DEVELOPMENT OF A STRATEGY FOR ISOLATING GENES INVOLVED IN THE Gα4-MEDIATED SIGNAL TRANSDUCTION PATHWAY OF *DICTYOSTELIUM DISCOIDEUM*

By

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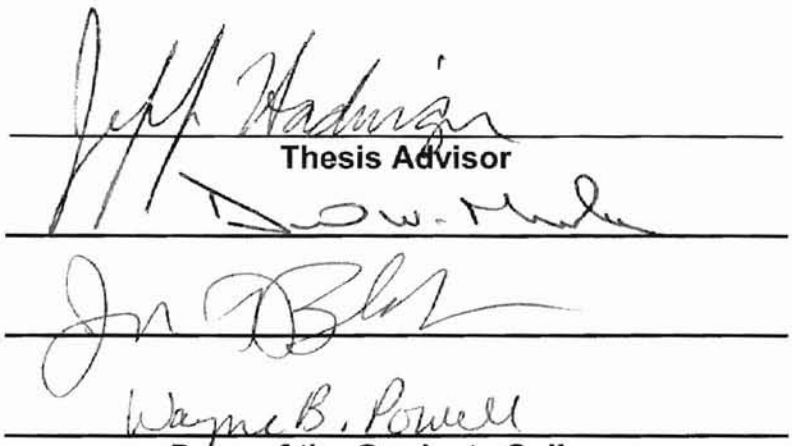
Thesis Approved:



Thesis Advisor

Dean of the Graduate College

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NOMENCLATURE

Bs^R	Blasticidin-resistant
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DNA	deoxyribonucleic acid
FA	folic acid
FAR	folic acid receptor
Gα4	G-protein #4 in *Dictyostelium discoideum*
$G\alpha4^{HC}$	Gα4 high-copy construct in WT *Dictyostelium* background
$g\alpha4^-$	Insertional knockout mutant of Gα4
GDP	guanosine diphosphate
GTP	guanosine triphosphate
ORF	open reading frame
REMI	Restriction Enzyme-Mediated Integration
WT	wild-type

CHAPTER 1

INTRODUCTION

An Introduction to G-proteins

G-proteins make up an important class of signal transduction proteins in eukaryotes. These have been discovered in most eukaryotic species surveyed, including humans. This ubiquitous class of signal transduction molecules has been shown to be involved in many processes, including sensory perception, chemotaxis, control of multicellular development, and hormonal regulation [1, 2, 3, 4, 5]. In addition, several motifs of G-proteins are highly conserved [6], making the use of model systems attractive for the study of these systems.

A Model of G-protein Function

G-proteins are heterotrimeric, having α,β and γ subunits. The β and γ subunits contain binding sites to each other and to the α subunit. The α subunit binds the β and γ subunits, GTP or GDP, a cell surface receptor, and intracellular effector(s). When a ligand binds to the receptor, a ligand-receptor-GαGDP-G$\beta\gamma$ complex forms. GTP then replaces GDP, and the complex dissociates into ligand, receptor, G$\beta\gamma$ dimer, and GαGTP. A GαGTP-intracellular effector complex then associates, a phosphate is cleaved from GTP, and the effector is activated and dissociates from GαGDP. The recycled GαGDP then associates with a G$\beta\gamma$ dimer to complete the cycle [1, 2, 3, 4, 5]. These steps are illustrated in Figure 1.

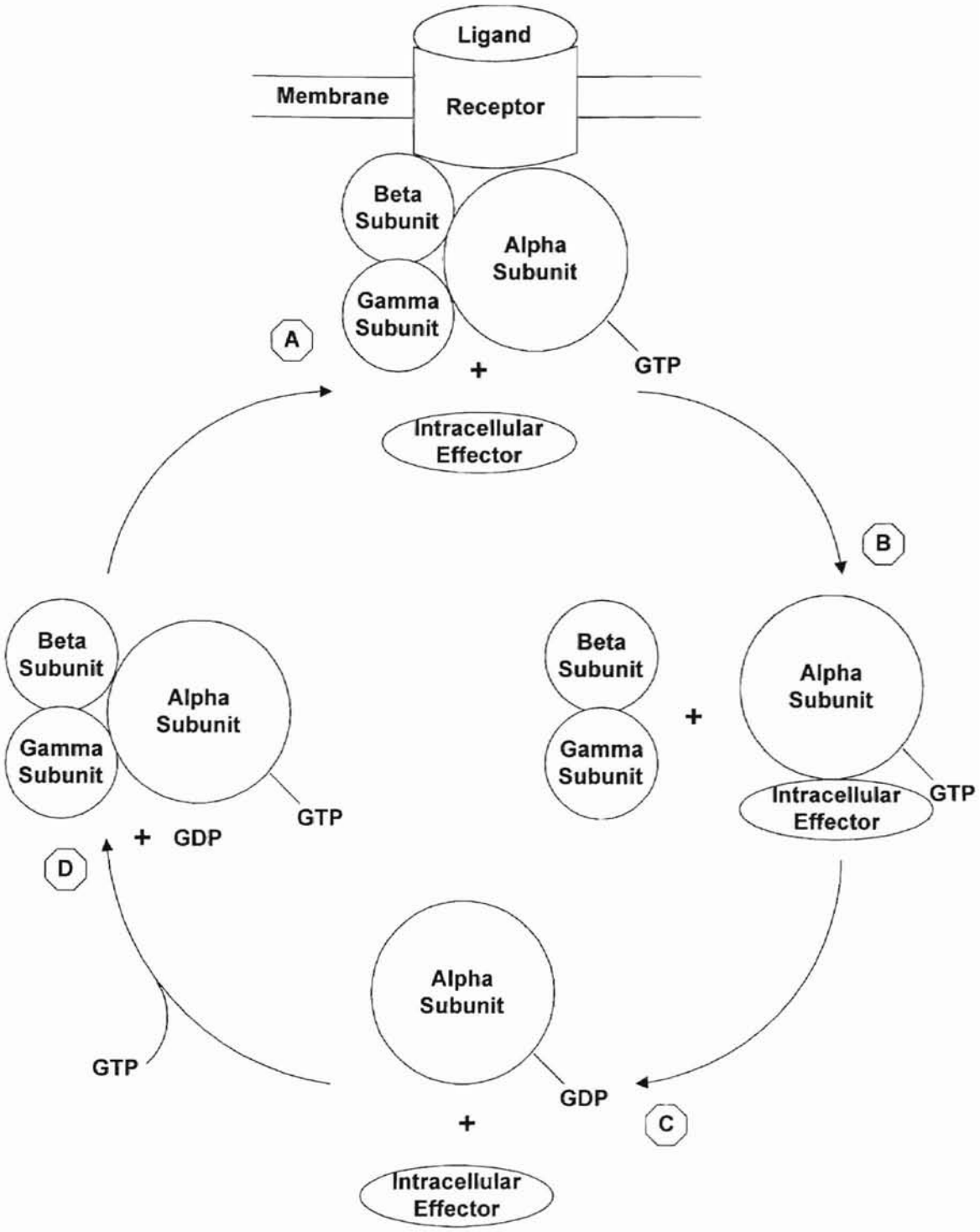

Figure 1. Model of G-protein function

G-proteins in humans

G-proteins have been shown to be essential for many functions in the human body. The sense of vision is mediated by at least two G-proteins, labeled $T_r\alpha$ (rod transducin) and $T_c\alpha$ (cone transducin) [7]. These receive signals from cell surface light receptors, then activate intracellular second messenger systems which prompt the discharge of electrical signals down the optic nerve.

Cell division control is also mediated in some instances by G-proteins. Mutations in the Gαs gene have been associated with growth hormone-secreting tumors in humans. Some research suggests that a subset of these tumors that have a constitutively active adenylyl cyclase also contain oncogenic mutations in the Gαs gene [8]. These mutations alter the intrinsic GTPase activity of Gαs, thereby allowing constitutive adenylyl cyclase activity, which in turn leads to uncontrolled cell division.

Leukocyte and macrophage migration is also mediated by receptor-linked G-protein-mediated signal transduction mechanisms [9,10]. Research has shown that the responses to the chemoattractants lysophosphatidic acid and platelet activating factor are mediated by G-protein mechanisms.

Dictyostelium discoideum as a Model System

Dictyostelium has many attributes that make it a good model system for the study of G-proteins and for comparison to the human system. Eight G-proteins have been found in *Dictyostelium* [6, 11, 12, 13]. Many similarities exist between these G-proteins and those of humans. Strains containing knockout

mutations in or overexpression constructs of some of these G-proteins exhibit developmental aberrations [14, 15, 16]. This suggests that some G-proteins are involved in developmental control and cell fate determination in this organism. All of these are believed to require a seven- transmembrane helix receptor protein in order to function, just as their human counterparts. In addition, the chemotactic movement of *Dictyostelium* amoebae is mediated by G-proteins, and is very similar to that of human macrophages, leukocytes and neutrophils [6, 10]. Some of the stimulants that elicit these responses are also common between humans and *Dictyostelium*, including platelet activating factor and lysophosphatidic acid [17. 18].

