CHARACTERIZATION OF A RAS AND A RAS-RELATED GENE AND THEIR DEVELOPMENTAL EXPRESSION IN THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

By

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ABSTRACT

Although it was previously reported that *Dictyostelium discoideum* possessed a single *ras* gene (Dd*ras*) that was maximally expressed during the pseudoplasmodial stage of development, a second *ras* gene (Dd*ras*G), has been isolated and characterized. It encodes a protein that is similar to the protein encoded by Dd*ras* and the human ras proteins. However, in contrast to Dd*ras*, the Dd*ras*G gene was only expressed during growth and early development. The two *ras* proteins may fulfill different functions: the DdrasG protein having a role during cell growth and the Ddras protein having a role in signal transduction during multicellular development. However, the expression of the Dd*ras*G gene throughout development did not appear to have a detrimental effect on differentiation. Although other eukaryotic organisms possess more than one *ras* gene, *D. discoideum* is thus far unique in expressing different *ras* genes at different stages of development.

Ras genes are members of a large *ras*-related multigene family that has been found in a wide variety of organisms. A *ras*-related gene was isolated from *D. discoideum* that hybridized to both the Dd*ras* and Dd*ras*G genes under low, but not under high stringency conditions. The predicted amino acid sequence shows a high degree of sequence identity with the human *rap* proteins and thus has been designated Dd*rap*1. During vegetative and early development a single 1.1 kb mRNA was present, but by aggregation this transcript was no longer detected and two new transcripts of 1.0 and 1.3 kb were observed and were present throughout the remainder of development. The maximum levels of the Dd*rap*1 specific mRNAs appeared during aggregation and culmination, developmental stages where the levels of Dd*ras*G and Dd*ras* messages were declining. The reciprocal nature of the Dd*rap*1 gene expression with respect to that of the two *ras* genes

suggests the possibility that the *ras* and *rap* gene products in *D. discoideum* have antagonistic roles. Antibodies that are specific for the Ddras, DdrasG and Ddrap1 proteins have been generated and can be used to help elucidate the biological functions of the individual proteins.

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LIST OF ABBREVIATIONS

A	absorbance, optical density
ATP	adenosine 5'-triphosphate
bp	base pairs
cAMP	adenosine 3', 5' -monophosphate
cDNA	complementary deoxyribonucleic acid
C-terminal	carboxy-terminal region of a protein
DEAE	diethylaminoethyl
dNTP	deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DNA	deoxyribonucleic acid
EDTA	disodium ethylene diaminetetraacetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kd	kilodaltons
µCi	micro-Curies
µg	micrograms
µl	microliters
mM	millimolar
M	molar
mRNA	messenger ribonucleic acid
M_r	apparent molecular weight
Pipes	piperazine-*N*, *N'* -bis(2-ethanesulfonic acid)
RNA	ribonucleic acid

SDS	sodium dodecyl sulfate
Tris	tri(hydroxymethyl)aminomethane
vol	volume
wt/vol	weight by volume

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CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 Oncogenes

Oncogenic retroviruses are in the family of viruses that contain an RNA genome and an RNA-dependent DNA polymerase. Almost all oncogenic retroviruses fall into two classes with regard to their efficiency and ability to transform cells. One group, the acutely oncogenic retroviruses contains a viral oncogene which is responsible for the rapid induction of tumours in animals and the efficient transformation of cells in culture. The other subclass does not contain a viral oncogene, lacks the ability to transform cells *in vitro*, but can cause tumourgenesis after a long latent period that appears to require a multistep process (for review see; Varmus, 1988).

The study of oncogenic retroviruses was enormously enhanced when it was discovered that viral oncogenes arose by the transduction of genetic loci from genomes of vertebrate cells (for review see; Bishop, 1983; Varmus, 1984; Bishop, 1987). Viral oncogenes exhibit a high degree of sequence conservation with their cellular counterparts, referred to as proto-oncogenes, but often contain mutations which at least in part are responsible for their oncogenic potential. The cellular proto-oncogenes are sometimes targets for somatic mutations or aberrant expression which can lead to malignancy. Although the intrinsic biochemical properties of most oncogenes have been identified, their physiological functions and the metabolic pathways which they influence remain largely unknown. The same is true for the functions of the proto-oncogenes although evidence supports the general premise that they are

important regulators of normal cell growth and development (for review see; Bishop, 1987).

Oncogenes can be categorized into at least six classes based on the proteins that they encode: growth factors (e.g., *sis*), growth factor receptors (e.g., *neu*, *erbA*, *fms*, *kit*, and *mas*), tyrosine protein kinases (e.g.,*src*, *fps* and *abl*), cytoplasmic serine and/or threonine kinases (e.g., *mos* and *raf*), guanosine triphosphate binding proteins (e.g., *ras*) and nuclear transcription factors (e.g., *myc*, *myb*, *fos* and *jun*). The proteins encoded by the oncogenes have a diverse range of biochemical functions that appear to be associated with signal transduction events that originate from the cell surface and end at the level of gene expression (for review see; Varmus, 1984; Bishop, 1987; Storms and Bose, 1989).

1.2 *Ras* genes

1.2.1 *Ras* genes and cancer

Of the 40 or so cellular oncogenes that have been described to date the *ras* genes have attracted considerable attention because of their strong association with carcinogenesis. In keeping with current nomenclature the gene will be referred to as *ras* and the gene product as ras, except when describing *Saccharomyces cerevisiae* which will be designated by the normal yeast capital letter nomenclature (*RAS* for the gene and RAS for the gene product). The *ras* genes were first described as the transforming genes of the Harvey and Kirsten murine sarcoma viruses. Both viruses cause a wide range of tumours including carcinomas, sarcomas and leukemias. The two viral oncogenes were generated by the transduction of two highly related cellular genes, now referred to as the Harvey (H-*ras*) or Kirsten (K-*ras*) genes (Ellis, *et al.*, 1980; Andersen, *et al.*, 1981).

