OVEREXPRESSION OF ACTIVATED RAS ALTERS CELL FATE DETERMINATION DURING THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

by

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ABSTRACT

The development of *Dictyostelium discoideum* provides an attractive model for studying the role of Ras in cell fate determination since amoebae differentiate into one of only two cell types, stalk or spore. RasD is the predominant Ras expressed during development and it is expressed in both cell types. It was previously demonstrated that a transformant overexpressing activated RasD (*Ddras*-Thr12) forms multi-tipped aggregates, is blocked from further morphogenesis, and exhibits marked changes in cell type specific gene expression. The morphological phenotype was reproduced in *rasD::rasG(G12T)* transformants. It was not known if the developmental defects induced by RasD(G12T) resulted from its expression in prestalk or prespore cells.

Transformants expressing RasG(G12T) from the prestalk specific *ecmAO* promoter formed single-tipped aggregates that produced outwardly normal slugs and almost normal cell type specific gene expression. However, prestalk cells were mislocalized and terminal morphology was abnormal: stalk cells were present in basal cell masses and there were no spore cells or stalk tubes. Thus, the formation of multi-tipped aggregates and deregulated cell type specific gene expression is not a consequence of activated Ras expression in prestalk cells.

Transformants expressing RasG(G12T) from the prespore specific *psA* promoter formed mounds with multiple tips and cell type specific gene expression was markedly altered. However, the tips extended to form finger-like and then slug-like structures which then formed stalks. Therefore, the formation of multiple tips and the deregulation of cell type specific gene expression, but not the block in

morphological development, results from expression of activated Ras in prespore cells.

Since limiting overexpession of activated Ras to either of the cell types did not completely recapitulate the phenotype of the *Ddras*-Thr12 transformant, cotransformants were generated which expressed both *ecmAO::rasG(G12T)* and *psA::rasG(G12T)*. These cotransformants formed aggregates with multiple protruding tips that were blocked from further morphogenesis. Thus, when activated Ras is expressed from the *rasD* promoter, the developmental aberrations result from expression in both cell types.

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LIST OF ABBREVIATIONS

ALC	Anterior like cells
BME	β-mercaptoethanol
BMP-4	Bone morphogenetic protein-4
BS	Bonner's salts starvation buffer
cAMP	Cyclic adenosine monophosphate
cAR	cAMP receptor
cDNA	Complementary deoxyribonucleic acid
[^{35}S]-α-dATP	[^{35}S]-labeled deoxyadenosine triphosphate
[^{32}P]-α-dCTP	[^{32}P]-labeled deoxycytosine triphosphate
DIF-1	Differentiation inducing factor-1
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetaacetic acid (disodium salt)
EGFR	Epidermal growth factor receptor
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GSK3	Glycogen synthase kinase 3
HBS	Hepes-buffered saline
HRP	Horseradish peroxidase
KK2	A potassium phosphate starvation buffer
LB	Luria broth

MAPK	Mitogen activated protein kinase
MOPS	Morpholinoprapanesulfonic acid
mRNA	Messenger ribonucleic acid
NAPS	Nucleic acid processing service
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI3K	Phosphoinositol 3-kinase
PKA	Protein kinase A
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SM VAN	A nutrient rich growth medium
SSC	Sodium chloride/sodium citrate buffer
TBE	Tris-buffered EDTA
TBST	Tris-buffered saline with tween-20
TGFβ	Transforming growth factor β
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylene-20-sorbitan monolaurate
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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To my mother and father, Karim, and Ma,
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CHAPTER ONE

INTRODUCTION

"...[one must have the ... patience to] 'hear what the material has to say to you, [the openness to] let it come to you, ... a feeling for the organism."

Barbara McClintock
(from Keller, 1983)

1.1 Pathways Regulating Development

Every aspect of multicellular development including cell growth and death, axis specification, cell and tissue differentiation, morphogenesis, healing, and regeneration is regulated by signal transduction. John Gerhart (1998) counted at least seventeen different pathways that orchestrate the development of multicellular organisms. Many components of pathways and even the interactions between components are well conserved amongst evolutionarily divergent species. It is this strong conservation that validates the use of model eukaryotic organisms in our attempts to understand the signaling pathways that specify developmental decisions. Some of the best understood of these pathways are briefly described below.

1.1.1 The Wnt Pathway

The Wnt signal transduction pathway (Wodarz and Nusse, 1998) is utilized by diverse organisms for varied developmental decisions such as the establishment of parasegment boundaries and cuticle pattern in the *Drosophila* embryo. A fruitfly Wnt signal, Wingless (Wg), is received by a 7-span transmembrane receptor of the

Frizzled family. Disheveled (Dsh) is a cytoplasmic protein required for Wnt signaling. The function of Dsh is not known but the protein is recruited to the plasma membrane in response to the Wg signal and contains protein interacting domains indicating that it may serve as an adaptor protein. Downstream of Dsh is Zeste-white 3 (Zw3) the homologue for the serine/threonine kinase, glycogen synthase kinase 3 (GSK3). Wg signaling represses Zw3 which otherwise constitutively represses Arm/β-catenin by shortening its half-life. With GSK3 activity suppressed, β-catenin accumulates, and forms a transcription factor complex with LEF-1 to regulate gene expression.

1.1.2 The TGFβ Pathway

The TGFβ superfamily signals have been shown to be involved in various developmental processes such as mesoderm ventralization in *Xenopus*. A distinguishing feature of this pathway (reviewed by Whitman, 1998) is that the TGFβ family ligands bind and induce dimerization of two distinct receptor serine/threonine kinases (Cho and Blitz, 1998). The type II receptor phosphorylates and activates the type I receptor which then relays the signal by phosphorylating specific receptor-activated members of the Smad family of proteins. Once phosphorylated, the specific Smads (e.g. Smad1, Smad2) complex with other Smad proteins that are shared amongst TGFβ pathways (e.g. Smad4). The Smad complex then translocates to the nucleus and regulates the expression of target genes. A third group of inhibitory Smad proteins (Smad6 and Smad7) antagonize TGFβ signaling, either by preventing the type I receptor-mediated phosphorylation of Smad1 and

Smad2 proteins or by preventing the association of phosphorylated Smad1 and Smad2 proteins with Smad4. In some cases, the inhibitory Smad proteins are induced by the signal they antagonize resulting in a negative feedback loop that limits signal duration. TGFβ signaling is also negatively regulated by factors secreted by the dorsal mesoderm. Noggin, Chordin and other factors in *Xenopus* bind to TGFβ family signals and prevent their interaction with receptors and consequently permit dorsal development.

1.1.3 The Hedgehog Pathway

Signaling via the Hedgehog (Hh) pathway has a demonstrated involvement in the patterning of various structures including the *Drosophila* wing and the chick limb. Two different transmembrane proteins, Smoothened (Smo) and Patched (Ptc), are part of the Hh signaling pathway (reviewed in Johnson and Scott, 1998). It appears that Ptc inhibits Smo, possibly through a physical association. This inhibition is relieved in the presence of the Hh signal, allowing Smo to relay the signal. The endpoint of the Hh pathway is the control of a transcription factor of the Gli family. The *Drosophila* transcription factor, Cubitus interruptus, Ci, exists in a complex and is released by Hh signaling. Activation of Ci results in the transcription of specific genes including *ptc*, as well as genes encoding members of other signaling pathways.

1.1.4 The Notch Pathway

The Notch pathway (reviewed in Weinmaster, 1998) acts in neurogenesis, myogenesis, and hematopoiesis to prevent progenitor cells from differentiating. In

addition, signaling via Notch can promote the specification of certain cell types. Notch is a cell surface receptor and is activated upon interaction with another cell expressing a specific ligand (Jagged 1 or 2, or Delta 1, 2, or 3). Once activated, Notch is proteolytically cleaved such that the intracellular portion of the protein is released. Truncated Notch protein then interacts with a transcription factor of the CSL group. This association results in the conversion of the CSL family protein from a transcriptional repressor to a transcriptional activator. Surprisingly, disrupting the Notch domain required for interaction with the CSL protein does not necessarily disrupt Notch-mediated signaling, indicating the presence of an alternate Notch pathway, independent of the CSL proteins.

1.1.5 The Receptor Tyrosine Kinase Pathway

Pathways downstream of receptor tyrosine kinases (RTK) (Gerhart, 1998) are involved in numerous developmental processes such as eye, limb, and muscle development. Ligand binding to the extracellular domain induces dimerization of receptor molecules and cross phosphorylation of their cytoplasmic tails. The activated receptors can then promote signaling via downstream pathways such as the Ras and the phosphoinositol-3 kinase (PI3K) pathways. The RTK-Ras pathway is addressed in more detail later in Section 1.3.

1.1.6 Signaling Networks That Organize Development

In recent years, much progress has been made in dissecting the various pathways, such as the ones described above, that are utilized to transmit extracellular signals to the nucleus. It has become apparent, however, that these

signaling pathways do not work in isolation, rather developmental decisions are made in response to a combination of various positive and negative signals transduced through multiple pathways. The process of muscle development provides one example of how signaling pathways converge to regulate development.

The MyoD family of proteins are basic helix-loop-helix transcription factors that interact with transcription factors of the MADS family and are essential for the specification and differentiation of vertebrate skeletal muscle (reviewed in Brand-Saberi and Christ, 1999; Chen and Goldhamer, 1999). In mice, two members of the MyoD family, MyoD and Myf-5 are required for muscle lineage determination and their overexpression can convert many cells to the myogenic fate. The other two family members, Myogenin and MRF4 are required for the differentiation of committed cells.

Investigations into the regulation of expression of the genes of the *myoD* family revealed the presence of complex signaling interactions. Wnt signals from the neural tube and the dorsal ectoderm function to induce the expression of *myoD* and *myf-5*. It is possible that different Wnt signals may induce the different *myoD* genes. Sonic hedgehog (Shh) signals from the notochord and the ventral neural tube also positively regulate myogenic specification. Ectopic *shh* expression results in an increased domain of *myoD*-expressing cells whereas *shh* knockout mice exhibit a block in *myf-5* expression. MyoD-dependent muscle cell specification is also regulated by negative signals such as Bone Morphogenetic Protein-4, BMP-4 (a TGFβ family signal), produced by the lateral plate mesoderm. A further complexity is that the negative influence of BMP-4 is antagonized by Noggin and Noggin is

downstream of both Wnt and Shh. These results indicate that the process of muscle development (and probably all other developmental processes) is coordinated by a complex network of signals that are only just beginning to be understood.

1.2 Repetitive Use of Signaling Pathways

Although numerous signal transduction pathways operate during multicellular development, these are used repeatedly by the same organism to specify very different outcomes. The *Drosophila* epidermal growth factor receptor (EGFR) homolog provides an excellent example to illustrate this point (Perrimon and Perkins, 1997; Freeman, 1998). In the embryo EGFR is involved in several processes including the establishment of ventral cell fates, the specification of cells of the nervous system, and the production of cuticle. It appears that in all instances, EGFR-mediated effects are dependent on signal transduction through a single signaling pathway, that involving Ras/Raf/MAPK. (This pathway will be discussed later in Section 1.3). Consequently, the question arises as to how one signaling pathway can specify such a diverse set of outcomes. In the next few sections, I will briefly present some of the common mechanisms by which various signaling pathways ensure the specificity of downstream effects.

1.2.1 Negative Feedback Mechanisms

In a recent review, Perrimon and McMahon (1999) discussed how negative feedback of incoming signals can regulate a cell's response either by regulating the level of induced activation, by spatially limiting the field of cells responding to the signal, or by modulating the effects of responding cells according to signal

concentration. Negative feedback can be cell autonomous, limiting an activated cell's response to a signal, or non-cell autonomous, also limiting the response of neighboring cells.

RTK signaling in *Drosophila* is subjected to cell autonomous negative feedback control by the cytoplasmic protein Sprouty (Sty). Sty was first observed to function as a negative regulator of FGFR signaling during tracheal development (Hachohen et al., 1998). Subsequently, Sty was also shown to inhibit EGFR signaling (Casci et al., 1999). Loss of *sty* results in eyes with too many photoreceptor, cone, and pigment cells whereas overexpression of *sty* is correlated with a decrease in the number of these eye cells. In the same study, Sty was also found to antagonize the effects of activated Torso (Tor) and Sevenless (Sev), two other *Drosophila* RTKs. Sty appears to regulate RTK signal transduction by preventing transmission of the signal to Ras. Sty binds to the Drk adaptor protein and to Gap1 indicating inhibitory mechanisms whereby Sty may bind Drk and prevent the recruitment of SOS. Alternatively, Sty may bind and recruit Gap1 to the plasma membrane to inactivate Ras. Since *sty* expression is dependent on the pathway it consequently inhibits (Hachohen et al., 1998; Casci et al., 1999) it functions as a cell autonomous negative regulator modulating the level of activity of the RTK/Ras pathway.

The importance of controlling the level of signal activity is illustrated by the work of Greenwood and Struhl (1997). Torso (Tor) is involved in specifying the terminal parts of the *Drosophila* embryo. Active Tor signals through the Ras pathway to induce expression of the genes for the Tailless (Tll) and Huckebein (Hkb) transcription factors. The level of Tor/Ras activity determines the expression of these two genes and the differentiation of terminal structures (Greenwood and

Struhl, 1997). A high level of Ras activity results in the expression of both *tll* and *hkb* and the consequent specification of terminal structures such as the anal pad and tuft. Lower Ras activity results in the expression of *tll* but not *hkb* and the specification of less terminal structures such as abdominal parts. How the intensity of Tor-mediated Ras activity is regulated is not known. It is possible that the level of Tor activation may itself be regulated by a gradient of the activating ligand. In light of the above-mentioned findings, it is conceivable that Sty or another negative regulator may fine-tune the level of Ras activity to specify different developmental structures.

Drosophila RTK signaling is also subject to non-cell autonomous negative feedback regulation. As described above, the EGFR functions in the specification of several cell fates. Argos (Aos) has been shown to modulate the level of EGFR signaling to specify several distinct outcomes (Freeman, 1996; Golembo et al., 1996a; Wasserman and Freeman, 1998). Aos contains an EGF motif (Howes et al., 1998) and is a secreted inhibitor of EGFR signaling (Schwietzer et al., 1995). The expression of *aos* is dependent on EGFR signaling (Golembo et al., 1996b) and this thus constitutes a negative feedback loop that influences EGFR signaling in neighboring cells.

In the developing eye, the EGFR is required for the specification of all cell types in successive waves of induction (Freeman, 1996). First, there is recruitment of the photoreceptor neurons, followed by recruitment of the cone cells, and finally recruitment of the pigment cells. (The requirement for EGFR in R8 photoreceptor cell differentiation is not known). Inhibition by Aos establishes the necessary waves of induction to ensure that in a particular subset of cells, EGFR is activated when the cells are competent to differentiate into the correct cell type. Initially, the EGFR

ligand, Spitz (Spi), is secreted by the central cells (R8, R2, R5). This results in the differentiation of neighboring cells into photoreceptor cells (R3, R4, R1, R6, R7). As the cells differentiate, they express and secrete Aos which diffuses outward to prevent more distal cells from assuming the photoreceptor fate. Later, these distal cells overcome Aos inhibition and produce Spi (Fig. 1-1). Aos thus spatially restricts the activation of the EGFR, enabling specific patterning during development.

1.2.2 Morphogen Gradients

Spatial concentration gradients in morphogen distribution can invoke different developmental decisions on a field of cells. One way in which such gradients are established is by regulating the spatial distribution of the receptor for a secreted morphogen (Perrimon and McMahon, 1999). Ptc is the transmembrane protein proposed to bind Hh (Chen and Struhl, 1996). Ptc expression is induced by the presence of Hh. The absence of Ptc correlates with an increase in the domain size of Hh-activated cells. Also, high levels of Ptc restrict the spread of Hh. Thus, by activating the expression of its own receptor, Hh uses a negative feedback inhibition mechanism to limit its ability to move through developing tissue. Variations in the ability of different cells to induce *ptc* expression in response to Hh may facilitate the establishment of different distribution ranges for the signal.

1.2.3 Asymmetric Cell Division

One mechanism that enables a diversity of cell fates to be established from a

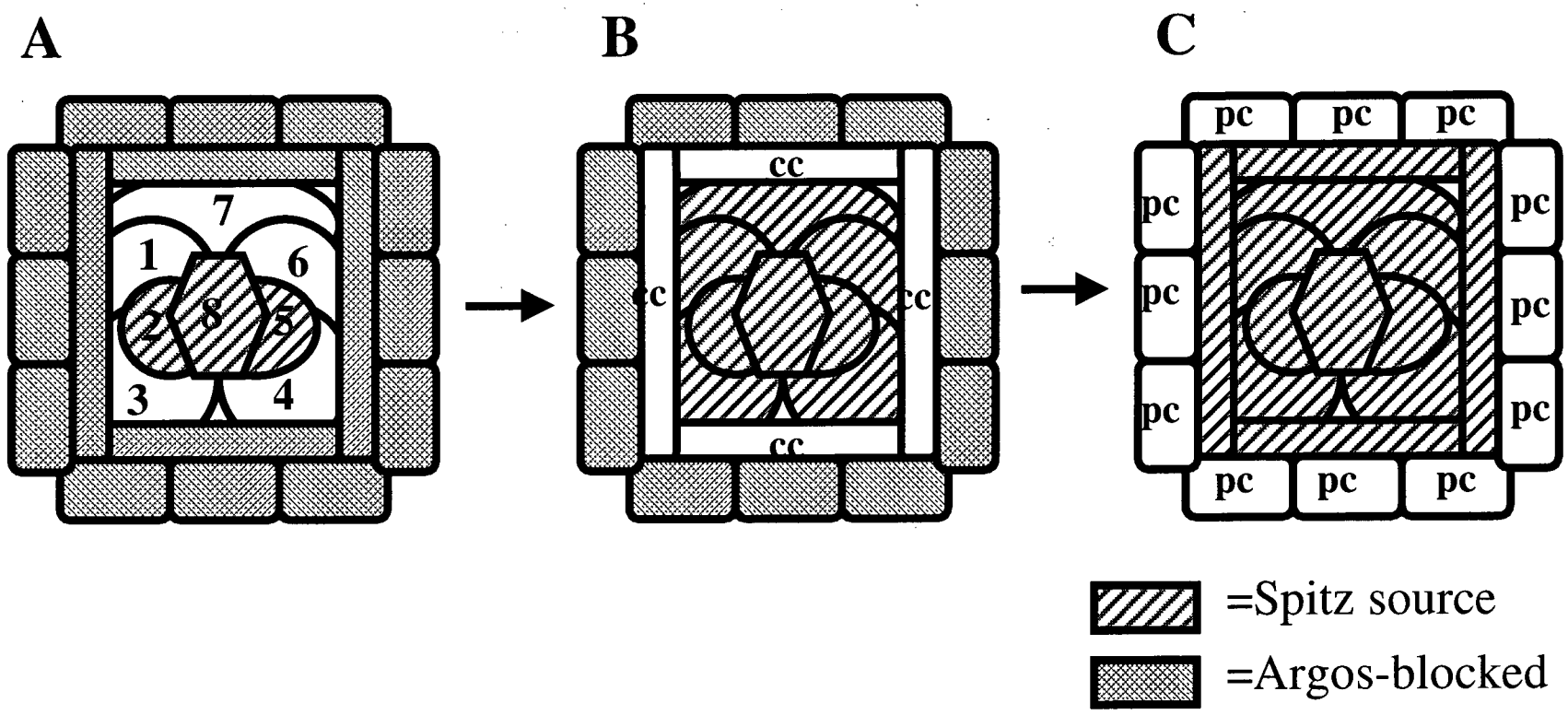


Figure 1-1: Spitz and Argos during eye development in *Drosophila*.
Cells secreting Spi are labeled with diagonal lines; those inhibited by Aos are spotted. The photoreceptors are numbered 1 through 8. In (A) the central photoreceptor cells are producing Spi. Cone cells (cc) and pigment cells (pc) are inhibited by Aos. In (B) all photoreceptor cells are producing Spi and the cc are recruited to differentiate. Only the pigment cells are inhibited by Aos. In (C) the photoreceptor and cone cells are producing Spi. None of the cells are inhibited by Aos during recruitment of the pigment cells.
(Adapted from Freeman, 1996).

single progenitor cell is that of asymmetric cell division (Rhyu and Knoblich, 1995). The result of such cell division is the generation of two daughter cells that are unequal with respect to some fate-specifying agent. For example, during embryonic *Drosophila* development, a sensory organ precursor (SOP) cell divides asymmetrically generating a neuron and glia precursor (NGP) cell and a shaft and socket precursor (SSP) cell. Another round of asymmetric cell division results in four distinct daughter cells: a neuron, a glia, a socket, and a shaft (hair) cell (Okano, 1995). One of the genes regulating this process is *numb* (Uemura et al., 1989; Rhyu et al., 1994). Loss-of-function *numb* mutants prevent the first asymmetric division and instead generate two SSP cells. Consequently, the differentiation of neuron and glia cells does not occur. Overexpression of *numb* results in the opposite phenotype and two NGPs are formed (Rhyu et al., 1994). Numb is a plasma membrane protein and its distribution is in the shape of a crescent that is limited to half of the SOP and overlies one of the centrosomes (Rhyu et al., 1994; Knoblich et al., 1995). Since cell division is asymmetric, only one daughter cell, the NGP, inherits the Numb protein indicating that it is the differential acquisition of Numb that distinguishes the lineage (Fig. 1-2). During the second round of asymmetric cell division, Numb is again localized to only half the cell membrane and is consequently only bequeathed to the neuron cell. The SSP cell that did not inherit Numb from the SOP, also divides asymmetrically. Before mitosis, Numb is synthesized and localized to half the cell membrane. Upon division, the daughter cell inheriting Numb becomes a shaft cell (Wang et al., 1998). The repetitive use of Numb indicates that it is a

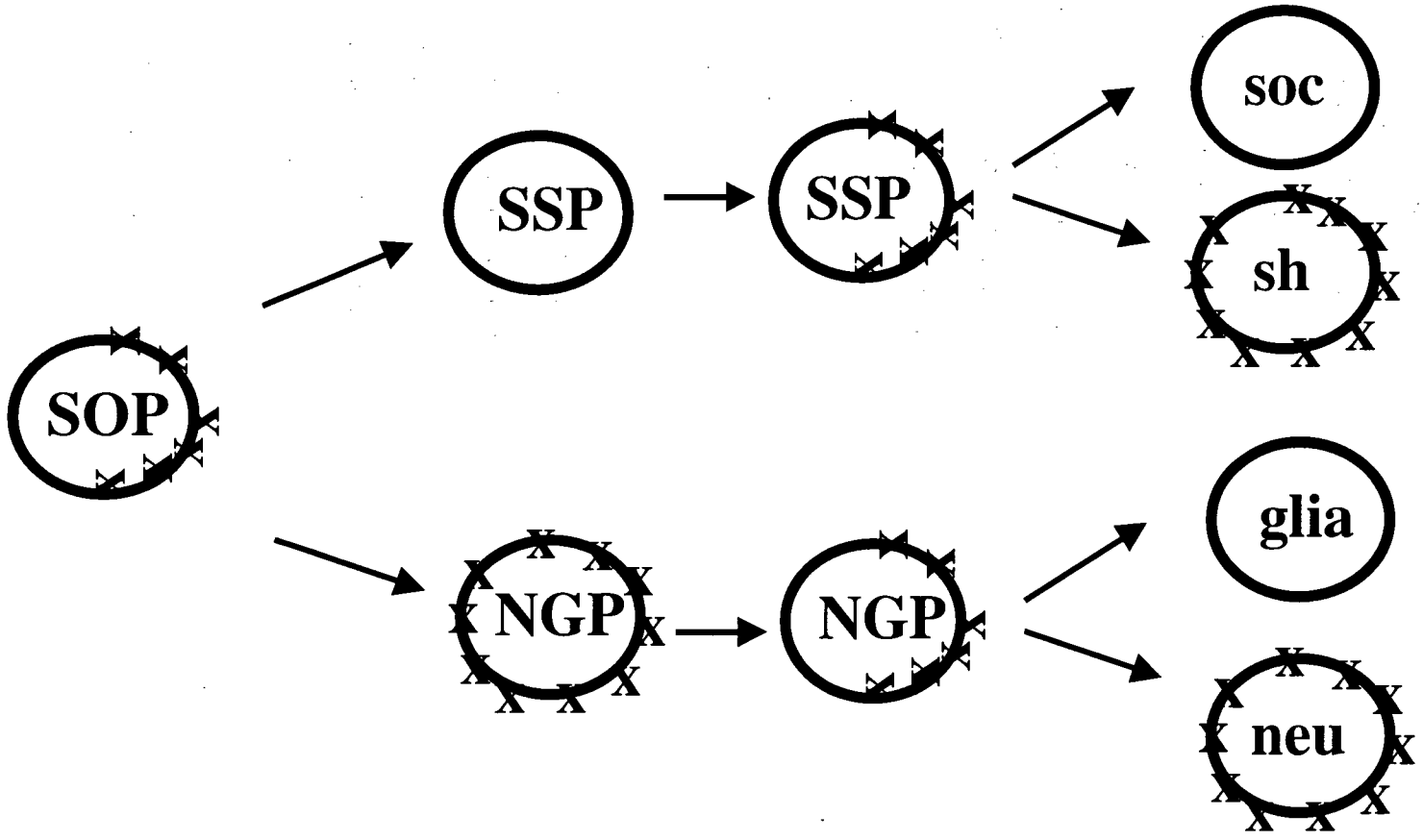


Figure 1-2: Asymmetric cell division of a sensory organ precursor (SOP) cell during Drosophila neurognesis. The 'X' markings indicate distribution of the Numb protein in the cell membrane. In the first cell division, the neuron and glia precursor (NGP) cell inherits Numb where as the shaft and socket precursor (SSP) does not and must therefore synthesize Numb before the second cell division. After the second division, the cells that inherit Numb differentiate as shaft (sh) and neuron (neu) cells. Daughter cells without Numb differentiate as socket (soc) and glia cells.
(Adapted from Lu et al., 1998).

common mediator for distinguishing two different daughter cell fates following cell division (Lu et al., 1998).

1.3 Ras Proteins

A key signaling intermediate involved in multiple cell fate decisions is Ras. The Ras proteins are small monomeric guanine nucleotide binding proteins (G proteins) associated with the inner surface of the cytoplasmic membranes of all eukaryotic cells. Ras was originally identified as the transforming oncogene in animal tumor viruses. Furthermore, the human Ras proteins, H-Ras, N-Ras, and K-Ras were found to be mutated in approximately 15% of all tumors (Bos, 1989). Wild type Ras proteins cycle between a GTP-bound 'on' state and a GDP-bound 'off' state in their function as molecular switches (Satoh et al., 1992). They are involved in the transduction of diverse signals from cell surface receptors via various effectors to regulate cell growth, differentiation, and apoptosis (reviewed in Khosravi-Far and Der, 1994; Denhardt, 1996; Campbell et al., 1998). Ras proteins possess intrinsic GTPase activity which is very low but which is enhanced by GTPase activating proteins or GAPs (Wittinghofer, 1998; Boguski and McCormick, 1993). The GAPs (p120GAP and NF1) are thus negative regulators of Ras activity. Amino acid substitutions at positions 12, 13, 61 interfere with GAP-stimulated GTP hydrolysis and thus render the mutant Ras protein constitutively active. The guanine nucleotide exchange factors (GEFs), including SOS1, SOS2, CDC25, RasGRF, postitively regulate Ras activity by promoting the exchange of bound GDP for GTP (Boguski and McCormick, 1993; Quilliam et al., 1995).

1.3.1 Role of Ras in Cell Proliferation and Transformation

Once activated, Ras induces proliferation in diverse cell types and constitutively active Ras has transforming potential (Lowe and Skinner, 1994). Ras proteins are activated by a variety of signals that bind to and activate receptor tyrosine kinases (RTKs), receptor-associated tyrosine kinases, as well as heterotrimeric G protein–coupled serpentine receptors (Khosravi-Far and Der, 1994). The best characterized signaling pathway that links a cell surface receptor to Ras is downstream of activated RTKs (reviewed in Schlessinger, 1993; Khosravi-Far and Der, 1994) (see Fig. 1-3). In response to ligand binding, individual RTK molecules dimerize and transphosphorylate their cytoplasmic tails. The adaptor protein, Shc, binds to the tyrosine phosphorylated receptor via Src homology 2 (SH2) domains (Mayer and Baltimore, 1993; Feig, 1994). Shc is then phosphorylated and directs the binding of another adaptor protein, Grb2 via its SH2 domain. Shc is not required for Grb2 recruitment since Grb2 can also interact directly with activated EGFR. Grb2 is associated with SOS; consequently then, the association of Grb2 with Shc (or with an activated RTK) effectively results in the recruitment of SOS to the plasma membrane where it can activate Ras (Feig, 1994; Khosravi-Far and Der, 1994).

An important downstream effector of active Ras is the serine/threonine kinase Raf, a mitogen activated protein kinase kinase kinase (MAPKKK or MEKK), (Moodie and Wolfman, 1994). When bound to GTP, Ras can interact with Raf which results in the translocation of Raf to the membrane where it is activated (Morrison and Cutler, 1997). Raf then phosphorylates a MAPK kinase, MEK1 or MEK2 (Crews and Erickson, 1993). The MEKs then phosphorylate tyrosine and threonine motifs in two MAPKs, ERK1 and ERK2 (Crews et al., 1992). These

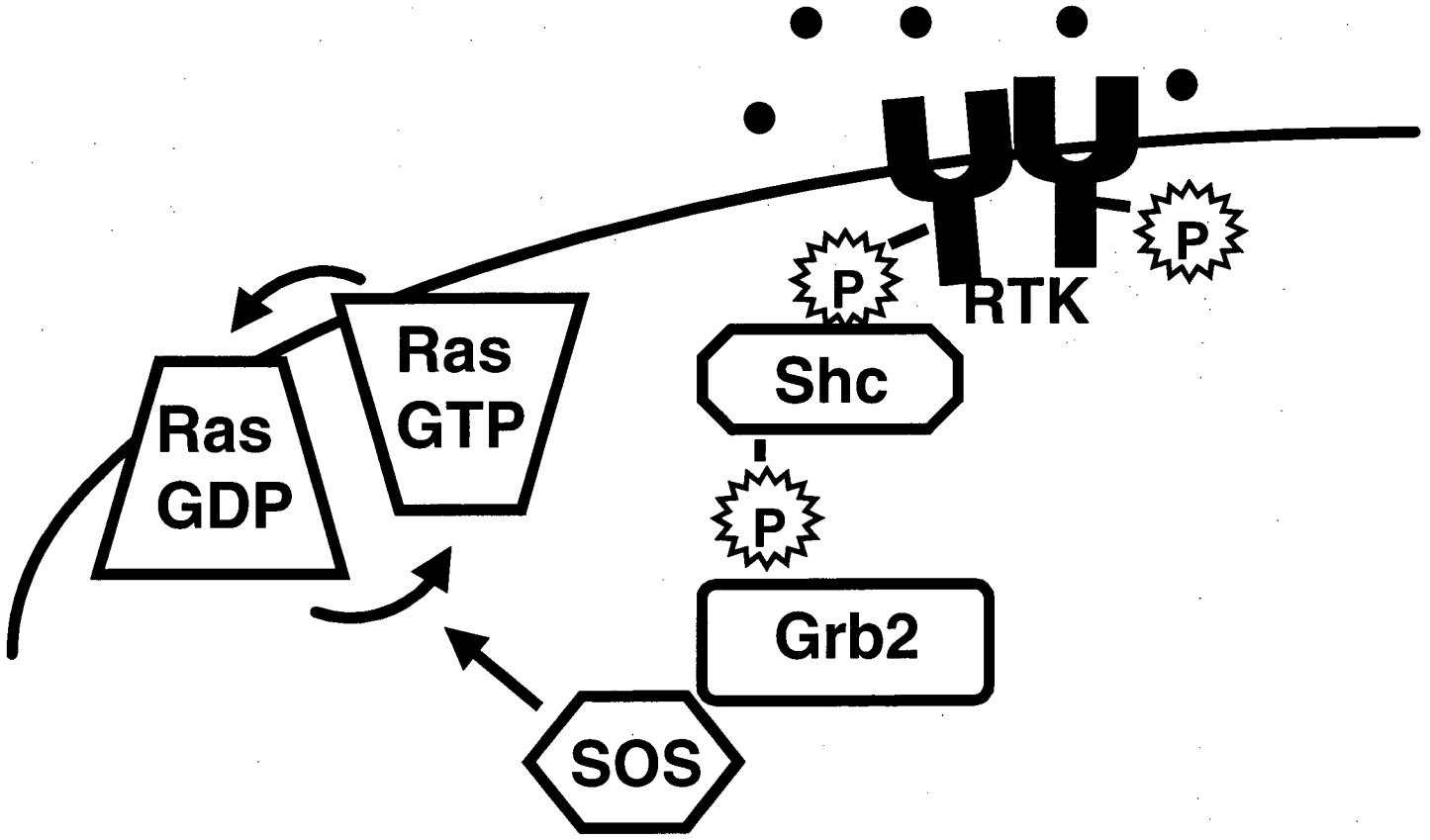


Figure 1-3: Model for RTK-mediated activation of Ras. Ligand binding induces the dimerization of RTK molecules followed by the phosphorylation of their cytoplasmic tails. (Phosphorylation is indicated by a 'P' enclosed in a jagged circle). The adaptor protein Shc binds to phosphorylated receptor and is itself phosphorylated and consequently serves as a binding site for Grb2. Since Grb2 is complexed with SOS, its binding to Shc results in the recruitment of SOS to the membrane where it can catalyze the exchange of bound GDP for GTP on Ras.

activated MAPKs then translocate to the nucleus where they activate kinases such as p90RSK (Blenis, 1993), or transcription factors such as Elk-1 (Marais et al., 1993), and thereby mediate the regulation of gene expression.

This linear Ras/Raf/MAPK signaling cascade has been shown to be conserved through evolution. However, it is only one of several signaling pathways downstream of Ras (Campbell et al., 1998). Effector domain mutants of activated Ras that are unable to interact with Raf (White et al., 1995) still retain some properties of Ras-mediated transformation (Khosravi-Far et al., 1996) indicating that additional effectors must function downstream of active Ras. It was subsequently shown that GTP-bound Ras activates two other MAPK cascades – the JNK/SAPK and the p38/HOG cascades (Kyriakis and Avruch, 1996) that are dependent on MEKK1 (Johnson et al., 1996) but independent of Raf (Minden et al., 1994; Olson et al., 1995). Thus, MEKK1 is likely another direct effector of Ras.

Although the Ras GAPs, p120 GAP and NF1 negatively regulate Ras activity (Boguski and McCormick, 1993), there is evidence supporting effector roles for both proteins (McCormick, 1995; Tocque et al., 1997). p120GAP knockouts (Henkenmeyer et al., 1995) and NF1 knockouts (Bollag, et al., 1996) are both embryonic lethal in mice. In addition, the N-terminal domain of p120GAP disrupts the actin cytoskeleton, possibly via an interaction with the GAP for another small GTPase, Rho (McGlade et al., 1993). NF1 probably also has additional signaling roles. Although it is unable to stimulate GTPase activity of activated Ras, NF1 inhibits transformation by v-Ras (Johnson et al., 1994).

Another enzyme that functions as an effector of Ras is phosphatidylinositol 3-kinase (PI3K) which catalyzes the phosphorylation of the 3′ position of the inositol

ring of phosphoinositides (Carpenter and Cantley, 1996a). Ras interacts with and activates multiple isoforms of PI3K (Rodriguez-Viciana, et al., 1997). Ras can activate PI3K *in vivo* and expression of activated Ras in COS cells results in an increase in the levels of PIP_3 (Rodriguez-Viciana et al., 1994). The pathways downstream of PI3K include the small GTPase Rac, p70S6 kinase, protein kinase B (PKB/Akt), and several atypical isoforms of PKC (Carpenter and Cantley, 1996b).

Evidence has recently been accumulating for the GEFs of another small GTPase, Ral, as effectors for Ras (Feig et al., 1996). Transient expression of Ras in COS cells has been shown to increase RalGEF activity (Urano et al., 1996). Furthemore, overexpression of the Ras-interacting domain (RID) of the RalGEFs inhibits Ras-mediated NIH 3T3 transformation and Raf activity. However, RID overexpression did not reduce Raf-mediated transformation, indicating that RID specifically binds to Ras and prevents transduction of the signal from Ras to Raf (Okazaki et al., 1996; Peterson et al., 1996). In addition, dominant negative Ral decreases Ras-mediated focus formation (Urano, et al., 1996).

The existence of a several downstream effectors for Ras raises a number of interesting scenarios (Campbell, et al., 1998). It is possible that some effectors may mediate the functions of normal Ras where as others are activated only in response to oncogenic Ras. Some of these putative effectors may actually be negative regulators that prevent productive interactions with signal transducing effectors. Other effectors may confer tissue specific consequences for Ras signaling. Perhaps different Ras proteins can activate different pathways via specific effectors. Also, given the very high degree of conservation amongst the proteins of the Ras family,

it is possible that some of the putative Ras effectors may actually function as effectors for Ras-related proteins under physiological conditions.