In addition to the fact that eight G-proteins are currently known in this organism, *Dictyostelium* displays a simple life cycle that includes aggregation of individual amoebae into multicellular fruiting bodies [19]. This provides many identifiable developmental checkpoints with which to identify the phenotypes of transformants.

Unique Developmental Cycle

Dictyostelium cells grow as individual free-living amoebae in the presence of a suitable bacterial or liquid carbon source. These amoebae clear plaques in bacterial lawns by consuming the bacteria in a radial fashion. The cells in the center of the plaque eventually become isolated from the bacteria outside the expanding plaque and begin to starve. At approximately four hours post-starvation, these cells start secreting cAMP. Surrounding cells are induced to

secrete cAMP as well, thereby radiating the cAMP signal throughout the plaque. Outlying amoebae chemotact toward the center of the plaque by following the cAMP concentration gradient. These amoebae form an aggregate at eight hours post-starvation. Mutational analysis of the Gα2 gene has suggested that its gene product is involved in mediating this aggregation process [14]. Expression of Gα4 and Gα5 increases upon aggregation [16, 20]. These proteins are important for the developmental fate of the cells in the aggregate, directing them to differentiate into either prespore or prestalk cells, respectively. The mass develops into a tipped aggregate by 12 hours post-starvation, and by 16 hours post-starvation becomes a mobile pseudoplasmodium, or "slug". By 24 hours post-starvation, this slug develops into a terminal fruiting body, with an elongated stalk and an apical spore mass. These spores are later released to complete the life cycle.

Haploid Genetics

Dictyostelium discoideum completes its entire life cycle as a haploid organism. *Dictyostelium* cells can be fused to make diploids, which are useful for crossover, complementation studies and maintenance of lethal mutations. However, this is not often utilized in *Dictyostelium* research. Maintaining these strains as haploid cells has distinct advantages, including the absence of crossover-type chromosomal rearrangement and the ability to produce noticeable phenotypic changes by knocking out single copies of genes. The strains used in this study were maintained in the haploid state throughout.

Transformation

A wide range of transformation strategies is available for use with *Dictyostelium*. Both homologous and heterologous integration are possible in these cells. Plasmids for integration and extrachromosomal maintenance have also been constructed that can be preserved in both vegetative cells and spores.

Restriction Enzyme-Mediated Integration (REMI) is a transformation technique recently developed for the *Dictyostelium* system. REMI allows mass gene tagging at specific restriction sites in the host genome. This strategy was first developed by Schiestl and Petes in *Saccharomyces cerevisiae*, and was soon adapted for use in *Dictyostelium* by Kuspa and Loomis [21, 22]. REMI mutagenesis allows one to direct integration of a linearized vector into specific restriction sites in the genomic DNA. The vector is first linearized with a restriction enzyme. The linearized vector is then combined with *Dictyostelium* cells and a restriction enzyme that produces single-stranded overhanging ends compatible with the ends of the linearized vector. Vector and restriction enzyme are co-electroporated and direct site-specific integration events at the approximate frequency of 1 in 10^4 cells.

Recent advances in the REMI vectors available have made this method more robust for large-scale gene tagging and retrieval. The pBsr2ΔBam vector used was produced specifically for both the ease of selection of transformants and cloning of vector and flanking genomic DNA [23, 24]. It contains a blasticidin S-resistance gene (Bs^R), which is driven by the *Dictyostelium* actin 15 promoter and an ampicillin-resistance gene driven by an *E. coli* promoter. We can

therefore add blasticidin S to the HL5 liquid medium [25] for selection against non-transformed cells. The blasticidin-resistant cells produce colonies that can be resuspended and plated onto bacterial lawns for isolation of clonal plaques. This strategy has been successfully used to produce and isolate cytokinesis mutants in *Dictyostelium* [26, 27]. REMI has also been successfully used to tag genes in *Candida albicans* [28] and the plant fungal pathogens *Cochliobus heterostrophus* [29], *Ustilago maydis* [30] and *Colletotrichum* species [31].

Considerations in the Use of REMI in *Dictyostelium*

REMI mutagenesis is useful for randomly tagging sites in the genome. In order to saturate the genome with REMI vector inserts, one must estimate the number of restriction sites present in that genome. This gives a rough estimate of the number of transformants that must be produced in order to achieve saturation.

Genomic DNA should contain restriction sites for any particular restriction enzyme once every 4^N base pairs, where 4 represents the possible bases (A, T, G or C) and N represents the number of bases in the restriction site. BamHI and DpnII enzymes were used as REMI mediators in this study. These have recognition sequences of 5'-GGATCC-3' and 5'-GATC-3', respectively. If one assumes equal parts of all nucleotides in the genomic DNA, corresponding restriction sites for BamHI should be present once every 4,096bp, and DpnII sites should occur once every 256bp. The *Dictyostelium* haploid genome size is approximately 40Mb [42]. Assuming equal ratios of the four nucleotides, one can

find the approximate number of restriction sites in the genome by dividing the genome size by the average distance between restriction sites. Using this method, there should be approximately 9.8×10^3 BamHI sites and 1.6×10^5 DpnII sites in the *Dictyostelium* genome. However, the G + C content of *Dictyostelium* genomic DNA is only ~22% [32, 33]. Furthermore, G + C content of poly(A) RNA is ~30%, and open reading frames vary between 30-40% G + C [34, 35, 36, 37]. In contrast, regions that do not code for DNA, such as introns, 5' and 3' untranslated regions of mRNAs, and rDNA spacers have much lower G + C content, which varies between 5-20% [35, 36]. Collectively, these observations would suggest that the total number of G-C rich BamHI and DpnII sites is much lower than the estimate above. In addition, a much higher proportion of these sites should lie inside translated regions of DNA than in untranslated regions, which also reduces the total number of transformants that must be screened through in order to assure saturation of the complete genome.

DNA methylation must also be considered when using REMI. Since integration occurs at restriction sites, one must assure that either the organism does not methylate its DNA or that the restriction enzyme mediating REMI is not sensitive to methylation. Methylation in *Dictyostelium* was addressed by looking for differences in digestion of genomic DNA by isoschizomers either sensitive or insensitive to methylation. No detectable methylation at either A or C was observed by this method [38, 39].

Genomic and cDNA Libraries

Once genes are isolated from the genome, one may wish to map it to a specific region of that genome. Many *Dictyostelium* libraries have been constructed for mutant identification and physical mapping of genes by complementation of mutant phenotypes. Dynes and Firtel have constructed a *Dictyostelium* library by integrating Sau3A genomic fragments into an extrachromosomally-replicating shuttle vector [40]. Kuspa and Loomis have created two different libraries. One is based on overlapping YAC contigs [41]. The other consists of RFLP fragments generated from genomic DNA containing a specially designed REMI vector [42, 43]. In addition, a group of Japanese *Dictyostelium* researchers is currently constructing a cDNA library.

Gα4-Mediated Signal Transduction

The *Dictyostelium* Gα subunit Gα4 is believed to be important for spore production during multicellular development. It is already known that when folate binds to an early-expressed αFAR or a late-expressed βFAR receptor that a Gα4-mediated response is elicited [20]. Based on knowledge of other G-protein-mediated systems, the Gα-GTP unit should complex with at least one intracellular effector protein. The Gα subunit's intrinsic GTPase activity then cleaves GTP into GDP and phosphate, at which time the activated effector protein is released. However, no effector associated with the *Dictyostelium* Gα4 subunit has been identified. In addition, relationships between folic acid

receptors, Gα4 expression and activation, and correlated biochemical and developmental markers are not well understood. Products other than these may also be involved.

Collectively, these unknowns present numerous targets for a random gene tagging strategy, such as REMI, coupled with a screen for phenotypes likely to be affected by a mutation in one of these genes.

CHAPTER 2

RESEARCH PROBLEM

Regulators and Effectors Involved in the Gα4-Mediated Pathway Unknown

As stated previously, neither upstream regulators nor downstream effectors involved in the Gα4-mediated signal transduction pathway have been isolated. The overall goal of this study is to develop methods for the discovery of these genes. To that end, the following strategies were devised.

We hypothesized that insertion of foreign DNA into the genes of upstream regulators or downstream effectors involved in the Gα4-mediated signal transduction pathway would interfere with Gα4-mediated signal transduction. This in turn would produce a phenotypic change similar to the phenotypes observed for known Gα4 mutants. The phenotypes for wild-type, $G\alpha4^{HC}$ (high-copy overexpressing construct), and $g\alpha4^{-}$ (null construct) strains are known and easily distinguishable from each other by visual and other means. These were used as controls when screening transformants.