In addition human cells contain a third *ras* gene (N-*ras*) for which there is no known viral counterpart (Hall, *et al.*, 1983; Shimizu, *et al.*, 1983; Taparowsky, *et al.*, 1983).

Overall about 30% of all human tumours thus far analyzed at the molecular level contain a mutated *ras* proto-oncogene that is capable of neoplastic transformation of NIH3T3 cells upon gene transfer (Barbacid, 1987). Mutated *ras* genes can be found in a variety of tumours with the highest incidences found in myeloid leukemia, thyroid tumours and in adenocarcinomas of the pancreas, colon and lung (Bos, 1989). In addition, the tumours from carcinogen treated animals often contain mutated *ras* genes (Barbacid, 1987). The direct involvement of *ras* genes in neoplasia has recently been demonstrated in transgenic mice bearing a mutated *ras* gene (Andres, *et al.*, 1987; Quaife, *et al.*, 1987). The transgenic mice developed tumours that were dependent on the spatial and temporal specificity of the specific promoters used to regulate the expression of the mutated *ras* gene, indicating that *ras* genes have a major role during the early stages of tumour formation.

1.2.2 Primary structure and evolutionary conservation of the *ras* genes

In addition to the three active *ras* genes, H-*ras*-1, K-*ras*-2 and N-*ras* (Chang, *et al.*, 1982b; Hall, *et al.*, 1983; McGrath, *et al.*, 1983; Shimizu, *et al.*, 1983; Taparowsky, *et al.*, 1983), two related pseudogenes, H-*ras*-2 and K-*ras*-1 have been identified in the human genome (Chang, *et al.*, 1982b; McGrath, *et al.*, 1983). The H-*ras*-1 and N-*ras* genes encode 189 amino acid proteins, while the K-*ras*-2 gene either encodes 189 or 188 amino acid proteins depending on the alternative use of two fourth exons (Capon, *et al.*, 1983; McGrath, *et al.*, 1983). The three different human *ras* genes encode proteins of 21,000 M_r (p21) and are approximately 85% conserved at the amino acid level (Barbacid, 1987).

The *ras* genes have been highly conserved throughout eukaryotic evolution. In addition to humans, *ras* genes have been identified in many other vertebrates including rat, dog, horse, chicken and goldfish (Nemoto, *et al.*, 1986; Barbacid, 1987). Highly related sequences have also been found in a number of invertebrate species including *Drosophila melanogaster* (Neuman-Silberberg, *et al.*, 1984; Mozer, *et al.*, 1985; Brock, 1987), the mollusk *Aplysia* (Swanson, *et al.*, 1986), in both the budding yeast *Saccharomyces cerevisiae* (Defeo-Jones, *et al.*, 1983; Powers, *et al.*, 1984) and the fission yeast *Schizosaccharomyces pombe* (Fukui and Kaziro, 1985) and the cellular slime mould *Dictyostelium discoideum* (Reymond, *et al.*, 1984). Most of the *ras* genes encode proteins of similar size to the 21 kd (p21) mammalian ras proteins with the exception of the *RAS1* and *RAS2* yeast genes, which encode proteins of 40 kd and 41 kd respectively. This widespread phylogenetic conservation of the *ras* genes suggests that they perform some general cellular function essential to all eukaryotes.

1.2.3 Structural and biochemical properties of the ras proteins

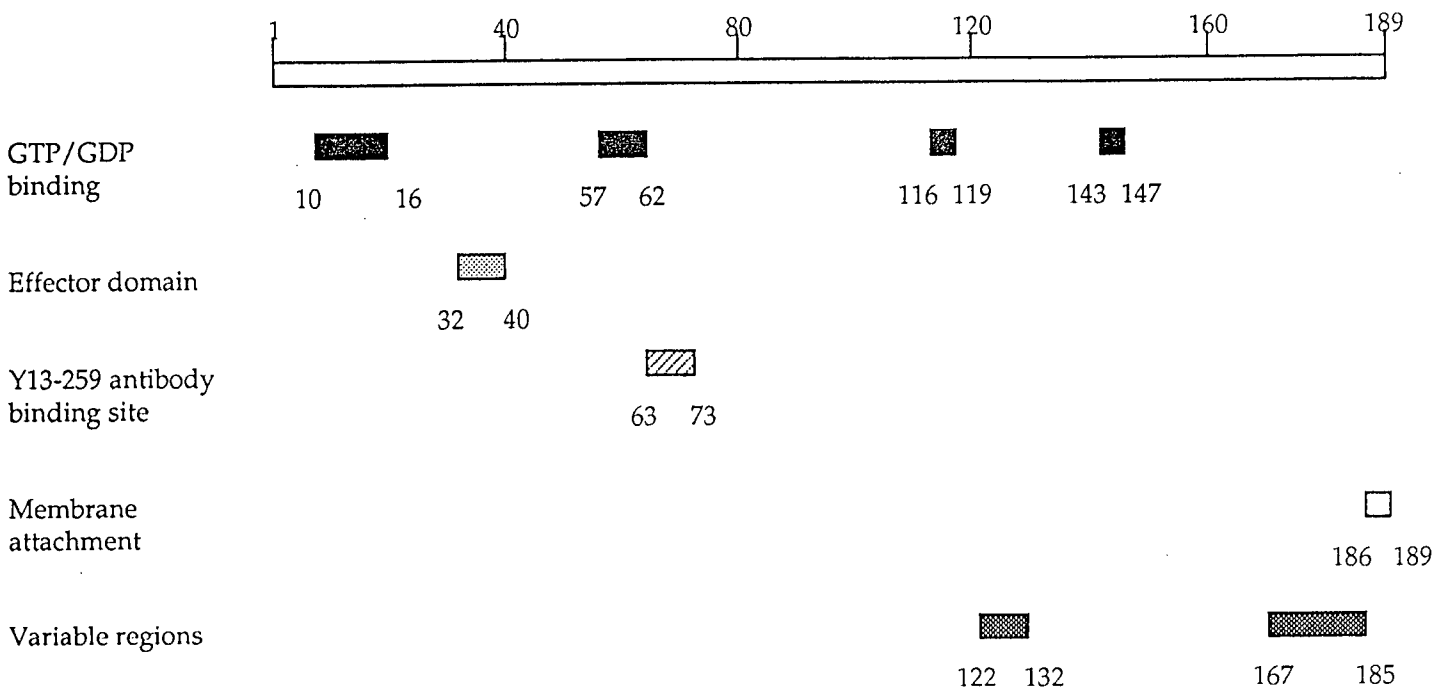
The *ras* genes encode biochemically and structurally similar proteins that bind guanine nucleotides (Shih, *et al.*, 1980), exhibit an intrinsic GTPase activity (Gibbs, *et al.*, 1984; McGrath, *et al.*, 1984; Sweet, *et al.*, 1984) and are associated with the inner surface of the plasma membrane (Willingham, *et al.*, 1980; Shih, *et al.*, 1982). These biochemical properties closely resemble those of the G proteins that are involved in mediation of signal transduction in a variety of systems (Gilman, 1987), and thus by analogy it has been postulated that the ras proteins regulate a signal transduction pathway (Hurley, *et al.*, 1984).