1.3.2 Roles for Ras in Development

One of the first indications for a role for Ras in differentiation came from experiments conducted with the PC12 phaeochromocytoma cell line. In this cell line, activated Ras can replace the requirement for nerve growth factor (NGF) for induction of neuronal differentiation (Szeberenyi et al., 1990). Since then, Ras has been shown to be involved in the regulation of developmental decisions in several organisms. Although the pathways mediating these decisions are not fully understood, it appears that the pathways defining Ras-mediated differentiation are the same as those that mediate proliferation.

1.3.2.(a) Invasive Growth of *Saccharomyces cerevisiae*

Haploid *S. cerevisiae* cells respond to nitrogen starvation by initiating a program of invasive filamentous growth (Roberts and Fink, 1994). Mosch et al. (1999) studied the role of Ras in this differentiation process. The *S. cerevisiae* Ras homologues, Ras1p and Ras2p (see Broach, 1991 for a review) are independently dispensable for growth but a double knock out is lethal. However, *ras2* null cells cannot induce filamentous growth. Clearly, endogenous Ras1p cannot substitute for Ras2p but, overexpression of *ras1* does rescue the loss-of-function ras2 defect. It was found that Ras2p induces the invasive growth response by activating both the MAPK and PKA pathways. Thus, in addition to regulating the mitosis (Morishita et

al., 1995) and longevity (Sun et al., 1994) of *S. cerevisiae* cells, Ras2p also regulates the ability of these cells to undergo starvation-induced differentiation.

1.3.2.(b) Embryonic Axis Formation in *Xenopus laevis*

During *Xenopus* embryogenesis, basic fibroblast growth factor (bFGF), activin, and BMP-4 have been identified as inducers of mesoderm development. All three mediate their effects via Ras (Whitman and Melton, 1992; MacNicol et al., 1993; Xu et al., 1996). BMP-4 is required for ventral mesoderm development, activin is required for dorsoanterior development, and bFGF is required for posterior and lateral development. Thus, Ras activity is implicated in multiple developmental pathways during *Xenopus* embryogenesis. Interestingly, although activin and BMP-4 are both proteins of the TGF-β family, BMP-4 requires both Ras and Raf function for its effects (Xu et al., 1996) while activin does not require Raf (Whitman and Melton, 1992). This distinction indicates that diverse Ras signaling pathways may lead to different developmental effects.

1.3.2.(c) Roles for Ras during *Caenorabhditis elegans* Development

The best characterized role for Ras during the development of *C. elegans* is vulval cell specification (Kayne and Sternberg, 1995; Sternberg and Han, 1998). Normally, six multipotent vulval precursor cells (VPCs) can differentiate into one of three fates (Kenyon, 1995; Sundaram and Han, 1996). Above the VPCs lies an anchor cell (AC) which induces vulval development. The VPC that lies beneath the AC generates the 1° lineage and the two adjacent VPCs generate the 2° lineage. Each of the 1° and 2° VPCs divide three times and produce vulval cells. The 1° VPC

produces 8 vulval descendents that become attached to the AC. The 2° VPCs produce 7 vulval cells each. In the 3° lineage, each of the three remaining VPCs divide to produce two epidermal cells (Sulston and White, 1980).

Without induction, all 6 VPCs assume the 3° epidermal fate and no vulva forms. Overinduction of the vulval signaling pathway results in a multivulva phenotype. The *C. elegans* homolog of Ras, Let-60, is involved in this pathway of vulval cell specification (Han and Sternberg, 1990). Dominant-negative *let-60* alleles were found to suppress a multivulva phenotype. Gain-of-function *let-60* alleles were found as multivulva mutants in an otherwise wild type background or as suppressors of a vulvaless mutant. The nematode homologue for the EGFR, Let-23, was shown to be upstream of Let-60 Ras. Downstream of Ras, this signaling pathway involves the *C. elegans* homologs of Raf (Han et al., 1993), MEK2 (Wu et al., 1995), and MAPK (Lackner et al., 1994; Wu and Han, 1994). Thus the RTK/Ras/Raf/MAPK pathway described earlier is conserved in *C. elegans* and regulates the decision of vulval fate specification.

There is evidence for a combined role of Ras and HOX genes in vulval fate specification. Let-60 Ras mediated vulval specification requires the product of the HOX gene *lin-39* (Maloof and Kenyon, 1998). *lin-39* is expressed at a low level in the VPCs (Clark et al., 1993). In response to Ras induction, *lin-39* expression is upregulated (Maloof and Kenyon, 1998). If Lin-39 is absent during Ras signaling, the VPCs do not divide and vulval cells are not formed. In addition to permitting Ras-mediated differentiation, HOX genes can confer specificity. Ras signaling is also involved in the differentiation of the pre-anal ganglion (PAG). The PAG precursors express the HOX gene *mab-5* (Salser et al., 1993). If *lin-39* is expressed in *mab-5*

animals, induced PAG precursor cells adopt vulval characteristics. Similarly, when *mab-5* was expressed in a *lin-39* deficient organism (using a temperature sensitive *lin-39* allele), the VPCs adopted the PAG fate. The results of this study indicate that one way in which specificity can be achieved downstream of Ras signaling is by the HOX gene product active in a given cell.

C. elegans Let-60 Ras is involved in other fate specification choices during development. The first *let-60* mutations identified were recessive lethal (Clark et al., 1988; Rogalski et al., 1982). Homozygotes carrying a null allele die by the fourth larval stage and exhibit a fluid-filled morphology (Han and Sternberg, 1991). A recent mosaic analysis has indicated that death does not result from a defect in cell proliferation at this stage but from the absence of excretory duct differentiation (Yochem et al., 1997). Normally one of two competent cells responds to a signal, activates Ras, adopts the excretory duct cell fate and at the same time, laterally inhibits the second cell from adopting the same fate. Gain-of-function Let-60 Ras can promote both cells to adopt the excretory duct cell fate. Thus, Ras is critical in establishing the excretory duct that is essential for viability.

1.3.2.(d) Roles for Ras During *Drosophila melanogaster* Development

The role for Ras during the development of photoreceptors in the *Drosophila* compound eye has been studied extensively. The eye imaginal disc, a set of undifferentiated multipotent cells differentiates to form the eye which consists of 800 ommatidia (reviewed in Wolf and Ready, 1993). Each ommatidium contains 8 photoreceptor cells (R1-R8), 4 cone cells, and 8 accessory cells. During the establishment of this complex structure, *Drosophila* Ras1 plays an integral role in

the establishment of cell fates (reviewed in Wassarman et al., 1995). Differentiation of the R7 photoreceptor is triggered by the activation of the Sevenless (Sev) RTK. Subsequently, Ras1 is activated via the SOS GEF and the Drk adaptor protein. Ras1 is upstream of a Raf homologue which is, in turn, upstream of a MAPK homologue. In addition, the Ras1 signaling cascade, which is activated by the EGFR, is required for the differentiation of all cell types in the eye (Freeman, 1996).

Ras1 also functions downstream of other RTKs during *Drosophila* development. The *Drosophila* FGFR1, the product of the *breathless* gene, is required for migration of tracheal cells (Klambt et al., 1992). Activated forms of Ras1 or Raf suppress the migration defect in *btl*⁻ animals (Reichman-Freid et al., 1994). I have already mentioned that Ras1 functions downstream of the Tor RTK in the specification of terminal structures of the developing embryo (Greenwood and Struhl, 1997). In addition, the *Drosophila* EGFR is involved in multiple developmental pathways, some of which have been mentioned previously. Ras signaling mediates EGFR-mediated differentiation in all instances (Freeman, 1998). The multitude of differentiation processes in *Drosophila* that require Ras signaling indicate the importance and versatility of Ras as a regulator of developmental decisions.

1.4 Development of *Dictyostelium discoideum*

Dictyostelium discoideum is a unicellular slime mold for which the processes of growth and differentiation are largely separate events. In the wild, vegetative amoebae feed on bacteria within the upper layers of the soil and divide by binary fission. When the nutrient source has been depleted, an interactive mode of

development is initiated (reviewed in Loomis, 1993). In response to pulses of cAMP, up to 10^5 cells aggregate to form a mound. A tip forms at the apex of the mound and then elongates to form a finger-like structure that can fall to the substratum to form a migrating slug. The slug sits on end to initiate the process of culmination which results in the formation of a fruiting body that consisting of a spore-filled sorus supported on a column of stalk cells (Fig. 1-4).

1.4.1 Cell Type Differentiation and Pattern Formation

As the aggregating cells compact into a tight mound, they assume the characteristics of either one of two cell types: prestalk or prespore. There is evidence that a cell's decision to become either prestalk or prespore is biased by an inherent heterogeneity in the vegetative cell population resulting from the cell's position in the cell cycle at the time of starvation (Maeda, 1993). It has been shown that cells starved late in the cell cycle preferentially differentiate as prespore cells and those starved early in the cell cycle preferentially differentiate as prestalk cells (Ohimori and Maeda, 1987). In addition, it is likely that the cell cycle position biases cell fate by affecting the speed with which cells enter the aggregate (Araki et al., 1997). Thus, cells starved early in the cell cycle enter the aggregate late and consequently, respond to positional cues directing their differentiation as prestalk cells. Although such biases exist in a natural population of starving amoebae, if cells are synchronized prior to starvation, both prestalk and prespore cells are formed suggesting that other factors exist to regulate differentiation (Maeda, 1997).

A key feature of *Dictyostelium* development is that cell type differentiation is regulative. Differentiation occurs such that by the slug stage, 20% of the cells are

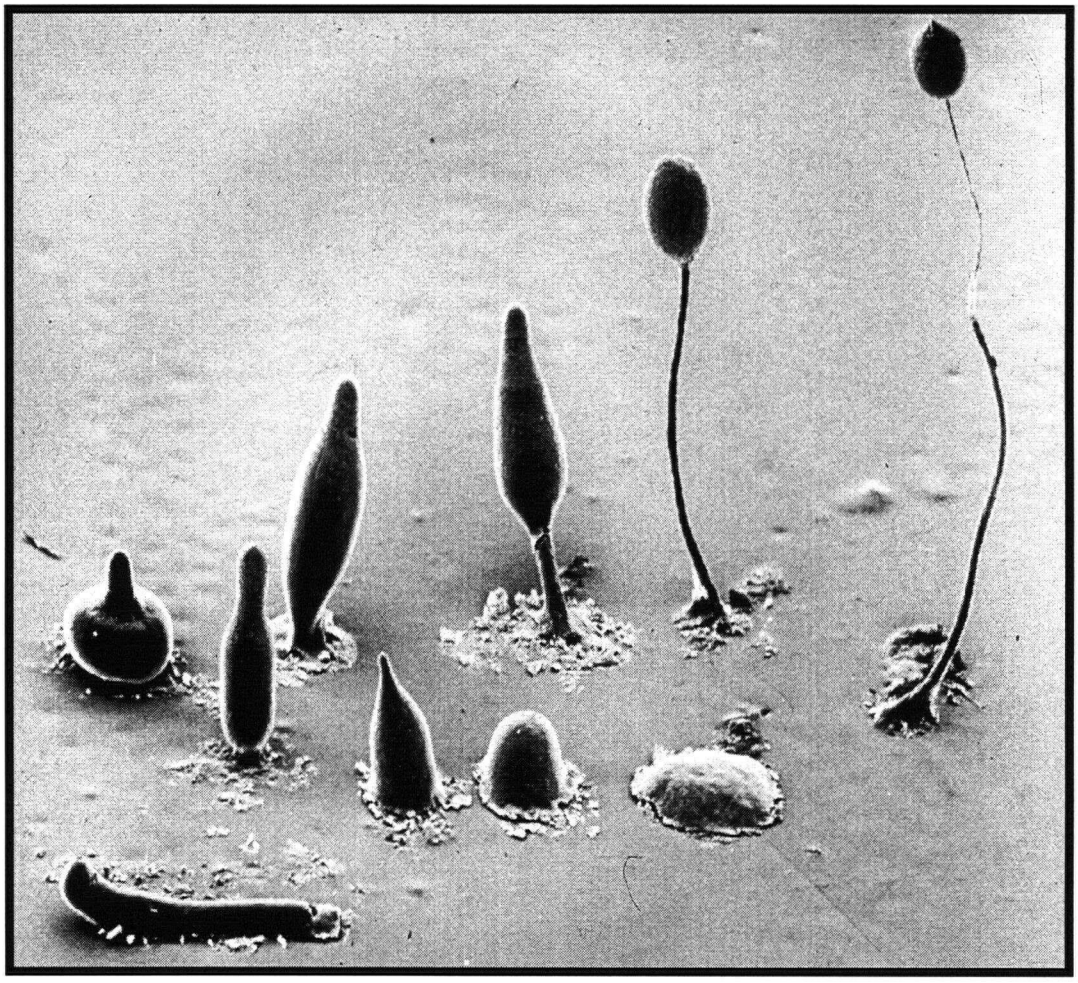


Figure 1-4: The developmental program of *Dictyostelium discoideum*. Development progresses clockwise in the figure beginning with the mound stage at the bottom right and culminating with formation of the fruiting body, top right. The structure in the bottom left corner is the migrating slug.

prestalk cells and 80% are prespore cells. This proportionality is maintained regardless of the size of the developing organism (Loomis and Cann, 1982). In fact, once differentiation has occurred, if the prestalk cells are removed, prespore cells will transdifferentiate to prestalk cells until the correct proportions are reestablished (Raper, 1940; Sakai, 1973). The mechanism by which the cells sense and maintain proportion homeostasis is not known. It has been suggested, however, that lateral inhibitors are employed by the differentiated cell types to maintain their proportions within the organism (MacWilliams and Bonner, 1979; Meinhardt, 1983; Loomis, 1993).

Although the prestalk population only comprises 20% of the cells in the developing organism, it is complex. As the aggregate forms, the different prestalk subtypes become identifiable based on the differential expression of prestalk specific genes, as detected by coupling the promoters of these genes to *lacZ*. Two classes of cells express the gene *ecmA* which encodes a prestalk cell specific extracellular matrix protein (Williams et al., 1987; McRobbie et al., 1988). The proximal region of the *ecmA* promoter directs expression in PstA cells and the distal region directs a comparatively lower level of expression in PstO cells (Jermyn and Williams, 1991; Early et al., 1993). In the multicellular structure, PstA and PstO cells are labeled with reporter constructs using the appropriate promoter element.

PstA cells are first visible at the periphery of the mound while PstO cells are first detected scattered in the mound (Early et al., 1995). As the mound compacts and a tip emerges, the PstA cells move from the periphery to the apex and populate the tip while the PstO cells sort to the region immediately below to the tip (Early et al., 1995). The differential sorting of the two cell types has been hypothesized to involve

differential chemotaxis with the PstA cells chemotaxing towards cAMP twice as fast as PstO cells (Early et al., 1995). During subsequent morphogenesis, as the mound elongates to form a finger and then a migrating slug, the PstA cells remain at the tip with the PstO cells forming a collar just behind them. Together, these two cell populations make up most of the anterior prestalk region of the developing organism (Fig 1-5).

Also during aggregate formation, another prestalk cell type, PstB, is detected. These cells are characterized by the expression of *ecmB*, which encodes another extracellular matrix protein (McRobbie et al., 1988; Ceccarelli et al., 1987). The PstB cells are initially scattered throughout the mound and then accumulate at the base (Jermyn et al., 1996). If the slug culminates in situ, these PstB cells form part of the basal disc (Jermyn et al., 1996). If the slug migrates, PstB cells form a rearguard region at the posterior from which cells are shed during migration (Sternfeld, 1992; Jermyn et al., 1996). A second distinct region of *ecmB* expression is detected in the anterior tip of the slug in a central cone of cells (Jermyn and Williams, 1991; Sternfeld, 1992). Since the cells in the cone express both the *ecmA* and *ecmB* genes, they have been referred to as PstAB cells (Jermyn and Williams, 1991). These cells lie at the position where the stalk tube formation is initiated. A final group of prestalk cells is found scattered throughout the prespore region of the developing slug. These cells, referred to as Anterior Like Cells (ALC), vary in their expression patterns of the *ecmA* and *ecmB* genes such that individual ALC may express either one or both of these genes.

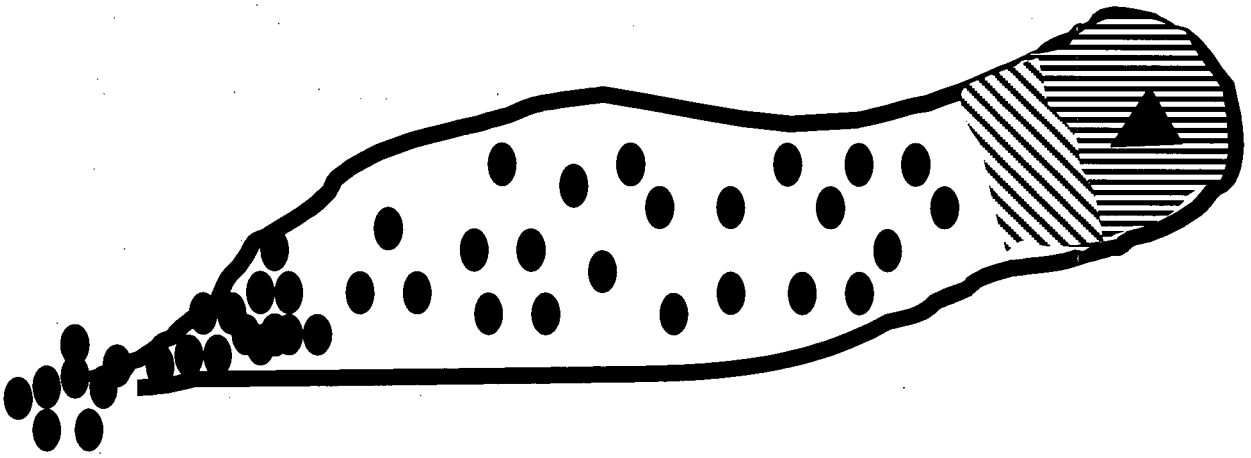


Figure 1-5: Diagram of the cell types within the slug. The horizontal lines in the anterior tip represent the PstA compartment and the diagonal lines represent the PstO collar. The triangle in the PstA zone represents the PstAB cells that form in the tip during migration. The scattered cells throughout the slug are the anterior like cells and the cluster of cells at the posterior are the PstB rearguard cells.

As the slug migrates, the PstB cells at the posterior are left behind in the slime trail (Sternfeld, 1992; Jermyn et al., 1996) and are replaced by the posterior movement of PstAB cells from the anterior cone (Sternfeld, 1992, Abe et al., 1994). The PstAB lost from the cone are replaced by PstA cells that initiate expression of the *ecmB* gene (Sternfeld, 1992; Abe et al., 1994). PstA cells are replaced by PstO cells, which are in turn replaced by ALC and these are subsequently replaced by the transdifferentiation of prespore cells (Sternfeld, 1992; Abe et al., 1994). Thus, the relative cell type proportions are maintained during slug migration, despite the loss of cells from the posterior of the slug.

As the slug rounds up for culmination, PstAB cells initiate stalk tube and stalk cell formation at the tip (Jermyn and Williams, 1991) and the PstA cells express *ecmB* as they enter the stalk tube (Jermyn and Williams, 1991). The stalk cells and stalk tube pass to the rear through the posterior prespore cells and the leading edge of the stalk embeds into the rearguard cells. The rearguard cells also differentiate into stalk cells to form part of the basal disc of the fruiting body (Jermyn and Williams, 1991; Sternfeld, 1992). Some ALC's move to the anterior region of the prespore cell mass to form the upper cup, and some move to the basal region of the prespore cell mass to form the lower cup and part of the basal disc (Ceccarelli et al., 1991; Jermyn and Williams, 1991; Jermyn et al., 1996). As the remaining PstA and PstO cells gradually enter the stalk tube and terminally differentiate into stalk cells, the nascent stalk elongates, lifting the prespore cell mass up. At this point, the prespore cells terminally differentiate into spores.

The tip region of the developing *Dictysotelium* organism is extremely important and has been referred to as the 'organizer' of development (Raper, 1940;

Rubin and Robertson, 1975; Schaap, 1986; Siegert and Weijer, 1995). Once a mound elongates to form a finger, the tip cells sense cues from the surrounding environment and respond either by initiating culmination in situ or by inducing the formation of a slug (Smith and Williams, 1980). During slug migration, the tip cells sense gradients of light and temperature and direct the migration of the slug accordingly (Fisher, 1997). The cells in the tip also initiate the process of culmination.

1.4.2 Regulators of Cell Type Differentiation in *Dictyostelium*

Although only a few of cell types are formed during *Dictyostelium* development, the regulation of their differentiation is complex. A number of gene products have been identified that promote the differentiation one cell type or the other (Aubry and Firtel, 1999) and many others probably await discovery. An understanding of how these various proteins, along with other low molecular weight factors, affect differentiation should enable us to appreciate how starving amoebae chose their fate. In this section, I will briefly describe some of the key regulators of differentiation in *Dictyostelium* (Table I).

1.4.2(a) DIF-1

Differentiation Inducing Factor-1 (DIF-1) is the most active member of a family of chlorinated alkyl phenones (Morris et al., 1987) and was identified based on its ability to induce stalk cell differentiation in isolated amoebae (Town et al., 1979). DIF-1 induces the transcription of prestalk genes and suppresses the transcription of prespore genes (Williams et al., 1987; Early and Williams, 1988).

Table I. Proteins that regulate differentiation during *Dictyostelium* development.

Protein	Effect on *Dictyostelium* differentiation
ERK2	Required for induction and maintenance of prepsore gene expression.
MEKKα	Required for induction and maintenance of prespore gene expression and correct cell type distribution.
RasGAP1	Possibly required for correct sorting and terminal differentiation.
Warai	Required for maintaining correct proportions of prespore and PstO cells.
Stalky	Required for prespore to spore differentiation.
TagB	Required for efficient prestalk gene expression and terminal differentiation of both cell types.
Gα1	Involved in PstB differentiation and cell type sorting.
Gα2	Involved in prestalk to stalk differentiation.
Gα4	Required for prespore gene expression.
Spalten	Required for prespore and prestalk gene expression.
cAR2	Involved in limiting prespore differentiation.
cAR3	Required for cAMP-mediated inhibition of stalk cell differentiation, via GSK3.
cAR4	Involved in differentiation and sorting of both cell types; antagonizes GSK3 activity.
GSK3	Required for prespore gene expression and for inhibition of *ecmB* expression and stalk cell differentiation.
PKA	Required for expression of genes in both cell types and for terminal differentiation.
rZIP	Involved in prespore gene activation and prespore to prestalk transdifferentiation.
STATa	Required for inhibition of *ecmB* expression and stalk cell differentiation.

It can also cause isolated prespore cells to form stalk cells instead of spore cells (Kay and Jermyn, 1983). These and additional findings led to the proposal that DIF-1 is the prestalk morphogen. However, the inductive properties of DIF-1 may not be straight–forward. There is evidence that within the multicellular organism, DIF-1 concentration is the highest in the prespore region (Brookman et al., 1987). Also, not all prestalk genes are induced by DIF-1 (Saxe et al., 1996; Shaulsky and Loomis, 1996). It is possible that DIF-1 cooperates with other extracellular morphogens to regulate differentiation.

The mechanism by which DIF-1 promotes stalk cell formation has not been elucidated. A cytoplasmic protein with affinity for DIF-1 has been identified (Insall and Kay, 1990) but not purified. Williams' group searched for targets of DIF-1 induction in the promoters of the *ecmA* and *ecmB* genes, both of which are rapidly induced by DIF-1 (Jermyn et al., 1987; Ceccarelli et al., 1987). They identified repeats of the TTGA sequence that constitute a DIF-1-response element (Kawata et al., 1996).

1.4.2(b) PSI

Recently, a novel glycoprotein factor has been identified that influences prespore differentiation (Oohata et al., 1997; Nakagawa et al., 1999). Prespore-inducing-factor (PSI or Ψ) is a factor purified from medium conditioned by the growth of amoebae at high density. PSI factor induces the differentiation of isolated amoebae into prespore cells and also induces cell division in prespore cells. The presence of DIF-1 antagonizes the activity of Ψ factor as it induces stalk cell formation even in the presence of Ψ factor. The importance of Ψ factor during multicellular development has not yet been ascertained.

1.4.2(c) G Protein Subunits

The *Dictyostelium* genome contains single genes encoding Gβ and Gγ subunits. Deletion of the gene encoding Gβ blocks the onset of development. Using temperature sensitive variants of this protein (Jin et al., 1998), it was found that Gβ is required for proper morphogenesis at all stages of development. However, it is dispensable for the expression of cell type specific genes. Nine different Gα subunits (Gα1 — Gα9) have been identified and these are expressed at various stages of the life cycle. Only those that have a demonstrated effect on cell type determination are discussed here.

The Gα1 subunit has been shown to be involved in the regulation of *ecmB* expression and stalk cell formation (Dharmawardhane et al., 1994). Cells in which *gα1* has been disrupted do not display visible defects in the expression of *ecmB* but form stalks that are longer and skinnier than wild type stalks. Cells overexpressing *gα1* do not express *ecmB* in the core of the anterior tip. These cells develop to form culminants with thick, short stalks. Cells overexpressing constitutively active Gα1 display defects in mound tip formation which has been correlated with the inefficient sorting of prestalk cells to the anterior of the mound (Rietdorf et al., 1997).

The Gα2 subunit is required early in development for aggregation. Later during development, expression of *gα2* occurs in prestalk cells. The expression of a dominant negative Gα2 in prestalk cells resulted in abnormal stalk differentiation at culmination indicating a role for Gα2 in stalk cell maturation (Carrel et al., 1994).

The Gα4 subunit appears to be expressed in the ALC population (Hadwiger et al., 1994) and is involved in morphogenesis and the differentiation of prespore cells (Hadwiger and Firtel, 1992). *gα4* null cells have reduced prespore gene expression and form few spores during development. The defect is partially rescued by the presence of wild type cells, indicating that mutant cells are deficient for a signal required for prespore differentiation.

The multidomain protein, Spalten, is not a true G protein subunit. However, the amino terminal domain is closely related to Gα subunits while the carboxy terminal domain contains a functional protein phosphatase (Aubry and Firtel, 1998). Spalten is required for development past the mound stage and for the expression of both prestalk and prespore genes. Null mutant cells are able to undergo prespore differentiation when developed in mixtures with wild type cells. Prestalk differentiation of the null is not corrected under this condition.

1.4.2(d) Stalky

Chang et al. (1996) reported the cloning of a putative transcription factor, STKA, which is required for the terminal differentiation of spore cells. Early development of *stkA*$^-$ cells appears normal although expression of the *ecmB* gene is upregulated. However, late in development, prespore cells appear to lose their specification and the culminant that forms is composed entirely of stalk cells. The defect is probably not due to the absence of an extracellular sporulation signal since the presence of wild type cells cannot induce the mutant cells to sporulate. Constitutive activation of PKA does not override the spore defect in the mutant, indicating either that STKA is downstream of PKA in a pathway promoting spore

formation or that STKA is a positive regulator of a prespore pathway not involving PKA. Alternatively, STKA may indirectly promote spore formation by inhibiting the expression of *ecmB*.

1.4.2(e) Warai

One of two *Dictyostelium* proteins with a homeodomain, Warai is postulated to function in a pathway regulating cell type proportions (Han and Firtel, 1998). A null mutation in *wri* results in an enlarged PstO zone at the expense of the prespore zone. Proportions of the other cell types are unaffected. Fruiting bodies of *wri* null cells have larger basal discs, consistent with the presence of additional stalk cells. Since *wri* is expressed in PstA cells, the results indicate a non-cell autonomous role in the regulation of PstO cell differentiation.

1.4.2(f) RasGAP

Faix and Dittrich (1996) isolated a *Dictyostelium* GAP homolog, DGAP1, with similarities to the IQGAPs. A null mutation in the corresponding gene resulted in the formation of multi-tipped aggregates during development. These aggregates proceeded to make abnormal fruiting bodies with thick, short stalks and irregular clusters of spore cells.

Lee et al. (1997) independently isolated the same protein and called it RasGAP1. Cells in which the *ddrasgap1* gene is disrupted exhibit apparently normal development till the migrating slug stage. At this time, the normal patterning of prestalk and prespore cells is lost and scattered localization of the two cell types is observed. No terminal fruiting bodies are constructed and the abnormal terminal

structures formed contain no stalk cells and few spore cells. Given the conflicting results, the role of this protein has yet to be resolved.

1.4.2 (g) TagB

The TagB protein contains a serine protease domain and a domain with sequence identity to the ABC transporters (Shaulsky et al., 1995). *tagB* is expressed in prestalk cells and knock out mutants arrest morphogenesis at the mound stage before tip formation. The *tagB*$^-$ cells are able to express prespore genes but prestalk gene expression is reduced. Mutant cells do not form stalk or spore cells during multicellular development. However, the mutant can differentiate into spore cells in the presence of wild type cells. Another protein, TagC, contains significant homology to TagB. Disruption of *tagC* results in a very similar phenotype to that of *tagB*.

1.4.2(h) MAPK Cascade Components

Slugs of null mutants in the gene encoding the putative MEK kinase homolog, *mekkα*, exhibit an increased PstO cell zone, a decreased prespore cell zone, and a less defined boundary between prestalk and prespore cells (Chung et al., 1998). MEKKα is required for the induction and maintenance of prespore differentiation and when developed in a chimaera with wild type cells, *mekkα*$^-$ cells preferentially differentiate into prestalk cells. Additional results indicate that the amount of MEKKα determines the fate of the cell; cells overexpressing MEKKα preferentially form prespore cells in mixtures with wild type cells.

A *Dictyostelium* MAPK, ERK2 has been isolated and shown to be required for aggregation (Segal et al., 1995). Later in development, ERK2 is required for the induction and maintenance of prespore gene expression but not for prestalk gene expression (Gaskins et al., 1996). ERK2 does not appear to be downstream of MEKKα (Chung et al., 1998).

1.4.2(i) Role of Membrane Bound cyclic AMP Receptors

The effects of extracellular cAMP are mediated by the 7-span transmembrane cAMP receptors, cAR1-4. The receptors have varied affinities for cAMP; cAR1 has the highest affinity, cAR3 has intermediate affinity, and cAR2 and cAR4 have low affinities (Saxe et al., 1991; Kim et al., 1998). During post–aggregative development, *car1* expression is enriched in prestalk cells whereas *car3* expression is observed only in prespore cells (Gollup and Kimmel, 1997; Yu and Saxe, 1996). Development of *car3* null cells is delayed but is otherwise normal (Johnson et al., 1993).

The cAR2 receptor is expressed preferentially in anterior prestalk cells (Ginsberg et al., 1995; Saxe et al., 1996). Cells deficient in cAR2 are blocked at the mound stage before tip formation (Saxe et al., 1993). Null cells overexpress prespore genes indicating that the binding of cAMP to the cAR2 receptor in prestalk cells may laterally control the differentiation of prespore cells. Expression of the *ecmA* gene is normal in *car2*$^-$ cells but expression of *ecmB* is reduced, possibly due to the inability of these cells to culminate.

cAR4 is expressed in both cell types, but expression is enriched in the prestalk population (Louis et al., 1994; Ginsberg et al., 1995). *car4* null cells overexpress prespore genes and underexpress prestalk genes. Patterning of the cell types is also

affected with prespore cells present in the anterior prestalk region (Ginsberg and Kimmel, 1997). When developed in chimaera with wild type cells, *car4*$^-$ cells can be induced to efficiently form prestalk cells. Moreover, wild type cells in these mixtures were not prevented from prestalk differentiation. Thus, the prestalk defect in the *car4*$^-$ null cells does not appear to result from a defect in either the production or reception of an extracellular signal. Similar experiments revealed that *car4*$^-$ prespore cells were capable of normal pattern formation in a chimaeric mixture containing a majority of wild type cells but, wild type prespore cells were mislocalized in a chimaeric mixture containing a majority of *car4*$^-$ cells. Thus, prespore cell localization is determined non-cell autonomously by the major population of cells in the mixture. Evidence was also presented for the existence of an extracellular DIF modulation factor (DMF) that appears to decrease the sensitivity of prespore cells to DIF. This factor is not produced by the *car4*$^-$ cells.

1.4.2(j) Role of GSK

The *Dictyostelium* GSK3 homolog is involved in regulating the differentiation of prespore cells and PstB cells (Harwood et al., 1995). Developing *gskA* null cells produce an enlarged basal disc supporting a short stalk and usually no sorus. They also exhibit drastically reduced prespore gene expression and elevated *ecmB* expression. In addition, the normal cAMP-mediated induction of prespore gene expression and repression of *ecmB* gene expression and stalk cell formation is not observed. These results indicate that GSK activation stimulates spore formation and inhibits stalk cell formation. When wild type cells are treated

with LiCl, an inhibitor of GSK3 activity, the *gsk3*⁻ phenotype is mimicked (Sakai, 1973; Van Lookeren Campagne, 1988).

In *car3*⁻ cells, GSK activity is not elevated at the time of cell type determination as it is in wild type cells (Plyte et al., 1999). In addition, *car3*⁻ cells do not exhibit cAMP-mediated repression of stalk cell formation (Plyte et al., 1999). Also, stimulation of wild type cells with cAMP results in an increase in GSK3 activity; this increase does not occur in cells lacking cAR3 (Plyte et al., 1999). However, multicellular development of *car3*⁻ cells is essentially normal (Johnson et al., 1993) whereas *gskA*⁻ cells form aberrant structures. It is possible that loss-of-function cAR3 may be compensated by another cAMP receptor. cAR1 is a good candidate since early in development, cAR3 can compensate for the absence of cAR1 indicating that some functional redundancy exists amongst these receptors (Insall et al., 1994).

cAR4 has also been implicated in the regulation of GSK3 activity (Ginsburg and Kimmel, 1997). At the level of gene expression, the phenotypes of *car4*⁻ cells and *gskA*⁻ cells are opposite. The treatment of developing *car4*⁻ cells with LiCl to inhibit GSK3 activity results in a correction of the defects in cell type specific gene expression. cAMP binding to cAR4 may negatively modulate the activity of GSK3 and in the absence of cAR4, GSK3 is hyperactive and prespore differentiation increases. Thus cAMP signaling through cAR4 might antagonize the effect of cAMP signaling through cAR3 with respect to the regulation of GSK3 activity.

GSK3 activity is also regulated by a novel tyrosine kinase, ZAK1 (Kim et al., 1999). cAMP-mediated ZAK1 activation is defective in *car3* null cells and GSK3 activity is reduced in *zak1* null cells. In addition, *zak1*⁻ cells have reduced ability to

form spores and do not exhibit cAMP-mediated repression of stalk cell differentiation, properties characteristic of *gskA* null cells. Also, ZAK1 phosphorylates and activates mammalian GSK3β in vitro. Together, these results place ZAK1 downstream of cAR3 in the pathway regulating GSK3 activity.

1.4.2(k) Role of PKA

The *Dictysotelium* cAMP-dependent protein kinase, PKA is required at multiple stages during development and plays an integral role in the differentiation of both spore and stalk cells (Loomis, 1998). When PKA is inactivated in prespore cells, using the *psA* promoter to drive expression of a dominant inhibitory form of the PKA regulatory subunit (PKA-Rm), spore formation does not occur (Hopper et al., 1993a). Also, as prespore cells accumulate sufficient PKA-Rm protein, the level of transcription of the spore coat genes becomes greatly reduced. This result is consistent with the fact that most prespore genes require PKA for maximum expression (Fosnaugh and Loomis, 1991; Hopper et al., 1995). However, the expression of *psA* is unaffected in this *psA::PKA-Rm* strain indicating that PKA does not regulate the expression of all prespore specific genes (Hopper et al., 1993a; Hopper and Williams, 1994).

Prestalk specific expression of the PKA-Rm protein, driven by the *ecmAO* promoter, results in the formation of defective slugs unable to culminate (Zhukovskaya et al., 1996). Expression of *ecmA* and *ecmB* are reduced, indicating a requirement for PKA in prestalk cells for efficient prestalk gene expression. It is believed that *ecmB* expression is repressed during the slug stage and that the repression is relieved by PKA activity during culmination (Loomis, 1998).

PKA activity is also required for the terminal differentiation of both cell types. During development, the catalytic subunit of PKA is preferentially expressed in prestalk cells but once culmination is initiated, expression is induced in the rising spore mass (Mann et al., 1994). Constitutive activation of PKA results in rapid development and a sporogenous phenotype suggesting that control of PKA activity is required to prevent premature sporulation. The signal pathway thought to activate PKA during culmination is described later in this chapter.

1.4.2(l) Role of rZIP

rZIP is a multidomain protein containing a RING-type zinc-binding domain, a leucine zipper, and an SH3 domain (Balint-Kurti et al., 1997). rZIP has similarities to the Cbl family proteins, that have been proposed to negatively regulate RTK-mediated signaling pathways (Yoon et al., 1995). When rZIP is overexpressed, prestalk gene expression is elevated and prespore gene expression is repressed, the opposite phenotype of an *rzpA* knock out (Balint-Kurti et al., 1997). Slugs of *rzpA* null cells are less motile than wild type slugs and mutant prespore cells are unable to transdifferentiate to prestalk cells.

When mutant cells are developed in a chimaera with an excess of wild type cells, the mutant cells display a gradient of expression of spore coat genes (*cotB* and *cotC*) such that expression is highest in the anterior of the prespore zone and decreases towards the posterior (Balint-Kurti et al., 1998). This gradient is corrected by the activation of PKA (using the membrane permeable cAMP analogue, 8-Br-cAMP, or the ectopic expression of the catalytic subunit of PKA). Based on these results, it was suggested that within the prespore zone of the slug, PKA activation

responds to an anterior to posterior graded signal and that *rzpA*⁻ cells have a reduced capacity to respond to this signal.

