide compatible ends of BSR cassette with the SLA872 plasmid digested with *Nsi* and *MunI*, the pBSR19 was double digested with *PstI* and *EcoRI*. After electrophoresis, the about 1.3 kb band of BSR cassette was seen (Fig. 55). The expected bands of digested SLA872 plasmid and BSR cassette were cut out of the 1%TAE agarose gel, gene cleaned and quantitated by EtBr dot assay. Then, ligation of SLA872 plasmid containing the *AP* cDNA with *Nsi* and *MunI* ends and BSR cassette with *EcoRI* and *PstI* ends were performed. A ligation reaction with the molar ratio of 4:1; insert:vector was performed at 15°C overnight and then electroporated into XLI blue bacteria with a time constant of 4.0. White colonies were selected and boiling plasmid preps were prepared. Each miniprep was digested with *Sall* (cut twice on a clone containing BSR cassette) and *NotI* (cut once on a clone containing BSR cassette) to release the BSR cassette and flanking *AP* cDNA. Three bands of about 850, 400 and 4,109 bp were obtained (Fig. 56, lane 2). The same miniprep was redigested with *KpnI* to confirm the insertion of the BSR cassette into the SLA872 plasmid. Fig. 56 (lane2) shows the expected 870 bp fragment from *KpnI* digestion.

In order to screen possible transformants, primers surrounding the *Nsi* and *MunI* restriction sites in the *AP* cDNA were designed. 5' primers are AP1F (5' ACA AAT AAT TCT ACT TCT TCA AAA 3'), SLA872 R1 (5' GTG ACT GTT GTG TTA TTG ATT GTG TTT T 3'), AP2F (5' TGG TCG TCA CAA CTC GTA TCT CT 3') and SLA872 R2 (5' TCG ATG TGA TAT TGG GTG GTG GTA AAC 3'); 3' primers are AP1R (5' CAC ATT TAC ATT TAG ACC CAT ATA 3'), AP2R (5' TAT TGG GTG GTG GTA AAC 3') and SLA 872 K1 (5' TAG TTA GAG AAT GGG CAG AGA AGG ATG G 3'). Fig. 57 shows the position of each primer and the *Nsi* and *MunI* restriction sites on the *AP* cDNA sequence. Optimum conditions for PCR reactions of each pair of primers was performed. Genomic DNA (100 ng) was used as template. A PCR product of 490 bp was amplified using AP1F and AP1R as primers (Fig. 58). Likewise the expected 870 bp product was obtained from SLA872 R1 and SLA872 K1 primers using genomic DNA as template (Fig. 59). The SLA872 plasmid containing the BSR cassette was then used as a template. The PCR conditions were 1, 1.25 and 1.5 mM Mg<sup>2+</sup>, amplified at 94°C for 2.5 min, 34 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 3 min and followed by extension at 72°C for 10 min. The expected 1,511 bp PCR product was obtained after electrophoresis (Fig. 56, lane 4-6).

The SLA872 knockout construct was digested with *SacI* and *NotI* to release the BSR cassette and flanking *AP* sequence. *Dictyostelium* cells were subjected to transformation as described above for the *5NU* construct. No blasticidin resistant colonies were detected. As with the *5NU* knockout attempts, either the mutation is lethal or the mutagenesis was not successful for technical reasons.