Comparison of the amino acid structure of the ras proteins with other GTP binding proteins such as bacterial elongation factor, transducin and the α-

subunit of G proteins demonstrated that they contain three consensus sequence elements with distinct spacings between them (Dever, *et al.*, 1987). These common domains were considered to confer the ability of the proteins to bind guanine nucleotides. In addition to the three consensus sequence elements the ras proteins contain a fourth domain that is also required for nucleotide binding. The importance of the four domains (amino acids 10-16, 57-62, 116-119 and 143-147) in guanine nucleotide binding in the mammalian ras proteins has been demonstrated by both random and site-directed mutagenesis (Feig, *et al.*, 1986; Willumsen, *et al.*, 1986; Clanton, *et al.*, 1987; Feig, *et al.*, 1987) (Figure 1). From the muational analyses it was established that amino acids 10-16 and 57-62 influence the binding and hydrolysis of GTP, whereas mutations in amino acids 116-119 only resulted in reduced binding affinities. X-ray crystallography has suggested that the fourth domain (amino acids 143-147), unique for the ras family of proteins is involved in the formation of the nucleotide binding pocket (McCormick, *et al.*, 1985; de Vos, *et al.*, 1988).

The oncogenic ras proteins require correct plasma membrane localization for transformation activity (Willumsen, *et al.*, 1984a; Lacal, *et al.*, 1988; Buss, *et al.*, 1989; Schafer, *et al.*, 1989; Mendola and Backer, 1990). For localization to the plasma membrane, the ras proteins undergo a series of complex post-translational modifications that require the conserved CAAX motif (C= cysteine, A= any aliphatic amino acid, X= any amino acid) located at the C-terminus of all ras proteins (Figure 1). The first series of modifications require the addition of a polyisoprenoid, probably a 15 carbon farnesyl moiety to the cysteine residue, the removal of the terminal three amino acids by proteolysis and methyl esterfication of the new C-terminal cysteine (Casey, *et al.*, 1989; Gutierrez, *et al.*, 1989; Hancock, *et al.*, 1989). These modifications are not sufficient for plasma

Figure 1. Schematic representation of the structural and functional domains defined within the ras proteins. The numbers indicate the amino acid residues defining the boundaries of the domains represented. See text for details.


1
40
80
120
160
189
GTP/GDP binding
10
16
57
62
116
119
143
147
Effector domain
32
40
Y13-259 antibody binding site
63
73
Membrane attachment
186
189
Variable regions
122
132
167
185

membrane localization since other proteins such as the lamins contain the CAAX motif, undergo similar modifications but are localized to the nucleus within the cell (Holtz, *et al.*, 1989). Although the farnesyl moiety increases the hydrophobicity of the ras proteins a further signal is required for targeting to the plasma membrane. The N-ras and H-ras proteins require the addition of palmitic acid at a cysteine residue near the C-terminus (Hancock, *et al.*, 1989), whereas a region rich in basic amino acids provides the additional signal for the K-ras protein (Hancock, *et al.*, 1990). Mutant ras proteins lacking the cysteine residue at amino acid 186 are not localized to the plasma membrane suggesting that all processing is dependent on the initial isoprenylation step (Willumsen, *et al.*, 1984b).

There are two other regions that are highly conserved amongst the ras proteins of phylogenetically diverse organisms (Figure 1). The region encompassing amino acids 63-73 forms the recognition sequence for the *ras*-specific monoclonal antibody, Y13-259, which is a diagnostic probe for the ras proteins (Furth, *et al.*, 1982; Sigal, *et al.*, 1986). In addition structure/function studies have identified amino acid 32-40 as being critical for biological activity (Sigal, *et al.*, 1986; Willumsen, *et al.*, 1986; Stone, *et al.*, 1988). Mutations of the nucleotides encoding this region disrupt biological activity of the proteins but do not affect the membrane localization or binding of guanine nucleotides. This region often referred to as the effector region, may interact with putative effector molecule(s). Recently a GTPase activating protein (GAP) has been identified which may represent a potential effector target for the ras proteins (Trahey and McCormick, 1987; Gibbs, *et al.*, 1988; Trahey, *et al.*, 1988). The GAP molecule stimulates the GTPase activity of the ras proteins by more than 100 fold (Trahey and McCormick, 1987; Gibbs, *et al.*, 1988). Mutations in the effector region that inhibit the biological activity of the ras proteins were found to not

respond to GAP stimulation of the intrinsic GTPase activity, suggesting that the interaction between GAP and the ras proteins is within the effector region of the ras proteins (Adari, *et al.*, 1988; Cales, *et al.*, 1988; McCormick, 1989). In addition antibodies that recognize the effector region of the ras proteins block the interaction with GAP (Rey, *et al.*, 1989).