An example of this would be the production of the spore mass. Wild-type cells produce a full spore mass at the anterior of the fruiting body, Gα4 overexpressors produce a large aggregate of prespore cells with little or no stalk, and null cells produce mostly stalk with few spores. An insertion into an effector gene may cause a wild-type or overexpressor cell line to stop producing prespore-type cells, to alter the ratio of prespore to prestalk cells or to alter the

rate of differentiation or organization of these cell types within the multicellular structure.

In order to promote insertion of the vector into the largest number of genes possible, Restriction Enzyme-Mediated Integration mutagenesis was employed. Cells containing such insertions could easily be identified and cloned based on the change of their developmental phenotype. After screening for developmental aberrations, the inserted vector and surrounding flanking sequences were subsequently excised by restriction digest from the mutant genomes. These isolated plasmids could then be homologously inserted into the genome of non-transformed cells to confirm the dependency of the mutant phenotype on the insertion. Sequencing of the flanking sequences and cloning of the intact genes could follow.

Aims of This Study

The overall aim of this study was to assess the feasibility of each of the steps outlined in the strategy above, both in terms of performing those steps and the validity and usefulness of the data gained from it. The following questions were specifically addressed:

1. Are plaques with mutant developmental phenotypes easily identified and separated from other plaques in mixed culture?
2. Which background (wild-type or overexpressor) is better to use for isolating the desired mutants?
3. Does REMI produce random mutations?

4. Does the REMI vector integrate singly, or do multiple copies insert in tandem?
5. Can vector + flanking sequence be isolated from mutant genomic DNA preparations?
6. What is the size range of the flanking sequences recovered by this method?
7. Can these plasmids homologously integrate back into gene sequence corresponding to the isolated flanking sequence, thereby reproducing the original mutant phenotype?

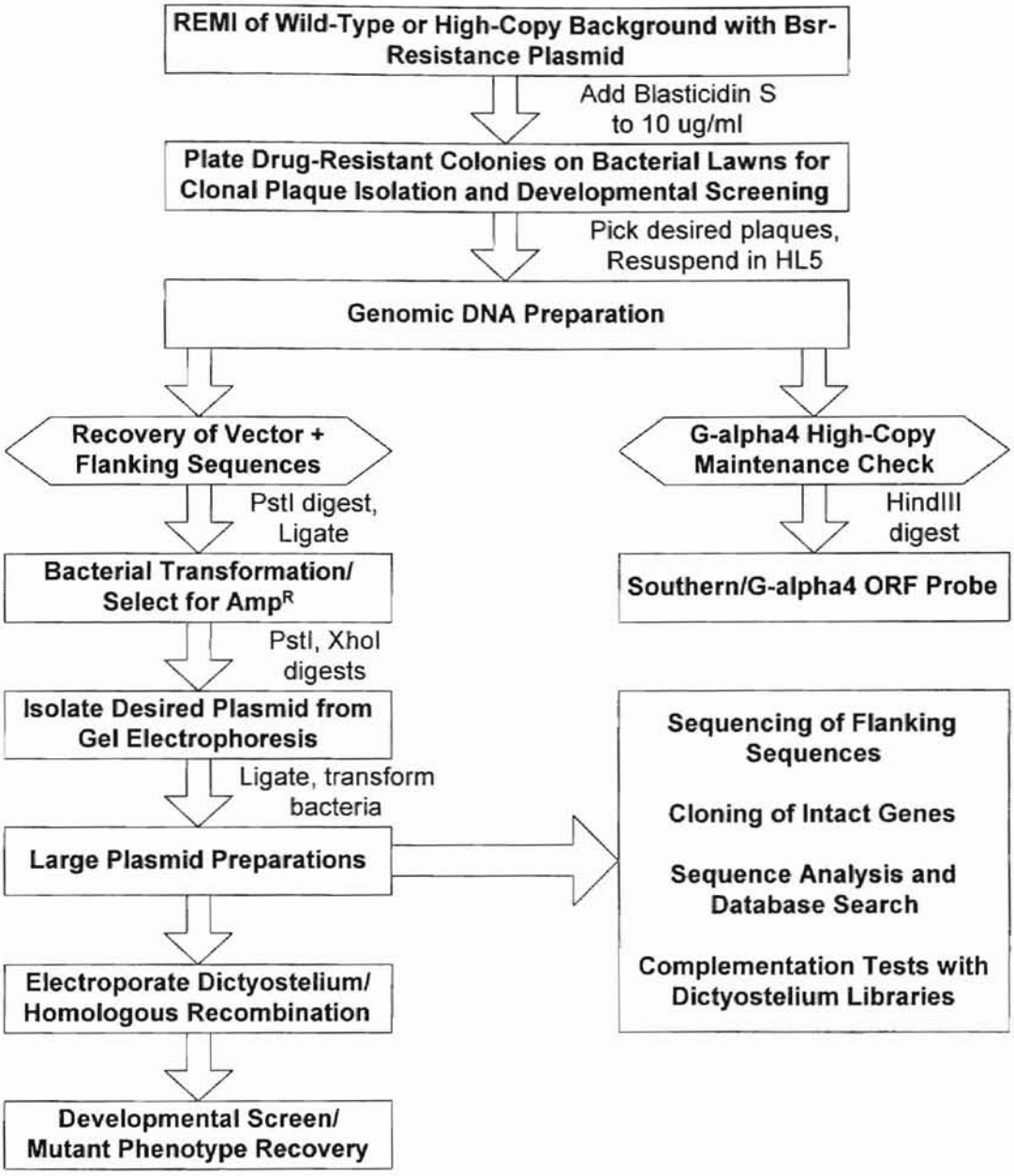

Figure 2. Overview of *Dictyostelium* REMI project and future experimentation

CHAPTER 3

MATERIALS AND METHODS

Strains

Dictyostelium discoideum and bacterial strains were used in this study. Wild-type (KAx3; courtesy of W. Loomis, UC San Diego) and Gα4^{HC} (JH384; J. Hadwiger, OSU) *Dictyostelium* strains were used as recipient cells in the REMI mutagenesis trials. A gα4^{-} strain (JH417; J. Hadwiger, OSU) was used as a control for comparison of transformant developmental phenotypes to that of the known null phenotype. A Thy^{-} auxotrophic strain (JH10; J. Hadwiger, OSU) was combined with transformed cells to produce chimeric fruiting bodies and spores if the clonal transformant line was unable to produce viable spores by itself. A *Klebsiella aerogenes* strain (011) was used as the food source for *Dictyostelium* when plated for isolated plaque development. The pBsr2ΔBam vector was supplied courtesy of K. Sutoh, University of Tokyo. Stable II ultracompetent bacteria were used for cloning of PstI-fragmented genomic DNA.

Preparation of pBsr2ΔBam Vector

100ml batches of *E. coli* cells transformed with the pBsr2ΔBam vector were grown in LB medium supplemented with 100μl of 45 mg/ml ampicillin solution overnight. Plasmid was then purified according to standard phenol/chloroform extraction and ethanol precipitation procedures. Prior to use,

these preparations were further purified by Sephadex G-50 spin column treatment.

Table I

A LIST OF STRAINS AND PLASMIDS USED AND THEIR GENETIC ATTRIBUTES

Strain / Contruct	Genetics	Source
***Dictyostelium discoideum*:**		
KAx3	Wild-type	W. Loomis, U.C. San Diego
JH 384	G-alpha4 high-copy	J. Hadwiger, Oklahoma State U.
JH 417	G-alpha4 null	J. Hadwiger, Oklahoma State U.
JH 10	Thy auxotroph	J. Hadwiger, Oklahoma State U.
Plasmids:		
pBsr2deltaBam	Blasticidin S resistance (*Dicty*) Ampicillin resistance (*E.coli*)	K. Sutoh, U. of Tokyo
p154	G-alpha4 overexpression vector	J. Hadwiger, Oklahoma State U.