1.4.2(m) Role of STATa

Analysis of the DIF-inducible *ecmA* gene revealed a promoter element required for DIF induction (Kawata et al., 1996). It was found that the necessary sequence is bound by the Dd-STATa protein (Kawata et al., 1997). Mammalian STAT (signal transducer and activator of transcription) proteins are activated in response to cytokine stimulation via tyrosine phosphorylation by Janus kinases (JAKs). Phosphorylated STAT proteins dimerize via C-terminal SH2 domains and translocate to the nucleus (reviewed in Ihle and Kerr, 1995).

The *Dictyostelium* STATa is activated in response to extracellular cAMP (Araki et al., 1998) and is required early in development for proper aggregation (Mohanty et al., 1999). STATa binds to a TTGA direct repeat in the *ecmA* promoter and inverted repeat repressor element in the *ecmB* promoter (Kawata et al., 1997). Deletion of the repressor element and insertional inactivation of the *STATa* gene both result in precocious expression of *ecmB* in the entire anterior prestalk domain (Kawata et al., 1997; Mohanty et al., 1999). Slugs of *STATa* null cells do not form fruiting bodies and do not produce stalk cells during multicellular development. Spore cells are formed but with reduced efficiency compared to wild type. The *STATa* null cells are more sensitive to DIF than wild type cells and require a lower DIF concentration for the induction of prestalk genes. The level of *ecmA* expression in *STATa* null cells is normal, indicating that while STATa can bind to the *ecmA* promoter, it is not required for induction of the gene. Precocious *ecmB* expression

in the null cells indicates that STATa is a negative regulator of *ecmB* expression and stalk cell differentiation. Although STATa is a repressor of stalk cell formation, its absence does not result in precocious stalk differentiation. This is most likely a consequence of the increased sensitivity of mutant cells to cAMP mediated inhibition of stalk cell formation.

1.4.3 Signals Regulating Terminal Differentiation of *Dictyostelium*

Results obtained over recent years have yielded insights into the regulation of terminal differentiation during culmination (reviewed in Loomis, 1998; Aubry and Firtel, 1999; Thompson et al., 1999). Late in the developmental program, when the slug ceases migration and rounds up prior to culmination, the prestalk cells at the apex secrete a sporulation factor (Anjard et al., 1998). This factor, SDF-2 (spore differentiation factor), is secreted via TagB and TagC and has an autocrine effect on prestalk cells triggering a burst of SDF-2 release. In prespore cells, SDF-2 activates DhkA, a membrane-bound histidine kinase (Wang et al., 1996). DhkA then inhibits RegA in a manner dependent on the MAPK, ERK2 (Shaulsky et al., 1998). RegA is a cAMP specific phosphodiesterase so its inhibition leads to a rise in intracellular cAMP and a concomitant rise in PKA activity which is required for prespore cells to become encapsulated. Stalk cell differentiation may also be initiated in response to activation of PKA as a consequence of RegA inhibition. In the basal disc, however, stalk cell formation may occur via a separate mechanism. In this case, low levels of cAMP (due to the physical distance from the anterior cAMP oscillator) lead to low levels of GSK-3 activity which in turn, induces stalk cell differentiation (Thompson et al., 1999).

1.5 Effects of *Dictyostelium* Ras on Development

At least six different *ras* sub-family genes are expressed by *Dictyostelium*, each with its own unique temporal expression pattern (Reymond et al., 1984; Robbins et al., 1989; Robbins et al., 1991; Daniel et al., 1993a; Daniel et al., 1993b). The protein products of these genes, RasD, RasG, RasB, RasC, and RasS, are related to the mammalian H-Ras whereas Rap1 is related to mammalian Rap. The RasG and RasD proteins are the most closely related to H-Ras, sharing 69% and 62% identity with the mammalian protein, respectively (Robbins et al., 1991). These two *Dictyostelium* Ras homologues are 82% identical and only differ in 3 positions over the first 100 amino acids (Robbins et al., 1989). Hence, both RasG and RasD have identical effector and effector-proximal domains (Figure 1-6). RasG and RasD have been studied the most intensely and activated forms of both have been shown to inhibit the normal developmental program.

1.5.1 *Dictyostelium* RasG

The *rasG* gene is expressed during vegetative growth and during the first few hours of development (Robbins et al., 1989). Transformants in which the activated *rasG(G12T)* is driven by the folate-repressible *discoidin* promoter fail to aggregate under all starvation conditions tested (Khosla et al., 1996). The aggregation defect occurs even when cells are grown in the presence of folate, indicating that a low level of the induced protein is sufficient to impair the onset of development (Khosla et al., 1996). The activated RasG transformants are impaired in the

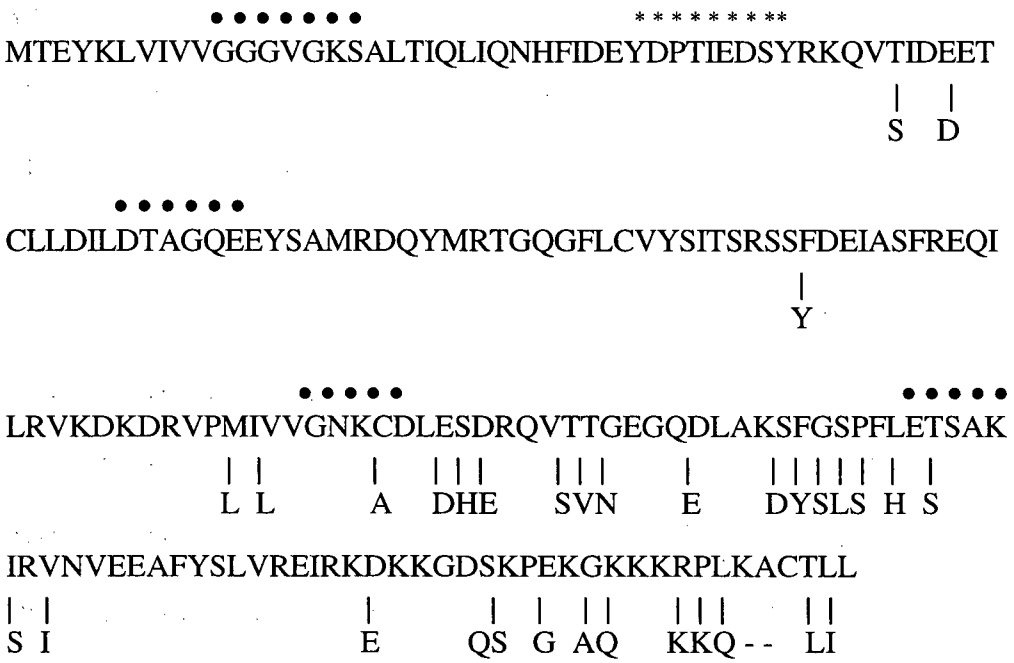


Figure 1-6: Amino acid sequence of RasG. The residues in RasD that differ from RasG are indicated below the RasG sequence. RasG has two additional amino acids relative to RasD at the C-terminal tail. These are indicated by dashes below the sequence. Residues involved in guanine nucleotide binding are labeled with dots above the sequence and residues involved with interactions with effector proteins are indicated with asterisks above the sequence. (Adapted from Robbins et al., 1989).

induction of two genes, *car1* and *pde*, both of which are expressed soon after the initiation of starvation (Khosla et al., 1996). When transformants are pulsed with cAMP while shaking in starvation buffer, the expression of these early response genes is increased and cells pulsed for 4 hours are able to complete the developmental program when plated on a solid substratum (Khosla et al., 1996). Since the pVEII-*rasG*-G12T transformants are capable of responding to cAMP pulses, the defect in aggregation could be due to a failure to generate cAMP. Consistent with this, the pVEII-*rasG*-G12T transformants produce only very small amounts of cAMP in response to a pulsatile stimulus but in the presence of wild type cells, they are able to participate in development (Khosla et al., 1996).

1.5.2 *Dictyostelium* RasD

The *rasD* gene was initially isolated as a prestalk specific gene (Reymond et. al., 1984) and the staining pattern for developing slugs expressing a *rasD::lacZ* reporter construct resembles the expression pattern for the prestalk cell specific *ecmA* gene (Esch and Firtel, 1991). This supports the prestalk specific designation for *rasD*. However, when prestalk and prespore cell populations are separated and assayed for gene expression by northern blot analysis, *rasD* mRNA is found to be only 3-fold enriched in prestalk cells, whereas both *ecmA* and *ecmB* mRNA's are more than 10-fold enriched in the prestalk cells (Jermyn et. al., 1987). Moreover, when β-galactosidase is assayed over longer time periods in slugs expressing *rasD::lacZ*, the prespore region also stained, confirming that prespore cells do express *rasD* but at a significantly lower level than prestalk cells (Jermyn and Williams, 1995). It was proposed that *rasD* is initially expressed in a non-cell type-specific

manner and then expression decreases in the prespore cells and as such, expression becomes prestalk enriched (Jermyn et. al., 1987). Consistent with this suggestion, the prespore region stains less intensely when slugs express a labile β–galactosidase (Jermyn and Williams, 1995).

A transformant overexpressing activated *rasD* (*Ddras*-Thr12) forms aggregates with multiple tips that do not proceed to form fruiting bodies (Reymond et al., 1986). Only 0.2% of the original population was found to form viable spores and upon disaggregation, stalk cells were not observed (Louis et al., 1997a). It was also found that the mRNA levels for 3 prestalk specific genes, *ecmA*, *ecmB*, and *tagB*, were dramatically elevated relative to control cells, while prespore specific *cotC* mRNA levels were reduced to barely detectable levels (Louis et al., 1997a). Consistent with this northern blot data, there was an increased expression of an *ecmA::lacZ* prestalk reporter and the entire aggregate gained the ability to express the β-galactosidase (Louis et al., 1997a). RasG and RasD have very similar sequences and when activated RasG was expressed from the *rasD* promoter, the *rasD::rasG(G12T)* transformants produced the same multi-tipped phenotype exhibited by the *Ddras*-Thr12 transformant (M. Khosla, unpublished observations).

When the *Ddras*-Thr12 transformant cells were mixed with wild type Ax3 cells in a 1:3 ratio and induced to develop, the wild type cells formed normal fruiting bodies that excluded the transformant cells (Louis et al., 1997). These results indicated that signals from the wild type cells were incapable of rescuing the development of the transformant and that the transformant cells did not negatively affect the development of the wild type cells. In the reverse scenario, when Ax3 cells were mixed with *Ddras*-Thr12 cells in a 1:3 ratio, multi-tipped aggregates

formed that did not proceed further and the wild type cells were found to be trapped within the deformed aggregates (Louis et al., 1997a). Thus, an excess of the transformant cells inhibited development of Ax3 cells.

1.6 Rationale and Research Objective

It had been hypothesized that the defect in the development of the *Ddras*-Thr12 transformant was due to expression of activated RasD in the prestalk cell population (Esch and Firtel, 1991). However, since *rasD* expression also occurs in the prespore cell population, there were several plausible alternative explanations that were consistent with the available data (Louis et al., 1997a). Activated RasD might exert its effects only in prespore cells, inhibiting prespore gene expression and enhancing prestalk gene expression, but blocking the conversion of prestalk cells to stalk cells. Alternatively, activated RasD might have effects in both cell types: preventing gene expression in prespore cells and enhancing gene expression in prestalk cells, but again inhibiting the conversion of prestalk cells to stalk cells. Finally, even if the defect in the *Ddras*-Thr12 transformant was due to an effect only in prestalk cells, as suggested by Esch and Firtel (1991), then this event must lead to the lateral inhibition of prespore gene expression. To distinguish between these possibilities, it was necessary to express the activated protein in a cell type specific manner, and this has been accomplished in the work described in this thesis.

To accomplish this task, I set out to express the activated *rasD* from the *ecmAO* and *psA* promoters. Considerable problems were encountered in attempting to construct the *ecmAO::rasD(G12T)* and *psA::rasD(G12T)* transformation plasmids. Since RasD(G12T) and RasG(G12T) have identical developmental

consequences, when expressed from the *rasD* promoter (M. Khosla, unpublished observations), I attempted to construct transformation plasmids containing *ecmAO::rasG(G12T)* and *psA::rasG(G12T)* and this was successful.

CHAPTER TWO

EXPERIMENTAL PROTOCOLS

A list of materials used to conduct these experiments, along with names of the suppliers is provided in Appendix I. Recipes for media and buffers are detailed in Appendix II.

2.1 DNA Preparation for Plasmid Construction

Small scale preparations of DNA were isolated from *Escherichia coli* cells using the miniprep protocol detailed in Sambrook et al. (1989). Large scale preparations of DNA for cloning and for *Dictyostelium* transformations were isolated by the $CsCl_2$ density gradient centrifugation method, except for the *act15::lacZ*, *psA::lacZ*, *psA::(his)lacZ*, and *psA::rasG(G12T)* plasmid DNAs which were isolated by the PEG precipitation method. Both methods are described in Sambrook et al. (1989). Recovery of DNA from agarose gels was accomplished using the Qiagen Gel Extraction Kit, except for DNAs used in the cloning of the *psA::rasG(G12T)* construct which were recovered using the GeneCleanII Kit. All kits were used according to their manufacturer's protocols.

2.2 Transformation of Bacterial Cells

E. Coli XL-1 cells were used for all transformations. Cells were made competent by the rubidium chloride technique (Sambrook et al., 1989) and then transformed using the $CaCl_2$ protocol (Sambrook et al., 1989). Selection for transformants was carried out on LB agar plates supplemented with 100 μg/ml

ampicillin. Rapid screening of transformants to detect successful cloning was performed by the Slot Lysis method (Sekar, 1987).

2.3 Vector Construction

2.3.1 *psA::rasG(G12T)* and *psA::rasG* Constructs

Using *ptZ19R-rasG(G12T)* (Khosla et al., 1996) as a template, the 5' portion of the activated *rasG* cDNA was modified by PCR to delete a stop codon located 5′ to the start codon (Fig. 2-1). (This was done to prevent any negative effect the stop codon may have had on translation). The Ras-5′ and Ras-3′ primers (Table II) were used in the amplification reactions. $Vent_R^{®}$ DNA polymerase was used for the PCR reactions along with 10 ng of template DNA, 20 pmol of each primer, and 400 mM of each dNTP per reaction. The conditions used for the PCR reactions were as follows: denaturation at 95°C for 30 seconds, annealing at 47°C for 50 seconds, extension at 72°C for 21 seconds, and cycles were repeated 25 times.

The PCR product from the *rasG(G12T)* template was digested with *BglII* and *AccI* and ligated with a similarly digested *ptZ19R-rasG* (Khosla et al., 1996) vector fragment in which a second *AccI* site in the polylinker had been destroyed by digesting with *SalI*, which recognizes an overlapping site, blunting the ends, and religating (Fig. 2-2). From this construct, *ptZ19R-rasG(G12T)ΔSalIΔstop*, the *rasG(G12T)* cDNA was released by digesting with *BglII* and *KpnI* and ligated to the vector fragment of the *BglII/KpnI* digested *psA-DdPK2* plasmid (Hopper et al., 1993b). The construct thus generated, *psA::rasG(G12T)3′PK2* contained the *rasG(G12T)* cDNA linked to the *psA* promoter but also contained a 3′ portion of the

```
TTTTTTTATATAATTTTTAAATTTTATATAATTAAACTTGTAATATATAACAACCCACACAAAAA 65

                                                      ******XXX
AAAAAAAAAAAAAACCAAATCAAATTATAAAACCCACACATATTTATATATAACAGATCTTAATTT 130

      √√√                                ACT
AAAAAAATGACAGAATACAAATTAGTTATTGTTGTTGGTGGTGGTGTCGGTAAAAGTGCCTTAAC 195

CATTCAATTAATCCAAAACCATTTCATTGATGAATACGATCCAACTATCGAAGATTCATACAGAA 260

AACAAGTTACCATTGATGAAGAAACTTGTTTATTAGATATTTTAGATACTGCTGGTCAAGAGGAA 325

                                                      ♦♦♦♦♦♦
TACTCTGCAATGAGAGACCAATATATGAGAACTGGTCAAGGTTTCCTTTGTGTCTACTCTATCAC 390
```

Figure 2-1: Sequence of the 5′ end of the *rasG* cDNA. The *BglII* and *AccI* restriction sites are indicated by asterisks and diamonds above the recognition sequence, respectively. The sequence for the stop codon is underlined and labeled from above with crosses. The sequence for the start codon is underlined and indicated with check marks from above. Numbers indicate the position of the rightmost nucleotide of that row.

Table II. Oligonucleotide primers* used for PCR and sequencing reactions.

Ras-5′	GTCTAGATCTTTTAAAAAAATGACAG
Ras-3′	CTTAGAGTTAAGGATAAGGA
S17N	GTCGGTAAAAGTGCCTTAACC
JD5	GATACTGCTGGTCAAG
SR3	CTTAGAGTTAAGGATAAGGA

*Sequences are given in 5′ to 3′ order.

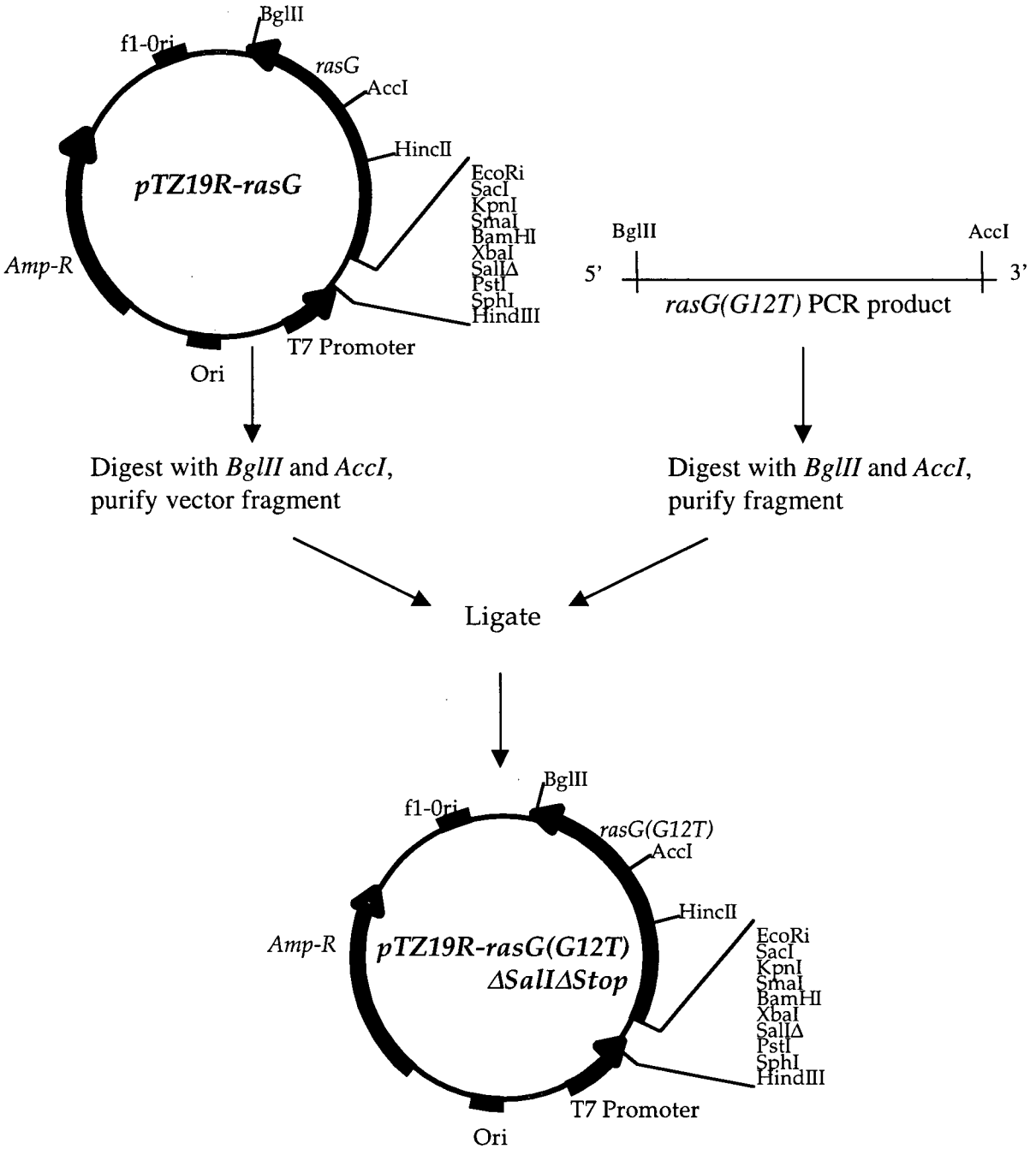


Figure 2-2: Partial cloning strategy for construction of the *psA::rasG(G12T)* vector DNA. The *ptz19R-rasG* vector, with the *SalI* site destroyed, was digested with *BglII* and *AccI* and the vector fragment isolated. This was ligated to a similarly digested PCR fragment of the 5′ end of the *rasG(G12T)* cDNA to generate the *ptz19R-rasG(G12T)ΔSalIΔStop* vector.

DdPK2 gene which was removed by digesting with *KpnI* and *XhoI*, blunting the termini, and religating (Fig. 2-3).

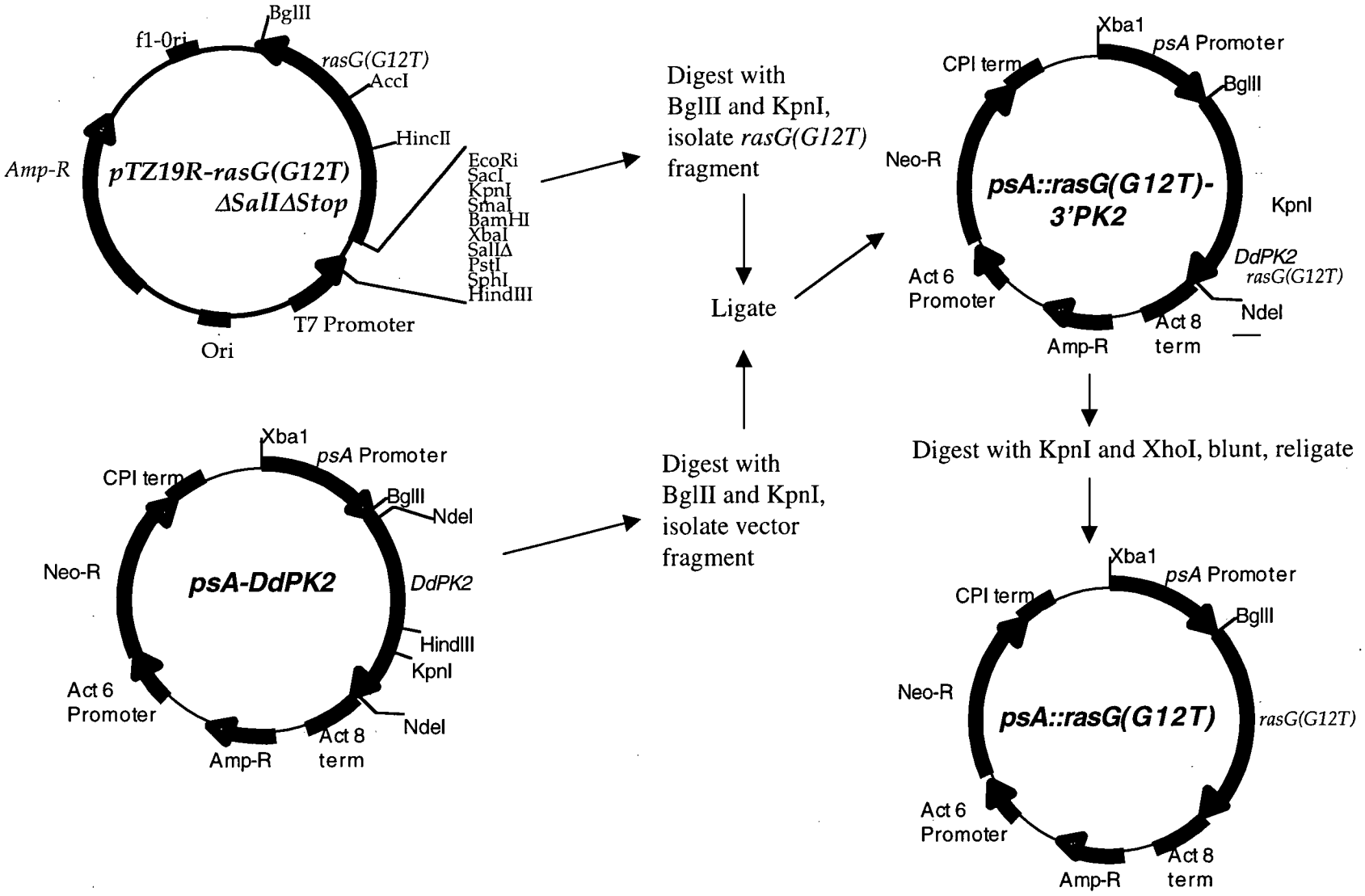
To clone the wild type *rasG* downstream of the *psA* promoter, the *ptZ19R-rasG* vector was used as a template in a PCR reaction identical to the one described above. The PCR product was digested with *BglII* and *AccI* and ligated to the vector fragment from a BglII/AccI digestion of the *psA::rasG(G12T)* construct. This cloning resulted in replacing the region containing the activating mutation with wild type coding sequence and thus generated the *psA::rasG* construct.

The generated vectors were isolated by the CTAB DNA preparation protocol (Del Sal et al., 1987) (for *psA::rasG(G12T)*) or using the Nucleobond Ax-20 DNA extraction kit (for *psA::rasG*) and the construction was verified by sequencing with the dideoxy chain termination method using the SN17, JD5, and SR3 primers (Table II). Reactions were carried out using the Sequenase2® kit according to manufacturer's protocol except that the DNA and primer were boiled for 5 minutes prior to the addition of the Sequenase buffer (Andersen et al., 1992). DNAs were labeled with [^{35}S]-α-dATP and electrophoresed on a 6% acrylamide gel buffered with TBE. The gel was then vacuum dried and exposed to X-ray film.

2.3.2 *ecmAO::rasG(G12T)* and *ecmAO::rasG* Constructs

The *psA::rasG(G12T)* and *psA::rasG* constructs were digested with *BglII* and ligated to a *BglII* fragment from the *ecmAO::lacZ* vector. This fragment contained the *ecmAO* promoter DNA. After verifying the orientation of the ligated promoter by restriction analysis, the generated constructs were digested with *KpnI* and *XbaI* to

Figure 2-3: Second half of cloning scheme for the *psA::rasG(G12T)* construct. The *ptz19R-rasG(G12T)ΔSalIΔStop* construct was digested with *BglII* and *KpnI* and the *rasG(G12T)* cDNA isolated. The *psA-Dd(PK2)* vector was also digested with *BglII* and *KpnI* and the vector fragment was isolated. These were ligated to generate the *psA::rasG(G12T)3'PK2* vector which was digested with *KpnI* and *XhoI*, blunted and religated to create the *psA::rasG(G12T)* vector.


BglII
f1-0ri
rasG(G12T)
AccI
HincII
Amp-R
pTZ19R-rasG(G12T)
ΔSalIΔStop
EcoRi
SacI
KpnI
SmaI
BamHI
XbaI
SalIΔ
PstI
SphI
HindIII
T7 Promoter
Ori
Digest with
BglII and KpnI,
isolate rasG(G12T)
fragment
Ligate
Xba1
psA Promoter
CPI term
BglII
Neo-R
psA::rasG(G12T)-
3'PK2
KpnI
DdPK2
rasG(G12T)
Act 6
Promoter
NdeI
Act 8
term
Amp-R
Digest with KpnI and XhoI, blunt, religate
Xba1
psA Promoter
CPI term
BglII
NdeI
Neo-R
psA-DdPK2
DdPK2
HindIII
KpnI
Act 6
Promoter
NdeI
Act 8
term
Amp-R
Digest with
BglII and KpnI,
isolate vector
fragment
Xba1
psA Promoter
CPI term
BglII
Neo-R
psA::rasG(G12T)
rasG(G12T)
Act 6
Promoter
Act 8
term
Amp-R

remove the *psA* promoter, blunt ended and religated to create the *ecmAO::rasG(G12T)* and *ecmAO::rasG* constructs (Fig. 2-4). The promoter/gene fusions were verified by sequencing. DNA was isolated using the Nucleobond Ax-20 kit and sequenced by the NAPS facility using the SN17, JD5, SR3 primers.

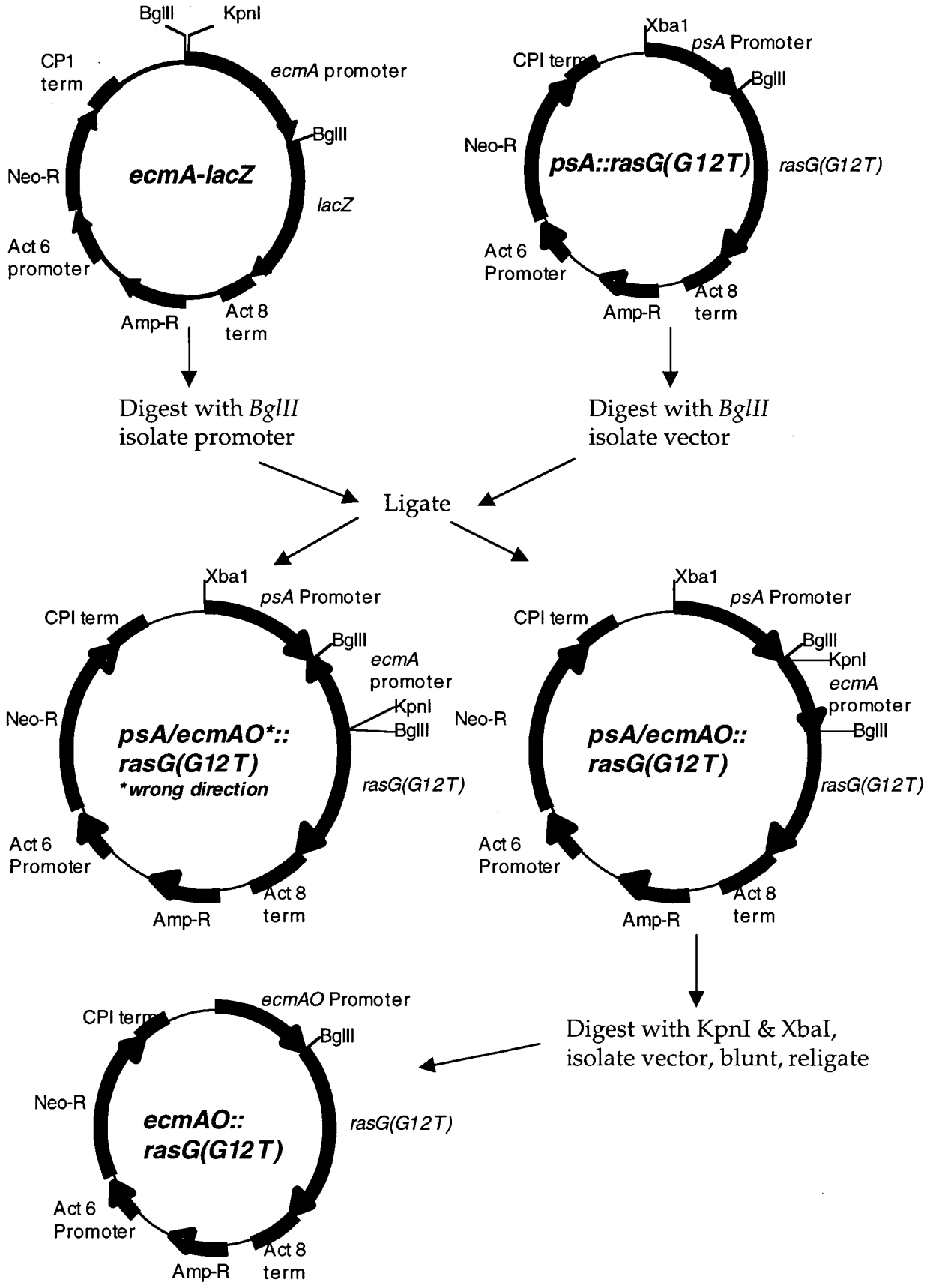
2.4 Growth of *Dictyostelium discoideum* Cells

Dictyostelium discoideum Ax2 cells were grown at 22°C in HL5 culture medium (Watts and Ashworth, 1970), containing 50 µg/ml streptomycin, either in shaking flasks (at 180 rpm) or in Nunclon™ (9 cm) dishes. Culture density was determined by counting cells using a hemocytometer. For clonal selection of transformants, *Dictyostelium* amoebae were seeded at low density in association with *Klebsiella oxytoca* on nutrient rich SM-VAN agar plates (modified from Sussman, 1987) and grown at 22°C. Vegetative amoebae from individual plaques were picked and transferred to HL5 medium for continued growth. For spore germination, a wire loop, prewetted with HL5 medium, was used to pick spores from fruiting bodies on SM-VAN plates and to transfer them to HL5 medium.

2.5 Development of *Dictyostelium discoideum* Cells

To initiate development, exponentially growing *Dictyostelium* cells were harvested and washed twice in Bonner's Salts (BS) by centrifugation for 5 minutes at 1200 rpm. Cell pellets were then resuspended in BS and 2.5×10^7 cells were plated onto white Millipore nitrocellulose filters (4.7 cm diameter) (Bonner, 1947). Black filters were used instead of white filters when unstained developing structures were

Figure 2-4: Coning strategy for the *ecmAO::rasG(G12T)* construct. The *ecmA::lacZ* vector was digested with *BglII* to release the *ecmAO* promoter which was ligated with a *BglII* digested *psA::rasG(G12T)* vector. After determining the orientation of the inserted *ecmAO* promoter by restriction analysis, the *psA* promoter was removed by digesting with *KpnI* and *XbaI*, blunting, and religating. An identical strategy was used to generate the *ecmAO::rasG* construct using the *psA::rasG* vector.


BglII
KpnI
CP1 term
ecmA promoter
BglII
Neo-R
ecmA-lacZ
lacZ
Act 6 promoter
Amp-R
Act 8 term
Xba1
psA Promoter
CPI term
BglII
Neo-R
psA::rasG(G12T)
rasG(G12T)
Act 6 Promoter
Amp-R
Act 8 term
Digest with BglII isolate promoter
Digest with BglII isolate vector
Ligate
Xba1
psA Promoter
CPI term
BglII
ecmA promoter
KpnI
BglII
Neo-R
psA/ecmAO*:: rasG(G12T)
*wrong direction
rasG(G12T)
Act 6 Promoter
Amp-R
Act 8 term
Xba1
psA Promoter
CPI term
BglII
KpnI
ecmA promoter
BglII
Neo-R
psA/ecmAO:: rasG(G12T)
rasG(G12T)
Act 6 Promoter
Amp-R
Act 8 term
Digest with KpnI & XbaI, isolate vector, blunt, religate
ecmAO Promoter
CPI term
BglII
Neo-R
ecmAO:: rasG(G12T)
rasG(G12T)
Act 6 Promoter
Amp-R
Act 8 term

to be photographed. Filters were placed atop BS-saturated pads in 4 cm Falcon® petri plates and incubated in a moist chamber at 22°C. Structures were viewed with a WILD M3Z dissecting microscope. Unless otherwise indicated, unstained structures were photographed with a DAGE-MTI CCD100 digital camera using Scion Image 1.62a software.

2.6 Spore Formation

To determine the spore formation, cells were allowed to develop for 36 to 48 hours to ensure fruiting body formation was complete. Cells were then washed off the filters in 5 ml of BS with 1% Triton X-100 and incubated for 30 minutes. Detergent-resistant spores were counted using a hemocytometer.

2.7 Calcofluor Staining

To label cellulose in stalk cell walls, cells were stained with calcofluor by a modification of the method described in Springer et al. (1994). Filters were incubated until development was complete. Sections of filters with several terminal structures were cut, and inverted onto a drop of calcofluor (5 µg/ml) on a glass slide to allow the structures to float off the filters and into the stain. The piece of filter was then removed and a coverslip placed on the drop containing the submerged structures. Stained structures were viewed with a Zeiss Azioplan2 fluorescence microscope and images captured with a digital camera (Spot Diagnostics Instruments, Inc.).

2.8 Transformation of *Dictyostelium discoidum* cells

Ax2 cells were transformed by the previously described $CaPO_4$ DNA precipitation and transformation method (Early and Williams, 1987). Between 2×10^6 and 5×10^6 Ax2 amoebae, passaged no more than twice after spore germination, were plated on Nunclon™ (9 cm) dishes. Cells were allowed to adhere to the plastic for approximately 30 minutes following which time the medium was replaced by bis-HL5 (supplemented with streptomycin). DNA was precipitated in an Eppendorf tube by mixing 400 µl dH_20, 10 µg plasmid DNA, 100 µl $CaCl_2$, and 500 µl 2X HBS. The mixture was incubated at room temperature for 30 minutes and then added to the cells in one dish in a dropwise manner. The cells were incubated at 22°C for 4-5 hours, the bis-HL5 removed, and the cells then subjected to a glycerol shock (15% glycerol in HBS) for 2 minutes at 22°C. The cells were then refed HL5 supplemented with streptomycin, and incubated overnight at 22°C. The following day, G418 was added at a concentration of 10 µg/ml. Growth medium was changed every 2-3 days until colonies appeared, usually after about 7 to10 days. Isolated colonies were picked and transferred to HL5 medium containing 10 µg/ml G418 in 4 cm wells of 6-well Nunclon™ plates. Once confluent, cells were transferred for continued growth, to 9 cm Nunclon™ plates or in shake culture. When the transforming plasmid DNA contained *lacZ*, β-galactosidase expressing colonies were selected for by the colony lift method described below. A complete list of the transformants generated for the investigations described in this thesis is provided in Table III.