1.2.4. Molecular model for ras protein activation

Oncogenic ras proteins are often described as activated ras proteins because of their ability to transform cells. The biological activation of the ras proteins can occur by at least two mechanisms which are both consistent with the G-protein hypothesis of ras function. Point mutations at codons 12, 13, 59 or 61 of the ras protein result in an activated protein that can transform a variety of cell types (Barbacid, 1987; Bos, 1989). These mutations block the ability of GAP to stimulate the GTPase activity of the activated ras protein resulting in a protein that is in a constitutively active GTP-bound state (Trahey and McCormick, 1987; Vogel, *et al.*, 1988). The increased expression of a normal *ras* gene can also produce the transformed phenotype (Chang, *et al.*, 1982a; Santos, *et al.*, 1983), presumably by increasing the levels of the active GTP-bound form of the ras protein. The overexpression of GAP can inhibit the morphological transformation of NIH3T3 cells that have increased *ras* expression, presumably by keeping the ras protein in an inactive GDP-bound configuration (Zhang, *et al.*, 1990a).

The GTP-bound form of the ras protein is required to promote neoplastic transformation of fibroblasts (Barbacid, 1987), maturation of *Xenopus* oocytes (Birchemeier, *et al.*, 1985) and differentiation of PC12 cells (Bar-Sagi and Feramisco, 1985; Satoh, *et al.*, 1987). In addition, mutants that preferentially bind GDP have a dominant negative effect on growth in mammalian cells and

S. cerevisiae (Feig and Cooper, 1988; Powers, *et al.*, 1989; Cai, *et al.*, 1990). Normally the ras proteins are predominantly in an inactive GDP-bound form. When an appropriate signal is recognized they are activated by the exchange of GDP for GTP, which stimulates a signal to a downstream effector(s). The active GTP-bound form of the protein is a transient intermediate since the biological activity of GAP cycles the protein back to the inactive GDP-bound state.

1.3 Physiological function of the ras proteins in yeast.

Genetic and biochemical analyses show that *S. cerevisiae RAS1* and *RAS2* genes regulate intracellular levels of cAMP by acting as positive regulators of adenylyl cyclase (Broek, *et al.*, 1985; Toda, *et al.*, 1985; Tamanoi, 1988). The *RAS1* and *RAS2* gene products appear to be biologically equivalent, although differential translational and transcriptional control mechanisms determine under what physiological conditions each will be expressed (Breviario, *et al.*, 1986; Breviario, *et al.*, 1988). The CDC25 gene product is involved in the regulation of the cAMP pathway in yeast by acting upstream of RAS (Broek, *et al.*, 1987; Robinson, *et al.*, 1987). The CDC25 gene is believed to encode a nucleotide exchange factor that regulates the amount of GTP bound to the ras protein. It remains to be determined if the RAS protein activates adenylyl cyclase directly or through an intermediary protein such as the adenylyl cyclase-associated protein (Field, *et al.*, 1990). Recently two genes, *IRA1* and *IRA2*, have been identified that function upstream of the *RAS* genes (Tanaka, *et al.*, 1990a; Tanaka, *et al.*, 1990b). The *IRA1* and *IRA2* gene products are similar to mammalian GAP and may be involved in the down-regulation of the yeast RAS proteins (Tanaka, *et al.*, 1990a; Tanaka, *et al.*, 1990b). Indeed, mammalian GAP can replace *IRA* function when expressed in *S. cerevisiae* (Ballester, *et al.*,

1989; Tanaka, *et al.*, 1990a). Although the major function of RAS in *S. cerevisiae* is to regulate cAMP metabolism, there is also genetic and biochemical data to suggest that the RAS proteins regulate other signalling pathways (Toda, *et al.*, 1987; Field, *et al.*, 1990).

1.4 Physiological function of ras proteins in higher eukaryotes

1.4.1 The involvement of ras proteins in cellular proliferation and differentiation

The mammalian *ras* genes are expressed in both immature dividing cells as well as certain differentiated cell types (Furth, *et al.*, 1987), indicating that they may have a role in both cellular proliferation and differentiation. The ability of the activated ras proteins to cause morphological transformation and unregulated growth control suggests that they are involved in the transmission of mitogenic signals. The microinjection of ras proteins into quiescent NIH3T3 cells stimulated DNA synthesis (Feramisco, *et al.*, 1984; Stacey and Kung, 1984). In accordance with these results, microinjection of the *ras*-specific monoclonal antibody, Y13-259 blocked DNA synthesis in fibroblasts stimulated by growth factors (Mulcahy, *et al.*, 1985; Kung, *et al.*, 1986). Furthermore, the Y13-259 antibody was able to block the proliferative signal from a wide variety of growth factors suggesting that the ras proteins transduce mitogenic signals that originate from a number of different cell surface receptors and membrane associated proteins (Smith, *et al.*, 1986; Yu, *et al.*, 1988).