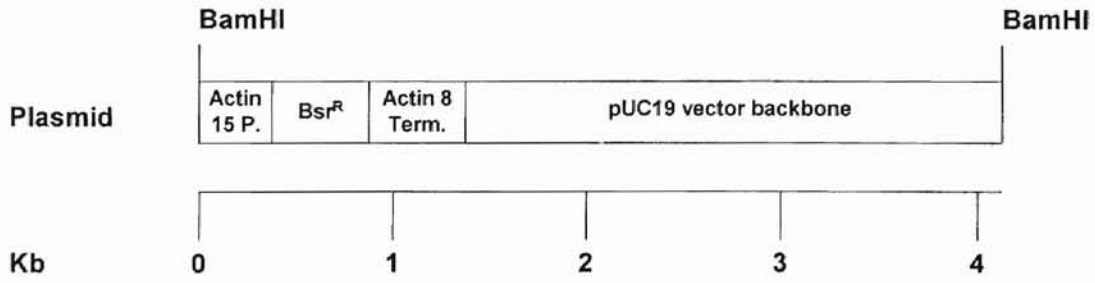

Figure 3. The pBsr2ΔBam vector for REMI mutagenesis in *Dictyostelium*

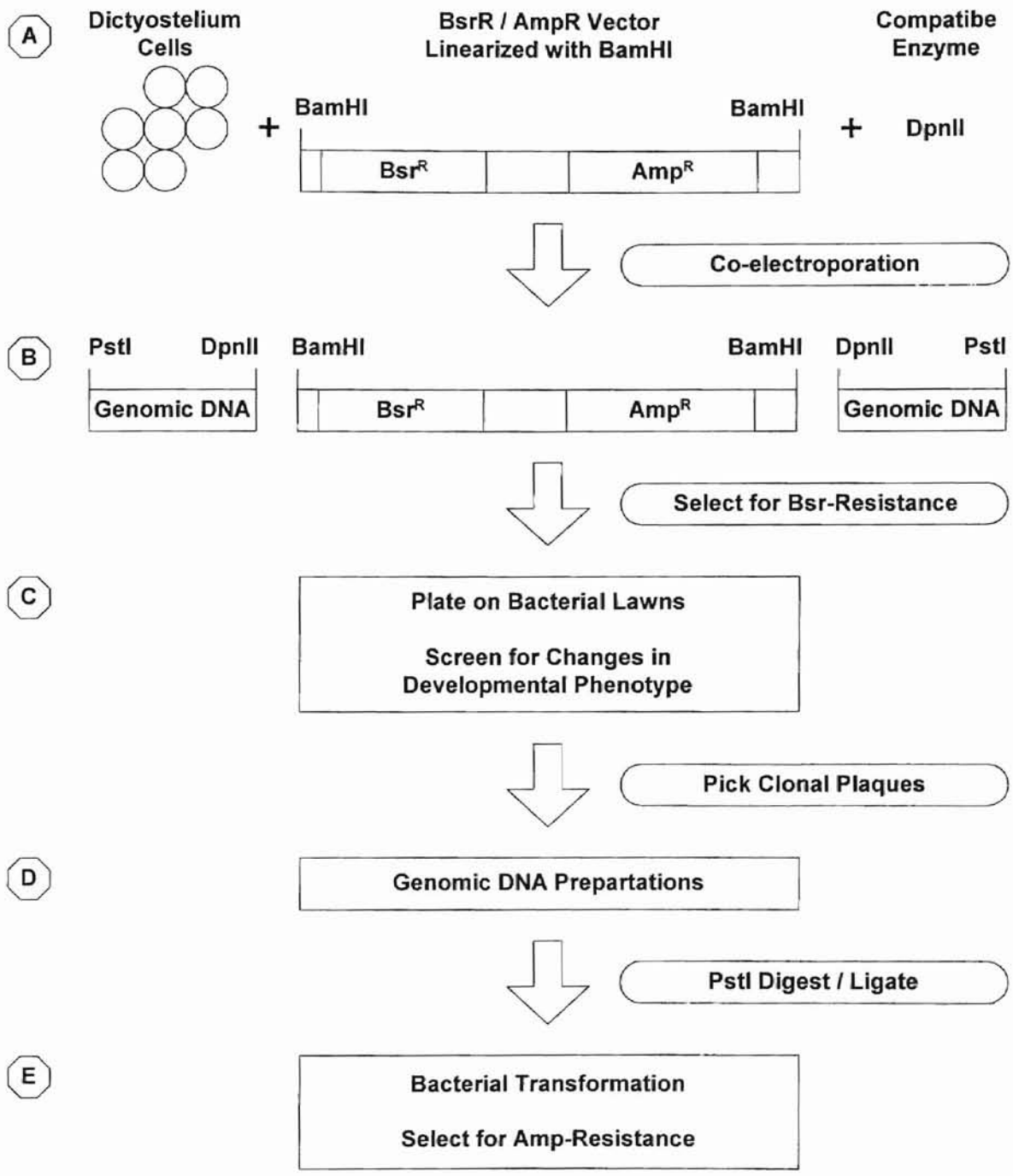

Figure 4. Overview of REMI mutagenesis and flanking sequence isolation

REMI Electroporation Procedure

Dictyostelium discoideum cells of desired background phenotype were grown to ~2 x 106 cells/ml in HL5 medium in shaking culture. 15ml of this cell suspension was combined with 35ml electroporation buffer at 4°C in 50ml conical tubes, and the cells were collected by centrifugation at 1500rpm in a Sorvall tabletop centrifuge. The supernatant was decanted, and the cells were then resuspended in 0.5ml cold electroporation buffer. All cell lots were combined, and 0.25ml aliquots were distributed into 0.2cm gap electroporation cuvettes. These were kept on ice until needed. 50-75 μl of pBsr2ΔBam solution previously linearized with BamHI restriction enzyme (Promega, Madison, Wisconsin) was added to each cuvette, save one no-DNA control. After mixing thoroughly, the cuvettes were left on ice for 10 minutes. 100U of DpnII restriction enzyme (NEB, Cambridge, Massachusetts) was then added to the cuvette and mixed thoroughly. The solution was then electroporated immediately (3.0μF, 2.0-2.4) and returned to ice for 10 minutes. 4μl healing solution was added to each cuvette, and the solutions were allowed to sit on ice for an additional 10 minutes. The solution from each cuvette was then aliquoted into 4-10 petri dishes, and 8-10ml HL5 medium was added to each dish. Blasticidin S was added after 24 hours to a concentration of 10 μg/ml. Visible colonies of Bs^R-transformants were produced in 6-8 days.

Phenotypic Screening of Bs^R Transformants

The Bs^R transformants from each plate was resuspended in 2ml HL5 medium. 1-5μl of this suspension was plated with 200μl log-phase *Klebsiella aerogenes* suspension on SM+/3 agar plates. Isolated plaques of Bs^R transformants arose in 3-5 days, and proceeded through development in 5-8 days. Transformants exhibiting desired phenotypes were picked with sterile toothpicks or small micropipette tips and resuspended in HL5 medium. This procedure was repeated to confirm isolation of a pure culture.

Chemotaxis Assays

2×10^6 log-phase cells were grown in HL5 shaking culture. 1mL of each sample (REMI mutants + JH384($G\alpha4^{HC}$), 417($g\alpha4^-$), and KAx3 (WT) controls) was transferred to microfuge tubes. These were spun at 3000rpm in a microfuge for 2 min to collect the cells. The supernatant was removed, and cells were washed in 1mL Na^+/K^+PO4^{3-} buffer. They were then spun at 3000rpm in a microfuge for 2 min to collect the cells. They were then resuspended in 100μL Na^+/K^+PO4^{3-} buffer. 1μL droplets of *Dictyostelium* suspensions were plated on Na^+/K^+PO4^{3-} plates in a radial pattern. 1μL droplets of folate solution were then plated 1-2mm away from each *Dictyostelium* droplet, and toward the center of the plate. Three groups which vary in folate solution concentration were usually produced (usually 1mM, 10mM and 25mM folate). The degree of chemotaxis was checked every hour for 6-8 hours and scored relative to the degree of chemotaxis produced in the controls.

Preparation of *Dictyostelium discoideum* Genomic DNA

Clonal isolates of Bs^R transformants were resuspended in HL5 medium and used to inoculate 15ml HL5 shaking cultures. These were grown to saturation (3-4 days). These cells were pelleted by centrifugation, washed with Na^+/K^+PO4^{3-} buffer, and pelleted again. The cells were then resuspended in 400μl lysis buffer and incubated for 2 hours at 65°C, then >8 hours at 37°C. The samples were then subjected to a phenol/chloroform extraction, ethanol precipitation, RNase treatment, and another ethanol extraction.

Isolation of pBsr2ΔBam Vector with Flanking Sequences

Samples of genomic DNA preparations were digested with PstI restriction enzyme (Promega, Madison, Wisconsin). This enzyme does not cut within the pBsr2ΔBam vector, therefore isolating genomic sequence flanking either side of the vector insert. After digestion, the PstI was heat-denatured at 65°C. The samples were then phenol/chloroform extracted and precipitated with 95% ethanol. The resultant pellets were resuspended to a concentration 1/3 that of the original sample (to promote intramolecular ligation versus ligation to other PstI fragments). The samples were then subjected to T4 ligase (NEB, Cambridge, Massachusetts). The resultant circularized fragments were then transformed into ultracompetent Stable II or E. coli NM522 cells. These cells were then plated onto LB + ampicillin plates to select for transformants containing the pBsr2ΔBam vector.

Recovery of Mutant Phenotypes in *Dictyostelium discoideum* Cells

Large preparations of the pBsr2ΔBam inserts + flanking sequences were made. Samples of each were linearized via PstI digestion. These were combined with wild-type Kax3 cells in a 0.2cm-gap electroporation cuvette and electroporated at 1.2V, 3.0μF. The cell suspensions were aliquoted into petri dishes and supplemented with 8-10ml HL5 medium. Blasticidin S was added after 24 hours to a concentration of 10 μg/ml. Visible colonies of Bs^R - transformants were produced in 6-8 days. These colonies were resuspended in HL5, and 1-5μl of this suspension was plated with 200μl log-phase *Klebsiella aerogenes* suspension on SM+/3 agar plates. Isolated plaques of Bs^R transformants arose in 3-5 days, and proceeded through development in 5-8 days. Transformants exhibiting desired phenotypes were picked with sterile toothpicks or small micropipette tips and resuspended in HL5 medium. This procedure was repeated to confirm isolation of a pure culture.