Table III. Strains used for the investigations described in this thesis.

Ax2	Parental axenic strain, the wild type control
act15::lacZ	Wild type strain, constitutive expression of *lacZ* in all cells
ecmAO::lacZ	Wild type strain, expression of *lacZ* in PstA/PstO cells
ecmA::lacZ	Wild type strain, expression of *lacZ* in PstA cells
ecmO::lacZ	Wild type strain, expression of *lacZ* in PstO cells
ecmB::lacZ	Wild type strain, expression of *lacZ* in PstB cells
ST::lacZ	Wild type strain, expression of *lacZ* in cells of the stalk and basal disc
psA::(his)lacZ	Wild type strain, expression of labile *lacZ* in prespore cells
ecmAO::rasG	Transformants expressing wild type RasG in PstA/PstO cells
ecmAO::rasG(G12T)	Transformants expressing activated RasG in PstA/PstO cells
ecmAO::rasG(G12T)/ act15::lacZ	Transformants expressing activated RasG in PstA/PstO cells and constitutively expressing *lacZ* in all cells
ecmAO::rasG(G12T)/ ecmA::lacZ	Transformants expressing activated RasG in PstA/PstO cells and expressing *lacZ* in PstA cells
ecmAO::rasG(G12T)/ ecmO::lacZ	Transformants expressing activated RasG in PstA/PstO cells and expressing *lacZ* in PstO cells
ecmAO::rasG(G12T)/ ecmB::lacZ	Transformants expressing activated RasG in PstA/PstO cells and expressing *lacZ* in PstB cells
ecmAO::rasG(G12T)/ ST::lacZ	Transformants expressing activated RasG in PstA/PstO cells and expressing *lacZ* in stalk and basal disc cells
ecmAO::rasG(G12T)/ psA::(his)lacZ	Transformants expressing activated RasG in PstA/PstO cells and expressing labile *lacZ* in prespore cells

Table III. Strains used for the investigations described in this thesis (continued).

psA::rasG	Transformants expressing wild type RasG in prespore cells
psA::rasG(G12T)	Transformants expressing activated RasG in prespore cells
psA::rasG(G12T)/ act15::lacZ	Transformants expressing activated RasG in prespore cells and constitutively expressing *lacZ* in all cells
psA::rasG(G12T)/ ecmAO::lacZ	Transformants expressing activated RasG in prespore cells and expressing *lacZ* in PstA/PstO cells
psA::rasG(G12T)/ ecmB::lacZ	Transformants expressing activated RasG in prespore cells and expressing *lacZ* in PstB cells
psA::rasG(G12T)/ ST::lacZ	Transformants expressing activated RasG in prespore cells and expressing *lacZ* in stalk and basal disc cells
psA::rasG(G12T)/ psA::(his)lacZ	Transformants expressing activated RasG in prespore cells and expressing labile *lacZ* in prespore cells
ecmAO::rasG(G12T)/ psA::rasG(G12T)	Transformants expressing activated RasG in PstA/PstO cells and in prespore cells
*rasD::rasG(G12T)**	Transformants expressing activated RasG from the *rasD* promoter (expression in both cell types)

*Strain obtained from M. Khosla.

2.9 Colony Lift for β-Galactosidase Expression

Dictyostelium cells were transformed with one of several reporter plasmids containing the *lacZ* gene. Once colonies became visible, the transformant cells were pooled and transferred to 4 cm wells in 6-well Nunclon™ plates to continue growth. A sample of the pooled cells was diluted and an aliquot, containing about 75 cells, was spread in association with *Klebsiella oxytoca* onto SM-VAN plates. After 4-5 days incubation, plaques from single *Dictyostelium* cells had formed and cells in the center of the plaques had begun differentiation. Clones were screened for expression of β-galactosidase as described by Buhl et al. (1993). Cells were adsorbed to Hybond-N+ nitrocellulose filters, the filters were fixed for 10 minutes in Z buffer containing 0.5% gluteraldehyde and then washed 4 times for 5 minutes each in Z buffer containing 2% Tween-20. The filters were then immersed in β–Galactosidase Staining Solution and incubated in a dark chamber overnight at 37°C.

Positive clones with wild type developmental morphology were selected and cells from the corresponding plaques were transferred to HL5 medium for growth. An identical protocol was followed to screen for cotransformants expressing both *lacZ* and *ras* constructs except that selected clones were positive for β-galactosidase expression and exhibited the developmental phenotype characteristic of the transforming *ras* construct.

2.10 In Situ Detection of β-Galactosidase Expression

Dictyostelium transformants expressing β-galactosidase were washed free of nutrients and set up for development on white Millipore filters using KK2 instead

of BS. At various times after aggregate formation, portions of the filters were cut and immersed for 10 minutes in Z buffer containing 1% gluteraldehyde to fix the cell masses (Dingermann et al., 1989). Samples were then washed twice with Z buffer and then incubated in β-galactosidase staining solution at 37°C overnight. Stained organisms were again washed with Z buffer and then photographed with a Nikon 35 mm camera and Kodak Ektachrome Color Slide Film.

2.11 cDNA Probe Preparation

Plasmid DNA containing the cDNA of the gene of interest was digested with the appropriate restriction enzymes to release the cDNA fragments. The digested DNAs were size fractionated by electrophoresis in a 0.7% agarose gel buffered with TBE. Electrophoresed DNA was stained with ethidium bromide and viewed on a UV transilluminator. The band containing the desired cDNA fragment was excised from the gel and purified using the Qiaquick Gel Extraction Kit. Approximately 100 ng of the cDNA, estimated by UV fluorescence relative to a standard concentration of a DNA mixture fractionated on the same gel, was labeled with [^{32}P]-α-dCTP by the random oligonucleotide primer method (Feinberg and Vogelstein, 1983). The labeled cDNA probes were purified from unincorporated nucleotides by passage through a Sephadex G–25 spin column (Sambrook et al., 1989).

2.12 RNA Isolation and Northern Analysis

The guanidinium isothyocyanate method (Chomczynki and Sacchi, 1987) was used to extract total RNA from *Dictyostelium* cells. Each 20 μg sample of RNA was

resuspended in RNA sample buffer and electrophoresed on a 1.25% agarose gel (containing 2.2 M formaldehyde). Ethidium bromide fluorescence of rRNA was used to ensure equal loading of RNA samples. The gel was then washed 4 times for 5 minutes each with distilled water. After washing, the gel was soaked in 20X SSC and then transferred by the capillary method to a Hybond-N+ nitrocellulose membrane (Sambrook et al., 1989). Transfer was permitted to occur overnight after which the RNA was fixed to the membrane by baking at 80°C for 2 hours. Blots were hybridized with radiolabeled cDNA probes in Hybridization Solution overnight at 42°C. Blots were washed first with 2X SSC, 0.1% SDS at room temperature and then with 0.5X SSC, 0.1% SDS at 60°C for 20 minutes before being exposed to x-ray film for varying times depending on signal intensity.

2.13 Protein Isolation and Western Blot Analysis

Dictyostelium cells were harvested and washed in BS and then lysed in 1% SDS in BS. A small volume was used to determine the protein concentration by measuring optical density at wavelengths 260 nm and 280 nm. The remainder of the lysate was mixed with an equal volume of 2X Protein Sample Buffer. Protein samples of 20 µg of each were then boiled for 3 minutes and subjected to SDS-PAGE analysis (Laemmli, 1970). A test gel for each set of protein samples was stained with Coomassie to confirm equal loading. For western analysis, following electrophoresis, proteins were transferred to Hybond-P PVDF membranes (Towbin et al., 1979). Prior to antibody probing, the blots were blocked with TBST (50mM Tris-HCl pH 7.5, 150 mM NaCl, 5% Tween-20) containing 5% non-fat dry milk for 1 hour at room temperature. Blots were then washed twice briefly in TBST and then

incubated with antibody specific for RasG (Khosla et al., 1994) (1:1000) in TBST containing 1% non-fat dry milk overnight. Probed blots were then washed 4 times for 5 minutes each in TBST and then incubated with secondary antibody, HRP-conjugated donkey-α-rabbit antibody (1:10,000) in TBST containing 1% non-fat dry milk for 1 hour. Blots were again washed 4 times for 5 minutes each with TBST. To detect bound secondary antibody, blots were treated with ECL reagents and exposed to X-ray film.

2.15 Phototaxis Assay

Cells were harvested and washed twice with BS, resuspended in BS at a density of $1x10^8$ cells/ml and spotted at one side of a 1% water agar plate (Dormann et al., 1996). The plates were then placed in a chamber lined with moist towels in an orientation such that the cells were placed furthest away from a single slit cut into one side of the chamber to allow light entry, and exposed to room light. Under these conditions, developing structures are maintained at the slug stage. Plates were incubated for 2 days and then examined for evidence of slug migration.

CHAPTER THREE

OVEREXPRESSION OF RASG(G12T) IN PRESTALK CELLS

3.1 Background

As described in the Introduction, wild type starving amoebae aggregate to form single tipped mounds which elongate and fall to the substratum to form migrating slugs. Starving amoebae of the *Ddras*-Thr12 transformant are different: they aggregate to form multi-tipped mounds that do not proceed to form slugs (Reymond et al., 1986). *rasD* was originally identified as a prestalk specific gene (Reymond et al., 1984) and its expression has been shown to be enriched in the *ecmA*-expressing subset of prestalk cells (Esch and Firtel, 1991; Jermyn and Williams, 1995). It has also been determined that the *Ddras*-Thr12 cells exhibit a defect in the desensitization of guanylyl cyclase which is involved in the chemotaxis to cAMP (Van Haastert et al., 1987). Given these results, and the fact that the *ecmA*-expressing cells sort to the tip (Early et al., 1995), which is the region responsible for generating the cAMP pulses that orchestrate development (Schaap, 1986), it was postulated that the overexpression of activated RasD in these prestalk cells was responsible for the developmental aberrations in the *Ddras*-Thr12 transformant (Esch and Firtel, 1991). I tested this possibility using activated RasG, which phenocopies activated RasD when expressed from the *rasD* promoter (M. Khosla, unpublished observations). Activated RasG was expressed in prestalk cells and the generated transformants characterized.

3.2 Developmental Phenotype of *ecmAO::rasG(G12T)* Transformants

The *rasG(G12T)* cDNA was cloned downstream of the *ecmAO* promoter (the complete promoter for the *ecmA* gene) (Early et al., 1993) in order to direct RasG(G12T) overexpression in *ecmA*-expressing prestalk cells. The construct was

transformed into *Dictyostelium* Ax2 cells. Multiple transformants were selected and maintained in 10μg/ml G418. Western blot analysis was used to detect overexpression of RasG(G12T) during development and the result for one transformant is given in Fig. 3-1. The transformant (Fig. 3-1B) contained a higher level of RasG relative to the wild type Ax2 control (Fig. 3-1A).

To examine the developmental characteristics of these *ecmAO::rasG(G12T)* transformants relative to wild type *Dictyostelium*, cells were starved on nitrocellulose filters to induce development. Wild type cells (transformed with a prespore specific *lacZ* construct as a control for transformation) aggregated to form a mound with a single apical tip (Fig. 3-2A) which first elongated to form a finger shaped structure (Fig. 3-2B) and then fell to the substratum to form a slug (Fig. 3-2C). Development culminated in the formation of a fruiting body. Fig. 3-2D shows a wild type culminating structure with the prespore mass raised above the substratum by a stalk.

When *ecmAO::rasG(G12T)* transformants were starved under similar conditions, the cells aggregated and formed mounds at approximately the same time as wild type cells. However, the subsequent development of each of the transformants studied was slow, asynchronous, and terminated with the formation of varied abnormal culminants. Due to the variation in developmental phenotypes exhibited by each of the transformants, only the most commonly observed morphologies are described.

Some aggregates did not progress beyond the mound stage. Other mounds produced a single small but distinct tip (Fig. 3-3A). In many cases, the tipped mounds formed by *ecmAO::rasG(G12T)* cells elongated vertically to form a finger (Fig. 3-3B) which subsequently fell to the substratum to form a slug (Fig. 3-3C). These early structures were morphologically similar to the wild type structures (Fig. 3-2A, B, C). However, by the end of the developmental program, no wild type fruiting bodies

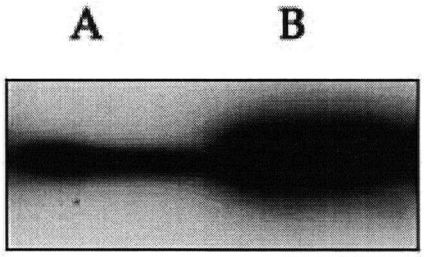


Figure 3-1: Western blot of cell lysates harvested at 16 hours of development and probed with an antibody specific for RasG. The lysate in lane (A) was made from wild type Ax2 cells. The lysate in lane (B) was made from an *ecmAO::rasG(G12T)* transformant.

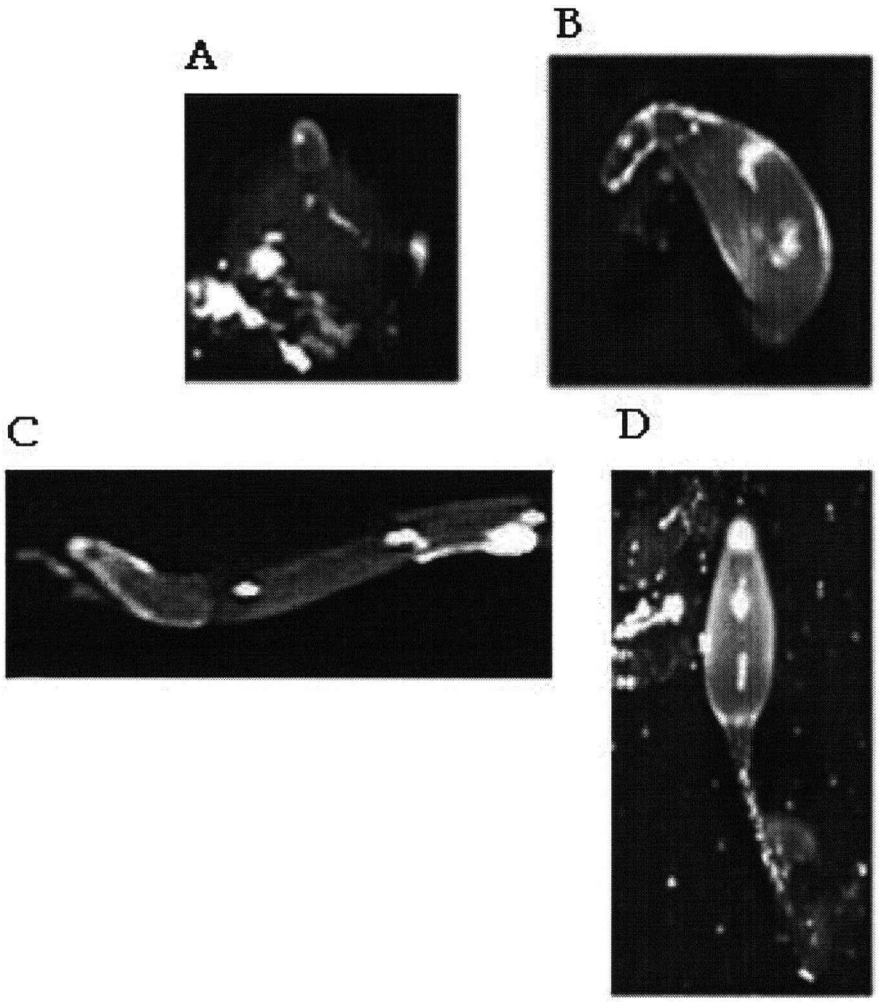


Figure 3-2: Developmental morphologies of *psA::lacZ* control transformant cells. Cells were starved on filters to induce development and were photographed at various stages. Cells formed a single-tipped mound (A), an elongated finger, (B), a slug (C), and a culminating fruiting body (D).

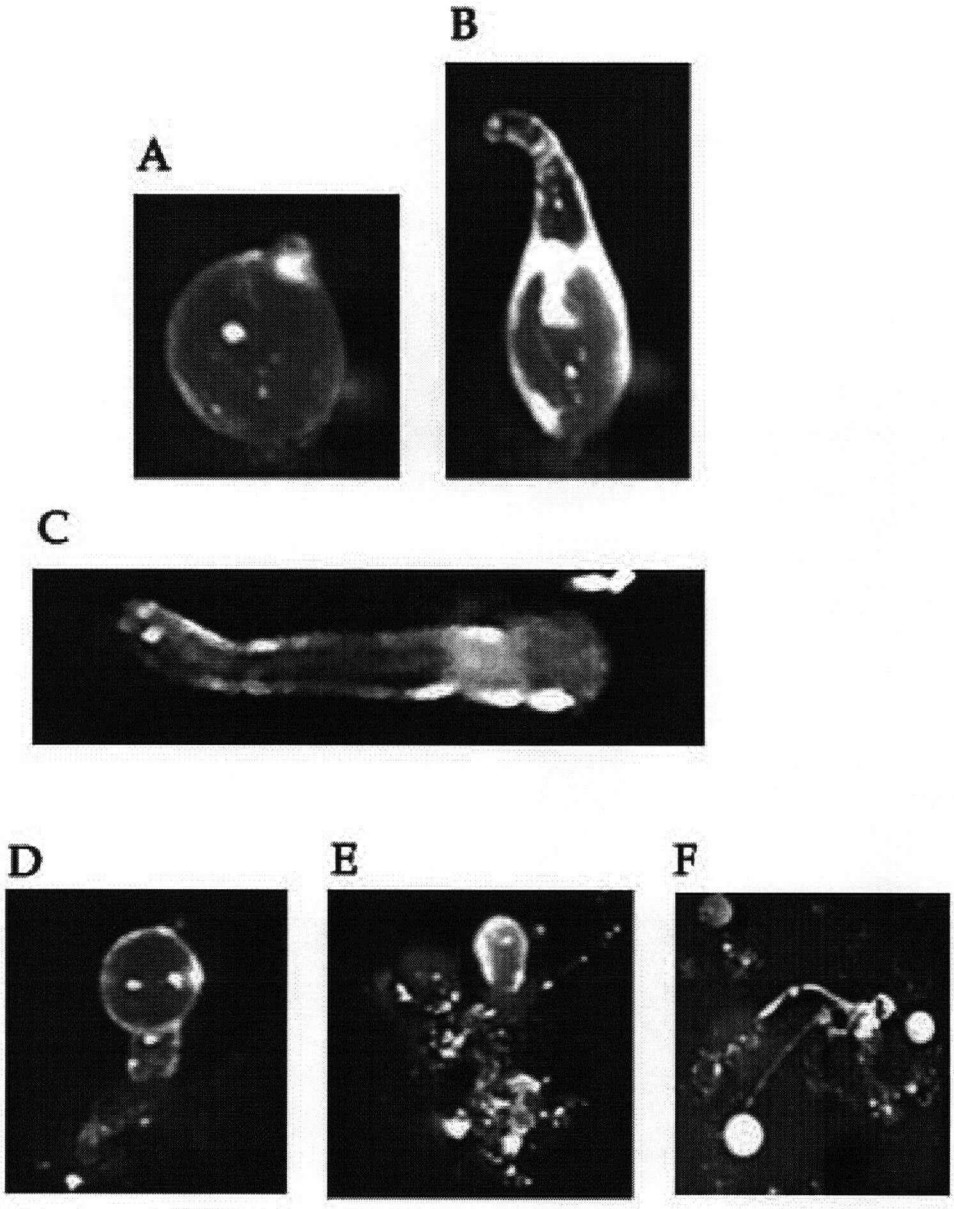


Figure 3-3: Developmental structures formed by *ecmAO::rasG(G12T)* transformants at different stages. Transformants were plated on filters for development and photographed at various stages. A mound of transformant cells formed a single tip (A). Some tipped mounds elongated to form a finger (B), which fell to the substratum to form a slug (C). Culminants were of various irregular morphologies (D) and (E). The culminant in (E) contained a cloudy and yellow sorus-like cell mass. Culminants of an *ecmAO::rasG* transformant are also shown (F).

were observed. Instead, a variety of morphologically abnormal culminants were produced by each of the *ecmAO::rasG(G12T)* transformants. Those that most resembled normal fruiting bodies consisted of a spherical sorus-like structure supported on a short irregular cell mass (Fig. 3-3D). However, other more aberrant culminant morphologies were also observed (Fig. 3-3E). The *ecmAO::rasG(G12T)* transformants required 36 to 48 hours to form these terminal structures whereas most wild type cells had completed development in 24 hours. Although the developmental morphology of the *ecmAO::rasG(G12T)* transformants was varied, no multi-tipped mounds were observed at any stage. This indicates that the expression of activated RasG (and by implication, RasD) in prestalk cells did not cause multi-tip formation.

Transformants that expressed wild type *rasG* from the *ecmAO* promoter were also generated. Developmental morphologies of these *ecmAO::RasG* transformants were also varied and irregular. But, as shown in Fig. 3-2F, these transformants were able to form fruiting bodies with sori raised on stalks.

3.3 Terminal Differentiation of *ecmAO::rasG(G12T)* Transformants

Although the terminal structures formed by the *ecmAO::rasG(G12T)* transformants were abnormal, some of the sorus-like cell masses were cloudy and yellow (Fig. 3-3E), indicating the possibility that they contained mature spores (Staples and Gregg, 1967). However, when filters of developed transformant cells were harvested in Bonner's salts containing Trition-X-100, the number of spores observed for the transformants was far less than for wild type strain. Table IV lists spore formation values for wild type (Ax2) cells and for one *ecmAO::rasG(G12T)* transformant. The transformant produced a very low number of spores. Spore formation data for an *ecmAO::rasG* transformant is also given. These cells also

Table IV. Spore formation by wild type cells and *ecmAO::rasG(G12T)* and *ecmAO::rasG* transformant cells.

Strain	Spore Formation (as % of cells induced to develop)*
Ax2	114 ± 10
ecmAO::rasG(G12T)	0.5 ± 0.4
ecmAO::rasG	66 ± 7

*Spore formation values are the average (and range) from two separate samples in one experimental trial.

produced fewer spores than the wild type but numbers were higher than for the *ecmAO::rasG(G12T)* transformants.

The *ecmAO::rasG(G12T)* culminants were also examined for the presence of stalk cells. Culminants were stained with calcofluor, which binds cellulose in spore coats and stalk cell walls (Harrington and Raper, 1968), and were then viewed by fluorescence microscopy. In a wild type culminant, the cell walls of cells in the basal disc and in the emerging stalk tube stained with calcofluor (Fig. 3-4A). Similarly stained cells were also observed in an *ecmAO::rasG(G12T)* culminant (Fig. 3-4B). The results indicate that these transformants were capable of differentiating into mature stalk cells. However, the structures formed were unlike the long, slender stalks formed by wild type cells (Fig. 3-4A) and stalk tubes were not observed. The arrow in Fig. 3-4B points to an apparently unstained mass of cells in the *ecmAO::rasG(G12T)* culminant that corresponded to a sorus-like cell mass. The lack of stain suggests the absence of spores, consistent with the fact that the transformants were defective in spore formation. In the wild type culminant shown in Fig. 3-4A, the spore head was outside the field of view but scattered, stained spore cells were visible.

3.4 Cell Type Specific Gene Expression During Development of *ecmAO::rasG(G12T)* Transformants

To further investigate the developmental characteristics of the *ecmAO::rasG(G12T)* transformants I examined the expression patterns of cell type specific genes. Northern blots of RNA isolated from *ecmAO::rasG(G12T)* transformant cells and control cells (transformed with the *ecmAO::lacZ* vector) (Early et al., 1993) at various developmental time points were probed with a labeled cDNA fragment of the prespore specific *cotC* gene (Fosnaugh et al., 1989). Compared to the *cotC* expression

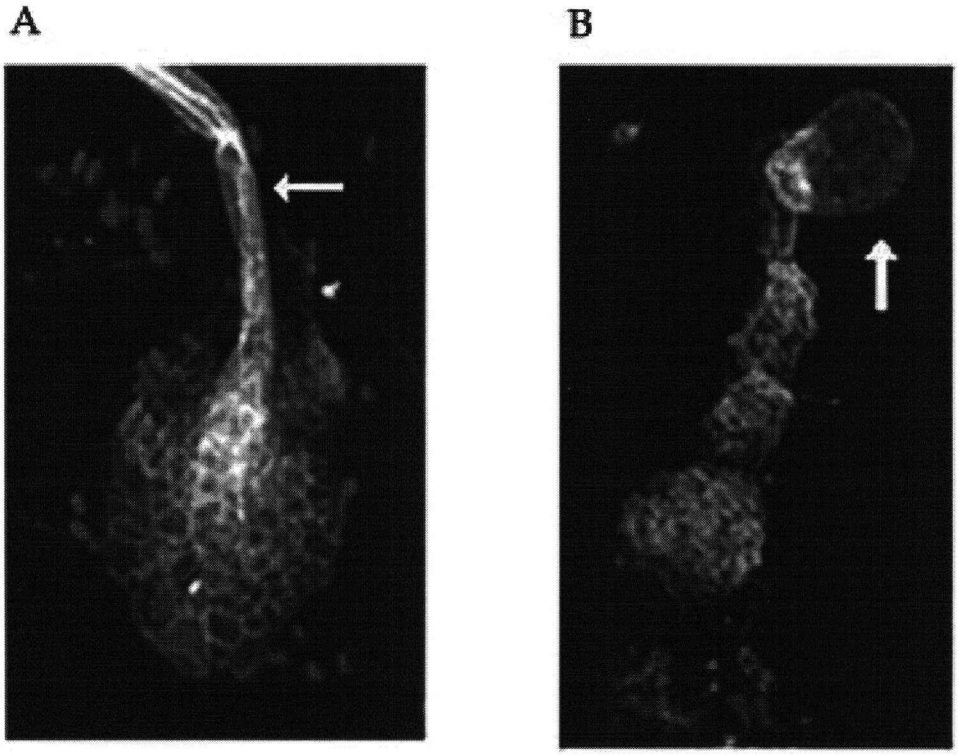


Figure 3-4: Calcofluor staining of wild type and *ecmAO::rasG(G12T)* structures. Culminants of wild type Ax2 cells (A) and *ecmAO::rasG(G12T)* transformant cells (B) were stained with calcofluor and viewed by fluorescence microscopy to identify cellulose in stalk cell walls. The arrow in (A) points to a wild type stalk tube. The arrow in (B) points to a sorus-like cell mass in the transformant culminant.

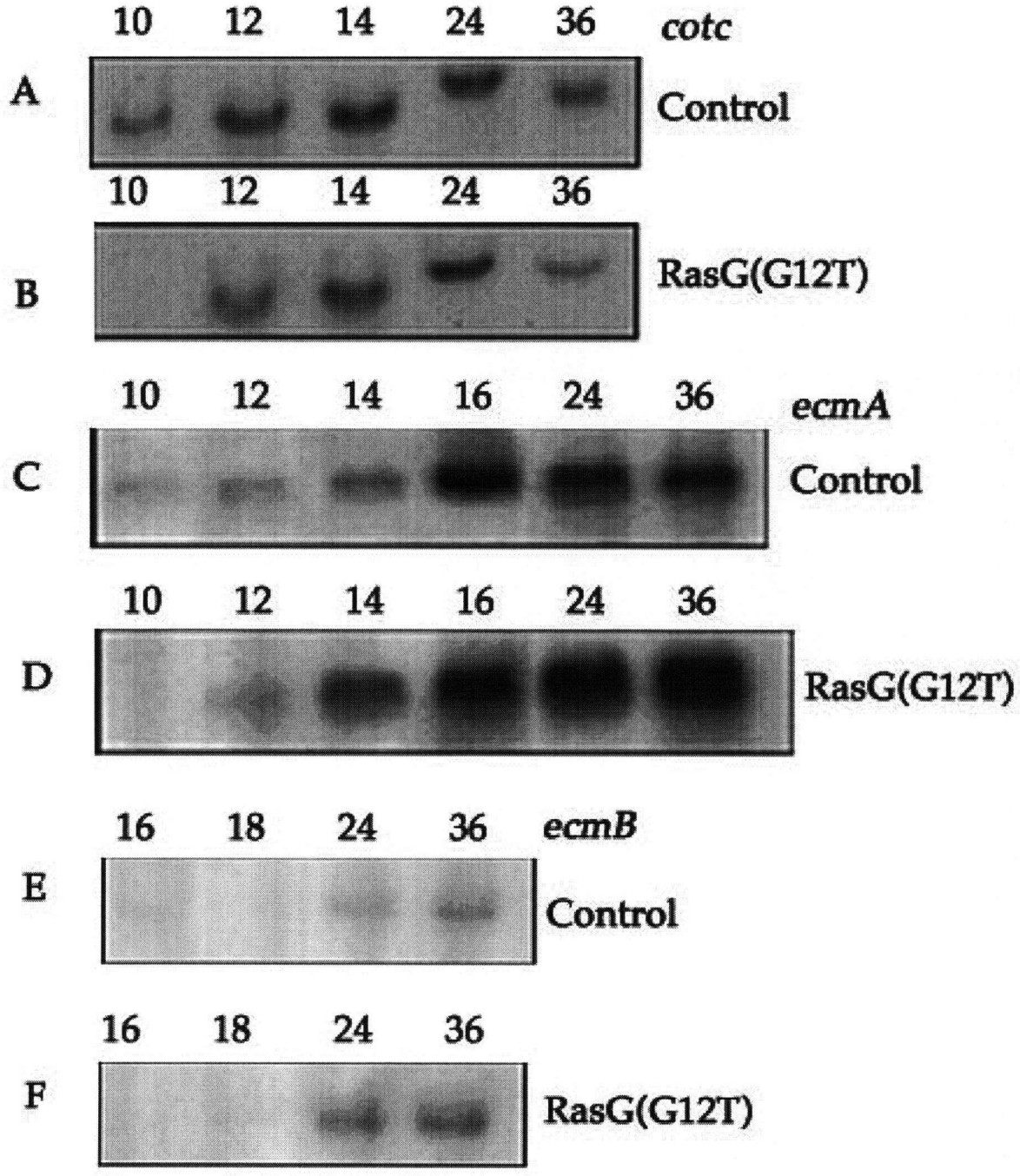


Figure 3-5: Northern blots of RNA of *ecmAO::lacZ* transformed control cells and of *ecmAO::rasG(G12T)* cells at various developmental time points. RNA was isolated, electrophoresed, and blotted as described in Chapter Two. The numbers above the lanes refer to the hour of development at which RNA was isolated. The blots in (A, C, E) are from *ecmAO::lacZ* control transformant cells. The blots in (B, D, F) are from *ecmAO::rasG(G12T)* cells. Blots in (A, B) were probed with the prespore specific *cotC* cDNA. Blots in (C, D) were probed with the prestalk specific *ecmA* cDNA. Blots in (E, F) were probed with the prestalk specific *ecmB* cDNA.

pattern in the control cells (Fig 3-5A), expression in the transformant was delayed by 2 hours but mRNA levels were only slightly reduced (Fig. 3-5B).

I also probed northern blots with two prestalk specific labeled cDNA's, *ecmA* and *ecmB* (Williams et al., 1987; Ceccarelli et al., 1987). Relative to the expression of *ecmA* in the control cells (Fig. 3-5C), the expression in the *ecmAO::rasG(G12T)* cells was delayed by 2 hours and the mRNA levels were slightly elevated (Fig. 3-5D). For the *ecmB* gene also, compared to the wild type cells (Fig. 3-5E), the transformant exhibited an increase in expression levels (Fig. 3-5F). These results demonstrate that the expression of activated RasG in prestalk cells resulted in a slight reduction of prespore gene expression and a slight enhancement of prestalk gene expression. In the *Ddras*-Thr12 transformant, prespore gene expression was drastically reduced and prestalk gene expression was drastically enhanced (Louis et al., 1997a). Thus, the *ecmAO::rasG(G12T)* transformant exhibited far less pronounced effects on cell type specific gene expression than those observed in the *Ddras*-Thr12 transformant.

3.5 PstA Cell Localization in *ecmAO::rasG(G12T)* Transformants

The slugs formed by the *ecmAO::rasG(G12T)* cells appeared morphologically similar to wild type slugs but did not go on to complete normal morphogenesis. One possible explanation was that the positioning of the various cell types within the slugs was unusual. I used cell type specific *lacZ* reporter constructs to determine the localization of different cell types in the developing *ecmAO::rasG(G12T)* organisms.

The *ecmA::lacZ* construct incorporates the proximal domain of the *ecmAO* promoter and drives expression of the linked gene in the PstA subset of prestalk cells (Early et al., 1993). When Ax2 cells transformed with this construct were developed, stained cells in mounds were concentrated in the center of the mound (as viewed from above) but were also observed scattered throughout the mound (Fig. 3-6A).

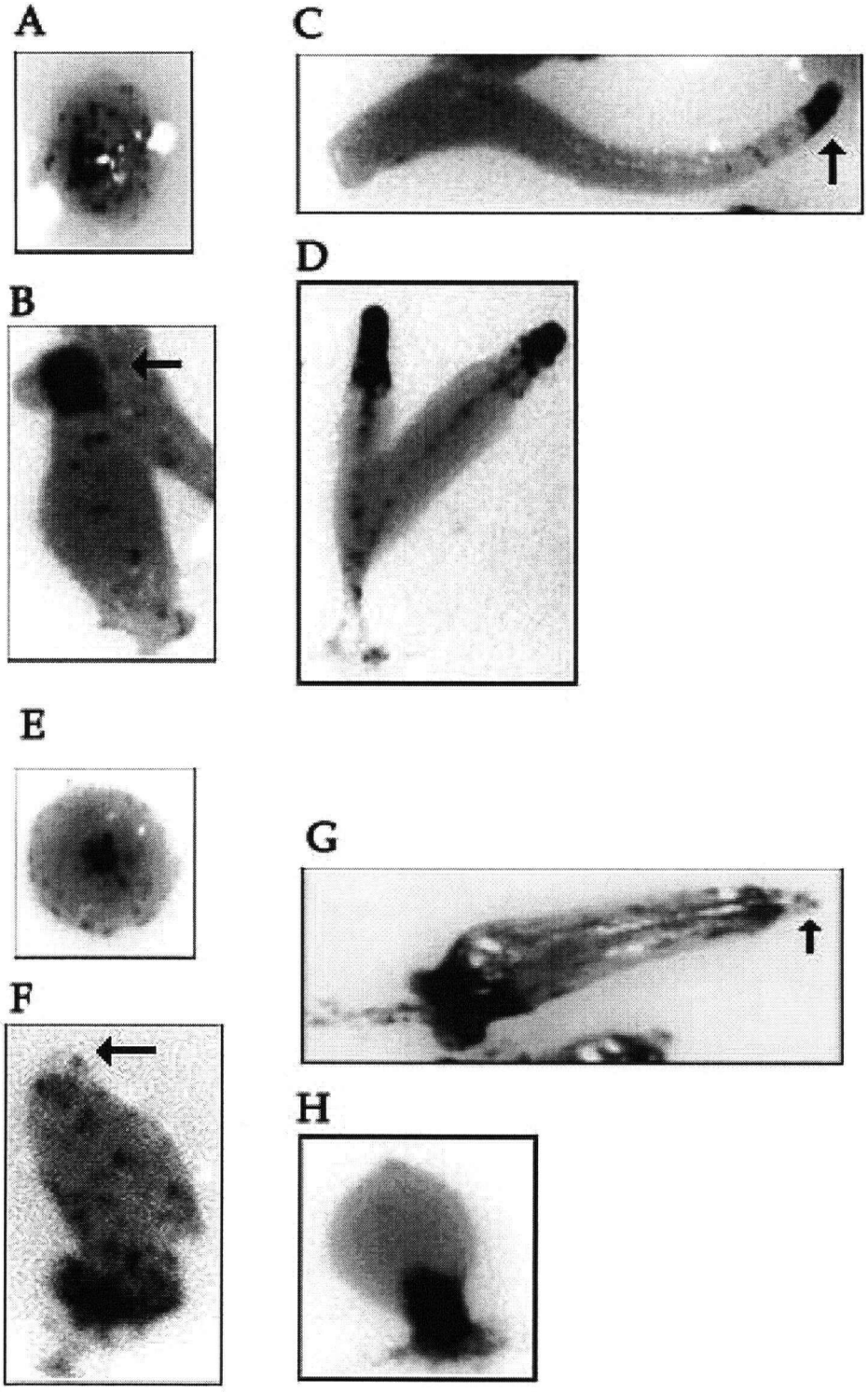


Figure 3-6: PstA cell localization in developing wild type and *ecmAO::rasG(G12T)* structures. A wild type mound (A), finger (B), slug (C), and culminant (D), and an *ecmAO::rasG(G12T)* mound (E), finger (F), slug (G), and culminant (H) were stained to detect the expression of β-galactosidase from the *ecmA* promoter. The arrows in (B, F) point to the tips of the fingers. The arrows in (C, G) point to the tips of the slugs.