In addition, the ras proteins appear to be involved in the differentiation of certain cell types. Activated *ras* genes can induce the terminal differentiation of the PC12 rat pheochromocytoma cell line, as evidenced by neurite outgrowth (Spandidos and Wilkie, 1984; Bar-Sagi and Feramisco, 1985; Noda, *et al.*, 1985;

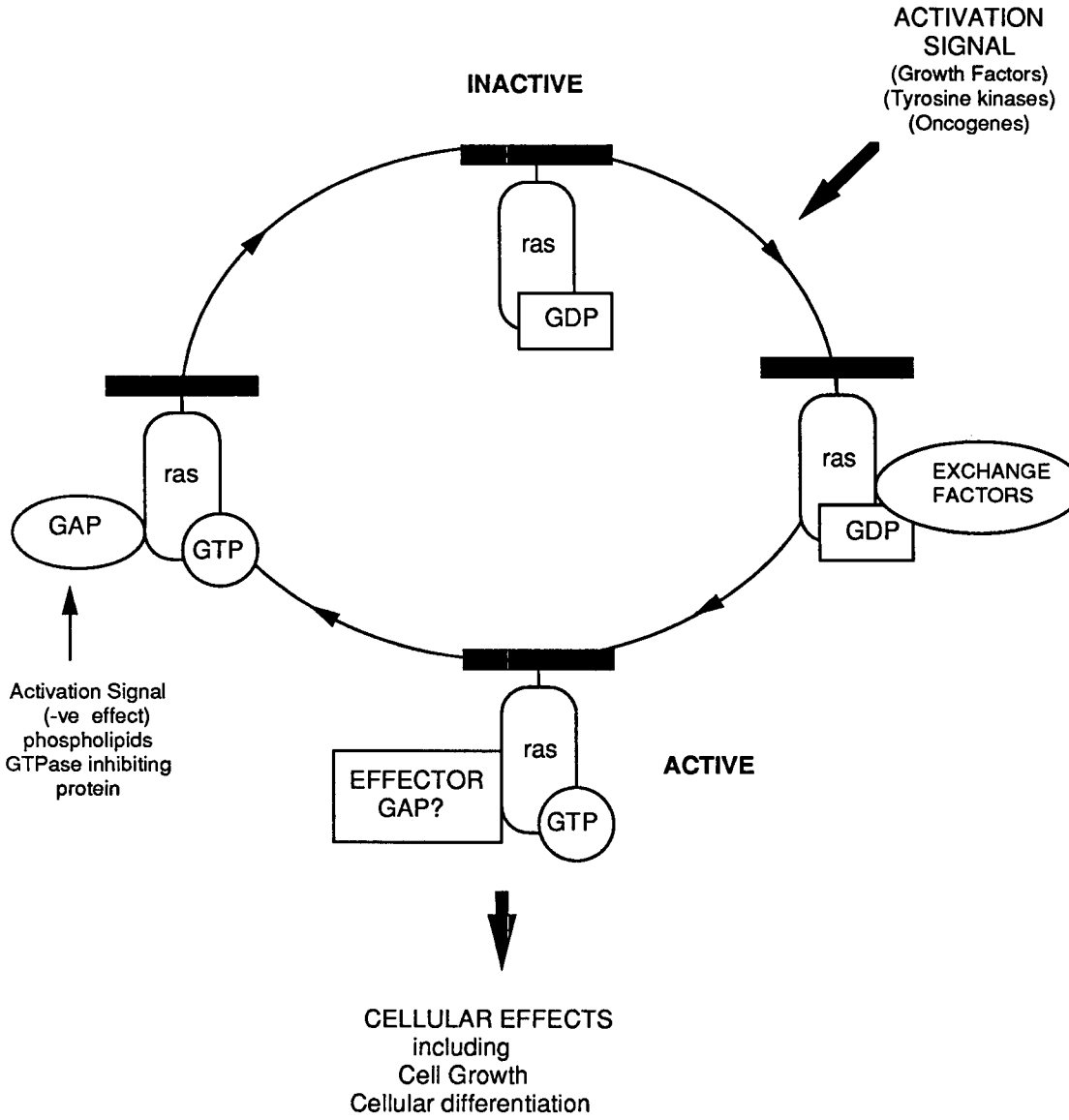
Satoh, *et al.*, 1987; Sassone, *et al.*, 1989). Nerve growth factor (NGF) and cAMP are both able to induce the differentiation process but they act through independent pathways. The microinjection of Y13-259 inhibits differentiation mediated by NGF but not by cAMP suggesting that the ras proteins may participate in the transduction of the NGF signal (Hagag, *et al.*, 1986). The ras proteins also promote the survival and fiber outgrowth of cultured embryonic neurons (Borasio, *et al.*, 1989). Induction of a differentiated phenotype by ras proteins has also been observed in a number of other cell types, suggesting that the ras proteins may fulfill a specialized role in cell type determination (for review see; Auersperg and Roskelley, 1990). Whether ras proteins have an effect upon differentiation or proliferation appears to be dependent upon the cell type and its differentiated state.

1.4.2 Ras proteins and signal transduction

Although the mammalian *ras* proteins can stimulate yeast adenylyl cyclase activity (Broek, *et al.*, 1985; DeFeo-Jones, *et al.*, 1985) it does not appear that their endogenous function is to regulate this activity (Beckner, *et al.*, 1985) and the search for an alternative function for ras proteins in mammalian cells is currently a very active area of investigation. A synopsis of the current proposals for the role of ras proteins in signal transduction is shown schematically in Figure 2.

Attempts have been made to characterize the upstream components of the ras signal transduction pathway that control the active GTP-bound state of the ras proteins. The amount of ras-GTP increases in cells that are treated with platelet derived growth factor (PDGF) or epidermal growth factor and in transformed cells carrying oncogene products with tyrosine kinase activity (Satoh, *et al.*, 1990a; Satoh, *et al.*, 1990b). These results suggest that receptors

Figure 2. A proposed model for the role of the mammalian ras proteins in signal transduction. Upon stimulation by the appropriate mitogenic signal ras protein exchanges GDP for GTP by the interaction with a specific nucleotide-exchange factor. The active membrane bound ras protein interacts with target molecules that result in pleiotropic cellular effects including cell growth and differentiation. The signal is turned off when GAP stimulates the intrinsic GTPase activity to convert ras-GTP to ras-GDP. The activity of GAP is negatively regulated by phospholipids that are produced as a consequence of mitogenic stimulation and by a specific GTPase inhibiting protein. GAP may also act as a downstream target for the activated ras protein, but it is probably not the only target molecule and therefore may not mediate all of the cellular effects. In activated *ras* transformed cells the GTP-associated form of the protein is favoured. See text for specific details.