Confirmation of Ga4HC Plasmid Maintenance

Genomic preparations of transformants, JH384($G\alpha4^{HC}$), and KAx3 (WT) controls were digested with HindIII restriction enzyme (Promega, Madison, Wisconsin). These were electrophoresed on 0.7% agarose gel. Southern blots were prepared from these gels, and were probed with ^{32}P- Gα4 ORF DNA. The blots were placed in exposure cassettes with X-ray film for ~4 days. Resultant band intensities were compared to those of the controls.

CHAPTER 4

RESULTS

Wild-Type REMI Trials

As stated in Chapter 2, two wild-type, BamHI-mediated REMI trials were conducted to assess the broad parameters of the REMI method as relates to the transformation efficiency versus concentrations of DNA and enzyme used and the voltage applied. Several more DpnII-mediated REMI trials were conducted to assess the ability of REMI to create transformants that produce plaques with a range of different developmental characteristics. Results of each trial are as follows:

BamHI-Mediated REMI Trials

These trials were conducted to better optimize the range of REMI transformation conditions, as well as to determine if the vector would integrate into restriction sites or via homology to the actin 15 promoter or actin 8 terminator contained in the REMI vector. 1.5×10^7 *Dictyostelium* cells were subjected to BamHI-mediated REMI. Varying voltages were used, from 0.5-2.4V. Trial 1 was composed of 0.5, 1.0, and 1.5V electroporation groups. 50μl of two different DNA concentrations (1.5 μg/ml and 3.0 μg/ml) were used at each voltage level. There was a general tendency for more Bs^R colonies to appear in higher voltage groups and at higher amounts of vector DNA. There was an ~10-fold increase in Bs^R colonies from the 1.0V to the 1.5V groups, following an ~2-fold increase from

0.5V to 1.0V. This effect was noted at both DNA concentrations. A small increase of transformation efficiency with increasing DNA concentration was also noted, reaching an ~1.5-to-2-fold increase in the 1.5V group. From these data, a second trial was planned to explore the observed correlation of voltage and Bs^R colony formation at higher voltages.

In order to assure that the REMI vector was integrating into restriction sites instead of other targets, the following experiment was conducted. A BamHI-digest of genomic DNA from Trial 1 transformants was separated on agarose gel electrophoresis and Southern blotted onto nitrocellulose. The blot was then probed with a ^{32}P-labelled Bsr fragment. The resultant autoradiograph is shown in Figure 5. If the vector integrated into BamHI sites, one would expect a single strong band at 4.1kb, which corresponds to the size of the vector. On the other hand, if the vector integrated into homologous or random sites, one would expect bands larger than 4.1kb and of varying size. All lanes exhibited a single strong 4.1kb band on the autoradiograph, suggesting that the vector inserted into available BamHI genomic sites rather than by other types of integration events. Note that this does not rule out multiple insertions of the REMI vector into single restriction sites, as multiple inserts would all digest to the single 4.1kb sized fragment.

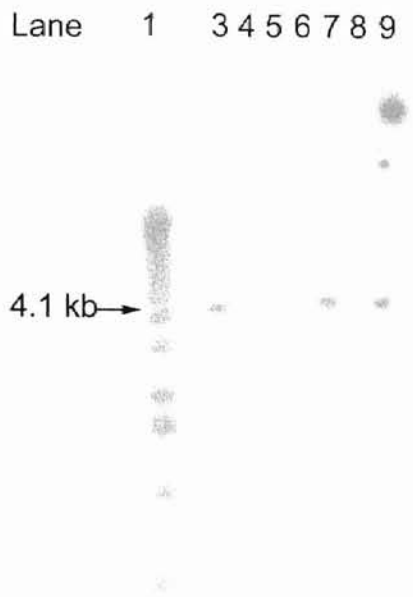

Figure 5: Genomic DNA blot of BamHI-REMI transformants probed for the BsR gene

Lane1: 1kb ladder DNA; Lanes 3-9, BamHI-digested genomic DNA from BamHI-REMI transformants

Blot was probed with 1.4 kb BamHI/HindIII fragment of pBsr2ΔBam vector containing all of the BsR ORF

For the second trial, 1.5 x 10^7 *Dictyostelium* cells and standard amounts of vector were subjected to BamHI-mediated REMI. Replicates of the cell + vector suspension were electroporated in the range of 1.0-2.4V in increments of 0.2V. Many of the colonies disappeared after one week of continuous drug selection. This was five days after the usual killing of non-drug-resistant cells. This suggests that not all of the colonies were drug resistant, or that they died due to other reasons. Unfortunately, the presence of these colonies precluded

obtaining accurate colony counts and transformation efficiency estimates. However, by observing the density of colonies after this second round of die-off it seemed evident that Bs^R colony formation increased several –fold with increased voltage.

DpnII REMI Trials

Three DpnII-mediated REMI trials were performed in the wild-type background in order to assess the variety of aberrant plaque development phenotypes that would be produced with the method. Approximately 10,000 Bs^R colonies were pooled as they were produced, plated onto bacterial lawns, and screened for aberrant plaque development. Of these, 54 plaques were shown to have some discernable developmental phenotype in clonal culture. These transformants could be arranged into five general categories of aberrations: (A) $g\alpha4^-$-like, which form small plaques and spore masses and malformed stalks, (B) $G\alpha5^{HC}$-like, which formed plaques containing an inordinate number of small fruiting bodies, (C) Bull's-eye, which form plaques that have a central zone of fruiting body formation surrounded by a clear band containing no fruiting bodies, (D) $G\alpha4^{HC}$-like, which form plaques with many terminal mounds, and (E) Slugger, which form plaques that precociously produce large slugs and very few fruiting bodies. There was also variety within these groups as to the timing of development, terminal plaque size, and multicellular structure size. This variety of mutant phenotypes suggests that the Bsr vector was integrating into many different sites in the *Dictyostelium* genome.

Suppressor of Gα4^{HC} REMI Trials

A different *Dictyostelium* genetic background was chosen for the rest of this study. A Gα4^{HC} strain (JH384) was used as a Gα4-overexpressing background. The critical assumption of this part of the study was that disruption of a gene involved in the Gα4-mediated signal transduction pathway would cause a suppression of the Gα4^{HC} phenotype. In other words, transformants containing these types of insertions would exhibit a phenotype similar to a wild-type or gα4^{-} strain during plaque development.

Trial 1

Two clonal isolates of strain JH384 cells were propagated as background cells for this trial. Identical numbers of cells were used in each electroporation group, as well as identical amounts of vector DNA. Four groups of each cell line were prepared, and one of each electroporated at 1.00V, 1.25V, 1.50V, and 1.75V. The resultant number of Bs^{R} colonies for each group is listed in Table II below. The results for isolate 1 show that transformation efficiency was extremely low, whereas isolate 2 produced Bs^{R} colonies in approximately the efficiency expected given the parameters of the electroporation. Isolate 2 was used for all subsequent REMI trials.

TABLE II

NUMBER OF BLASTICIDIN-RESISTANT COLONIES PRODUCED BY REMI IN TWO *DICTYOSTELIUM* ISOLATES

384 Isolate	Voltage	# Bsr-resistant
Isolate 1	1.00	2
	1.25	0
	1.50	0
	1.75	0
Isolate 2	1.00	2
	1.25	38
	1.50	146
	1.75	277