Subsequently, PstA cells were strongly localized to the tips of the fingers (Fig 3-6B) and slugs (Fig. 3-6C) that were formed. As developing structures culminated, PstA cells were detected in the anterior tip of the culminant as well as in the stalk tube (Fig. 3-6D). This staining pattern is similar to that previously observed (Early et al., 1993 and 1995).

When mounds of an *ecmAO::rasG(G12T)/ecmA::lacZ* cotransformant were viewed from above, cells staining for β-galactosidase expression were observed to be concentrated in the center (Fig. 3-6E), similar to the pattern seen in wild type mounds (Fig. 3-6A)). However, the central stained cells did not rise upward to localize to the tip of elongating fingers (Fig. 3-6F). Transformant slugs varied with respect to the amount of anterior staining, but the tips of the slugs contained relatively few PstA cells (Fig. 3-6G) compared to wild type slugs (Fig. 3-6C). The majority of PstA cells in *ecmAO::rasG(G12T)* slugs were ectopically localized to the posterior (Fig. 3-6G). Cells expressing the PstA marker were also shed from the posterior of some slugs and expression was detected in scattered cells throughout the slug (Fig. 3-6G). In the culminants that contained a sorus-like structure, the irregular mass of supporting cells stained for *lacZ* expression (Fig. 3-6H). These results indicate that activated RasG expression in PstA cells resulted in their mislocalization in the developing organism.

3.6 PstO Cell Localization in *ecmAO::rasG(G12T)* Transformants

A fusion between the distal portion of the promoter for the *ecmA* gene that had been linked to *lacZ*, was used to identify PstO cells (Early et al., 1993). In slugs of Ax2 cells carrying the *ecmO::lacZ* marker, *lacZ*-expressing cells were located in an anterior collar just posterior to the tip and in ALC throughout the prespore region (Fig. 3-7A). During culmination, *lacZ*-expressing cells were observed mostly in the region forming the upper cup and some were also present in the lower cup (Fig. 3-7B). These results

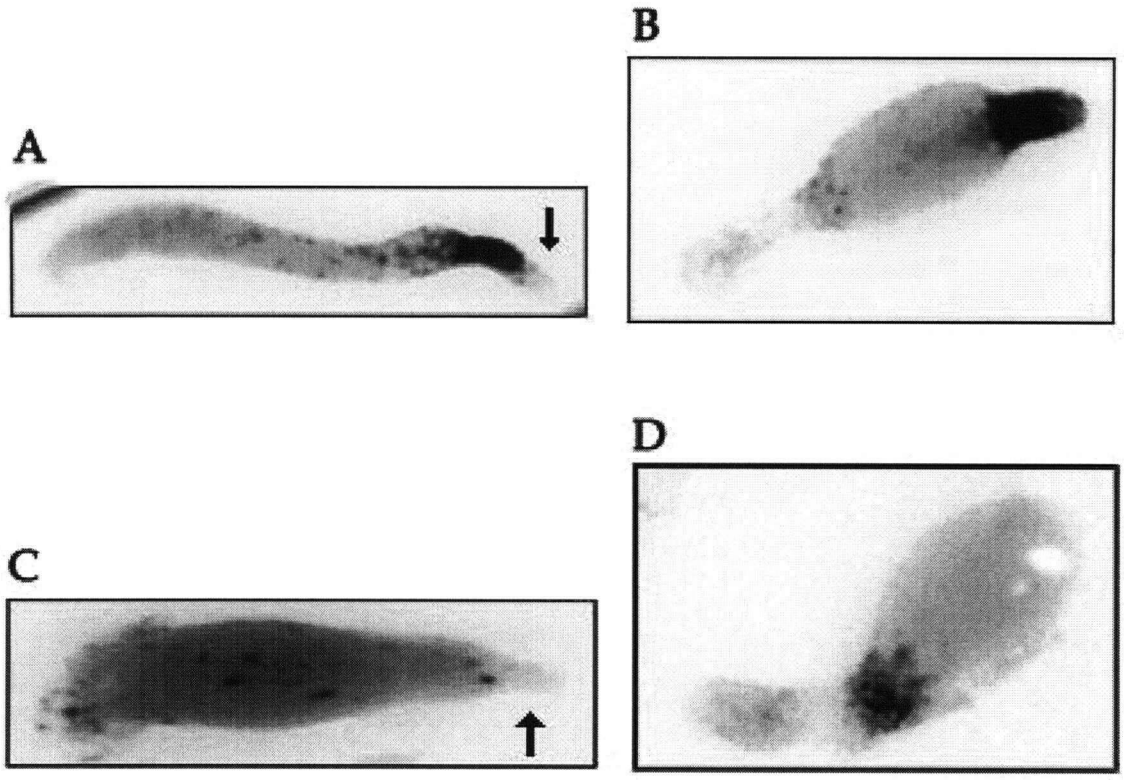


Figure 3-7: PstO cell localization during wild type and *ecmAO::rasG(G12T)* development. A wild type slug (A) and culminant (B) and an *ecmAO::rasG(G12T)* slug (C) and culminant (D) stained to detect expression of β-galactosidase from the *ecmO* promoter. The arrows in (A, C) point to the tips of the slugs.
are similar to staining patterns previously observed for PstO cells (Early et al., 1993 and 1995).

When cells cotransformed with *ecmAO::rasG(G12T)* and *ecmO::lacZ* were induced to develop, the usual collar of PstO cells was not observed in the slugs; only a low level of expression occurred and this was limited to a few scattered cells (Fig. 3-7C). Although β-galactosidase expression increased slightly as development progressed in culminants, the β-galactosidase expression was limited to a small region at the posterior of the organism (Fig. 3-7D). These results indicate that activated RasG expression inhibited the differentiation and localization of PstO cells.

3.7 PstB Cell Localization in *ecmAO::rasG(G12T)* Transformants

The promoter for a second prestalk gene, *ecmB*, was also used to drive *lacZ* expression in order to identify the location of PstB cells (Jermyn and Williams, 1991). PstB cells were observed to be scattered throughout wild type mounds (Fig. 3-8A) In slugs, PstB cells were found in the posterior rearguard region of the slug, as anterior like cells scattered throughout the prespore region of the slug, and in a group of cells in the anterior prestalk region (Fig. 3-8B). At culmination, *lacZ* expression was observed in the stalk, the basal disc, and in the upper and lower cups (Fig. 3-8C). The wild type staining patterns observed for β-galactosidase expression were consistent with those reported previously (Ceccarelli et al., 1991; Jermyn and Williams, 1991; Jermyn et al., 1996).

In *ecmAO::rasG(G12T)* mounds, PstB cells were observed scattered throughout the mound and concentrated in the center (Fig. 3-8D), a pattern that was identical to the mound stage localization of PstA cells (Fig. 3-6E). This staining pattern is unusual since in wild type mounds PstB cells were not concentrated in the center (Fig. 3-6A). The central PstB cells did not rise to the tip and in transformant slugs the anterior

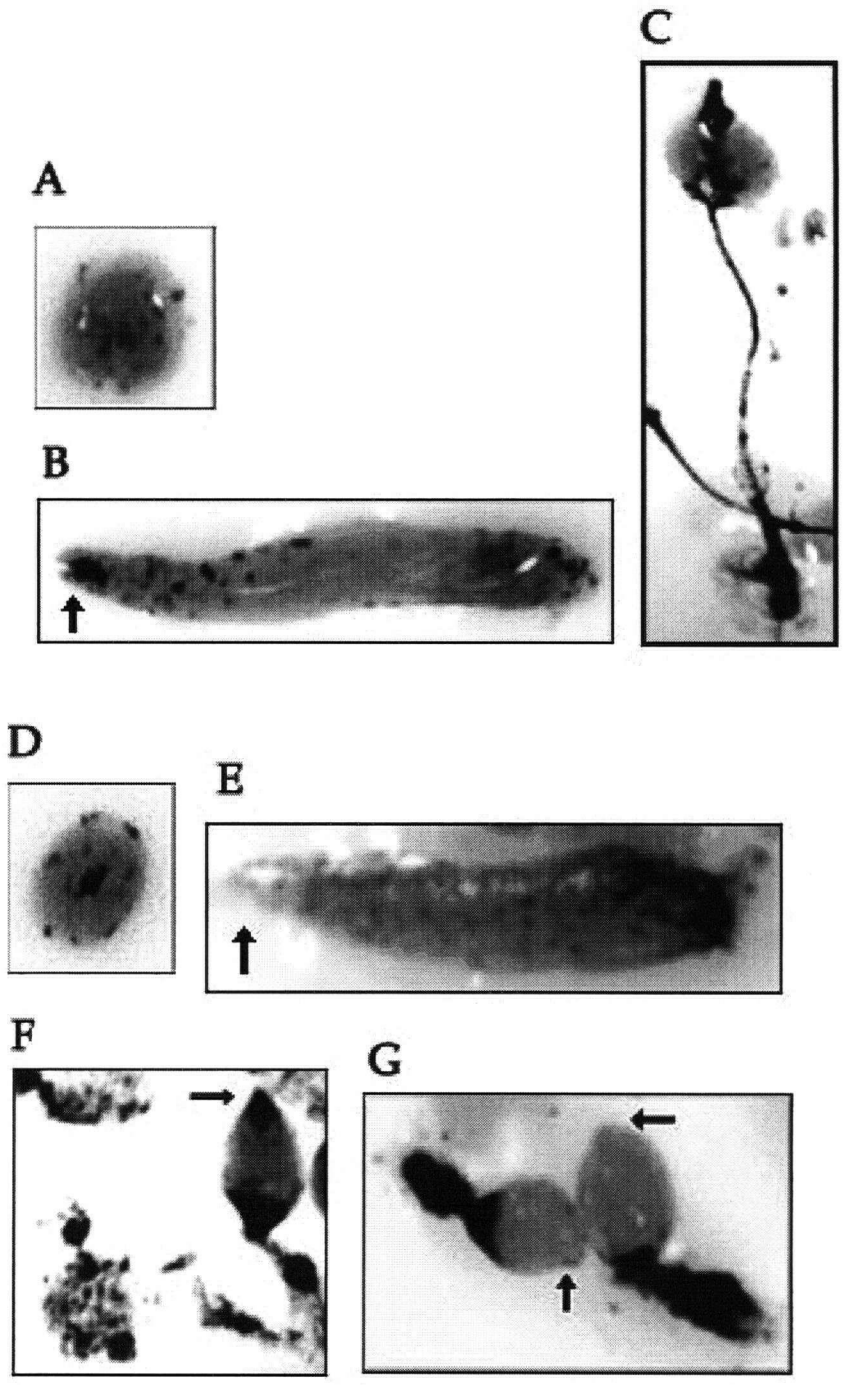


Figure 3-8: PstB cell localization in wild type and *ecmAO::rasG(G12T)* structures. A wild type mound (A), slug (B), and culminant (C), and an *ecmAO::rasG(G12T)* mound (D), slug (E), and culminants (F, G) stained to detect β-galactosidase expression from the *ecmB* promoter. The arrows in (B, E) point to the slug tips. The arrows in (F, G) point to the tips of the culminants.

region remained free of stained cells (Fig. 3-8E). β-galactosidase expression in the slugs was observed in the posterior rearguard cells and in scattered cells in the prespore region (Fig. 3-8E). By the time culminants were formed, some structures had gained the ability to express *ecmB::lacZ* in anterior tips. In the culminant depicted in Fig. 3-8F, PstB cells were localized in the tip as well as in the posterior of the structure and in the discarded sheath. In other culminants such as those in Fig. 3-8G, PstB cells were localized exclusively at the posterior in the irregular shaped cell mass that supports the sorus-like structure.

The *ecmB* promoter has been dissected and a region termed *ST* has been shown to be responsible for directing expression only in the cells that form the stalk and the basal disc (Ceccarelli et al., 1991). Ax2 slugs expressing β-galactosidase from this promoter fragment exhibited staining in a few cells within the anterior tip region (Fig. 3-9A). During culmination, expression was detected in the extending stalk in and the basal disc (Fig. 3-9B).

In the developing *ecmAO::rasG(G12T)* cells marked with the *ST::lacZ* construct, β-galactosidase expression was delayed relative to expression in wild type cells and stained cells were only observed in the posterior of the developing slugs (Fig. 3-9C). During the subsequent morphogenesis of these transformants, *lacZ* expression remained restricted to the posterior of the organism. Culminants expressed β-galactosidase in the irregular cell mass that supported the sorus-like structure (Fig. 3-9D). There was no indication of a developing stalk tube within the sorus-like structures of the transformants. The pattern of *ecmB::lacZ* staining was consistent with that of calcofluor staining since both indicate that stalk cells were formed in the basal regions of *ecmAO::rasG(G12T)* culminants.

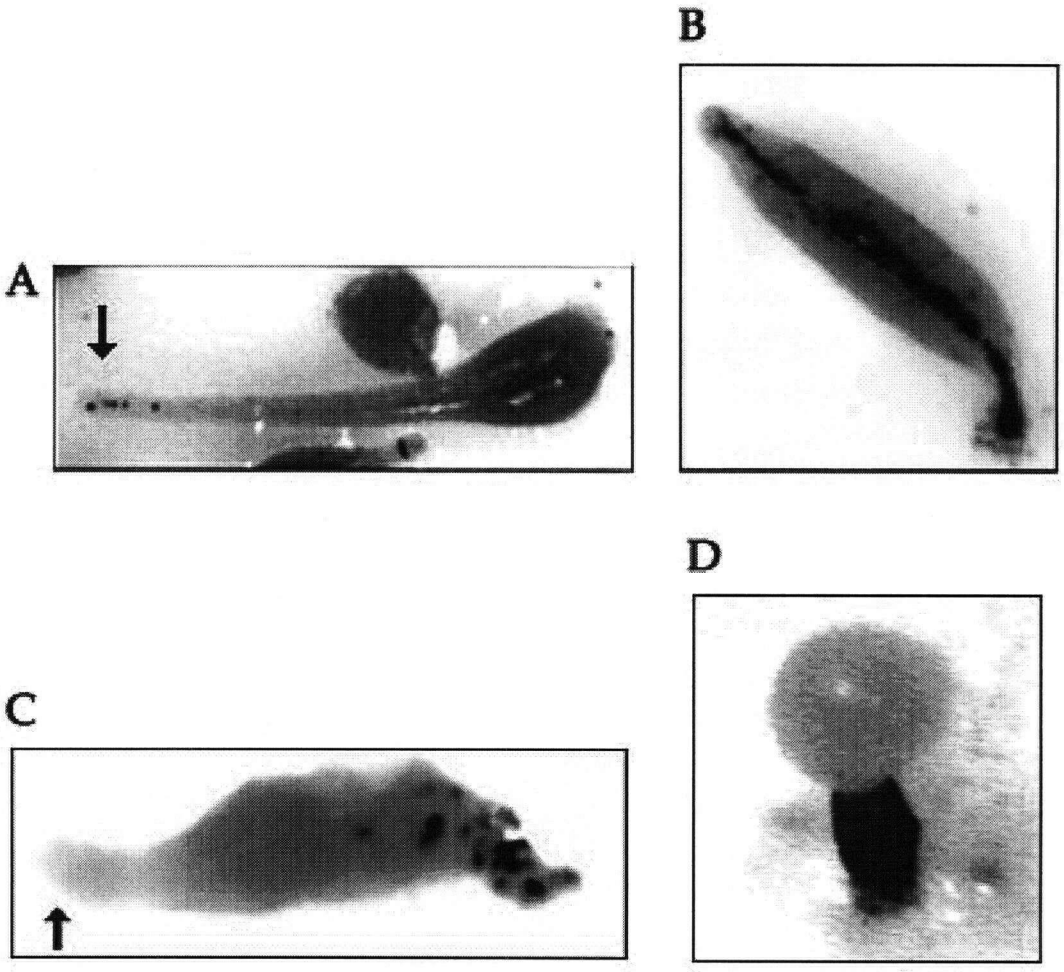


Figure 3-9: Localization of cells expressing the *ST::lacZ* construct during wild type and *ecmAO::rasG(G12T)* development. A wild type slug (A) and culminant (B) and an *ecmAO::rasG(G12T)* slug and culminant stained to detect β-galactosidase expression from the *ST* promoter fragment. The arrows in (A, C) point to the tips of the slugs.

3.8 Prespore Cell Localization in *ecmAO::rasG(G12T)* Transformants

To determine prespore cell localization, I used the prespore specific *psA* promoter (Early et al., 1988) to drive expression of *lacZ*. Since prespore gene expression was slightly reduced in the *ecmAO::rasG(G12T)* transformants (see Section 3.4), it was possible that the number of prespore cells within multicellular structures decreased during the developmental time course. To examine this possibility, I used a *psA::(his)lacZ* construct encoding a labile β-galactosidase (with a half-life of approximately 3 hours (Detterbeck et al., 1994)) so that a loss of prespore cells would correlate with a decrease in the proportion of stained cells. When wild type slugs expressing this labile β-galactosidase from the *psA* promoter were fixed and stained, the majority of the slug was stained blue except for the prestalk region in the anterior of the slug and the posterior rearguard region (Fig. 3-10A). In culminating wild type structures, the developing sorus was intensely stained for *lacZ* expression while the anterior tip and developing stalk remained unstained (Fig. 3-10B). These results are consistent with the expression pattern previously reported (Detterbeck et al., 1994).

In *ecmAO::rasG(G12T)* slugs, *psA*-driven *lacZ* expression was observed in the prespore region and also in the posterior rearguard region while the anterior region remained unstained (Fig. 3-10C). Culminants formed by the *ecmAO::rasG(G12T)* transformants contained small patches of stained cells amongst irregular masses of unstained cells (Fig. 3-10D). These results demonstrate that initial prespore cell localization was essentially normal in the *ecmAO::rasG(G12T)* slugs. The results also indicate that by the end of development, the proportion of prespore cells was reduced in the transformant structures.

3.9 Spore Formation in Chimaeras of Wild Type and *ecmAO::rasG(G12T)* Cells

Since prespore cell specific gene expression and slug stage prespore cell

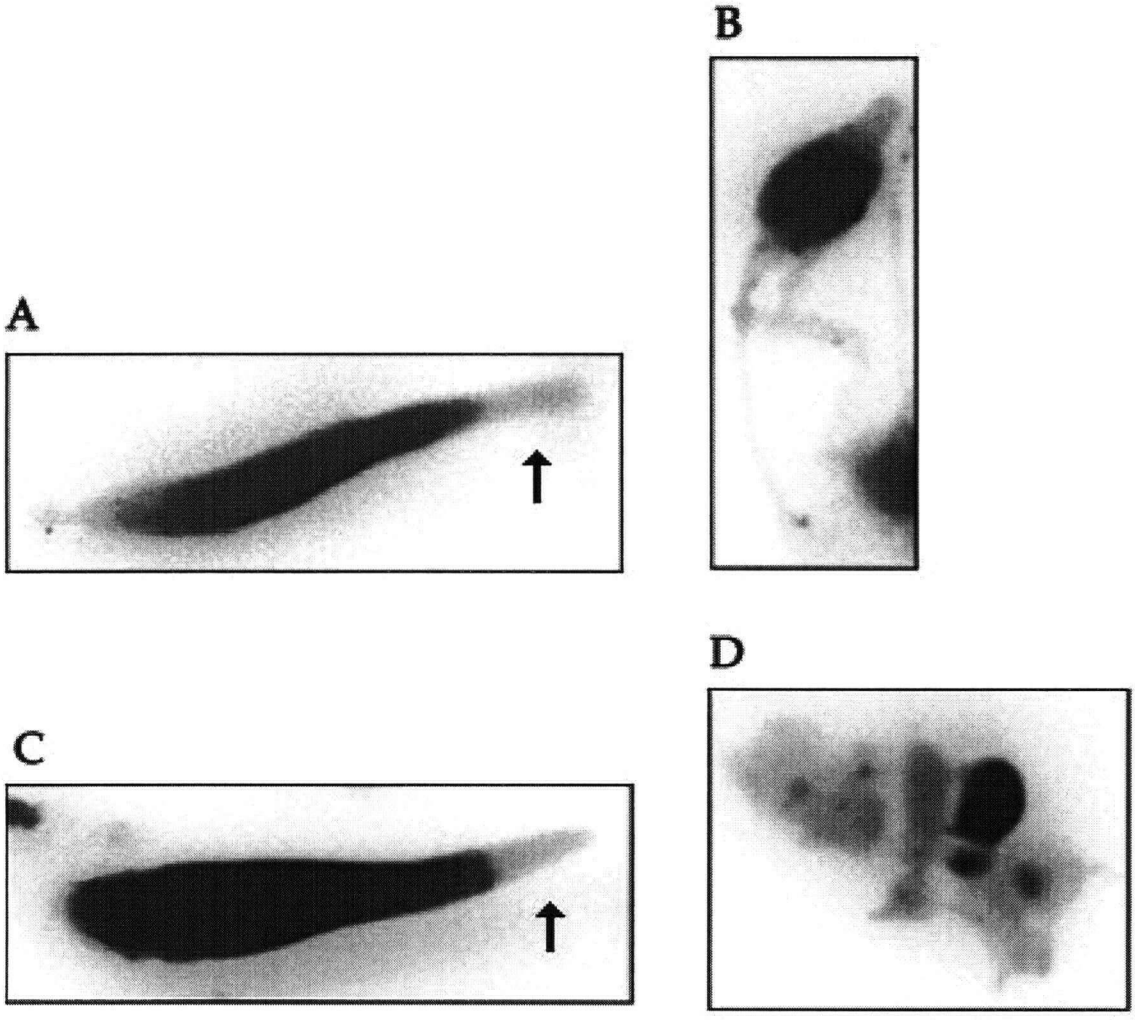


Figure 3:10: Prespore cell localization in wild type and *ecmAO::rasG(G12T)* structures. A wild type slug (A) and culminant (B) and an *ecmAO::rasG(G12T)* slug (C) and culminant (D) stained to detect expression of a labile β-galactosidase from the *psA* promoter. The arrows in (A, C) point to the slug tips.

localization were both relatively normal in the *ecmAO::rasG(G12T)* transformants, I reasoned that the lack of spore formation likely resulted from a defect at a late stage of development. It was possible that the transformant cells were defective in the production of a sporulation signal, in which case the phenoypte might be rescued by providing the wild type signal. To test this hypothesis, I mixed *ecmAO::rasG(G12T)* cells and wild type cells, induced them to develop in chimaera, and assayed for spore formation. Harvested spores were grown in medium with or without G418 selection to determine their respective genotype.

As shown in Table V, the number of spores formed during development of chimaeric mixes was less than the number of spores formed during development of wild type cells alone. However, of the spores formed by the chimaeras, the majority were produced by the *ecmAO::rasG(G12T)* transformant cells. These results indicate that the transformant prespore cells were capable of terminally differentiating into spore cells and that during *ecmAO::rasG(G12T)* development, a signal inducing this differentiation was absent.

3.10 Cell Sorting in Chimaeras of Wild Type and *ecmAO::rasG(G12T)* Cells

The number of spores formed by chimaeric mixtures of wild type and *ecmAO::rasG(G12T)* cells was much less than would be expected if the fraction of wild type cells in the chimaera were induced to develop alone. This indicates that the transformant cells may have an inhibitory effect on wild type spore formation. Alternatively, wild type cells may preferentially form the prestalk and stalk cell regions of a mixture. To test this possibility, *ecmAO::rasG(G12T)* cells were mixed in a 9:1 ratio with wild type cells marked with the *act15::lacZ* construct. In these chimaeras, wild type cells expressed the *lacZ* gene throughout development. As shown in Fig. 3-11A, in chimaeric slugs, wild type cells were preferentially localized to the tip and

Table V. Spore formation by chimaeras of wild type and *ecmAO::rasG(G12T)* transformant cells.

Chimaera	Spore Formation (as % of cells induced to develop)*	*ecmAO::rasG(G12T)* Spores (as % of total spores formed)
100% Ax2	90 ± 11	0
25% Ax2 75% *ecmAO::rasG(G12T)*	19 ± 2.7	61
10% Ax2 90% *ecmAO::rasG(G12T)*	2 ± 0.3	83
100% *ecmAO::rasG*	<1	100

*Spore formation values are the average (and range) from two separate samples in one experimental trial.

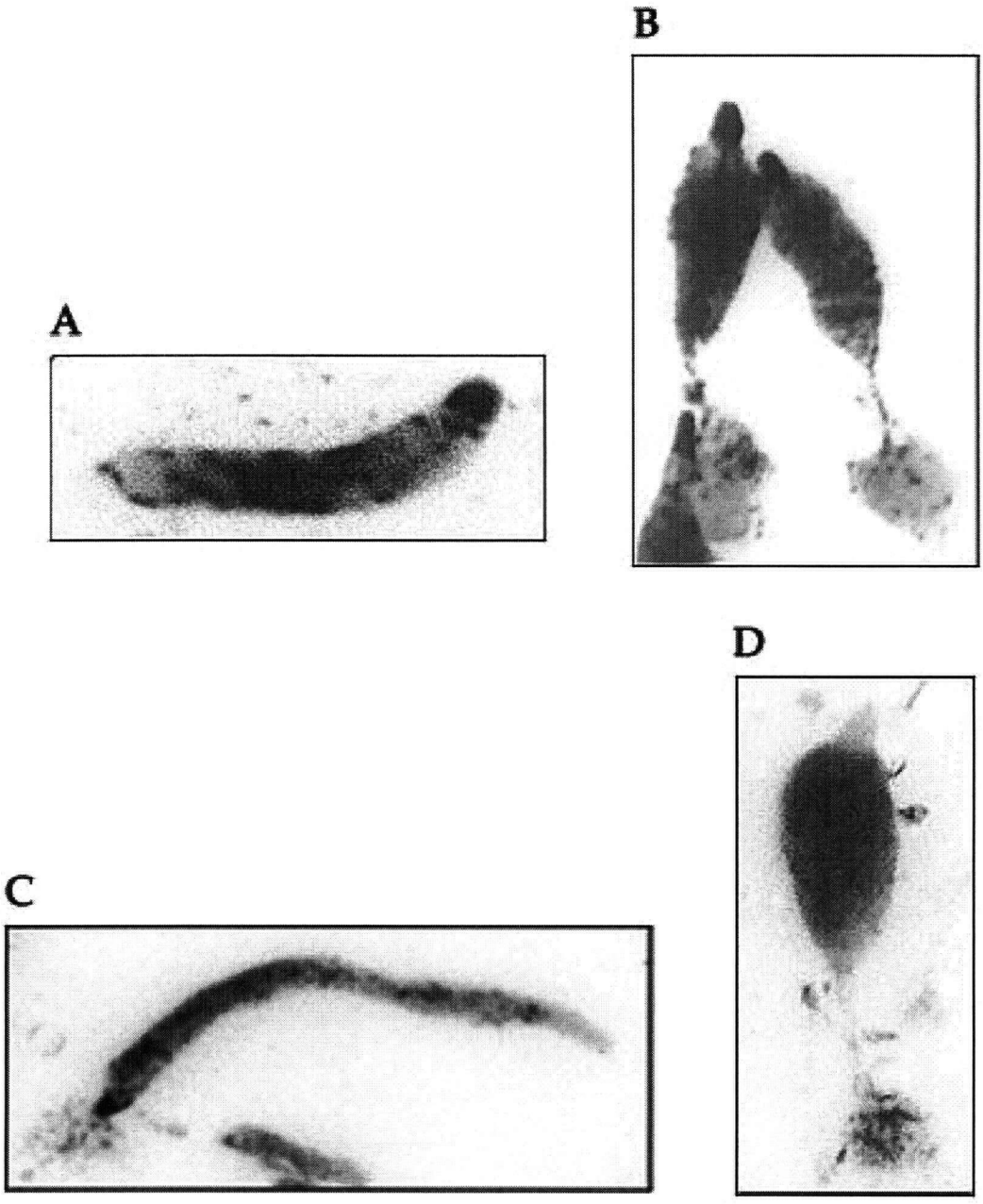


Figure 3-11: Cell sorting in chimaeras of Ax2 and *ecmAO::rasG(G12T)* cells. A slug (A) and culminants (B) formed by mixtures of 10% *act15::lacZ* labeled Ax2 cells mixed with 90% unlabeled *ecmAO::rasG(G12T)* cells and a slug (C) and culminant (D) formed by 10% *act15::lacZ* labeled *ecmAO::rasG(G12T)* cells mixed with 90% unlabelled Ax2 cells.

were also found scattered in the prespore region. In chimaeric culminants (Fig. 3-11B), the tips and stalk tubes were stained indicating that wild type cells constructed stalks during mixed development. Often, a mound of unstained cells was observed at the base of the chimaeric culminant, indicating that the *ecmAO::rasG(G12T)* cells were either forming an enlarged basal disc or being excluded from the developing structure (Fig. 3-11B).

When unmarked wild type cells were mixed in a 9:1 ratio with *act15::lacZ* labeled *ecmAO::rasG(G12T)* transformants, stained cells were localized to the rearguard region and throughout the prespore region of the slug, but very few were present in the anterior (Fig. 3-11C). This result indicated that again the tip of the chimaeric slug was formed by wild type cells. Thus, the *ecmAO::rasG(G12T)* transformants are inherently defective in the formation of normal tip cells. In culminants, *ecmAO::rasG(G12T)* cells were observed in the basal disc and in the prespore region, but seldom in tip or in the stalk tube (Fig. 3-11D).

3.11 Phototaxis and Motility of *ecmAO::rasG(G12T)* Slugs

It has been shown that the tip region is responsible for directing slug motility and phototaxis (reviewed by Fisher, 1997). Given that the *ecmAO::rasG(G12T)* transformant slugs exhibited mislocalization of PstA cells which normally occupy the tip, I examined the ability of the *ecmAO::rasG(G12T)* slugs to migrate towards light. After two days of exposure to unidirectional light, Ax2 slugs had clearly migrated away from the point of deposition and towards the light (Fig. 3-12A). In contrast, *ecmAO::rasG(G12T)* slugs had migrated in random directions for only very short distances (Fig. 3-12B). These results indicated that the *ecmAO::rasG(G12T)* slugs were not phototactic and exhibited reduced motility compared to wild type slugs. When chimaeric slugs with 10% Ax2 cells and 90% *ecmAO::rasG(G12T)* cells were exposed to

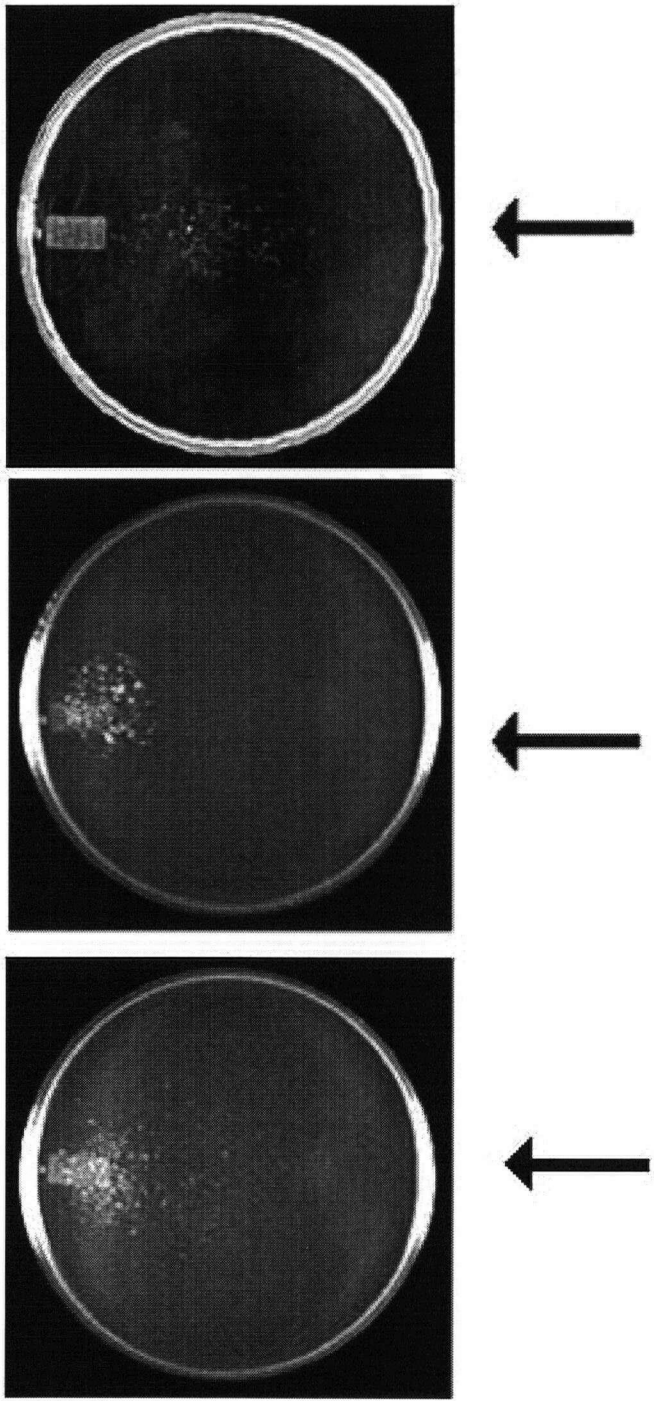


Figure 3-12: Results of phototaxis assay. Slugs of Ax2 cells (A), *ecmAO::rasG(G12T)* cells (B), and chimaeric mixtures of 10% Ax2 and 90% *ecmAO::rasG(G12T)* cells were tested for their ability to migrate towards a unidirectional light source. The arrows indicate the direction of light entry. Cells were deposited on the right side of the rectangle on the bottom of each plate.

unidirectional light, some slugs migrated towards the light, indicating that the presence of wild type cells was able to partly rescue the phototactic defect (Fig. 3-12C).

3.12 Discussion

As discussed previously, overexpression of activated Ras from the *rasD* promoter deranges normal morphogenesis and cell type differentiation (Reymond et al., 1986; Louis et al., 1997a). Since *rasD* expression is enriched in the prestalk cells, it was hypothesized that the overexpression of activated RasD in this population of cells caused the abnormal development (Esch and Firtel, 1991). The results presented in this chapter indicate otherwise. The developmental abnormalities seen in the *ecmAO::rasG(G12T)* transformants were varied, however, the transformants did not produce mounds with multiple tips. Although some mounds produced no tip, those that did produced single tips that were able to elongate and form slugs. In addition, although cell type specific gene expression was slightly altered in the *ecmAO::rasG(G12T)* transformants, the drastic deregulation that occurs in the *Ddras*-Thr12 transformants (Louis et al., 1997a) was not observed. I cannot dismiss the possibility that activated RasG and activated RasD would induce different developmental phenotypes if expressed from the *ecmAO* promoter. However, expression of either protein from the *rasD* promoter induces the same developmental phenotype (M. Khosla, unpublished observations). Therefore, the results obtained with the *ecmAO::rasG(G12T)* transformants suggest that the overexpression of activated RasD in the *ecmA*-expressing prestalk cells was not solely responsible for the developmental phenotypes of the *Ddras*-Thr12 transformants.

The normal progression of prestalk differentiation is summarized in Fig. 3-13. Progenitor amoebae differentiate into four distinct prestalk subtypes, PstA, PstB, PstO, and ALC. The majority of PstA cells in the tip become PstAB cells prior

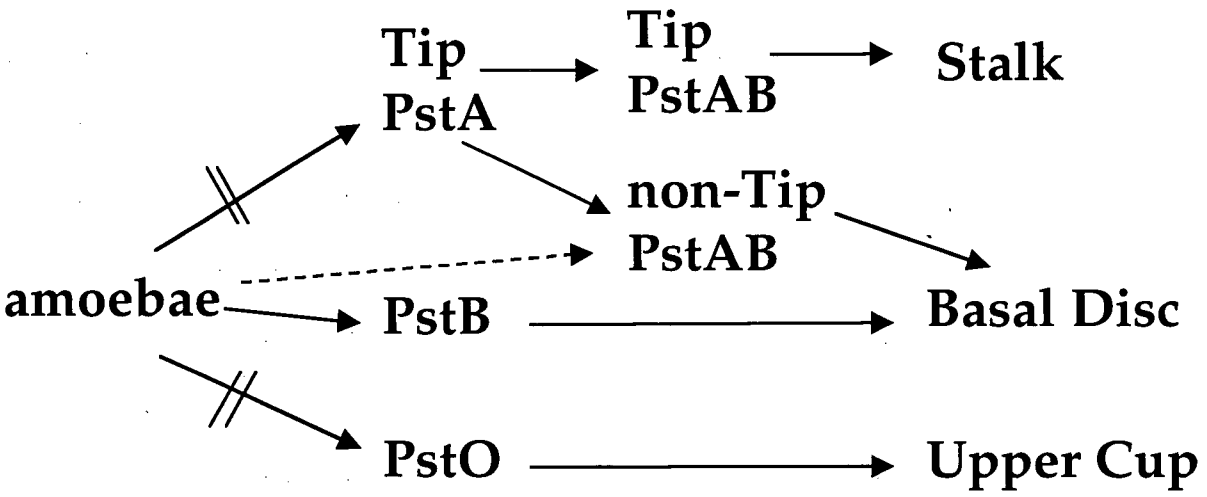


Figure 3-13: A schematic diagram depicting the differentiation of the various prestalk cell types during development and the effects of *ecmAO::rasG(G12T)* expression. Cross bars indicate differentiation steps that are inhibited in the transformant cells. The dotted line indicates a differentiation step that does not normally occur but is favored in the transformant cells. Wild type amoebae that differentiate into PstO cells in the mound form the upper cup of the mature culminant. The differentiation of this cell type is largely inhibited in the *ecmAO::rasG(G12T)* transformants. Wild type PstA cells sort to the tip where they subsequently form PstAB cells and initiate formation of the stalk. This differentiation pathway was also inhibited in the *ecmAO::rasG(G12T)* transformants. Some wild type PstAB cells move to the posterior during slug migration and subsequently participate in basal disc formation during culmination. In the *ecmAO::rasG(G12T)* transformants, posterior PstAB cells were observed and believed to contribute to the formation of an extended basal disc. However, these cells did not arise from anterior cells. Wild type PstB cells at the posterior of the slug contribute to basal disc formation. Although PstB cell differentiation and localization was affected in the transformants, this normal pathway was still observed. The ALC, which contribute to the lower cup formation, have not been depicted for the sake of simplicity.

to terminal differentiation, at which point they form stalk cells and build the stalk tube (Jermyn and Williams, 1991; Sternfeld, 1992). A subset of tip PstA cells become PstAB cells during slug migration. These become localized to the posterior of the slug, and contribute to the formation of the basal disc (Sternfeld, 1992). PstB cells are localized mostly in the posterior of the slug and terminally differentiate as stalk cells and also contribute to the formation of the basal disc, although some are discarded during slug migration (Jermyn and Williams, 1991; Sternfeld, 1992). Cells that differentiate as the PstO subtype are ultimately found in the upper cup at culmination (Early et al., 1993; Abe et al., 1994). Some progenitor cells differentiate into ALC, but this conversion is not shown in Fig. 3-17 since it is not relevant to the following discussion.