ACTIVATION
SIGNAL
(Growth Factors)
(Tyrosine kinases)
(Oncogenes)
INACTIVE
ras
GDP
ras
EXCHANGE
FACTORS
GDP
ras
GAP
GTP
Activation Signal
(-ve effect)
phospholipids
GTPase inhibiting
protein
ras
EFFECTOR
GAP?
GTP
ACTIVE
CELLULAR EFFECTS
including
Cell Growth
Cellular differentiation

with tyrosine kinase activity may transduce a mitogenic signal via the ras proteins. Recently it has been demonstrated that the stimulation of T lymphocytes through the antigen receptor results in a rapid increase in the amount of ras-GTP (Downward, *et al.*, 1990a). The identification of nucleotide exchange factors that may act as positive regulators of ras-GTP levels may be involved in the transduction of mitogenic signals. Recently several mammalian proteins, ras guanine nucleotide exchange factor (rGEF), *ras*-exchange promoting protein (REP) and ras-guanine nucleotide-releasing factor (GRF), have been identified that promote the exchange of guanine nucleotides on the ras proteins and thus may be analogous to the yeast CDC25 gene product (Downward, *et al.*, 1990b; Huang, *et al.*, 1990; Wolfman and Macara, 1990). It is not known whether the three factors represent identical proteins. Very little is known about the control of the nucleotide exchange factors, however it is conceivable that their regulation is dependent on an activation signal.

GAP can act as a negative regulator of ras protein activity by greatly stimulating the endogenous GTPase activity of the protein and may be involved in the transduction of mitogenic signals by returning the protein to the inactive GDP-bound state. After stimulation of mammalian cells with a variety of growth factors and oncogene products, GAP is phosphorylated on tyrosine residues (Molloy, *et al.*, 1989; Ellis, *et al.*, 1990). GAP has also been shown to physically associate with activated PDGF receptors (Kaplan, *et al.*, 1990; Kazlauskas, *et al.*, 1990). The significance of these protein-protein interactions and the tyrosine phosphorylation of GAP is unknown, but it is conceivable that tyrosine kinases modulate ras activity through GAP by a yet to be determined mechanism. Phospholipids produced in cells as a consequence of mitogenic stimulation are able to inhibit GAP activity (Tsai, *et al.*, 1989; Yu, *et al.*, 1990), as well as increase the activity of a GTPase inhibiting protein (Tsai, *et al.*, 1990).

The interaction of lipids with these two regulatory proteins would tend to increase the biological activity of the ras proteins by favouring the association with GTP. In fibroblasts the overexpression of GAP suppresses neoplastic transformation induced by the ras proteins suggesting that GAP is a principal regulator of ras activity (Zhang, *et al.*, 1990a). Furthermore the increase in ras-GTP upon activation of the T lymphocytes appears to be due to an inactivation of GAP activity (Downward, *et al.*, 1990a).

The determination of downstream effects of ras signal transduction has been difficult because of the pleiotropic effects associated with *ras* transformation. Since cellular transformation and cellular proliferation are accompanied by the rapid metabolism of phosphatidylinositol it has been suggested that ras proteins regulate phospholipase C (PLC) activity. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylgylcerol (DAG). DAG activates protein kinase C, and IP_3 mediates the release of calcium from intracellular stores (Berridge, 1987). These second messengers are critical regulators of cellular proliferation (Berridge, 1987). Several groups have looked for changes in phosphatidylinositide (PI) turnover in activated *ras* transformed cells, but the results have been inconsistent. Some groups have reported elevated levels of IP_3, whereas others have observed no changes in PI turnover (Fleischman, *et al.*, 1986; Wakelam, *et al.*, 1986; Seuwen, *et al.*, 1988). However, a consistent observation has been that *ras* transformation is associated with elevated levels of DAG (Fleischman, *et al.*, 1986; Lacal, *et al.*, 1987a; Lacal, *et al.*, 1987c; Wolfman and Macara, 1987; Wilkison, *et al.*, 1989) and the subsequent activation of protein kinase C is necessary for the stimulation of DNA synthesis by ras proteins (Lacal, *et al.*, 1987b; Lloyd, *et al.*, 1989). Recent data suggests that the ras proteins specifically regulate an alternative pathway for the generation of DAG

(Lacal, *et al.*, 1987c; Price, *et al.*, 1989; Lacal, 1990), most probably by the activation of a phosphatidylcholine (PC) hydrolyzing PLC (Diaz-Laviada, *et al.*, 1990). In agreement, PC hydrolysis yields DAG molecular species that are distinct from those that originate from PI degradation (Bocckino, *et al.*, 1985; Pessin, *et al.*, 1990) and the fatty acid composition of DAG from *ras* transformants differs from that of cells with an increased PI turnover (Moscat, *et al.*, 1989). Interestingly, protein kinase C is not required for *ras*-induced morphological transformation suggesting that the activated ras proteins are involved in the transmission of more than one signal (Lloyd, *et al.*, 1989).

In addition to being an upstream negative regulator of ras protein activity, other evidence suggests that GAP may act as a downstream target (McCormick, 1989; Hall, 1990). Mutations in the effector region (amino acids 32-40) of the ras proteins prevent neoplastic transformation and also prevent the protein from interacting with GAP (Adari, *et al.*, 1988; Cales, *et al.*, 1988). Furthermore, it has been demonstrated that ras-GTP prevents the coupling of a G protein to the M_2 muscarinic acetylcholine receptor. This effect is prevented by GAP specific antibodies and ras antibodies that are known to block the interaction between GAP and the ras proteins, suggesting that GAP is necessary for the ras protein to act in this system (Yatani, *et al.*, 1990). The idea that GAP can act as a downstream target of ras in some systems is intriguing but it is unlikely that GAP is the sole target molecule for ras mediated signal transduction (Hall, 1990).