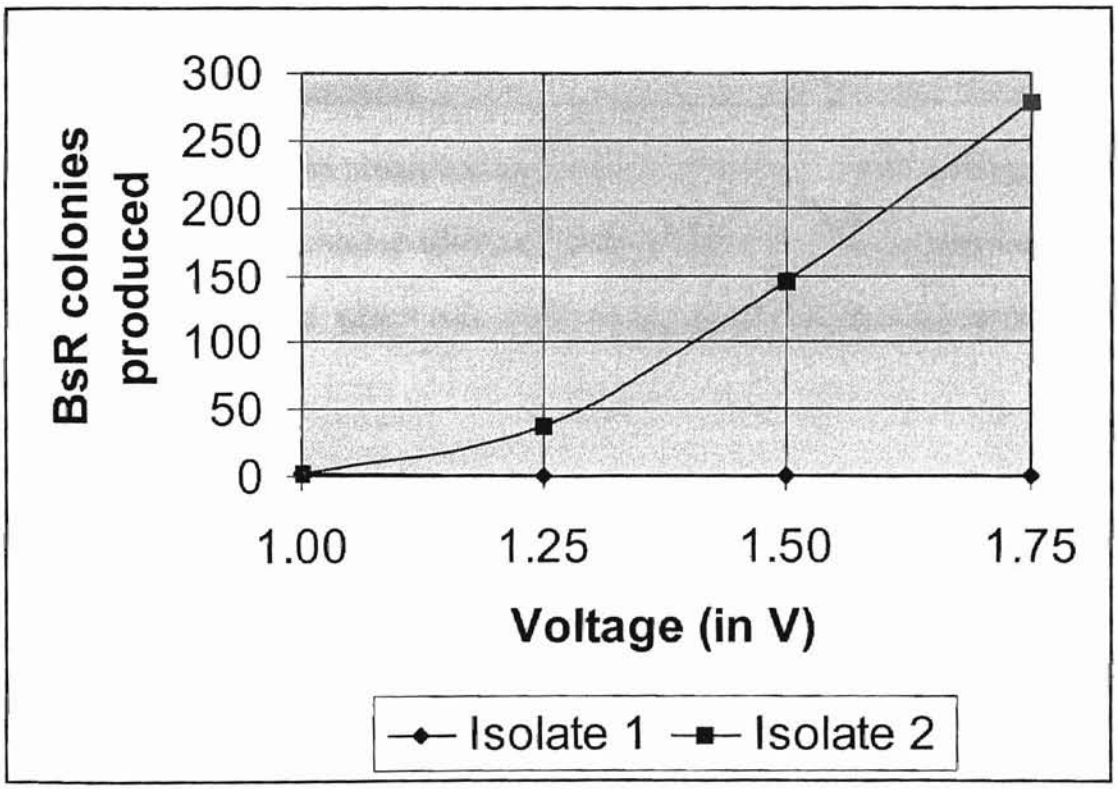

Figure 6: Number of BsR colonies produced/Voltage

Trial 2

This trial was designed to assess transformation efficiency at higher voltages. Paired replicates consisting of identical concentrations *Dictyostelium* cells, DpnII enzyme and vector DNA in solution were exposed to the same voltage, and identical replicate pairs were electroporated at 1.75V, 2.00V, and 2.25V. Results are as shown below in Table III. The only results from this trial that are consistent with Trial 1 are the 1.75V,B and 2.25V,B groups, which produced approximately equivalent numbers of Bs^R colonies as similar group. There were no variations in the concentrations of cells, DNA, or DpnII between the replicates, which suggests that other variables may account for the discrepancies. One variable in particular comes to mind, namely that there may have been some contaminant or defect in individual electroporation cuvettes. A fungal contaminant was present in two of the HL5 plates. Other colleagues in the lab also had similar contamination problems at this time. The problem was soon traced to our Pipet-Aid, which was sterilized to prevent future occurrences of this problem.

Analysis of Trial 1 and 2 Mutants

Trial 1 and 2 transformants were pooled and plated onto *Klebsiella aerogenes* bacterial lawns for plaque development screening. A total of six plaques exhibited a suppressor-like phenotype. These were isolated for further study.

Table III

NUMBER OF BLASTICIDIN-RESISTANT COLONIES PRODUCED IN TRIAL 2

Voltage/Group	# Bsr-resistant	Comments/Notes
1.75V, A	1	
1.75V, B	300	
2.00V, A	8	(1 plate of 3 contaminated)
2.00V, B	7	
2.25V, A	10	
2.25V, B	1000	(1 plate of 3 contaminated)

Table IV

CHEMOTAXIS ASSAY TO FOLATE, TRIAL 1 AND 2 MUTANTS

Date: 2/12/98 Time started: 11:40AM Conc. Of folate: 1uM

Strains:	~ dist. @3hrs	~ dist. @4hrs	~ dist. @5hrs	~ dist. @6hrs	~ dist. @7hrs	~ dist. @8hrs
Controls:						
384	N/A	1.5	1.5	1.5	2.0	2.5
KAx3		1.5	1.5	1.5	1.5	2.0
417		0	0	0	0	0
Exps:						
SR1-1		0.5	0.5	1.0	1.0	1.5
SR1-2		1.0	1.0	1.5	1.5	2.0
SR1-3		1.0	1.5	2.0	2.5	2.5
SR1-4		2.0	2.0	2.0	2.5	2.5
SR1-5		2.0	2.0	2.5	2.5	3.0
SR1-6		2.0	2.0	2.0	2.5	2.5

Chemotaxis Assays: Disruption of a gene involved in the Gα4-mediated signal transduction pathway may alter the cell's chemotactic behavior toward folate, which is mediated by Gα4. Therefore, a chemotaxis assay to folate was performed on these six mutants in order to compare their chemotactic behavior to that of the $G\alpha4^{HC}$ background strain. The results are shown in Table IV. These data show that mutants 1 and 2 chemotact toward folate at approximately the same rate as do wild-type cells. This may be interpreted in one of two ways. Either these cells contain gene disruptions which interfere with chemotaxis toward folate, or these cells have lost copies of the $G\alpha4^{HC}$ construct. Mutants 3-6 exhibited chemotaxis similar to the high-copy cells, which suggests that they have maintained copy number of the $G\alpha4^{HC}$ construct and do not contain disruptions that interfere with folate chemotaxis.

$G\alpha4^{HC}$ Construct Copy Number Maintenance Check: Since the resultant phenotype from these transformants is dependent on both the gene disruption produced by the REMI insert and the maintenance of high copy number of the $G\alpha4^{HC}$ construct, a check of high-copy maintenance was necessary. An autoradiograph from a Southern blot of a HindIII digest of the genomic DNA from these six showed that isolates 1 and 2 did not maintain high copy of the JH384 $G\alpha4^{HC}$ plasmid (Figure 7 below). Due to this, these two isolates were not included in subsequent analysis.

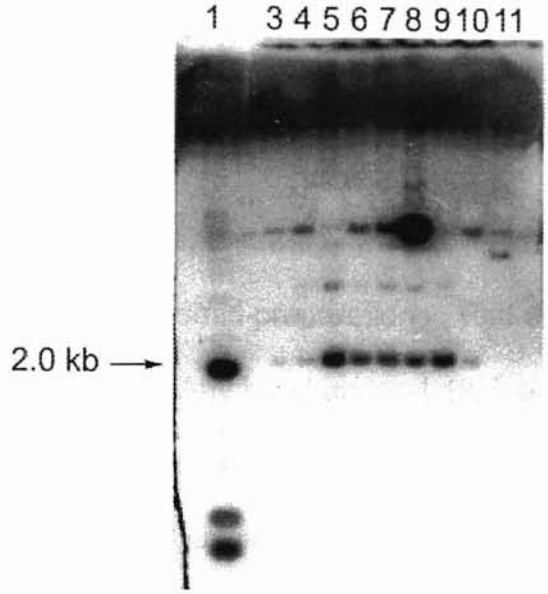

Figure 7: Genomic DNA blot of Trial 1 and 2 DpnII-REMI mutants probed for Gα4^{HC} construct

Lane 1: λ-HindIII DNA marker; Lanes 3-8: HindIII digest genomic DNA from *mutants* 1-6; Lane 9: *HindIII* digest of JH384 genomic DNA; Lane 10: HindIII digest of wild-type genomic DNA; Lane 11: HindIII digest of JH417 genomic DNA

Blot was probed with 3.0 kb BamHI/EcoRI fragment of p154 vector containing the majority of the Gα4 ORF from the 5' end

Isolation of Vector and Flanking Sequences: The next step of the strategy was to isolate the REMI inserts with flanking sequences from these transformants. To accomplish this, genomic DNA is digested with PstI, an enzyme which does not digest the REMI vector. It cuts outside the vector, thereby isolating flanking genomic sequences. PstI fragments of the genomic DNA from isolates 3-6 were produced, self-ligated, and used to transform Stable

II cells. Ampicillin-resistant colonies were isolated, and large-scale plasmid preparations were made.

Since both the REMI vector and the $G\alpha4^{HC}$ construct contain the ampicillin-resistance gene, a strategy was devised for isolating the REMI constructs from these plasmid preparations. PstI enzyme should linearize all plasmids in the solution. Any band under 4.1kb (size of the REMI vector) would be immediately ruled out. PstI cuts the p154 vector into three fragments. Bands exhibiting this pattern are also ruled out. A second comparison is made between XhoI and the uncut bands. XhoI cuts the REMI vector once, but does not cut the $G\alpha4^{HC}$ vector. Thus, bands that are linearized by both PstI and XhoI are deemed likely to contain the REMI vector versus the $G\alpha4^{HC}$ vector.

Uncut, PstI-digested, and XhoI-digested samples of each plasmid preparation were run out on an agarose gel electrophoresis. Two bands were isolated from each uncut lane that were <4.1kb and that were linearized by XhoI and PstI. The DNA from these were purified with a Gene-Clean kit, and Stable II cells were transformed. Ampicillin-resistant colonies were then recovered.