Overexpression of activated RasG from the *ecmAO* promoter induced a number of dramatic changes in prestalk differentiation. In developing *ecmAO::rasG(G12T)* transformants, most PstA cells were not positioned in the tip of the slug, but instead were localized in the posterior. PstO cells were reduced in number and of these, most were scattered throughout the slug rather than localized in an anterior collar. The proportion of PstB cells appeared to be greater and these cells were found in the posterior of the slugs and later in development, were often found in the anterior regions of culminants also. Although stalk cells were formed, they were confined to the posterior region of the terminally differentiated organism and no stalk tube was observed.

I propose that the major direct effect of Ras(G12T) in these transformants is the mislocalization of PstA cells and that the remaining developmental aberrations are consequences of this mislocalization. The tip cells have been referred to as the organizers of multicellular development in *Dictyostelium* (Raper, 1940; Rubin and Robertson, 1975; Durston, 1976; Schaap, 1986; Siegert and Weijer, 1995) and the tip is

normally comprised of PstA cells (Jermyn and Williams, 1991; Abe et al., 1994; Early et al., 1995). Since the tip is responsible for directing slug migration (Fisher, 1997), initiating formation of the stalk tube (Jermyn and Williams, 1991; Sternfeld, 1992), and orchestrating the movements necessary for culmination (Dormann et al., 1996; Chen et al., 1998), it is not surprising that the slugs of the *ecmAO::rasG(G12T)* transformant exhibited abnormalities in these processes. My results show that the outwardly normal slugs formed by these transformants were compromised in both phototaxis and migration, could not initiate stalk tube synthesis, and formed a variety of disorganized terminal structures.

During wild type development, the PstA cells arise at the periphery of the mound and then sort to the tip (Early et al., 1995). The mechanism of PstA cell sorting has not been fully elucidated but differences in the chemotactic response of the cells may be involved. The PstA cells chemotax towards cAMP more quickly than the PstO or prespore cells and this difference may facilitate PstA cell migration from the mound periphery to the mound apex (Early et al., 1995). Activated RasD has previously been shown to affect desensitization of guanylate cyclase (Van Haastert et al., 1987) which is involved in chemotaxis. However, cells expressing activated Ras still chemotax towards cAMP. In addition, PstA cells in the *ecmAO::rasG(G12T)* transformants were observed in the mound center from where the tip would rise. Thus, the cells did initially sort in the expected manner. In addition, the localization defect of the *ecmAO::rasG(G12T)* transformants was cell autonomous. When a small number of labeled transformant cells were mixed with wild type cells, the transformants were excluded from the tip. This indicates that the localization defect of the transformants was not a consequence of the lack of a localization signal. Also, the presence of *ecmAO::rasG(G12T)* cells did not prevent the localization of wild type cells to the tip in

a chimaeric slug indicating that the transformant slugs did not produce a signal that interferes with this process.

I propose that RasG(G12T) induces a more rapid conversion of PstA to PstAB cells and as a consequence, the PstA cells do not localize in the tip. In support of this hypothesis is the observation that wild type anterior PstA cells that have become PstAB move to the posterior region of the slug from where they are discarded during slug migration (Sternfeld, 1992). In addition, there are notable similarities between the staining patterns for *ecmA::lacZ* and *ecmB::lacZ* expression in the *ecmAO::rasG(G12T)* transformants at the slug stage and even earlier at the mound stage. All the regions that stain for *ecmA* expression also stain for *ecmB* expression. I suggest that RasG(G12T) induces PstA cells to express *ecmB* and hence become PstAB cells. As a result, these cells migrate to the posterior region of the organism. Further support for this hypothesis comes from the observation that although *ecmAO::rasG(G12T)* slugs do not migrate extensively, they discard large clumps of *ecmA* and *ecmB* expressing cells from the posterior.

Although all the regions of the *ecmAO::rasG(G12T)* slug that stained for *ecmA* expression also stained for *ecmB* expression, there were cells in the tip region of some structures that only stained for *ecmB* expression and were therefore classified as PstB cells. Localization of PstB cells in the anterior is not observed during normal development and the ectopic localization of PstB cells in the anterior of the transformant may also be a consequence of the mislocalization of PstA cells. During normal development, the PstA cells at the tip are believed to be the source for cAMP oscillations. Since *ecmAO::rasG(G12T)* slugs did not have a normally functioning tip, cAMP oscillations from the tip would likely have been significantly altered. cAMP inhibits expression from the *ecmB* promoter (Ceccarelli et al., 1987), so the absence of PstA cells at the tip may result in the expression of *ecmB* in that region.

A role for Ras in modulating cell migration during development has been described in other systems. In *C. elegans*, Ras has been implicated in the migration of sex myoblasts. A pair of sex myoblasts (SMs) migrate anteriorly during the second larval stage to the mid-body to become positioned on either side of the gonad. In loss of function *let-60-ras* mutants and in gonad-ablated organisms, the SMs still migrate anteriorly but their final position is altered such that a broad range of positions were observed (Sundaram, et al., 1996). Constitutively active *let-60-ras* transgenes displayed no phenotype in a wild type background but allowed proper positioning of the SMs in gonad-ablated organisms, suggesting that Ras alters the SM signal response (Sundaram, et al., 1996).

In *Drosophila*, a group of anterior follicle cells, called border cells, migrate from the anterior tip of the egg chamber to the nurse cell - oocyte border during stage 9 of oogenesis. Expression of dominant negative Ras prior to the migration period induces early migration whereas activated Ras expression inhibits migration. When expressed during the migration period, both dominant negative and activated Ras inhibit migration (Lee et a., 1996; Lee and Montell, 1997). Increasing Ras activity during early oogenesis induces expression of posterior markers in the anterior follicle cells indicating an alteration of cell fate that may consequently affect the migration of these cells (Lee and Montell, 1997). This situation is similar to that observed in *Dictyostelium* where activated Ras changes PstA cells to PstAB cells and consequently alters their localization.

The PstB cells observed in the tips of some structures were clearly not able to rescue normal tip functions. Notably, they were unable to initiate synthesis of a stalk tube. Hence, staining for neither the *ST::lacZ* reporter nor calcofluor was observed in the anterior region of the *ecmAO::rasG(G12T)* organisms. These results also indicated that the anterior prestalk cells did not create the upper cup, which normally appears

after stalk formation. Thus, it appears that the anterior PstB cells did not terminally differentiate.

Cells expressing *ecmB* at the posterior of the organisms were able to terminally differentiate. Based on the intense expression of both *ecmA* and *ecmB* reporter constructs in this region, these cells were most likely PstAB cells. Normally, this rearguard region participates in formation of the basal disc (Jermyn and Williams, 1991; Sternfeld, 1992). Cells in this region were the only ones to express *lacZ* from the *ST* promoter and to stain with calcofluor, suggesting that the stalk cells formed during *ecmAO::rasG(G12T)* development were extended basal disc structures.

Spore formation was also inhibited during *ecmAO::rasG(G12T)* development. Since prespore cell gene expression and prespore cell localization was not drastically altered in these transformants, prespore differentiation was probably normal. I conclude that the lack of spore formation is a result of a block in the conversion of prespore to spore cells at culmination. Prestalk cells have been shown to release factors that signal prespore cells to induce sporulation (Anjard et al., 1998). It is believed that the signal is generated from the tip since sporulation occurs progressively from the anterior to the posterior of the sorogen (Richardson et al., 1994). The absence of spore formation in *ecmAO::rasG(G12T)* transformants was therefore likely to be another consequence of the defective tip.

Additional evidence supporting the idea that mislocalization of tip cells was the primary defect in the *ecmAO::rasG(G12T)* transformants comes from the results of chimaeric development. When mixed with a majority of transformant cells, Ax2 cells preferentially sorted to the tip region. The presence of wild type cells in the tip enabled chimaeric organisms to sense and respond to a light gradient, construct a stalk tube, and form spores.

Since activated RasG would be expected to be simultaneously expressed in PstA cells and PstO cells, I cannot rule out the possibility that Ras(G12T) had a primary effect in PstO cell differentiation which in turn lead to the later developmental defects. Since very few PstO cells were formed during *ecmAO::rasG(G12T)* development it is unlikely that activated RasG stimulated the production of an inhibitor in PstO cells. However, lack of PstO cells could have contributed to the abnormal phenotypes.

It is more likely that activated RasG simply shut off expression from the *ecmO* specific regions of the *ecmAO* promoter and cell autonomously inhibited PstO differentiation. Alternatively, it is also possible that PstA cells expressed activated RasG and laterally inhibited the differentiation of PstO cells. Lateral regulation of PstO specification by PstA cells has been previously observed for the *warai* null cells (Han and Firtel, 1998). The *Dictyostelium* HOX (*hox*) gene, *warai*, is expressed in PstA cells. *warai* null cells exhibit an enlarged PstO compartment, indicating that PstA cells regulate the proportion of PstO cells. In Chapter One, I presented a few examples of Ras-mediated HOX gene regulation. It is possible that Ras(G12T) could be a positive inducer of Warai or of another homeotic gene specifying PstAB or PstB cell fate.

The work presented in this chapter was intended to determine if expression of activated RasG in the *ecmA*-expressing prespore cells could recapitulate the *Ddras*-Thr12 phenotype. The results illustrate that RasG(G12T) expression from the *ecmAO* promoter resulted in the formation of single-tipped aggregates and only slightly altered cell type specific gene expression patterns. The *ecmAO::rasG(G12T)* transformants did exhibit alterations in prestalk cell localization and consequently the tips formed lacked normal organizing capacity. Thus, it is unlikely that activated RasD expression in the prestalk population induces the multi-tipped aggregates and

the drastically deregulated cell type specific gene expression patterns characteristic of the *Ddras*-Thr12 transformant.

CHAPTER FOUR

OVEREXPRESSION OF RASG(G12T) IN PRESPORE CELLS

4.1 Background

I expected that overexpression of activated RasG in the *ecmA*-expressing prestalk cells would recapitulate the phenotype of the *Ddras*-Thr12 transformant. However, as described in Chapter Three, this was not the case. The *ecmAO::rasG(G12T)* transformants did not form multi-tipped mounds, were not blocked from slug formation, and did not exhibit drastic alterations in the expression of cell type specific genes. Given these results, it was important to determine if the overexpression of activated RasG in prespore cells would produce a phenotype similar to that of the *Ddras*-Thr12 transformant.

Although *rasD* was originally identified as a prestalk specific gene (Reymond et al., 1984), its expression is not limited to the prestalk population. Whereas *ecmA* expression is ten–fold enriched in prestalk cells over prespore cells, *rasD* expression is only three–fold enriched (Jermyn et al., 1991). Using a labile β-galactosidase expressed from the *rasD* promoter, Jermyn and Williams (1995) demonstrated that an appreciable level of *rasD* expression occurs in the prespore cells of the aggregate. Although prespore specific expression decreases as development progresses beyond the tipped mound stage, it is conceivable that the overexpression of activated RasD in the prespore population causes the developmental abnormalities observed by Reymond et al. (1986). To test this possibility, I used a prespore specific promoter to overexpress RasG(G12T) specifically in prespore cells and characterized the resulting transformants.

4.2 Developmental Morphology of *psA::rasG(G12T)* Transformants

PsA is a cell surface glycoprotein expressed only in prespore cells (Early et al., 1988). The promoter for the *psA* gene was used to construct a vector to drive overexpression of *rasG(G12T)* exclusively in prespore cells. The *psA::rasG(G12T)* construct was transformed into *Dictyostelium* Ax2 cells and stable transformants were selected. Overexpression of activated RasG during development was demonstrated in several independently isolated transformants by western blot analysis and data for two of the transformants is shown in Fig. 4-1. Both *psA::rasG(G12T)* transformants clearly overexpressed RasG protein compared to the vector control transformant at 16 hours of development. The level of overexpressed protein was higher in clone A than in clone B. The overexpressed protein in the transformant was slightly larger than the endogenous RasG because the transgene encodes additional amino acids derived from the 5′ end of the *psA* gene.

Upon starvation, wild type cells formed mounds with single tips such as the one shown in Fig. 3-2A, whereas all of the *psA::rasG(G12T)* transformants formed mounds with multiple tips (Fig. 4-2A). The tips extended to form finger-like projections that then fell to the substratum and appeared to attempt migration, as would be typical of wild type slugs. However, the slug-like structures remained anchored to the basal mound. Many of the mounds formed by the developing *psA::rasG(G12T)* transformant had two or three emerging protrusions (Fig. 4-2B) while some produced more protrusions (Fig. 4-2C). When the further differentiation of these transformants was monitored by time-lapse video microscopy, it was observed that additional tips continued to emerge from the mounds (data not shown). These structures were formed at the same time that wild type cells had formed slugs (Fig. 3-2C).

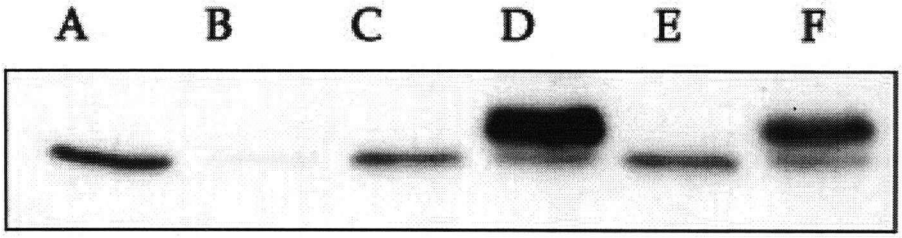


Figure 4-1: Western blots of lysates from cells harvested at 0 hours (lanes A, C, E) and 16 hours (lanes B, D, F) of development and probed with an antibody specific for RasG. Lanes A and B contain lysates from a control strain transformed with *psA::lacZ*. Lysates from two independent *psA::rasG(G12T)* clones were separated in lanes C, D (clone A) and E, F (clone B).

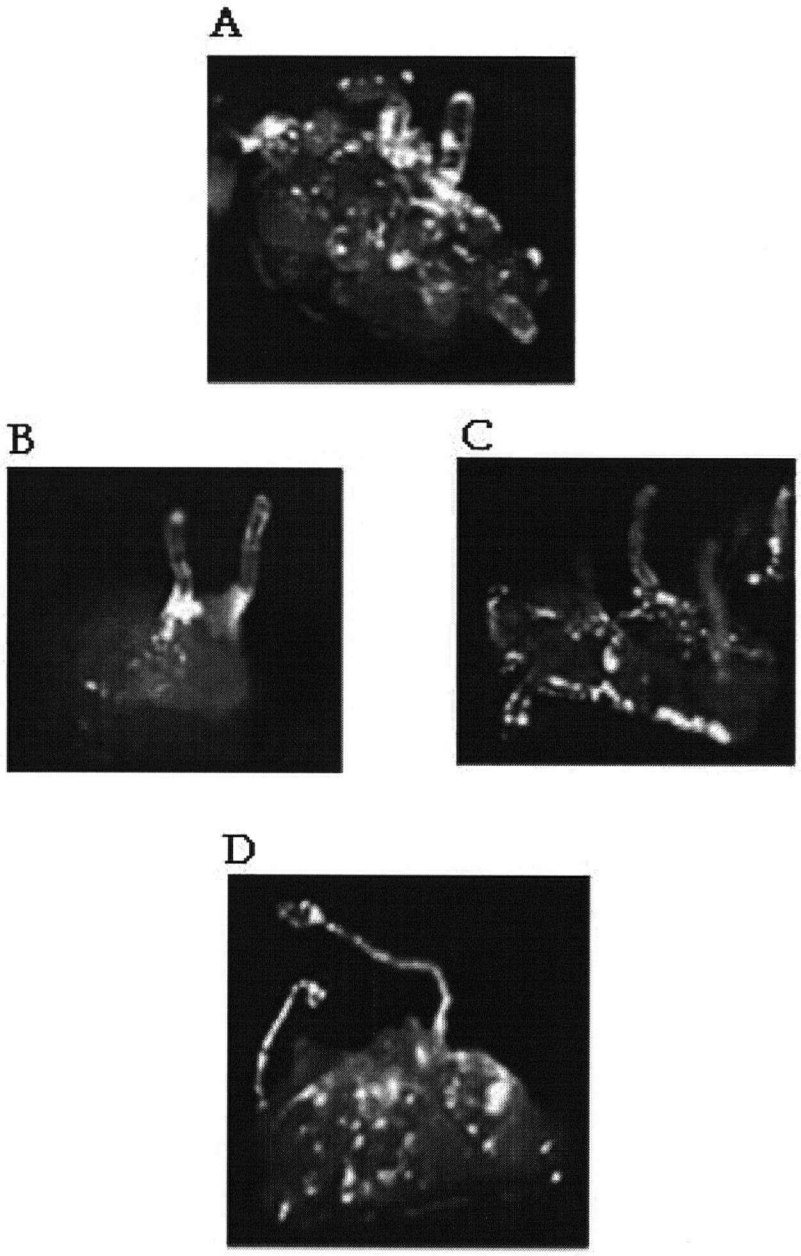


Figure 4-2: Developmental structures formed by *psA::rasG(G12T)* cells. Cells formed mounds with multiple tips (A) which elongated to form finger-like structures (B, C). Transformant culminants contained a basal mound with emerging stalk-like protrusions and multiple small tips (D).

The *psA::rasG(G12T)* transformants formed aberrant culminants at about the same time that wild type cells formed normal fruiting bodies. The aberrant culminants consisted of a basal mound to which stalk-like structures were attached (Fig. 4-2D). Time lapse photography indicated that the stalk-like structures were formed from the protrusions that were produced initially at the mound stage. Multiple small tips were also visible on basal mound (Fig. 4-2D). Time lapse photography indicated that these tips emerged from the mound late in development and did not extend further. Although most of the stalk-like structures lacked a sorus, a few did produce an apical pseudo-sorus, which was very small and translucent compared to the wild type sorus (Fig. 3-2C). Thus, the prespore specific expression of activated RasG did result in multi-tip formation, similar to that observed for the *Ddras*-Thr12 transformant. However, the multiple tips of the *psA::rasG(G12T)* mounds were not blocked from further morphogenesis. These results suggest that in the *Ddras*-Thr12 transformant, expression of activated RasD in the prespore cells causes multiple tips to form but is not sufficient to inhibit elongation of the tips.

Transformants overexpressing wild type RasG in prespore cells were also generated and examined for their developmental morphology. Fig. 4-3 shows culminant morphologies for wild type cells, *psA::rasG(G12T)* cells, and *psA::rasG* cells. Wild type fruiting bodies consisted of a cloudy sorus raised on a stalk (Fig. 4-3A). The *psA::rasG(G12T)* culminants contained mounds with multiple emerging stalk-like structures (many of which had fallen to the substratum) and no sori (Fig. 4-3B). The *psA::rasG* culminants contained some basal cell masses and translucent sori raised on stalks (Fig. 4-3B).

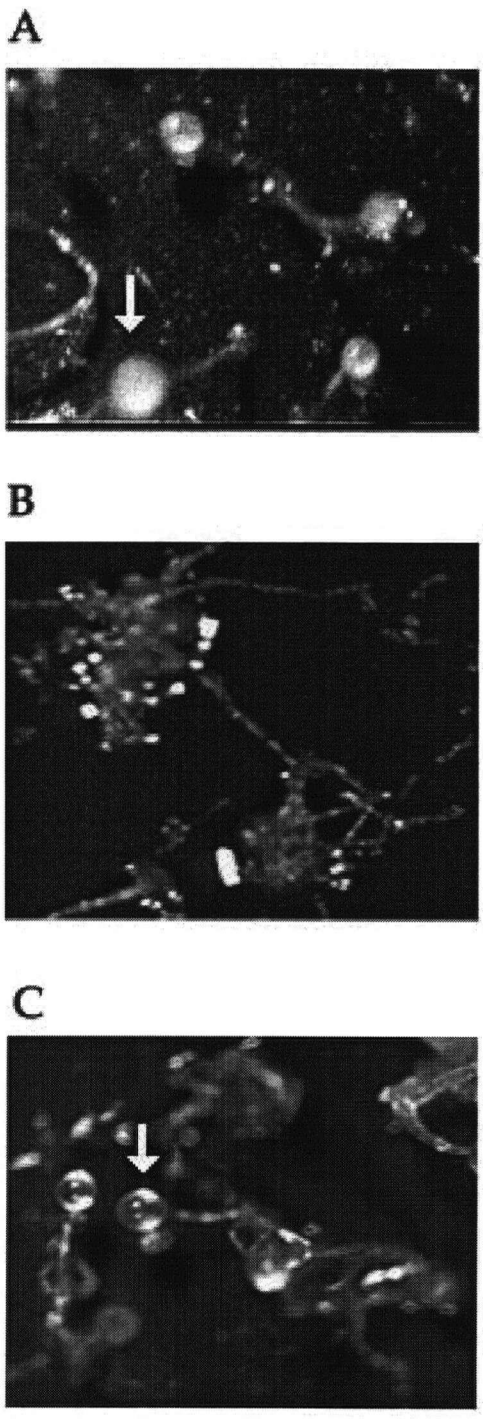


Figure 4-3: Culminants of wild type cells (A), *psA::rasG(G12T)* cells (B), and *psA::rasG* cells (C). The arrow in (A) points to a cloudy sorus whereas the arrow in (C) points to a translucent sorus. Photographs were taken with a Nikon 35 mm camera and Kodak TMAX-400 black and white film.

4.3 Terminal Differentiation of *psA::rasG(G12T)* Transformants

Given that the *psA::rasG(G12T)* transformants produced infrequent and small sori, it seemed unlikely that they were capable of producing mature spore cells. Several of the *psA::rasG(G12T)* transformants were tested and although they varied slightly in their ability to sporulate, spore formation was always much less than for the controls. Values for spore formation for wild type cells and two *psA::rasG(G12T)* transformants are given in Table VI. The two *psA::rasG(G12T)* transformants assayed for spore formation were the same for which western blot results are given in Fig. 4-1. I observed that the transformant that expressed a higher level of RasG protein produced fewer spore cells. In addition, as indicated in Table VI, cells expressing *psA::rasG* (wild type RasG) were also defective in spore formation.

Since the culminants formed by the *psA::rasG(G12T)* transformants contained stalk-like structures, I examined them for the presence of mature stalk cells by staining with calcofluor. Wild type culminants contained calcofluor stained stalk cells in the basal disc and the emerging stalk tube (Fig. 4-4A). When viewed by phase contrast microscopy, these cells were observed to be highly vacuolated (Fig. 4-4B) as is characteristic of stalk cells. When culminants of *psA::rasG(G12T)* cells were stained with calcofluor, the entire basal mounds and the emerging stalk-like structures were composed of fluorescing cells (Fig. 4-4C). When these structures were viewed by fluorescence microscopy, the stalk-like structures were observed to contain vacuolated cells (Fig. 4-4D), similar to the wild type stalk tube (Fig. 4-4B). However, since the mound cell mass was densely packed, it was difficult to determine if individual cells within the mass stained with calcoflour and were vacuolated. I therefore tapped the coverslip on the microscope slide in order to break apart the structures. Once *psA::rasG(G12T)* culminant mounds were disrupted, I observed that cells within the mounds were labeled with calcofluor (Fig. 4-4E) and were highly vacuolated in

Table VI. Spore formation by wild type cells and *psA::rasG(G12T)* and *psA::rasG* transformant cells.

Strain	Spore Formation* (as % of cells induced to develop)
Ax2	128 ± 39
psA::rasG(G12T), clone A	0
psA::rasG(G12T), clone B	0.8 ± 0.2
psA::rasG	3.8 ± 3.2

*Spore formation values are the average (and range) of three separate samples from one experiment.

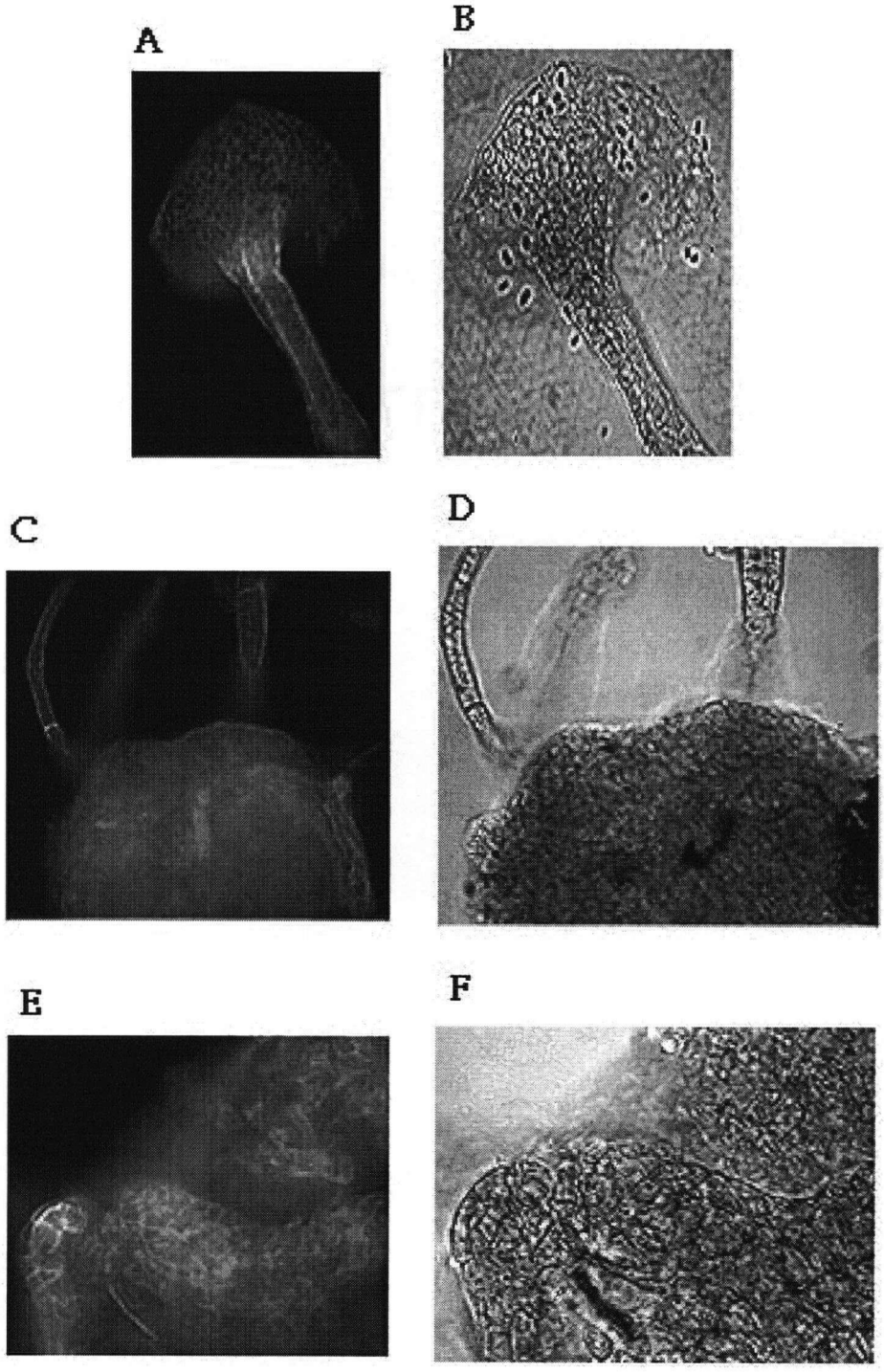


Figure 4-4: Culminants of Ax2 cells (A, B), and *psA::rasG(G12T)* cells (C, D, E, F) were stained with calcofluor and viewed by fluorescent microscopy (A, C, E) or by phase contrast microscopy (B, D, F). Images in (E, F) are from cells released after a transformant culminant was smashed apart.

appearance (Fig. 4-4F). Thus, the majority of cells in the *psA::rasG(G12T)* culminant terminally differentiated into stalk cells.

In Chapter Three, I presented results demonstrating that stalk cell formation was not inhibited when activated RasG was overexpressed in prestalk cells. Together with the data presented here, these results contradicted expectations based on an earlier report that stalk cell formation did not occur in the *Ddras*-Thr12 transformants (Louis et al., 1997a). I therefore examined cells that express RasG(G12T) from the *rasD* promoter (M. Khosla, unpublished results) by staining culminants with calcofluor. Fig. 4-5 shows data comparing a wild type culminant (Fig 4-5A, B) with a *rasD::rasG(G12T)* culminant (Fig. 4-5 C, D, E, F). The *rasD::rasG(G12T)* culminant emitted fluorescence throughout the mound and the multiple protruding tips (Fig. 4-5C). Although this indicates the presence of stalk cells, the density of the structure prevented the observation of cells within the mound by either fluorescent microscopy (Fig. 4-5C) or phase contrast microscopy (Fig. 4-5D). Therefore, the structures were broken apart as described above. Cells released from the *rasD::rasG(G12T)* culminant mounds were stained with calcofluor (Fig. 4-5E) and were vacuolated (Fig. 4-5F). Thus, overexpression of activated RasG from either the *psA* or *rasD* promoters resulted in stalk cell differentiation in the majority of cells in the population.

4.4 Cell Type Specific Gene Expression in *psA::rasG(G12T)* Transformants

To further investigate the aberrant development of *psA::rasG(G12T)* cells, northern blots of mRNA isolated from one of the transformants and from control cells (transformed with *psA::lacZ*) at various times of development were probed with cell type specific cDNA's. As seen in Fig. 4-6A, expression of the prestalk specific gene, *ecmA* (Williams et al., 1987), was markedly elevated during the development of the

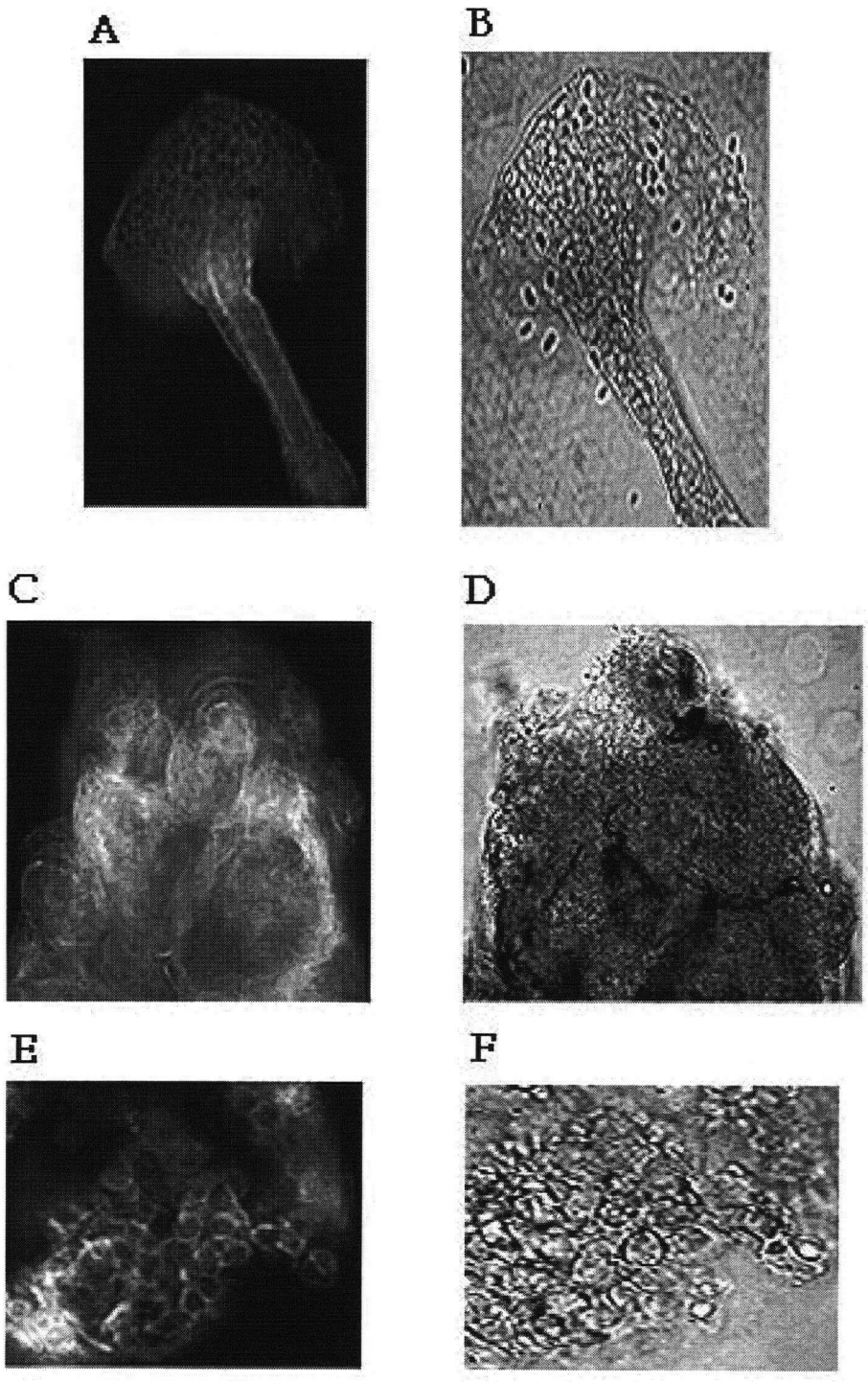


Figure 4-5: Culminants of Ax2 cells (A, B), and *rasD::rasG(G12T)* cells (C, D, E, F) were stained with calcofluor and viewed by fluorescence microscopy (A, C, E) or by phase contrast microscopy (B, D, F). Images in (E, F) are from cells released after a transformant culminant was smashed apart.

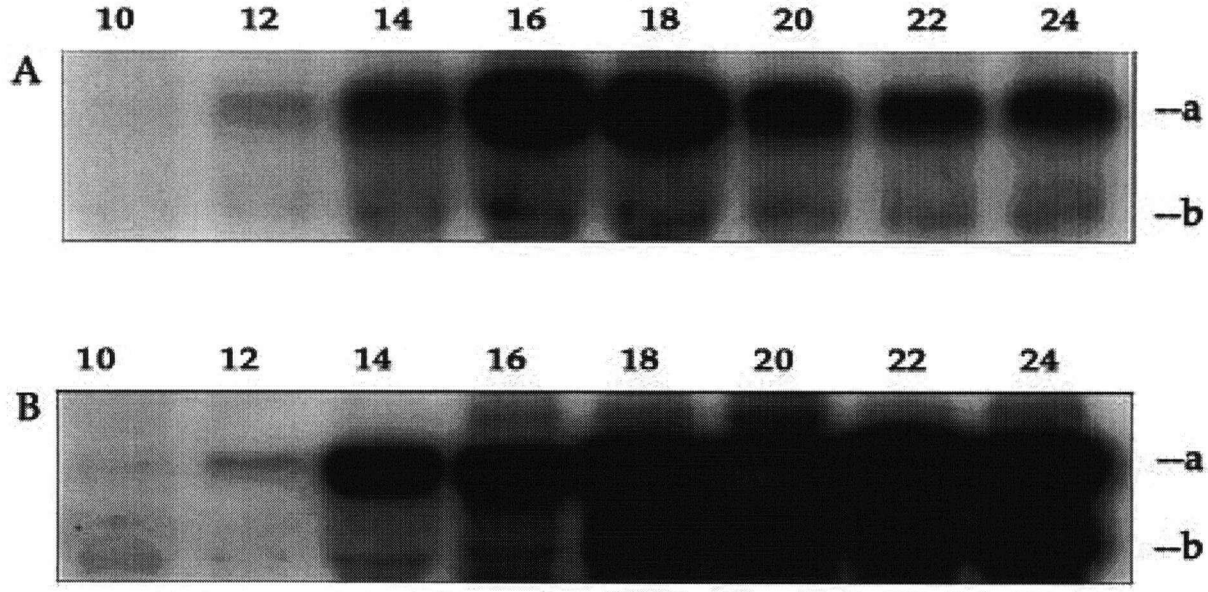


Figure 4-6: Northern blots of mRNA isolated from *psA::lacZ* transformed control cells (A) and *psA::rasG(G12T)* cells (B) at the times (in hours) during development indicated above the lanes. Blots were probed with a labeled cDNA fragment of the *ecmA* gene. The message indicated 'a' corresponds to *ecmA* mRNA and the message indicated 'b' corresponds to the cross-hybridized *ecmB* mRNA.

psA::rasG(G12T) cells. In control cells, *ecmA* mRNA was first observed at 12 hours of development, increased to a maximal level at 16-18 hours and declined thereafter (Fig. 4-6A). In the *psA::rasG(G12T)* cells, the *ecmA* mRNA expression pattern was at first similar to the wild type pattern but there was no decrease in *ecmA* mRNA late in development (Fig. 4-6B). The *ecmA* probe cross–hybridized with a similar prestalk specific mRNA, *ecmB* (Ceccarelli al., 1987), and this allowed for the coincident determination of *ecmB* expression patterns. In both control cells and *psA::rasG(G12T)* cells, *ecmB* expression was detected by 14 hours of development (Fig. 4-6A, B). However, the *psA::rasG(G12T)* cells expressed a considerably higher level of *ecmB* mRNA by 18 hours than the control cells and this elevated level persisted throughout development (Fig. 4-6A, B).