1.5 *Ras* gene superfamily

It is now evident that *ras* genes are part of a much larger superfamily and members of this superfamily have been found in a variety of organisms (Table 1) (Chardin, 1988; Sanders, 1990). Many of these genes were identified because

TABLE 1. The ras protein superfamily

Ras family		Rap family		Rho family		Rab family	
Mammalian	Other	Mammalian	Other	Mammalian	Other	Mammalian	Other
H-ras	ScRAS1	rap1A[b]	Dras3	rhoA	ScRHO1	rab1A	ScYPT1
K-ras	ScRAS2	rap1B	Ddrap1[a]	rhoB	ScRHO2	rab1B	ScSEC4
N-ras	SpRAS	rap2A	ScRSR1	rhoC		rab2	DdSAS1
	Dras	rap2B				rab3A	DdSAS2
	Ddras			rac1	ScCDC42	rab3B	
	DdrasG[a]			rac2		rab4	
R-ras	Dras2					rab5	
ralA						rab6	
ralB						rab7	
						BRL-ras	

[a] sequences described in this thesis
[b] also called Krev1 and smg21

Abbreviations: Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; D, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum*

Was adapted from Downward (1990)

they hybridize with nucleotide sequences derived from *ras* genes or because their encoded products bind GTP. These *ras*-related GTP-binding proteins appear to be involved in a wide range of biological processes. The major sequence conservation within the superfamily is the preservation of the four domains implicated in the binding of guanine nucleotides (Chardin, 1988; Sanders, 1990). There are additional regions of conservation and the individual members of the *ras* gene superfamily vary in the amount of this additional identity. The ral, R-ras and rap proteins are more closely related to the ras proteins with approximately 50% amino acid identity, whereas the rho, rac, and rab proteins have approximately 30% identity.

The function of the ral, R-ras and rap proteins is not known. However, it is interesting to note that the overexpression of the rap1A protein can morphologically revert the transformed phenotype associated with *ras*-transformed cells (Kitayama, *et al.*, 1989).

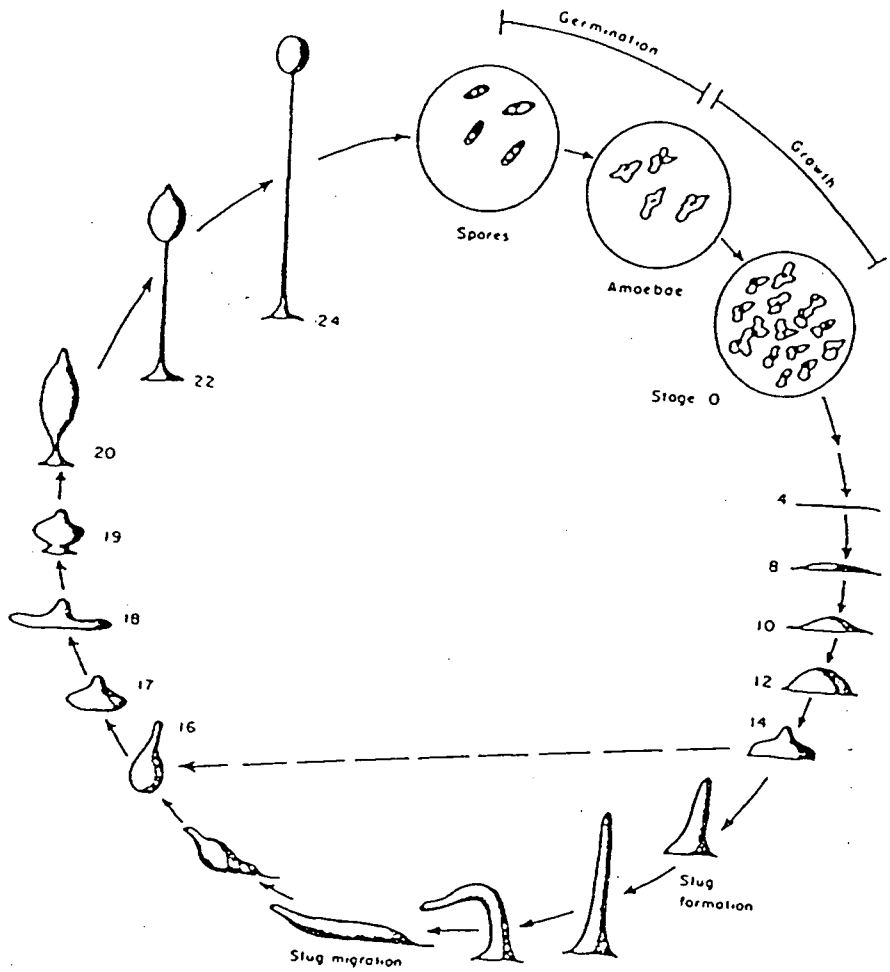
Experiments with the rho protein family in both yeast and mammalian cells suggest that they are involved in regulating some aspect of the cytoskeletal network that affects cell shape (Madaule, *et al.*, 1987; Paterson, *et al.*, 1990). The mammalian rab family of proteins are similar to the yeast YPT1 and SEC4 proteins. Genetic evidence demonstrates that the yeast YPT1 and SEC4 genes are essential genes involved in intracellular vesicle transport (Salmien and Novick, 1987; Segev, *et al.*, 1988). Since the *rab* genes can complement YPT1 deletions in yeast it is proposed that the rab protein family regulate intracellular trafficking of vesicles (Segev, *et al.*, 1988). The analysis of both the rho and rab protein families illustrates the diverse functions of the ras protein superfamily within eukaryotic cells.