Large-scale plasmid preparations of these eight transformants were then used to transform $G\alpha4^{HC}$ cells in order to attempt to recreate the original mutant phenotypes. Samples of each were linearized with PstI, then electroporated into wild-type and $G\alpha4^{HC}$ cells. These cells were subsequently exposed to blasticidin. The small fragment isolated from mutant 4 and the large fragment from mutant 5 genomic DNA produced blasticidin-resistant colonies in the $G\alpha4^{HC}$ background. These were spread plated onto bacterial lawns in order to observe the resultant

developmental phenotypes and compare them to the original mutants. The mutant #5 group exhibited transient similarity to the original mutant phenotype, but this similarity was not observed after replating these cells onto fresh bacterial lawns. Mutant #4 DNA did not reproduce the original mutant phenotype.

Subsequently, a check was performed on the isolated plasmids to see which contained the BsR gene. A blot of uncut plasmid preparations was made and probed for the BsR gene. The results are shown in Figure 8 below. Every plasmid isolated seems to contain the BsR gene. This suggests two things. First, adjustment of the electroporation conditions or production of many electroporation replicates may be needed to ensure recovery of BsR colonies. And second, by isolating a high and a low band from each uncut lane in the uncut/PstI/XhoI comparison, both supercoiled and relaxed circle conformations of the same plasmid were probably excised.

Check for integration of REMI vector into genome: In order to determine if the REMI vector integrated into the genome versus existing as an extrachromosomal element, the following procedure was performed. A blot of mutant genomic DNA digested with PstI was made and probed for the BsR gene. If the REMI vector is maintained as an extrachromosomal element, it should be detected as a 4.1 kb fragment. However, integrated vector would pick up flanking sequence, and so would light bands corresponding to larger fragments. No bands of 4.1 kb in size were found. Several higher bands could be observed, but they were too faint to be observable in a printed figure (data not shown).

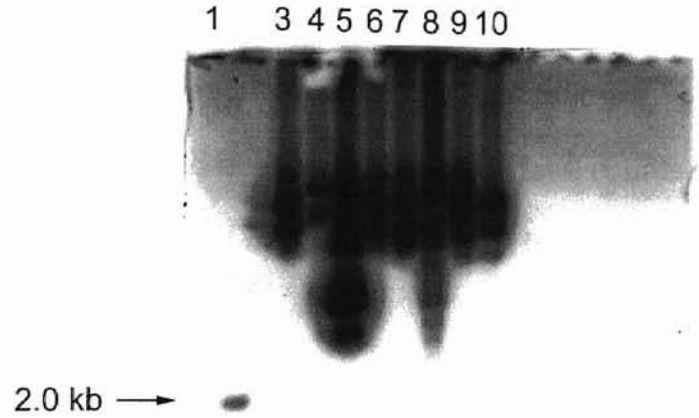

Figure 8: DNA blot of isolated Trial 1 and 2 plasmids probed for the BsR gene

Lane 1: 1 kb ladder; lane 3: mutant SR1-3, low band; lane 4: mutant SR1-3, high band; lanes 5-10: SR1-4 through SR-6

Blot was probed with 1.4 kb BamHI/HindIII fragment of pBsr2ΔBam vector containing all of the BsR ORF and *Dictyostelium* actin 15 promoter and actin 8 terminator

Check for tandem integration of REMI vector: In order to determine if the REMI vector was integrating singly into the genome or in tandem repeats, the following strategy was employed. A blot of mutant genomic DNA digested with HindIII was produced and probed for the BsR gene. Since a single HindIII site exists in the REMI vector, two bands should light if the vector integrates as a single copy. Three bands, one of which would be 4.1 kb, would light if tandem integration occurred. As with the aforementioned PstI/BsR blot, the bands were

too faint to be observable in a printed figure. However, no bands were observed at 4.1 kb by visual review of the autoradiograph. This suggests that tandem integration did not occur. However, total counts of bands were not possible. In addition, a band at 4.1 kb may have been missed at this low concentration of DNA (data not shown).

Trial 3

This was the first large-scale suppressor-REMI trial conducted in for project. Sixteen electroporation groups were carried out at 1.75V, 2.00V, and 2.25V. The same increase in transformation frequency with increased voltage that was observed previously was seen in these groups. To accommodate this volume of work, some groups were double-sized and electroporated in 0.4cm-gap cuvettes (as opposed to the standard 0.2cm-gap cuvette usually used). Transformant yield was from 3- to 20-fold lower in these groups compared to the 0.2cm-gap cuvette groups at the same voltages. Unfortunately, two of the electroporation groups were contaminated, which lowered the transformant yield. However, approximately 4000 Bs^R colonies were obtained from the untainted groups for further study.

The Bs^R colonies were pooled and plated onto *Klebsiella aerogenes* bacterial lawns for plaque development screening. A total of six plaques exhibited a phenotype resembling suppression of $G\alpha4^{HC}$. These were isolated for further study. An autoradiograph from a Southern blot of a HindIII digest of the genomic DNA was obtained, but did not yield useable results as the DNA

concentrations between the lanes were not equal, and were overall too low to obtain any strong signal.

Trial 4

Nine electroporations were carried out at 2.25V, as per previous conditions, with the exception that the *Dictyostelium* cell number/cuvette was high (2.6×10^7 compared to 1.5×10^7). The resultant HL5 plates showed extremely dense Bs^R colony development. Due to this density of growth, colony estimates were based on a sample of the area of the plate, which was then factored for the entire area. Estimates put the colony count for all groups combined between 50,000 and 65,000 transformants. These were pooled and plated onto *Klebsiella aerogenes* bacterial lawns for plaque development screening. Of these, 238 plaques were picked for further analysis. However, when G418 was added to the HL5 medium, all but 40 of these died. The remaining samples did not exhibit any developmental aberrations when replated onto bacterial lawns at low density.

Trial 5

Nine electroporations were carried out as per previous procedure, with the exception that there were 9×10^6 *Dictyostelium* cells/cuvette. Just over 4,000 Bs^R colonies arose from this treatment. These were pooled and plated onto bacterial lawns for plaque development assays. 58 plaques were picked for further study. Fifteen of these were picked from a secondary spread plating as

clonal isolates representative of all of the phenotypes observed. Genomic DNA preparations were made from these isolates for further experimentation.

Gα4^{HC} Construct Copy Number Maintenance Check: A check of copy number maintenance was made in the same fashion as with *Trial 1 and 2 mutants. The autoradiograph is shown in Figure 9 below. The mutant DNA in lane 4 shows a possible loss of some copy number, and lanes 9 and 11 exhibit DNA degradation. All others appear to have maintained high copy number of the Gα4^{HC} construct.

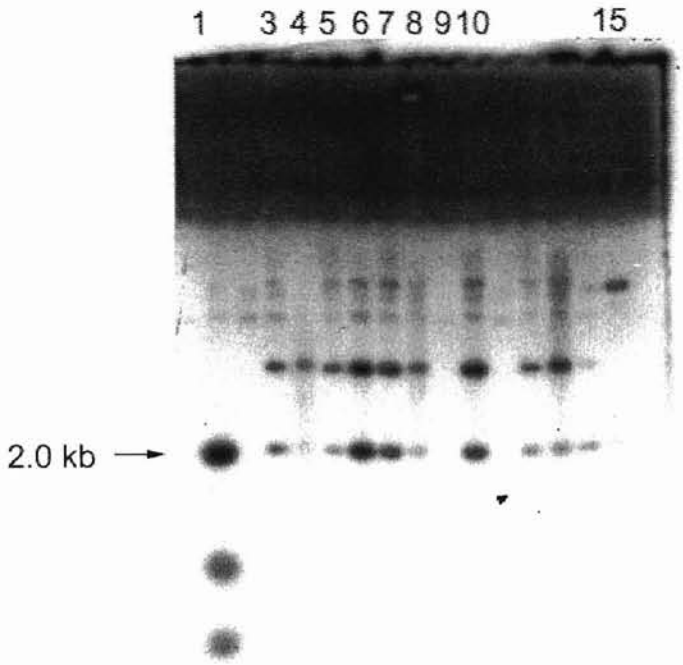

Figure 9: Genomic DNA blot of Trial 5 DpnII-REMI mutants probed for Gα4HC construct

Lane 1: 1 kb ladder; lane 2: empty; lanes 3-13: Trial 5 mutants 1-11; lane 14: JH 384 DNA; lane 15: KAx3 wild-type DNA

Blot was probed with 3.0 kb BamHI/EcoRI fragment of p154 vector containing the majority of the Gα4 ORF from the 5' end

Check for integration of REMI vector into genome: This check was performed in a similar manner to the check of Trial 1 and 2 mutants. The resultant autoradiograph is shown in Figure 10 below. As with the Trial 1 and 2 blot, many of the bands are very faint. However, no bands are visible at 4.1 kb, which supports integration of the REMI vector into the genome rather than maintenance as an extrachromosomal element.