Northern blots were also probed for prespore specific mRNA's. As illustrated in Fig. 4-7B, expression of *cotC* mRNA (Fosnaugh and Loomis, 1989) was delayed by 2 hours during the development of *psA::rasG(G12T)* cells, and the level of expression was dramatically reduced relative to wild type cells (Fig. 4-7A). Additionally, *cotC* mRNA levels in the transformant rapidly decreased as development progressed and transcripts were barely detectable by 20 hours post-starvation (Fig. 4-7B). Panels C and D of Fig. 4-7 compare the expression of another prespore specific gene, *psA*. The level of *psA* expression was also lower in the transformant cells relative to the control cells. In addition, *psA* expression in the transformants rapidly declined to undetectable levels. The northern blot analysis clearly demonstrated that in a population of developing *psA::rasG(G12T)* cells, prestalk gene expression was greatly enhanced while prespore gene expression was significantly reduced. This is similar to the phenotype of the *Ddras-Thr12* transformant which also overexpresses prestalk genes and underexpresses prespore genes (Louis et al., 1997a).

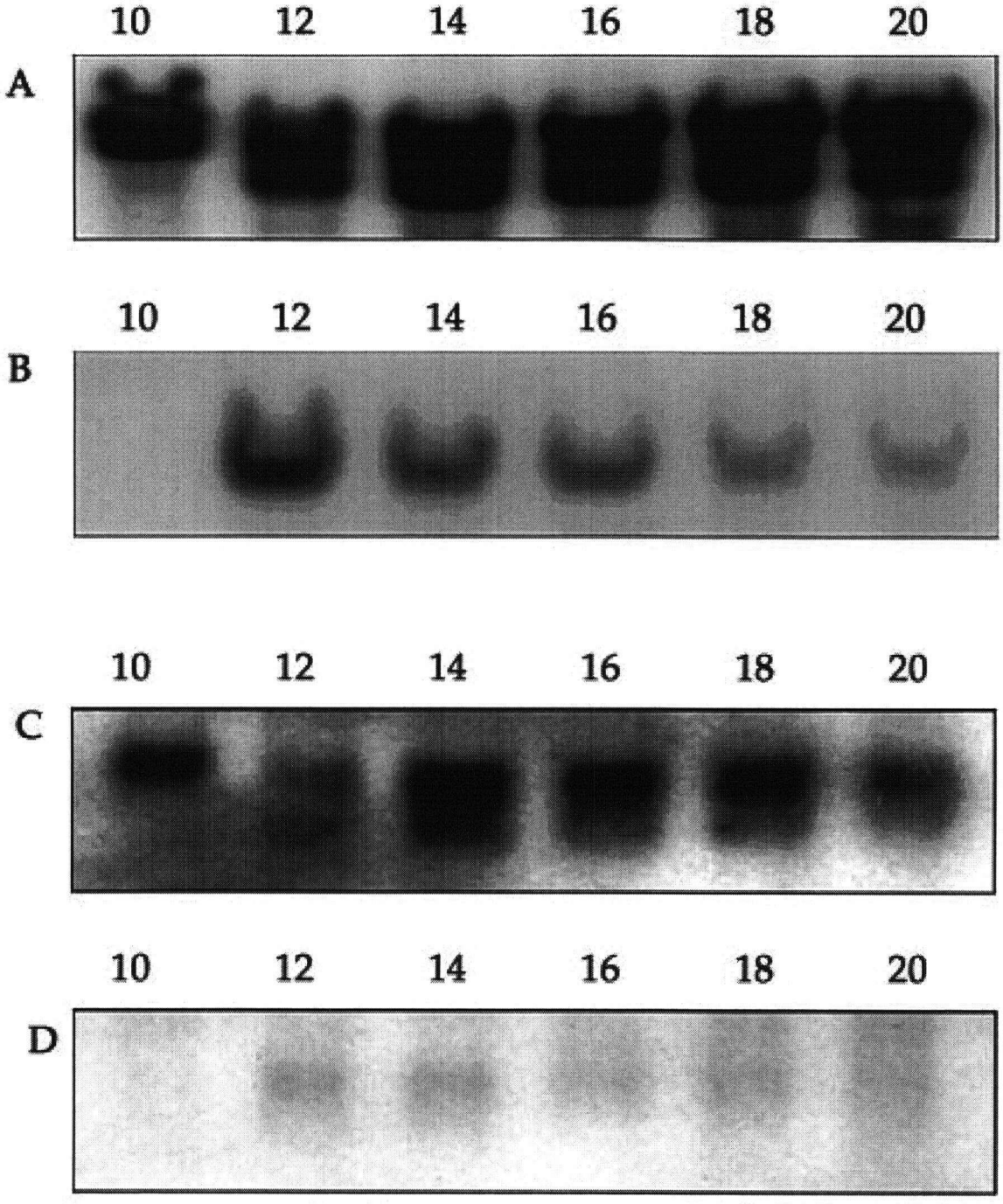


Figure 4-7: Northern blots of mRNA isolated from *psA::lacZ* transformed control cells (A, C) and *psA::rasG(G12T)* cells (B, D) at the times (in hours) during development indicated above the lanes. Blots were probed with a labeled cDNA fragments for the *cotC* gene (A, B) and the *psA* gene (C, D).

4.5 Prestalk Cell Localization in Developing *psA::rasG(G12T)* Organisms

The increase in prestalk gene expression and the decrease in prespore gene expression during development could be due to an increase in the number of prestalk cells and a concomitant decrease in the number of prespore cells within developing *psA::rasG(G12T)* structures. To test this possibility, Ax2 cells were cotransformed with the *psA::rasG(G12T)* construct and with one of several constructs containing *lacZ* expressed under the control of cell type specific promoters.

When wild type cells were transformed with the *ecmAO::lacZ* construct and induced to develop, staining was observed in the anterior region of the slugs that were formed (Fig. 4-8A). In culminants, stained cells were observed in the stalk tube, basal disc, and upper and lower cups (Fig. 4-8B). When *psA::rasG(G12T)* cells expressing *ecmAO-lacZ* were induced to develop and stained with x-gal, the tips of the multiple finger-like projections and a few scattered cells within the mound stained blue (Fig. 4-8C). As the multiple tips extended, stained cells were observed throughout the lengths of the finger like structures (Fig. 4-8D) and as development progressed, an increasing proportion of *psA::rasG(G12T)* cells expressed β-galactosidase and entire culminant structures stained blue (Fig. 4-8E).

The *ST* region of the *ecmB* promoter drives expression in cells that either enter the stalk tube or those that form the basal disc. Wild type slugs stained for *ST::lacZ* expression in few scattered cells within the anterior of the slug (Fig. 4-9A). The level of anterior staining had increased in structures initiating culmination (Fig. 4-9B) and in culminants, staining was evident in the stalk tube and basal disc (Fig. 4-9C). In developing *psA::rasG(G12T)* structures containing the *ST-lacZ* construct, *lacZ* expression was first detected in scattered cells within the basal mound (Fig. 4-9D). As development progressed, more of the mound cells and some cells in the finger-like projections expressed the *ST-lacZ* marker (Fig. 4-9E). The pattern of the stained cells

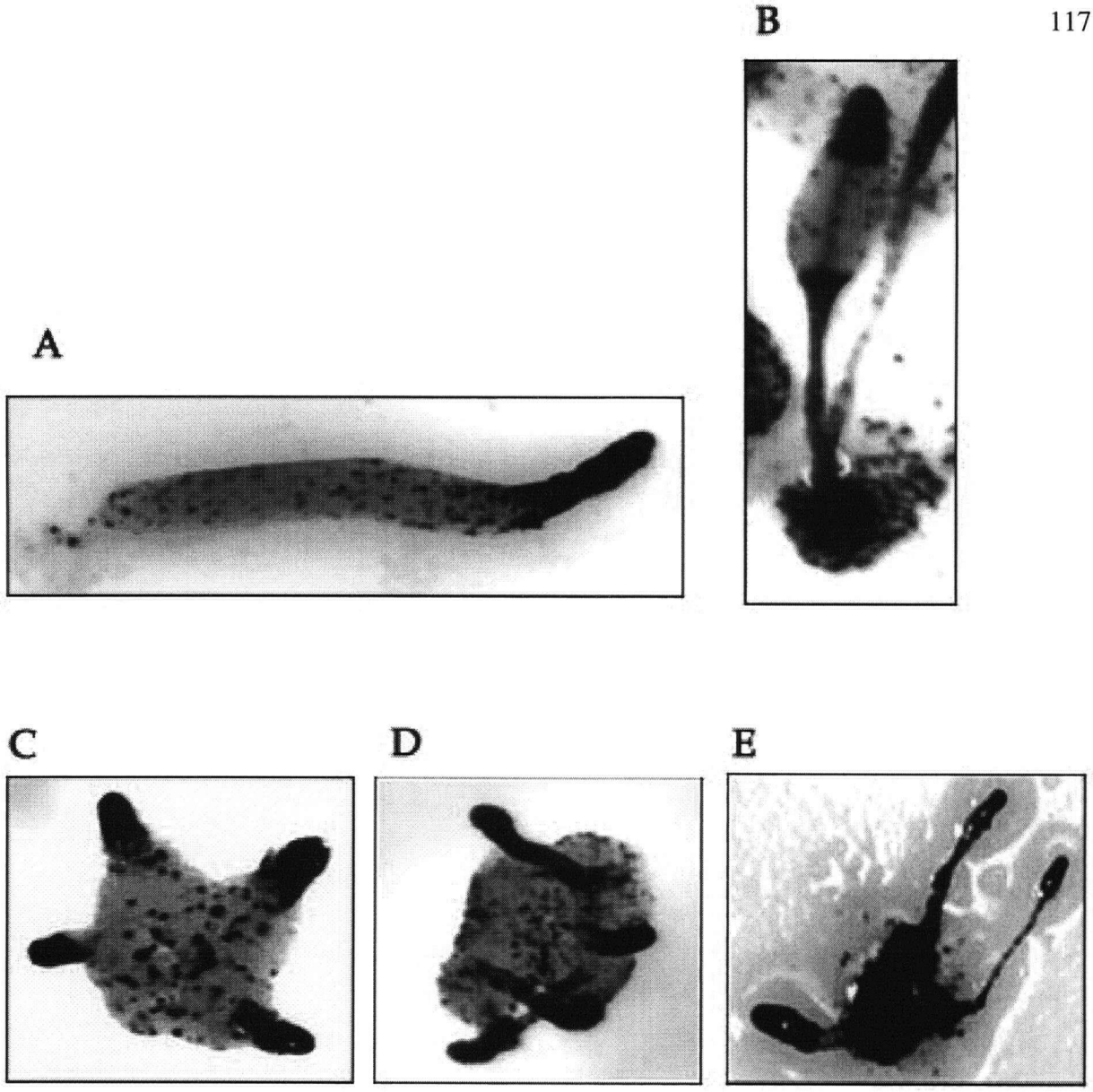


Figure 4-8: Developmental structures of wild type and *psA::rasG(G12T)* cells carrying the *ecmAO-lacZ* reporter. A wild type slug (A) and culminant (B) and a *psA::rasG(G12T)* multi-tipped mound (C) intermediate stage mound (D) and culminant (E) stained with x-gal.

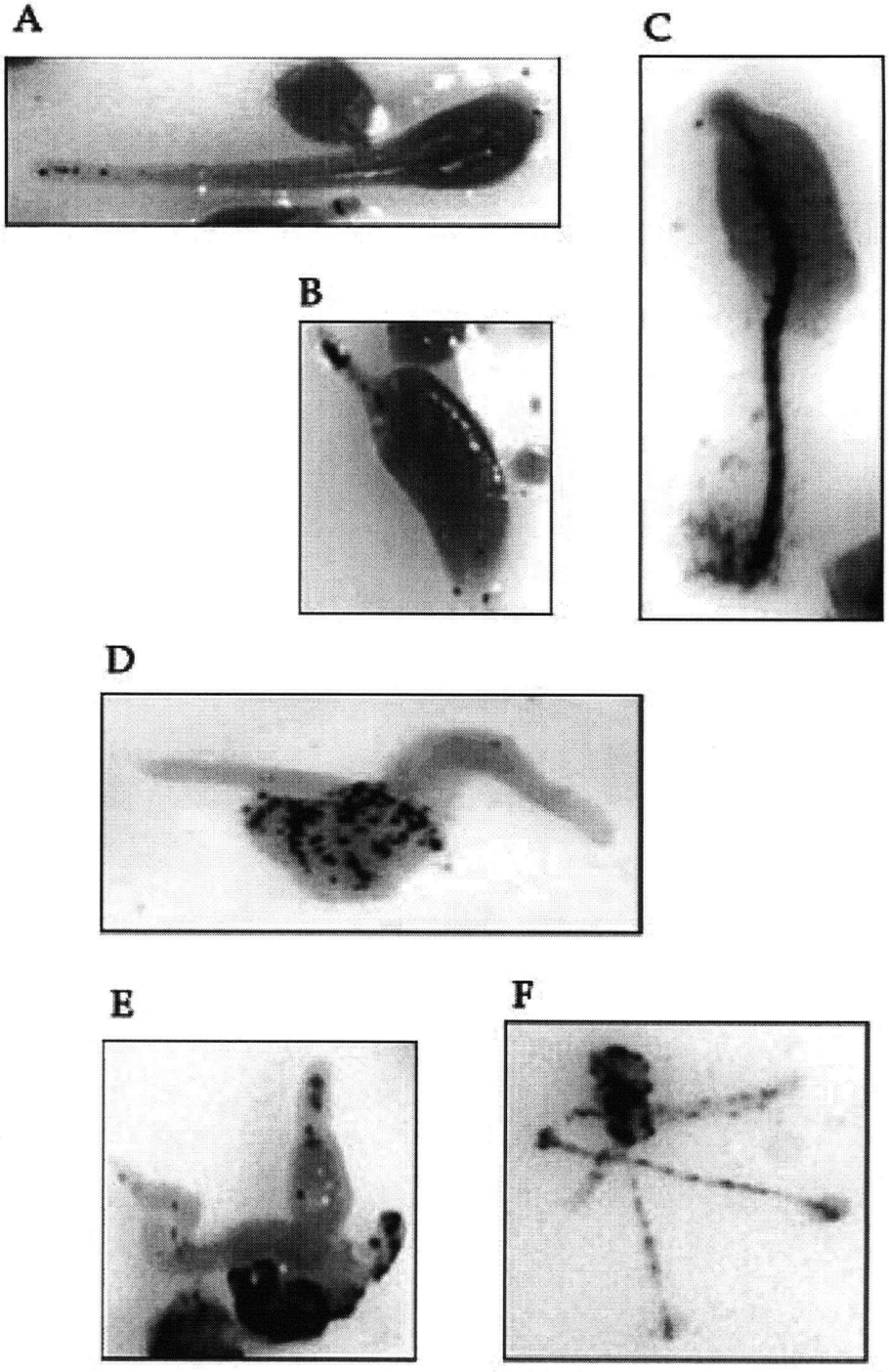


Figure 4-9: Developmental structures of wild type and *psA::rasG(G12T)* cells carrying the *ST-lacZ* reporter. A wild type slug (A), a structure initiating culmination (B) and a culminant (C) and a *psA::rasG(G12T)* mound with elongated fingers (D), an intermediate stage (E) and a culminant (F) stained to detect β-galactosidase expression.

within the finger-like projections resembled that of a wild type slug (Fig. 4-9A). In *psA::rasG(G12T)* culminants, the mounds were almost completely blue (Fig. 4-9F). The protruding stalk-like structures also expressed the *ST-lacZ* reporter. This staining pattern is consistent with the observation that in terminally developed *psA::rasG(G12T)* organisms, the mound mass, as well as the protruding stalk-like structures, are composed mainly of terminally differentiated stalk cells.

4.6 Prespore Cell Localization in Developing *psA::rasG(G12T)* Organisms

In view of the fact that *psA* mRNA levels rapidly declined during the development of *psA::rasG(G12T)* transformant cells (Fig. 4-6B), a labile *lacZ* reporter construct was used to determine the spatial expression from the *psA* promoter. Wild type slugs stained for expression of *psA:(his)lacZ* in the prespore region while the anterior and posterior regions remained unstained (Fig. 4-10A). In wild type culminants, β-galactosidase expression was observed in the sorus region (Fig. 4-10B). *psA::rasG(G12T)* cells containing the *psA::(his)lacZ* construct expressed β-galactosidase in the basal mounds but not in the finger-like projections (Fig. 4-10C). As the development of these transformants continued, there was a decrease in the proportion of *psA*-positive cells so that by the time culminants were formed, the level of *psA::(his)lacZ* expression had declined dramatically and only a few scattered cells stained positive for β-galactosidase (Fig. 4-10D). These results are consistent with the idea that in developing *psA::rasG(G12T)* structures, most cells in the mound initially assumed a prespore fate but subsequently lost the prespore specification and transdifferentiated to the prestalk lineage.

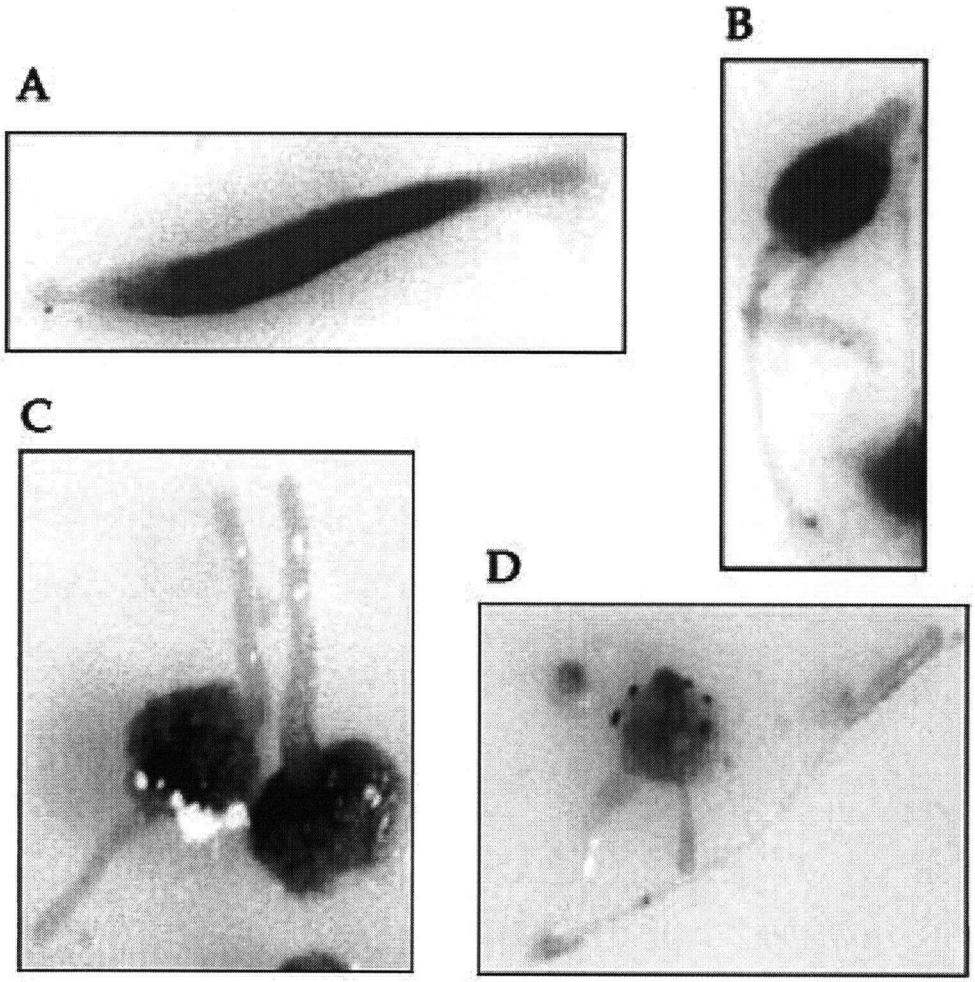


Figure 4-10: Developmental structures of wild type and *psA::rasG(G12T)* cells carrying the labile *psA::(his)lacZ* reporter. A wild type slug (A) and culminant (B) and a *psA::rasG(G12T)* mound with elongated fingers (C) and culminant (D) stained with x-gal.

4.7 Chimaeric Development with Wild Type Cells

I wished to test whether the presence of wild type cells would enable the *psA::rasG(G12T)* transformants to complete the spore development and produce viable spore cells. *psA::rasG(G12T)* cells were mixed in various proportions with Ax2 cells and starved to induce development. Development was allowed to progress for 48 hours and spore formation was then assessed. The number of spores produced by the chimaeric culminants reflected the number of Ax2 cells in the mix (Table VII). Furthermore, all of the germinated spores exhibited a wild type developmental phenotype. From these results, I concluded that the presence of wild type cells during development was not sufficient to enable spore formation. These results also indicated that the *psA::rasG(G12T)* induced sporulation defect was cell autonomous since the presence of the transformants did not hinder the ability Ax2 cells to make spores. This suggests that the *psA::rasG(G12T)* transformants did not produce a negative signal that inhibited sporulation.

4.8 Cell Sorting in Chimaeras

The results presented in Table VII indicated that *psA::rasG(G12T)* cells could not contribute to the spore population during chimaeric development. To test if they were capable of contributing prestalk cells to chimaeric organisms, chimaeras of Ax2 cells and *psA::rasG(G12T)* cells, in which one of the strains was carrying a *lacZ* reporter construct, were created.

Ax2 cells were transformed with a construct in which the *act15* promoter constitutively drives *lacZ* expression in all cells and these cells were mixed with *psA::rasG(G12T)* cells in a 1:9 ratio. When the mixture was induced to develop, the wild type cells sorted away from the transformant cells (Fig. 4-11A) and progressed through development to form a culminant, leaving behind a mound of

Table VII. Spore formation by chimaeras of wild type and *psA::rasG(G12T)* transformant cells.. Columns 1 and 2 indicate the proportion of Ax2 and *psA::rasG(G12T)* cells in the chimaera. Column 3 indicates the spore formation of the cell mixture as a percentage of total cells. Column 4 presents the spore formation as a percentage of Ax2 control cells in the chimaera.

% Ax2 Cells	% Ras Cells	Spore Formation* (as % of cells induced to develop)	Ax2 Spore Formation (as % of Ax2 cells induced to develop)
100	0	154	154
75	25	118	157
50	50	74	148
25	75	34	137
0	100	2	---

*Spore formation values are the average of three separate samples from one experiment.

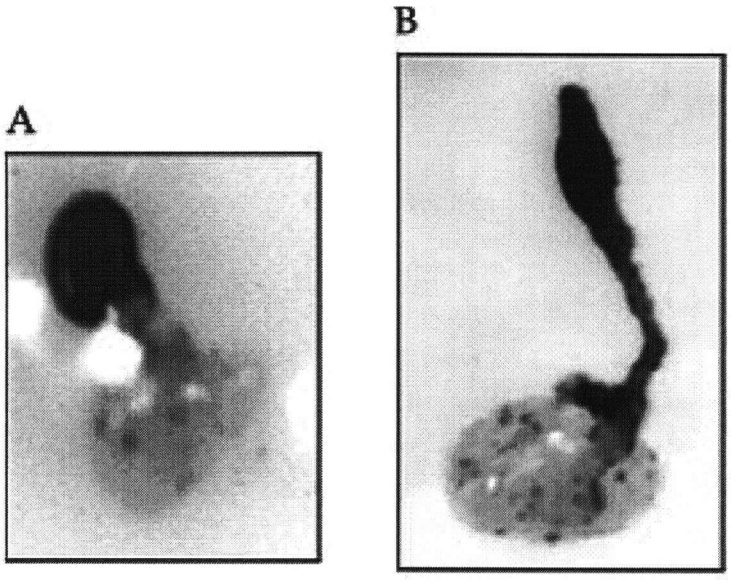


Figure 4-11: Cell sorting during chimaeric development. Mixtures of 10% *act15::lacZ* labeled wild type cells and 90% unlabeled *psA::rasG(G12T)* cells were induced to develop and stained with x-gal at intermediate (A) and culminant (B) stages.

psA::rasG(G12T) cells (Fig. 4-11B). The fact that the mound was for the most part devoid of stained cells indicated that very few wild type cells had been trapped. Although I cannot rule out the possibility that a few *psA::rasG(G12T)* cells were carried along with the developing wild type cells (since unstained cells would not be readily visible within a majority of stained cells), it was clear that the majority of wild type cells had segregated away from the majority of *psA::rasG(G12T)* cells.

psA::rasG(G12T) cells containing the *act15-lacZ* construct were mixed with unlabelled Ax2 cells in a 1:9 ratio. In this case, the *psA::rasG(G12T)* cells were able to participate in development and sorted to the prestalk regions of the chimaeric organisms. By the slug stage, the majority of the *psA::rasG(G12T)* cells were found in the anterior prestalk zones although some remained at the posterior and as the slug migrated, some *psA::rasG(G12T)* cells were shed onto the substratum (Fig. 4-12A). At culmination, the *psA::rasG(G12T)* cells were present in the basal disc, the stalk, and the upper and lower cups (Fig. 4-12B). I also created similar chimaeras in which the *psA::rasG(G12T)* cells contained the prestalk specific *lacZ* reporter constructs, *ecmAO::lacZ* (Fig. 4-12C) or *ecmB::lacZ* (Fig. 4-12D). In these cases, I found similar staining patterns to that just described which verifies that the *psA::rasG(G12T)* cells that sort to the prestalk regions in chimaeras do in fact express prestalk markers.

4.9 Overexpression of RasG(G12T) in Prespore and Prestalk Cells

It had been hypothesized that the defects in the *Ddras*-Thr12 transformants could be accounted for by the fact that the majority of *rasD*-expressing cells are prestalk (Esch and Firtel, 1991). However, as I have shown, expression of activated RasG in the prestalk cell population alone was not sufficient to induce the defects observed in *the Ddras*-Thr12 transformants. Although the *ecmAO::rasG(G12T)* transformants exhibited defects in PstA cell localization, slug behavior, culmination,

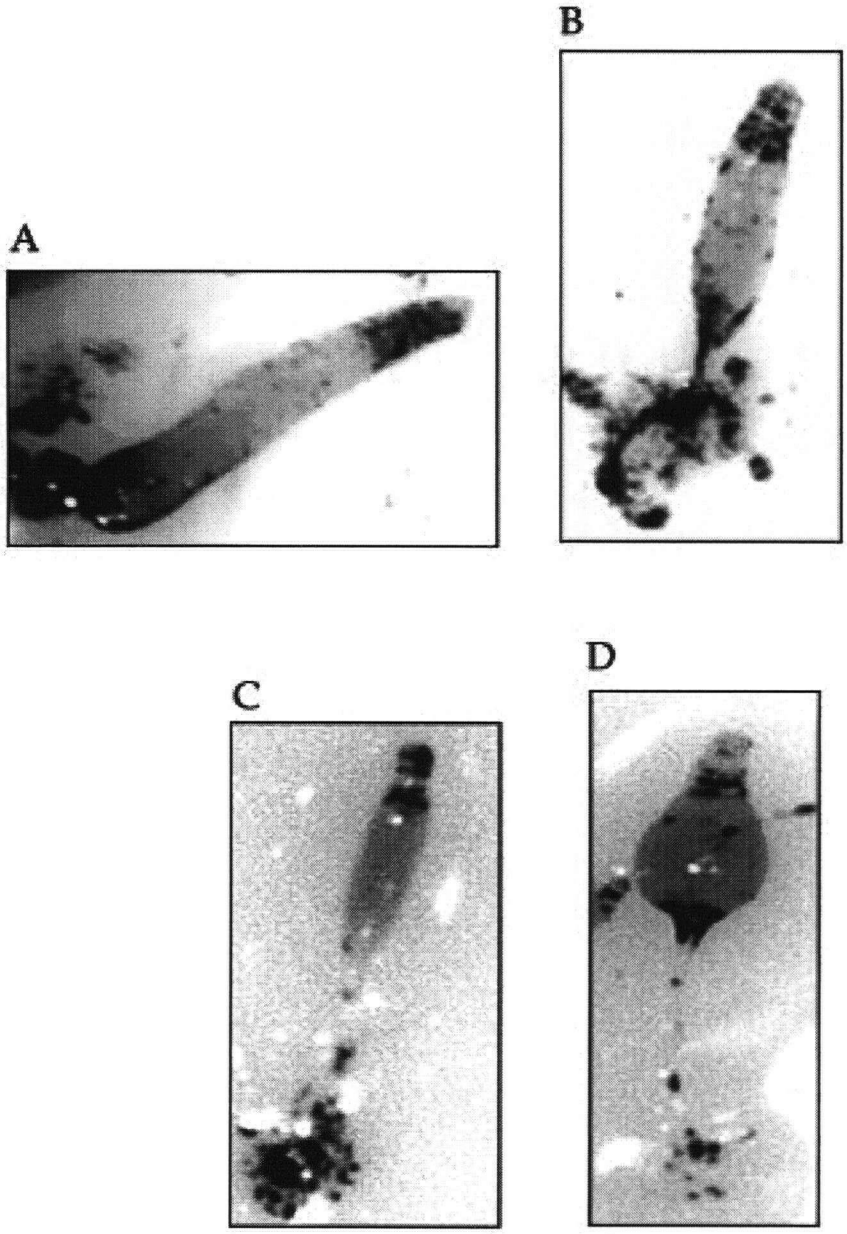


Figure 4-12: Cell sorting during chimaeric development. Mixtures of 10% *act15::lacZ* labeled *psA::rasG(G12T)* and 90% unlabeled wild type cells were induced to develop and stained with x-gal at the slug (A) and culminant (B) stages. Culminants of similar mixes using *ecmAO::lacZ* labeled *psA::rasG(G12T)* cells (C) or *ecmB::lacZ* labeled *psA::rasG(G12T)* cells (D) are also shown.

and spore formation, the transformants did not form multi-tipped mounds nor did they exhibit drastic deregulation of cell type specific gene expression. However, the *psA::rasG(G12T)* transformants did form multi-tipped mounds in which cell type specific gene expression was grossly distorted and spore formation was inhibited. However, the finger-like projections were capable of morphogenetic movements and formed stalk tubes. In combination, these results suggested that the developmental phenotype of the *Ddras*-Thr12 transformant resulted from the combined overexpression of activated Ras in both prestalk and prespore cells.

To test this possibility, I cotransformed Ax2 cells with the *psA::rasG(G12T)* and the *ecmAO::rasG(G12T)* constructs. When starved to induce development, the cotransformants formed mounds with multiple protruding tips (Fig. 4-13A) that did not form finger-like projections or stalk tubes (Fig. 4-13B) and thus were reminiscent of the mounds formed by either *Ddras*-Thr12 (Reymond et al., 1986) or *rasD::rasG(G12T)* (M. Khosla, unpublished observations) transformants. These cotransformants were also defective for spore formation (only $5.2 \pm 0.8\%$ of cells induced to develop produced spores).

Although no stalk tubes were observed in the culminants of the *ecmAO::rasG(G12T)/psA::rasG(G12T)* cotransformants, based on the results in Section 4.3 of this thesis, I expected that stalk cells would be present. When stained with calcofluor, the mounds and protruding tips of the cotransformant culminants were observed to fluoresce. Again, due to the density of these structures, neither calcofluor staining nor phase contrast microscopy was sufficient to establish whether the structures in fact contained stalk cells (Fig. 4-14C, D). (For comparison, a wild type culminant is shown in Fig. 4-14A, B). Once the structures were broken apart, individual cells that stained with calcofluor (Fig. 4-14E) and were vacuolated (Fig. 4-14F) were observed, indicating that the cotransformant cells differentiated into stalk

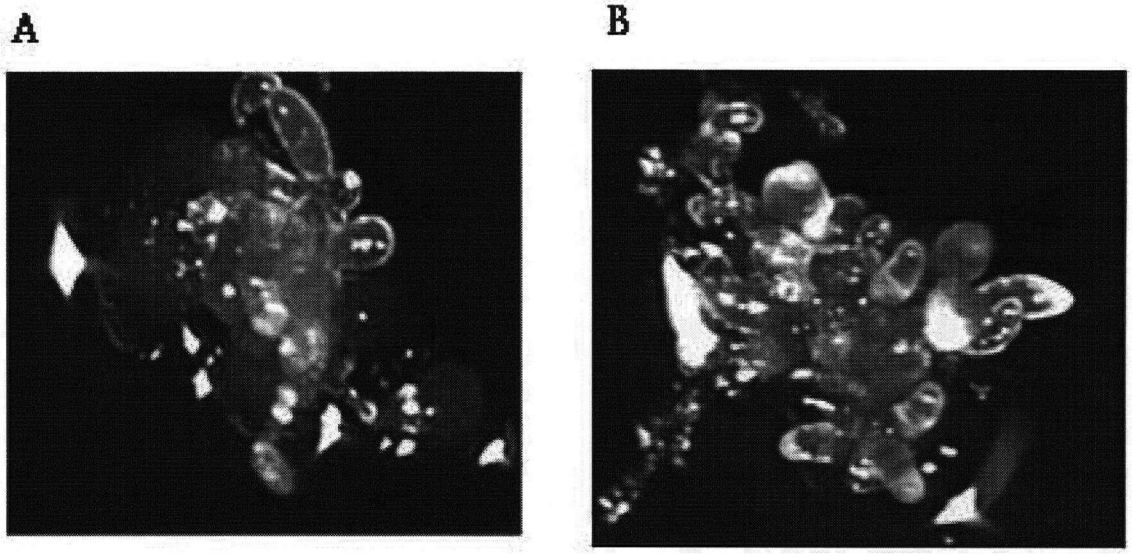


Figure 4-13: Developmental morphologies of cotransformants. *ecmAO::rasG(G12T)/psA::rasG(G12T)* cells formed multi-tipped mounds (A) which did not extend to form finger or slug like structures (B).