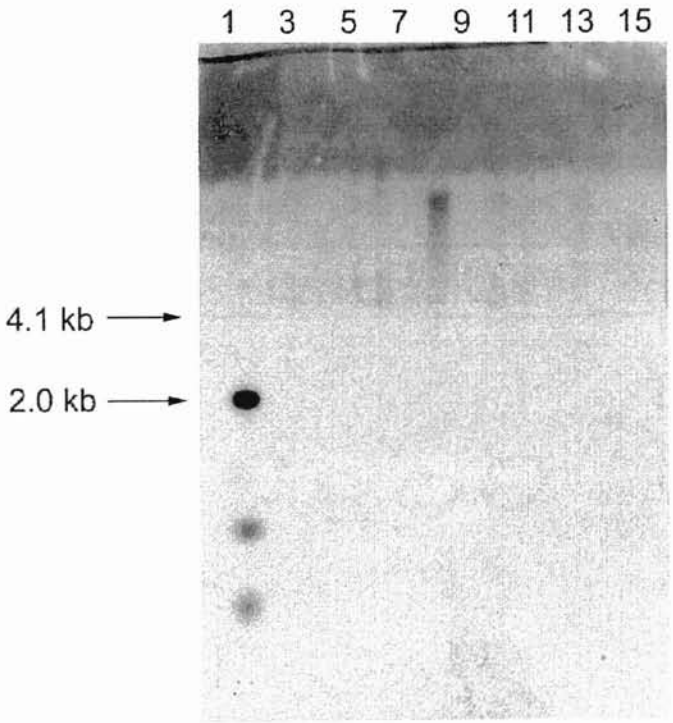

Figure 10: Blot of Trial 5 mutant genomic DNA digested with PstI and probed for BsR gene

Lane 1: 1 kb ladder; lane 2: empty; lanes 3-13: Trial 5 mutants 1-11; lane 14: JH 384 DNA; lane 15: KAx3 wild-type DNA

Blot was probed with 1.4 kb BamHI/HindIII fragment of pBsr2ΔBam vector containing all of the BsR ORF

Check for tandem integration of REMI vector: This check was performed in the same manner as the check of Trial 1 and 2 mutants. The resultant autoradiograph is shown in Figure 11 below. These bands are also rather faint. However, no bands of 4.1 kb are observed. In addition, all lanes that contain observable bands exhibit either one or two bands, but not three or more. Together, these lines of evidence support single integration events in the genome, and not multiple tandem integration when using DpnII-REMI.

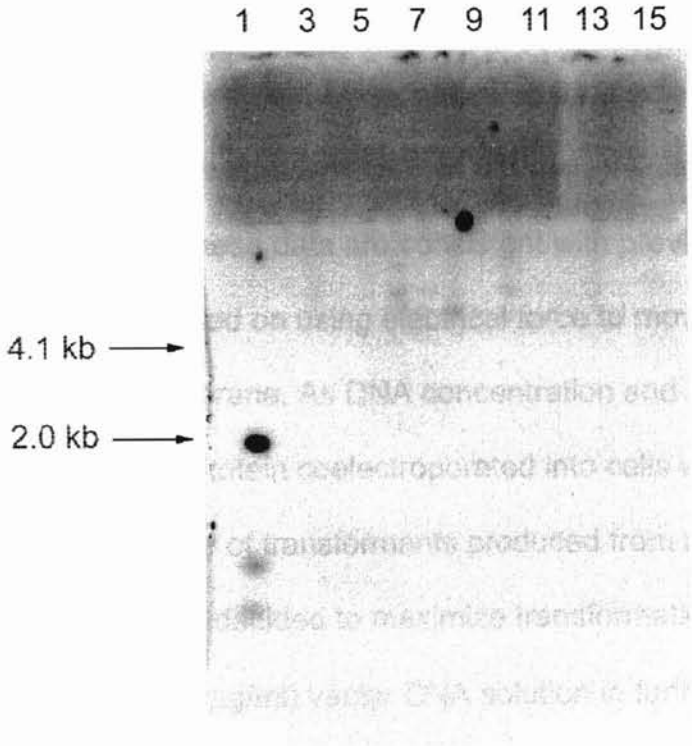

Figure 11: Blot of Trial 5 mutant genomic DNA digested with HindII and probed for the BsR gene

Lane 1: 1 kb ladder; lane 2: empty; lanes 3-13: Trial 5 mutants 1-11; lane 14: JH 384 DNA; lane 15: KAx3 wild-type DNA

Blot was probed with 1.4 kb BamHI/HindIII fragment of pBsr2ΔBam vector containing all of the BsR ORF and actin 15 promoter and actin 8 terminator

CONCLUSIONS

BamHI-mediated Wild-type REMI Trials

These trials were conducted to generally assess transformation frequency with variable amounts of DNA and levels of voltage, as well as to assess whether the REMI vector was inserting at restriction sites or integrating by other means. Observation of the number of Bs^R colonies produced in groups containing differing amounts of vector DNA shows that there is a general increase in transformation efficiency as DNA concentration increases. This effect is seen at all voltages tested. Likewise, it was observed that increasing voltage dramatically increases transformation frequency, yielding an ~100-fold increase from 0.5V to 2.4V. These data are consistent with previous findings. Electroporation is based on using electrical force to move DNA and protein through the cell membrane. As DNA concentration and voltage increase, the amount of DNA and protein coelectroporated into cells will increase, thereby increasing the number of transformants produced from a given number of cells. From this data, it was decided to maximize transformation frequency by using the high concentration (3 μg/ml) vector DNA solution in further trials, and voltages within the 1.75-2.4V range.

The autoradiograph in Figure 5 shows that the flanking restriction sites aroung the REMI vector are maintained. A 4.1kb band is observed in every lane of transformant DNA. This is the size of the REMI vector. Since the REMI vector is not maintained extrachromosomally, this evidence suggests that the BamHI-

linearized vector did insert into genomic BamHI sites, and that those BamHI sites were maintained after the integration event. However, this finding does not rule out the possibility that multiple vectors integrated into the same site in tandem, or that multiple integration events occured.

DpnII-mediated Wild-type Trials

These were performed for the purpose of assessing the variety of mutant phenotypes that could be recovered from a REMI trial. Out of ~10,000 transformants produced with this method, 54 exhibited an aberrant phenotype that was detectable via the plaque development screen. These could be arranged into five distinct classes of phenotypes, with some variation within each class. In addition, one must consider that there are some unaccounted groups of transformants that would contain other gene disruptions not found by the plaque screen. Many mutations would not cause a change in *Dictyostelium* plaque development, and transformants with lethal mutations would die out before the plaque development stage. The variety of mutants recovered combined with these arguments collectively suggest that many different genes were being interrupted in these transformants, which would suggest that the REMI vector was integrating into random restriction sites.

Suppressor-REMI Trials

Just over 10,000 Bs^R colonies were produced through trials 1-3 and 5. Of these, over thirty exhibit some suppressor-like phenotype in clonal culture. This

suggests that suppressors of Gα4 function may be produced by the REMI method. These suppressor-like transformants were also easily found in plaque development screens. It has also been shown through Gα4-specific probing of Southern blots of HindIII-digested Trial 1 and 2 genomic DNA that 4 of the 6 phenotypes are unlikely to be due to loss of the $G\alpha4^{HC}$-plasmid. This suggests that the REMI vector inserts are responsible for the change in phenotype from the background. To prove that the inserts really did cause the changes in phenotype will require that the vectors + flanking sequences be transformed back into background cells. If homologous integration takes place, the wild-type gene will become knocked out, and the resultant cell line will reproduce the original mutant phenotype.

The evidence from the Southern blots of mutant genomic DNA suggests that the REMI vector is integrating into the genome and in single copy at least the majority of the time. These findings are complimentary to those of Morio et al. [24].

Future Research

The next step in this process should be the sequencing of recovered tagged genes which are shown to reproduce a mutant phenotype upon homologous recombination into background cells. Once the sequences are known, they should be compared to other sequences in database searches, such as GenBank, Entrez, Blast, etc. If unique or interesting results come forth from this comparison, the whole gene should be cloned and sequenced. Once

cloned, the gene can be physically mapped with the *Dictyostelium* libraries that are available. Also, epistatic control, double null mutation, and other genetic experiments may be necessary to confirm a direct role of any particular mutant in the Gα4-mediated pathway. In addition, biochemical studies of the protein product and its function could be performed.

If this overall method of screening through REMI transformants is to be used in the future to saturate the *Dictyostelium* genome in search of $Ga4^{HC}$ suppressors, one should consider a change to the REMI vector used. Both the pBsr2ΔBam vector and the $Ga4^{HC}$ expression vector in strain JH384 contain the ampicillin-resistance cassette for selection in bacteria. If another drug resistance cassette were inserted into the polylinker of the pBsr2ΔBam vector, REMI fragments could be positively selected for versus fragments containing the JH384 $Ga4^{HC}$ plasmid. This would help to avoid a major source of error in isolating the REMI vector, as well as save considerable time in bacterial screening and running electrophoreses of DNA digests.

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