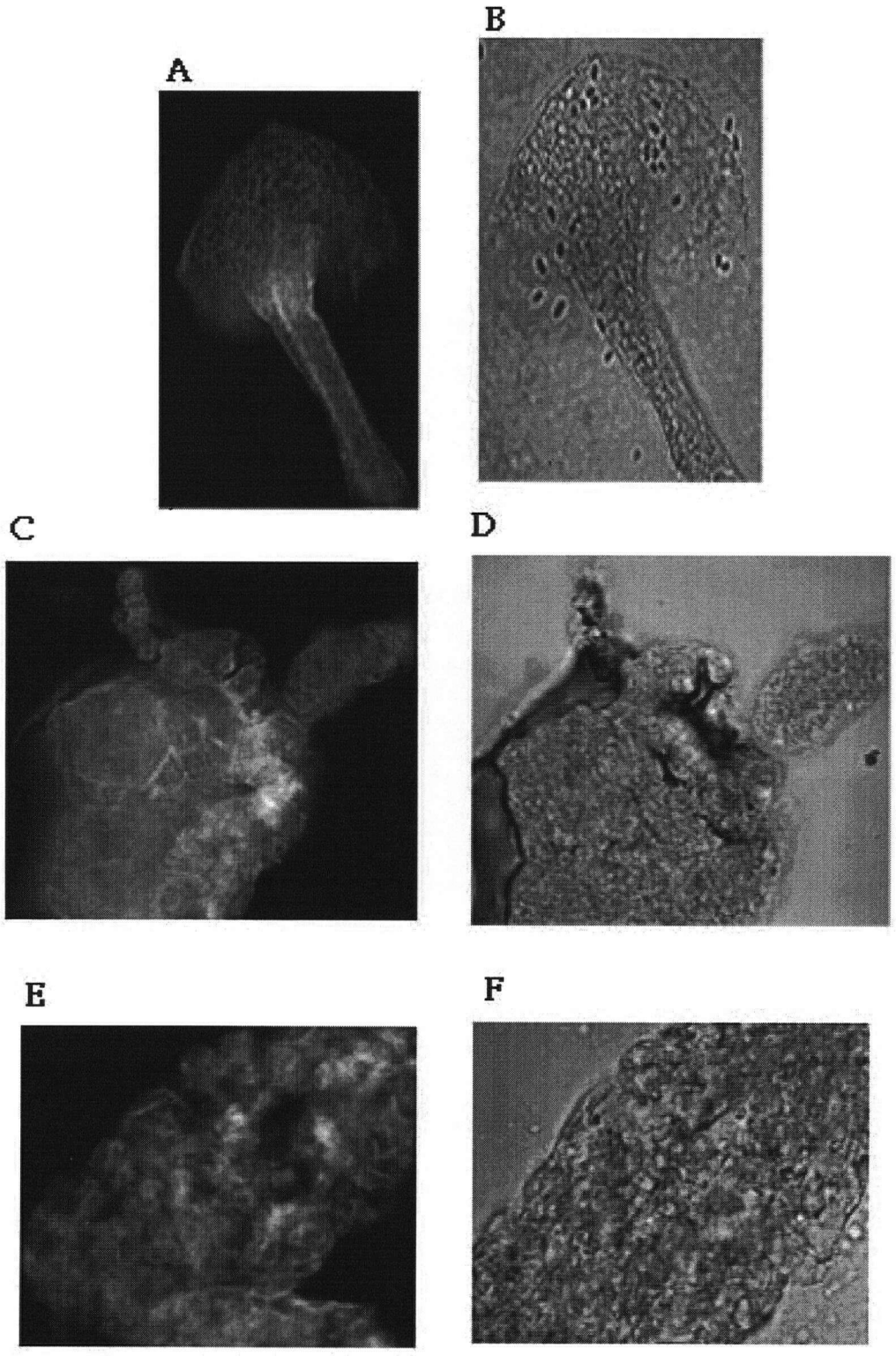


Figure 4-14: Culminants of Ax2 cells (A, B), and *ecmAO::rasG(G12T)/psA::rasG(G12T)* cells (C, D, E, F) were stained with calcofluor and viewed by fluorescence microscopy (A, C, E) or by phase contrast microscopy (B, D, F). Images in (E, F) are from cells released after a cotransformant culminant was smashed apart.

cells. Thus, based on the developmental characteristics of the *ecmAO::rasG(G12T)/psA::rasG(G12T)* cotransformants, I conclude that the developmental defects in the *Ddras*-Thr12 cells are the result of the combined expression of activated Ras in both prestalk and prespore cells.

4.10 Discussion

In Chapter Three, I presented results demonstrating that the overexpression of activated RasG in prestalk cells did not result in the developmental aberrations characteristic of the *Ddras*-Thr12 transformant. In this chapter, I have presented results illustrating that it is the overexpression of activated RasG in prespore cells that mimic the *Ddras*-Thr12 induced defects. The *psA::rasG(G12T)* transformant mounds resemble those of the *Ddras*-Thr12 transformants in that both produce multiple tips. A distinction between the two transformants is that the *psA::rasG(G12T)* transformant mound tips did extend to form finger and slug like structures. The *psA::rasG(G12T)* transformants also exhibited drastic deregulation of cell type specific gene expression similar to that observed with the *Ddras*-Thr12 transformant (Louis et al., 1997a). These results indicate that during the development of *Ddras*-Thr12 transformant, it is the presence of activated Ras in the prespore cells that makes a major contribution to the observed phenotype.

However, the *psA::rasG(G12T)* transformants were not morphogenetically inhibited at the multi-tipped mound stage, indicating that the overexpression of activated Ras in prespore cells is not sufficient to induce all of the defects exhibited by the *Ddras*-Thr12 strain. It is therefore likely that the block at the tipped mound stage is the result of the presence of activated Ras in both cell types. The phenotype of the *ecmAO::rasG(G12T)/psA::rasG(G12T)* transformants is consistent with this prediction.

Cotransformant cells produced multiple tipped mounds and were blocked at that stage.

The deregulation of cell type specific gene expression is consistent with the fact activated Ras induced transdifferentiation of the prespore cells to prestalk cells in the *psA::rasG(G12T)* and *Ddras*-Thr12 transformants. The formation of multi-tipped aggregates and the alterations in gene expression patterns could be the consequence of activated Ras interfering with a single pathway. However, it is more likely that the two phenotypes are due to two separate pathways since the concurrent overexpression of *rasD(G12T)* and the *rap1* gene resulted in a correction of cell type specific gene expression, although multiple tip formation persisted (Louis et al., 1997b). Thus, the effect of RasD(G12T) on cell type specific gene expression was antagonized by Rap, whereas the misregulation of tip formation was independent of Rap.

Tip formation is a pivotal event during *Dictyostelium* development since the tip is believed to orchestrate the remainder of the developmental program. However, the process of tip formation is complex and poorly understood. Characterization of mutant strains deficient in tip formation has indicated that proper tip formation involves extracellular cAMP-mediated signaling, cell movement, cell sorting, and cell adhesion (Saxe et al., 1993; Stege et al., 1997; Tsujioka et al., 1999; Jiang et al., 1998; Vasiev and Weijer, 1999). There is evidence that the tip acts as an oscillating center, responsible for the generation of cAMP waves. As the mound compacts, the cAMP waves are observed as spirals or concentric circles originating from a single center that then becomes the tip (Siegert and Weijer, 1995; Rietdorf et al., 1998). Multiple competing oscillating centers can occasionally arise in the same mound but eventually one center dominates and gives rise to the single tip. It is possible that the expression

of activated Ras in the prespore cells of either *Ddras*-Thr12 or *psA::rasG(G21T)* may alter the cAMP signaling in the mound and consequently, multiple tips arise.

A number of other *Dictyostelium* strains exhibit a multi-tip phenotype. A double knockout of two of the PI3K isoforms (Zhou et al., 1995), as well as the disruption of a ubiquitin-conjugating enzyme (Clark et al., 1997), or the overexpression of a phosphotyrosine phosphatase (Howard et al., 1992) all compromise the ability to control tip number. In addition, multi-tipped mounds are formed during the development of strains with disruptions in any one of four *tip* genes that encode proteins with as yet no homologues in the data base (Stege et al., 1997; Stege et al., 1999). Finally, the *scrA* null strain also forms multi-tipped aggregates during development (Bear et al., 1998). The *scrA* gene was isolated as a suppressor of the *carB* null strain (Saxe et al., 1993) that lacks cAR2, one of the four cAMP receptors. The fact that there is no obvious connection between the disrupted or deregulated proteins in the various multi-tipped strains, indicates that the suppression of multiple tips in the wild type mound depends upon a number of signaling pathways. It seems unlikely that activated Ras simply downregulates any of the proteins that are required to suppress multiple tip formation because the subsequent development of the activated Ras expressing strains is unlike that of any of the null strains.

During chimaeric development of *psA::rasG(G12T)* and wild type cells, only the wild type cells produced spores. This indicates that wild type signals were unable to correct the sporulation defect of the *psA::rasG(G12T)* transformants. This also indicates that the transformant cells did not produce an inhibitory signal preventing sporulation of the wild type cells. When a small number of *psA::rasG(G12T)* cells were mixed with wild type cells, the transformants were able to participate in development and formed prestalk cells. When the proportions were reversed and a small number of wild type

cells were mixed with transformants, the wild type cells formed culminants without the participation of the transformants. These results are different from those in which wild type cells were mixed with *Ddras*-Thr12 cells. Expression from the *rasD* promoter occurs earlier than from the *psA* promoter and it is possible that this temporal difference results in the altered ability of the transformants to participate in chimaeric development.

As mentioned in Chapter One, differentiation in *Dictyostelium* is regulated such that the relative proportions of the various cell types are maintained. Cell type proportions were deregulated by the overexpression of activated Ras in prespore cells. Although the majority of *psA::rasG(G12T)* transformant cells initially adopted a prespore fate, they transdifferentiated into prestalk cells. If we assume that lateral inhibitors function to maintain cell type proportion homeostasis during normal development, several possible perturbations of such a system could potentially explain the observed *psA::rasG(G12T)* phenotype. Activated Ras in prespore cells could either interfere with the production of a prespore activator within the prespore cells or alter the cells' response to a prespore activator or inhibitor produced by the prestalk cells. Either of these effects could potentially induce prespore transdifferentiation into prestalk cells. However, if the *psA::rasG(G12T)* cells were deficient in activator production, then the presence of wild type cells during chimaeric development should provide activator and rescue the prespore development of the transformants. Since this was not the case, it is unlikely that Ras(G12T) inhibited the production of a prespore activator, but rather that the prespore cells were altered in their response to signals from prestalk cells.

Transdifferentiation has been shown to occur during the normal course of development as a consequence of slug migration (Sternfeld, 1992; Abe et al., 1994). As slugs migrate, PstB cells are lost from the posterior. These are replaced by anterior

PstA cells in which expression of the *ecmB* gene is induced. These cells are referred to as PstAB cells. The PstA cells are in turn replaced by the conversion of PstO cells to PstA cells. The decrease in PstO cells is compensated for by the recruitment of anterior like cells (ALC's, another prestalk subtype). Finally, ALC's are replaced by prespore cells that transdifferentiate into ALC prestalk cells. Thus the sequence of transdifferentiation during normal development has a specific directionality: from prespore to ALC to PstO to PstA to PstAB. However, in wild type cells, these cell type conversions must be reversible since, as mentioned previously, isolated prestalk compartments can reestablish correct proportions and form complete slugs (Raper, 1940). Given the number of prestalk cell subtypes, a complex network of activators and inhibitors regulating the relative proportions of each of the cell types must exist.

Since the transdifferentiated *psA::rasG(G12T)* cells expressed both the *ecmA* and *ecmB* genes, I conclude that the cells became PstAB cells. As discussed above, one possible explanation for the *psA::rasG(G12T)* phenotype is that expression of activated Ras in the prespore cells eliminated their sensitivity to inhibition of transdifferentiation. An alternative possibility is that activated Ras is a positive inducer of the PstAB cell fate and that the observed transdifferentiation does not involve lateral inhibitors. In fact, I have shown in Chapter Three that in the *ecmAO::rasG(G12T)* transformants, activated Ras induced the transdifferentiation of PstA cells to PstAB cells. Activated Ras may therefore be a positive inducer of the PstAB cell fate regardless of which cell type it is expressed in. However, I cannot discount the possibility that in the *ecmAO::rasG(G12T)* transformants, the PstA to PstAB conversion may be a consequence of a Ras-G12T mediated loss of inhibition rather than a positive induction of PstAB differentiation.

Since differentiation to PstAB is a commitment step prior to terminal stalk cell differentiation, it is possible that Ras(G12T) actually induces terminal stalk cell

formation. This conclusion is supported by the fact that the entire population of *psA::rasG(G12T)* cells appeared to differentiate into stalk cells, based on the expression of *ST::lacZ* and staining with calcofluor. Further support is provided by the finding that culminants of the *rasD::rasG(G12T)* transformant cells also contained terminally differentiated stalk cells. In addition, during the development of *psA::rasG(G12T)* transformants, the cells in the basal mound expressed *ST::lacZ* before expression was detected in the extended slug-like structures. Since the cells in the basal mound expressed activated Ras whereas the cells in the slug-like structures did not, as determined by the *psA::(his)lacZ* expression pattern, it appears that activated Ras accelerates terminal stalk cell differentiation.

The *psA::rasG(G12T)* transformants exhibited a notable difference from cells overexpressing RasG(G12T) from either the *rasD* or *ecmAO* promoters. Only when activated Ras was expressed from the prespore promoter was the formation of stalk cells encased in stalk tubes observed. Wild type stalk formation is initiated in the tip of a culminating organism and extends basipetally. When activated Ras is overexpressed from the *ecmAO* promoter, the tip that forms during development is not a functional developmental organizer. The *ecmAO::rasG(G12T)* tip cells do not initiate stalk tube synthesis; cells that differentiate as stalk cells are at the posterior of the organism and may represent enlarged basal disc structures. In the *Ddras*-Thr12 transformants activated Ras was expressed in both cell types and it is therefore likely that the expression of activated Ras in the prestalk cells prevented the formation of functional tips. As a result, no stalk tubes were observed. The stalk cells in the terminal mounds of *Ddras*-Thr12 transformants also probably represent extended basal disc structures.

In contrast, during the development of *psA::rasG(G12T)* aggregates, two populations of prestalk cells were specified. The first was the population of prestalk

cells that was specified at the time of initial cell type divergence and which did not express the *psA*-driven *rasG(G12T)* transgene. These prestalk cells formed the initial multi-tips and since these tip cells were in essence wild type, they were able to organize the later developmental processes. As a result, these tips extended to form finger-like and slug-like structures that even attempted migration. The tip cells were also able to initiate the synthesis of a stalk tube during the later stages of differentiation. The second population of prestalk cells arose as a result of transdifferentiation of prespore cells. These cells expressed activated Ras during their short period as prespore cells and were therefore defective as organizers due to the presence of residual activated Ras. Thus, the tips that formed late during the development of *psA::rasG(G12T)* transformants, did not have the organizing capacity of wild type tips and were thus morphogenetically blocked.

CHAPTER FIVE

PERSPECTIVES

Data was presented by Reymond et al. (1986) indicating that activated RasD overexpression resulted in the formation of multi-tipped mounds that were morphogenetically inhibited at that stage. The transformant was examined in greater detail by Louis et al. (1997a), who discovered that the transformant cells underexpressed prespore cell specific genes, overexpressed prestalk cell specific genes, and did not form either terminally differentiated stalk or spore cells. It had originally been suggested that it was the expression of RasD(G12T) in prestalk cells which induced these developmental phenotypes (Esch and Firtel, 1991). However, endogenous *rasD* expression was demonstrated to occur in prespore cells as well (Jermyn and Williams, 1995), leading to other possible hypotheses to explain the consequences of RasD(G12T) overexpression (Louis et al., 1997a).

All of the hypotheses suggested by Louis et al. (1997a) required an explanation that included a block in prestalk to stalk cell formation. Since I have found that stalk cells were produced by the *rasD::rasG(G12T)* transformants, the number of possible hypotheses was reduced. It was possible that in the *Ddras*-Thr12 transformant, activated Ras might have exerted its effects in prestalk cells, activating prestalk gene expression and repressing prespore gene expression by a lateral mechanism. Alternatively, activated RasD might have acted in the prespore population, decreasing prespore gene expression and enhancing prestalk gene expression. Finally, it is possible that activated RasD may have functioned in both cell types to produce the developmental defects of the *Ddras*-Thr12 transformant.

The experiments described in this thesis were designed to dissect these effects and attempt to determine which of the possible scenarios, if any, was correct.

The hypothesis that activated RasD functioned in prestalk cells was tested by examining the developmental characteristics of the *ecmAO::rasG(G12T)* transformants. During development, these transformants did exhibit a slight increase in prestalk gene expression and decrease in prespore gene expression as assessed by northern blot analysis. However, the effects on gene expression were minimal compared to the drastic alterations exhibited during the development of the *Ddras*-Thr12 transformant (Louis et al., 1997a). These results indicate that activated RasG expression, and by analogy activated RasD expression, in prestalk cells does not have a major effect on prestalk gene expression or prespore gene expression.

In addition, these transformants did not form multi-tipped aggregates, indicating that the morphological defect of the *Ddras*-Thr12 transformant did not result from RasD(G12T) expression in prestalk cells. However, the *ecmAO::rasG(G12T)* transformants were not able to construct stalk tubes, suggesting that the absence of stalk tubes in the *Ddras*-Thr12 culminants results from activated Ras expression in prestalk cells. Also, the *ecmAO::rasG(G12T)* transformants exhibited some morphogenetic defects; some structures were blocked at the mound stage and the slugs were impaired in motility. These results suggest that the morphogenetic block in the *Ddras*-Thr12 transformant might have resulted in part from the expression of activated RasD in prestalk cells.

Since most of the developmental defects in the *Ddras*-Thr12 transformants were not recapitulated in the *ecmAO::rasG(G12T)* transformants, it was important to

test the second hypothesis. Prespore specific expression of activated RasG in the *psA::rasG(G12T)* transformants did result in drastic alterations in cell type specific gene expression. These transformants greatly underexpressed prespore genes and overexpressed prestalk genes, similar to the *Ddras*-Thr12 transformant. In addition, by culmination, most *psA::rasG(G12T)* cells no longer expressed a prespore specific reporter construct and instead expressed a prestalk reporter. Evidence of such prespore to prestalk transdifferentiation was also observed during the development of the *Ddras*-Thr12 transformant (Louis et al., 1997a). These results suggest that in the *Ddras*-Thr12 transformant, activated RasD expression in prespore cells induced their transdifferentiation to prestalk cells and as a consequence, resulted in altered patterns of cell type specific gene expression.

The *psA::rasG(G12T)* transformants also formed multi-tipped aggregates during development, suggesting that this morphological defect during *Ddras*-Thr12 development was also a result of prespore specific RasD(G12T) expression. However, the *psA::rasG(G12T)* transformants were not blocked at this stage. The tips extended to form finger-like and slug-like structures and finally formed stalk tubes. The most likely explanation for this is the fact that during the development of the *psA::rasG(G12T)* cells, the originally determined prestalk population never expressed activated RasG and was able to organize tip elongation and stalk tube construction. Thus, prespore specific expression of activated RasG was not sufficient to recapitulate all of the phenotypic features of the *Ddras*-Thr12 transformant.

Since expression of RasG(G12T) in either cell type alone did not explain the full phenotype of the *Ddras*-Thr12 cells, it was likely that activated RasG expression in both cell types was necessary. This possibility was examined by characterizing the

developmental morphology of the cotransformants. Indeed, the *ecmAO::rasG(G12T)/psA::rasG(G12T)* cotransformants developed to produce mounds with multiple tips. These mounds were morphogenetically inhibited and no stalk tubes were constructed. Hence, the developmental phenotype of the *Ddras*-Thr12 transformant was most likely the combined result of RasD(G12T) expression in both cell types.

In Chapter One, I briefly described some known regulators of cell type determination in *Dictyostelium*. Although few biochemical or genetic interactions between the known regulators of cell type determination in *Dictyostelium* have been demonstrated, some of the *Dictyostelium* proteins induce developmental consequences similar to those produced by activated RasD or activated RasG expression and possible interactions are therefore worth considering.

Cells disrupted in *gskA* exhibit a developmental phenotype (Harwood et al., 1995) that is similar to that of the *psA::rasG(G12T)* transformants. Both strains form large mounds with emerging stalks (only one stalk in the *gskA* null strain). In both cases, the cells in the mounds differentiate as stalk cells and spore cell formation is severely compromised. Similarities between the two strains also exist at the level of gene expression. However, the *psA::rasG(G12T)* cells overexpress both the *ecmA* and *ecmB* genes where as the *gskA*$^-$ strain overexpressed the *ecmB* gene without altering the expression of *ecmA*. So, while *psA::rasG(G12T)* cells appear to differentiate as PstA cells as an intermediate step in their transdifferentiation from prespore to PstAB cells, the *gsk* null cells appear to differentiate as PstB cells without inducing expression of the *ecmA* gene. Thus, although the majority of cells differentiate as stalk cells in both cases, the molecular events leading to terminal

differentiation are likely to be different. In addition, the *gskA*$^-$ cells do not exhibit cAMP-mediated repression of stalk cell formation. The *Ddras*-Thr12 transformant cells were prevented from stalk cell formation in the presence of cAMP (Louis et al., 1997a). Therefore, stalk cell formation mediated by activated RasD most likely occurs through a pathway that does not involve GSK3 inhibition.

Slugs of *STATa* null cells (Mohanty et al., 1999) resemble those of the *ecmAO::rasG(G12T)* transformants in some respects. Developing *STATa*$^-$ cells overexpress *ecmB* and in mutant slugs, PstB cells are observed throughout the whole anterior prestalk region. *ecmAO::rasG(G12T)* cells exhibit a slight increase in *ecmB* expression and in some transformant slugs, PstB cells were observed in the anterior. However, the PstB cell localization varied and many slugs exhibited no anterior expression of the *ecmB::lacZ* marker indicating that important differences exist between the two strains in the regulation of PstB cell differentiation. In addition, PstA cell differentiation is also dissimilar in the two strains since the cell type was mislocalized in the *ecmAO::rasG(G12T)* slugs but normally localized in the *STATa* null slugs. STATa has the properties of a negative regulator of stalk cell formation but in the multicellular organism, null cells do not differentiate as stalk cells, probably because of increased sensitivity to cAMP-mediated inhibition. It is possible that activated RasG may act upstream of STATa in a pathway regulating *ecmB* gene expression. However, other pathways must exist downstream of RasG(G12T) which cause mislocalization of the prestalk cell types and allow for stalk cell differentiation to occur.

Prespore specific expression of the dominant inhibitor of PKA results in the inability of cells to maintain prespore gene expression and the absence of spore

formation (Hopper et al., 1993a). Although this phenotype resembles that of the *psA::rasG(G12T)* transformants, it is unlikely that activated RasG signals only via PKA. In the *psA::PKA-Rm* transformants, expression of the spore coat genes is drastically reduced but expression of *psA* is not. The *psA* gene does not depend on PKA for expression whereas the spore coat genes require PKA activity for maximum expression. Thus, in this transformant, the PKA-sensitive prespore gene expression pathways are effected. In contrast, in the *psA::rasG(G12T)* transformants, expression levels of both the spore coat gene, *cotC*, and of *psA* are were drastically reduced. This indicates that in the strain expressing activated RasG, the inhibition of prespore gene expression is a more general effect than in the strain expressing PKA-Rm. Thus, although PKA-sensitive gene expression is reduced in the *psA::rasG(G12T)* transformants, the defect in prespore cells likely occurs at a step upstream of PKA and affects multiple pathways.

PKA is also required for terminal differentiation. Presumably, PKA can still be activated in activated RasG or activated RasD expressing cells since all transformants tested form stalk cells and PKA activity is required for stalk cell differentiation (Zhukovskaya et al., 1996). In addition, in the *ecmAO::rasG(G12T)* transformants, it is apparent that PKA activity is regulated in the prespore cells. Although these transformants do not form spore cells on their own, they are capable of being induced to form spores by signals from wild type cells. Thus, within the prespore cells, this response pathway is functional. These results indicate that the *ecmAO::rasG(G12T)* transformants may be defective in the production of a sporulation signal. SDF-2 has been shown to induce spore formation in isolated cells and is most likely produced from the prestalk cells during culmination (Anjard

et al., 1998). Thus, activated RasG expression in prestalk cells may interfere with the production of SDF-2 or another such factor.

The existence of a MAPK cascade downstream of Ras has not yet been demonstrated in *Dictyostelium*. A MAPK, ERK2 has been identified and shown to be required early in development for aggregation (Segal et al., 1995) and later in development for the induction and maintenance of prespore gene expression but not for prestalk gene expression (Gaskins et al., 1996). In wild type cells, ERK2 has been shown to be transiently activated by cAMP (Maeda et al., 1997; Knetsch et al., 1996; Kosaka et al., 1998). In addition, during starvation, ERK2 is required for the activation of adenylyl cyclase and therefore, for the production of cAMP (Segall et al., 1995).

The role of Ras in the activation of ERK2 has been investigated by several laboratories. In one report, the presence of constitutively activated RasD(G12T) was shown to increase the basal level of ERK2 activity (Knetsch et al., 1996). However, Maeda et al. (1997) determined that activated RasD(T61Q) inhibited the cAMP-induced activation of ERK2 and a similar result was obtained by Kosaka et al. (1998) using RasG(G12T). Also, the expression of activated RasG inhibits the production of cAMP (Khosla et al., 1996). While the discrepancy between the positive and negative effects of activated Ras on ERK2 activation remains to be resolved, these results indicate the possibility that Ras is a negative regulator of the MAPK cascade or that activated Ras is acting as a dominant negative in the cascade.

If activated Ras is a negative regulator of ERK2 activity during aggregation, it also follows that RasD may negatively regulate ERK2 during later development. Consistent with this idea, activated RasG, expressed in prespore cells drastically

reduces prespore gene expression and loss of ERK2 function during development also reduces prespore gene expression levels. It is possible that activated RasG or activated RasD inhibits the expression of prespore genes by inhibiting ERK2 activation. However, since loss of ERK2 during development does not result in increased prestalk gene expression, activated Ras must be having additional effects that do not involve the regulation of ERK2 activity.

Alternatively, if activated Ras is a positive regulator of ERK2 (Knetsch et al., 1996), this could explain the multi-tipped phenotype induced by activated RasD or activated RasG. Wild type aggregates treated with cAMP produce multiple tips (Nestle and Sussman, 1972) and *erk2* null cells are defective in the activation of adenylyl cyclase (Gaskins et al., 1995). Therefore, if activated Ras stimulates ERK2 activity, the downstream effect could be an overstimulation of adenylyl cyclase and consequently, an overproduction of cAMP. Thus, the multi-tip formation induced by activated RasD or activated RasG could be a consequence of ERK2 activation.

The putative *Dictyostelium* MEK kinase homolog, MEKKα, is required for maintaining cell type proportions in the developing organism. Null mutants exhibit an enlarged PstO zone and a reduced prespore zone (Chung et al., 1998). In addition, MEKKα is required for induction and maintenance of prespore gene expression. Although the requirement for prespore gene expression is similar to that of ERK2, there is no evidence linking the two proteins in a single pathway.

Since activated RasG and activated RasD have negative effects on prespore differentiation, if Ras and MEKKα lie in the same pathway, Ras-GTP would be expected to negatively regulate MEKKα. This is the opposite of what has been observed in other systems. But, given the results indicating a negative regulation of

ERK2 by activated Ras (Maeda et al., 1997; Kosaka et al., 1998), it is possible that in *Dictyostelium*, Ras may inhibit activation of MAPK cascade components or that activated Ras has a dominant negative effect on the signaling cascade.

It is difficult to imagine that a Ras/MAPK cascade does not exist in *Dictyostelium* since it is such a well conserved signaling circuit amongst many other organisms including lower organisms such as *S. cerevisiae*. However, most of the available data do not suggest that active Ras postitively regulates any of the MAPK cascade components. The MAPK pathway components thus far identified in *Dictyostelium* do not constitute a single pathway, indicating that multiple such pathways exist and that the remaining components have yet to be isolated. Perhaps when these pathways are better characterized, we will be better able to assess the role of Ras proteins in their regulation.

The PI3K1 isoform of *Dictyostelium* interacts with activated RasG and activated RasD (Lee et al., 1999). However, a double knockout of the PI3K1 and PI3K2 isoforms results in the formation of multi-tipped mounds (Zhou et al., 1995) suggesting that Ras might be a negative regulator of PI3K. In contrast, in mammalian systems, activated Ras positively regulates PI3K activity.

Although a large number of small GTPases have been described in *Dictyostelium*, limited interactions with GEFs or GAPs have been identified. Several putative Ras GEFs have been identified (Insall et al., 1996; www.csm.biol.tsukuba.ac.jp/cDNAproject.html) but interactions with Ras family GTPases have not been demonstrated. Null mutations in *aimless*, which encodes a Ras GEF homologue, result in cells that are impaired in chemotaxis and do not aggregate (Insall et al., 1996). Although proper GDP/GTP cycling of RasG is required

for aggregation, both cells expressing activated RasG and cells with null mutations in *rasG* are able to chemotax (Khosla et al., 1996; Tuxworth et al., 1997). It is possible that the activity of a *Dictyostelium* Ras protein other than RasG is regulated by Aimless.

An IQGAP-related protein has been identified independently by two groups and found to have different developmental consequences when knocked out (Faix and Dittrich, 1996; Lee et al., 1997; Faix et al., 1998). Although Lee et al. (1997) detected an interaction between the putative IQGAP homologue and RasD, the developmental phenotypes of loss-of-function GAP and gain-of-function RasD are very different. Faix and Dittrich (1996) demonstrated that loss-of-function GAP resulted in cells with multi-tipped fruiting bodies. However, they did not detect an interaction between the IQGAP homologue and RasD (Faix et al., 1998). Instead, a biochemical interaction was detected with *Dictyostelium* Rac1 but no increase in GTPase activity was observed (Faix et al., 1998). A second IQGAP related protein, GAPA, has been identified by Adachi et al. (1997). An interaction between GAPA and a *Dictyostelium* GTPase has not yet been demonstrated. Since the mammalian IQGAPs do not interact preferentially with Ras subfamily proteins, it is likely that the *Dictyostelium* homologues will not have GAP activity for the *Dictyostelium* Ras proteins.

The possible effects of activated Ras signaling in *Dictyostelium* appear to contradict what is known from other systems. However, the putative interactions are well worth investigating. Although multiple Ras pathways have been identified in mammalian systems, it is clear that additional Ras-mediated interactions and effects have yet to be uncovered. It is possible that in an organism such as

Dictyostelium, if the effectors for Ras are different, or if the interactions between Ras and its effectors are different from those identified in other organisms, such findings would reveal novel aspects of Ras signaling.

The work described by Reymond et al. (1986), Louis et al. (1997a), and in this thesis illustrates that the expression of either activated RasD or activated RasG induces significant defects during the development of *Dictyostelium*. Recently, mutants have been created in which the *rasD* gene was ablated (Wilkins et al., 2000). These *rasD*$^-$ mutants exhibit normal development with no observable defects in morphology, patterning, cell type proportions, or terminal differentiation. Mutant slugs were, however, compromised with regards to their phototactic and thermotactic responses. The mechanisms by which slugs detect and respond to gradients in temperature and light have not been elucidated. However, one of the second messengers involved in regulating these processed is cGMP. Activated RasD was shown to affect the desensitization of guanylyl cyclase during chemotaxis (Van Haastert et al., 1987). Recently, a Ras Interacting Protein (RIP3) has been identified that interacts with activated RasG and, to a lesser extent, with activated RasD (Lee et al., 1999). RIP3 is necessary for proper activation of guanylyl cyclase during aggregation. It is possible that during normal development, RasD regulates guanylyl cyclase activity and consequently enables normal slug migration, but RasD is not the Ras protein that directly regulates aggregation since aggregation is apparently normal in *rasD* null slugs.

The finding that *rasD* null cells exhibit normal differentiation brings into question the previously proposed significance of RasD as a regulator of essential developmental processes. Is the regulation of slug migration RasD's only function?

Since there are a large number of Ras homologs in *Dictyostelium*, it is possible that RasD does have a function during normal development but this function is compensated for by the presence of other Ras proteins. It is also possible that RasD(G12T) and RasG(G12T) interfere with the normal function of other Ras or Ras-superfamily proteins that are essential for pattern regulation. To establish which of these possibilities is correct will require the construction of other *ras* null strains and an analysis of upstream and downstream Ras pathway components in order to identify the level of cross talk between the various Ras proteins.

With the results in this thesis, I have shown that activated RasG expression in prespore cells results in the formation of multi-tipped aggregates, the transdifferentiation of prespore cells to prestalk cells, and the terminal differentiation, of most cells into stalk cells. Also, the overexpression of activated RasG in prestalk cells prevents the correct localization of prestalk cells and although these cells differentiate into stalk cells, the differentiation of prespore cells to spore cells is inhibited. Although the phenotypes of these two transformants are quite different with respect to morphology and gene expression patterns, they are similar with respect to terminal differentiation. It appears that all cells expressing activated Ras are induced to differentiate as stalk cells regardless of whether they initially were of the prestalk or prespore specification. Ras has been shown to regulate choice of cell fate in other systems, some of which are described in the Introduction to this thesis. In *Dictyostelium*, stalk cell formation is under tight control by multiple regulators including GSK3, STATa, and PKA. It is possible that the pathway(s) downstream of activated Ras may constitute another mechanism whereby stalk cell formation is specified. Further investigation into activated Ras signaling and

elucidation of downstream effectors should shed more light on the complex question of how cells choose their fate.

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APPENDIX I

Materials Used For This Thesis

All chemicals and reagents were purchased from either Fisher Scientific or Sigma Chemical Co., unless otherwise indicated. Restriction enzymes and DNA modifying enzymes were purchased from Life Technologies unless otherwise indicated.

MATERIALS	SUPPLIER
Agar	Difco
Bacteriological peptone	Oxoid
Calcofluor	Sigma Chemical Co.
($^{\alpha 35}$S)dATP	NEN Scientific
($^{\alpha 32}$P)dCTP	NEN Scientific
ECL (Enhanced Chemiluminescence kit)	Amersham
G418 (Geneticin)	Life Technologies
GeneCleanII Kit	Bio 101, Inc.
HRP-conjugated-donkey-α-rabbit IgG	Amersham
Hybond-N+ nitrocellulose	Amersham
Hybond-P PVDF	Amersham
Nucleobond Ax-20 kit	Machery-Nagel
Nunclon™ plates	Nalge Nunc Int.
Oligonucleotides	NAPS
Qiagen DNA kits	Qiagen
Sequenase2®	United States Biochemical
TritonX-100	Fisher Scientific
Vent$_R$®	New England Biolabs
XL-1 *E. coli* cells	Stratagene
X-ray film	Eastman Kodak Co.
X-gal	Life Technologies
Yeast extract	Oxoid

Additional Materials

The α-RasG-GST fusion protein antibodies were generated by Dr. S. Robbins (Robbins et al., 1989) and by L. Duncan. The *rasD::rasG(G12T)* transformant was obtained from M. Khosla. The *psA::Dd-PK2* was obtained from N. Hopper. The *ecmAO::lacZ, ecmA::lacZ, ecmO::lacZ, ecmB::lacZ, ST::lacZ,* and *psA::lacZ* constructs were all obtained from Drs. K. Jermyn and J. Williams. The *psA::(his)lacZ* was obtained from Dr. H. MacWilliams.

APPENDIX II

Culture Media and Buffer Recipies

II.i. *Dictyostelium discoideum* Growth Media

HL5 Medium:

14.3 g Neutralized bacteriological peptone
7.15 g Yeast extract
0.96 g Na_2HPO_4
0.48 g KH_2PO_4
Per liter of distilled water
(The medium was supplemented with 3 ml of 30.8% glucose after autoclaving.)

SM-VAN Agar:

10 g Glucose
10 g Neutralized bacteriological peptone
1 g Yeast extract
1 g $MgSO_4 \cdot 7H_20$
1.55 g $NaH_2 \cdot PO_4H_20$
1 g K_2HPO_4
10 g Agar
Per 980 ml of distilled water

II.ii. *Dictyostelium discoideum* Starvation Media

1% Water Agar Plates:

1 g Agar
Per 100 mls of distilled water

Bonner's Salts (BS):

(For 10x stock solution)
6 g NaCl
7.52 g KCl
3 g $CaCl_2 \cdot 2H_20$
Per liter of distilled water

KK2:

(For 10x stock solution)
200 mM K_2HPO_4
200 mM KH_2PO_4
(The potassium phosphate dibasic is titrated with potassium phosphate monobasic until the solution reaches a pH of 6.0.)

II.iii. *Dictyostelium discoideum* Transformation Buffers

Bis-HL5:
2.1 g Bis-Tris
10 g Peptone
5 g Yeast extract
Per liter of distilled water
(The medium was supplemented with 3 ml of 30.8% glucose after autoclaving.)

2X HBS:
0.27 M NaCl
10 mM KCl
1.4 mM Na_2HPO_4
42 mM Hepes
10 mM Glucose
pH to 7.1 with 0.5 N NaOH
Filter sterilize and store at $^{-}20°C$

II.iv. Bacterial Media

LB Agar:
10 g Bacteriological peptone
10 g NaCl
1 g Yeast extract
10 g Agar
Per liter of distilled water

II.v. Buffers for Northern Analysis

TBE:
(For 10X stock solution)
108 g Tris base
7.4 g EDTA
55 g Boric acid
Per liter of distilled water

RNA Sample Buffer:
(For each 20 µg RNA sample)
2.0 µl 5x RNA Gel Running Buffer
3.5 µl Formaldehyde
10.0 µl Deionized formamide
2.0 µl RNA loading dye
0.5 µl Ethidium bromide (10mg/ml)
4.5 µl Distilled water

5X RNA Gel Running Buffer:
0.2 M MOPS (pH 7.0)
50 mM Sodium acetate
5 mM EDTA (pH 8.0)

RNA Loading Dye:
50% Glycerol
1 mM EDTA (pH 8.0)
Bromphenol blue and xylene cyanol to color

SSC:
(For 20X stock solution)
175.25 g NaCl
88.25 g Sodium citrate
pH to 7.0 with 10 N NaOH

Hybridization Solution:
5X SSC
1X Denhardt's Reagent
50 mM Sodium phosphate buffer, pH 6.5
0.5% SDS
30% Deionized formamide
250 µg/ml ssDNA
30 µg/ml polyA

50X Denhardt's Reagent:
for 100 ml
1 g Ficoll (type 400)
1 g Polyvinylpyrrolidone
1 g Bovine serum albumin (fraction V)

II.vi. Buffers for Western Analysis

2X Protein Sample Buffer:
0.5% BME
0.5% SDS
50 mM Tris pH 6.8
12.5% Glycerol
0.04% Bromphenol blue

TBST:
50 mM Tris-HCl pH 7.5
150 mM NaCl
5% Tween-20

II.vii. Solutions for β–galactosidase Assays

Z Buffer:
60 mM Na_2HPO_4
40 mM NaH_2PO_4
10 mM KCl
1 mM $MgSO_4$
2 mM $MgCl_2$

β–Galactosidase
Staining Solution:
(Made in Z buffer)
1 mM X-gal (in DMF)
5 mM $K_3[Fe(CN)_6]$
5 mM $K_4[Fe(CN)_6]$
1 mM EGTA