1.6 *Dictyostelium discoideum*

1.6.1 Life Cycle

The cellular slime mould *Dictyostelium discoideum* is a useful developmental system for examining various aspects of signal transduction during cellular proliferation and differentiation. In this simple eukaryotic organism cell division and differentiation are mutually exclusive. The vegetative amoebae feed on bacteria and divide by binary fission as long as a bacterial food source is available. Upon starvation cell division ceases and a relatively simple developmental process occurs (Figure 3) (Loomis, 1975). With the onset of starvation, a few cells begin to secrete spontaneously the chemoattractant cAMP, attracting other cells which then gain the capacity to relay a synchronous pulse of cAMP. Cells adhere to one another by means of cell adhesion molecules forming aggregates of approximately 10^5 cells. Each aggregate forms a single 'tip' and elongates to form a pseudoplasmodium which then behaves as an organized multicellular individual and migrates over the substratum until conditions favour the formation of the final fruiting body. Within the migrating pseudoplasmodium differentiation into distinct prestalk cells at the anterior region and prespore cells at the posterior region become discernible. The final fruiting body consists of a sorus of mature spores, supported off the substratum by a fibrous stalk comprised of vacuolated stalk cells.

Figure 3. The life cycle of the cellular slime mould *Dictyostelium discoideum*. The stages of development are shown along with the times (hours) at which they occur after the onset of starvation. In the pseudoplasmodium, cell differentiation takes place into either pre-stalk cells in the anterior region or pre-spore cells in the posterior region. After approximately 24 hours the final fruiting body is formed and consists of a sorus of mature spores that are supported by a fibrous stalk comprised of stalk cells.

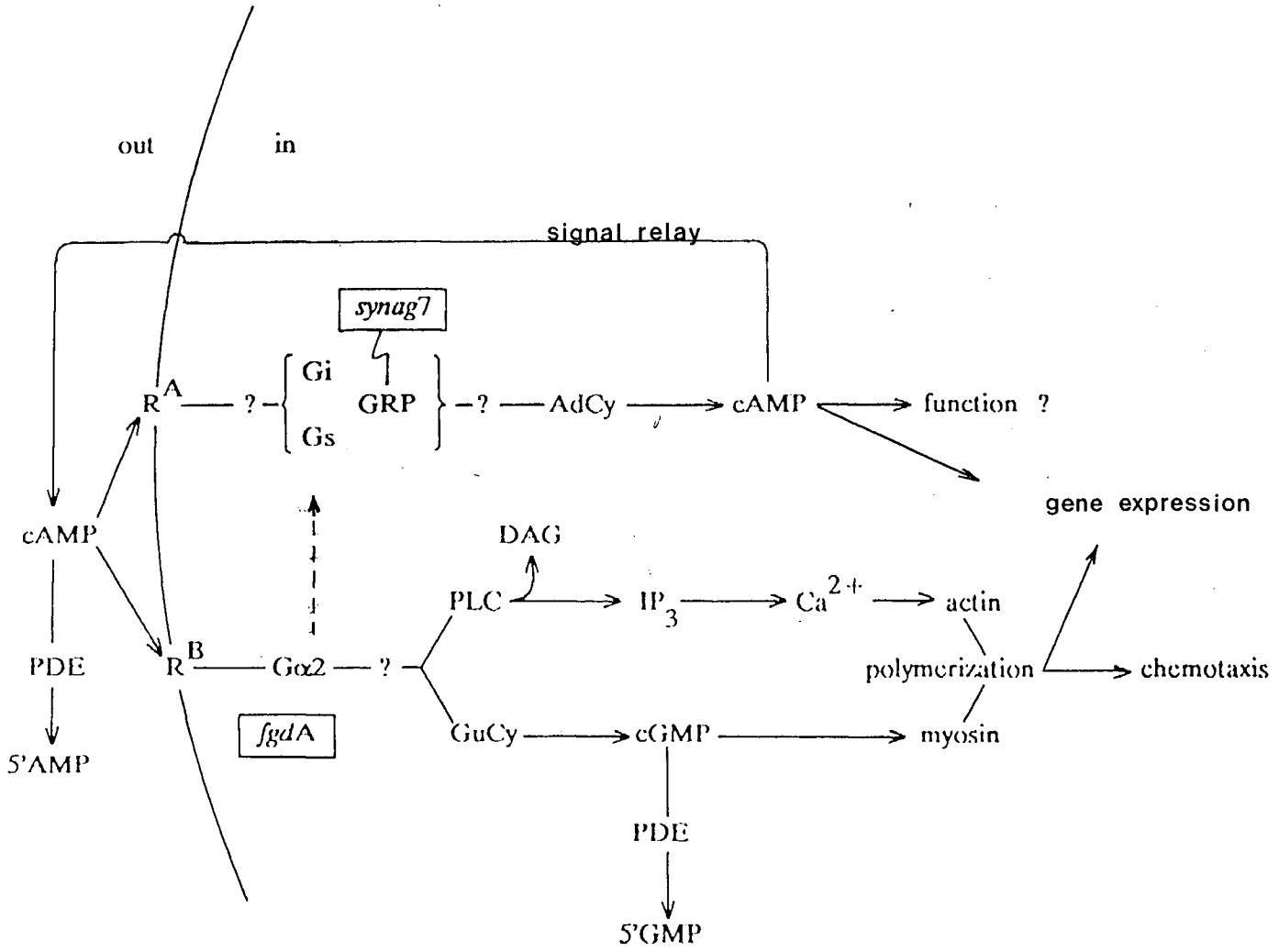


1.6.2. Signal transduction in *D. discoideum*

Differentiation in *D. discoideum* represents the simplest form of developmental regulation in that the original amoebae differentiate into either stalk cells or spore cells. The differentiation process is dependent upon G protein mediated signal transduction pathways which have a striking similarity to the signal transduction systems in higher organisms (Firtel, *et al.*, 1989). Signal transduction pathways regulate chemotaxis, aggregation and gene expression during the development of *D. discoideum.*

During aggregation cells respond chemotactically towards extracellular cAMP. The cAMP interacts with specific cell surface receptors which activate two well defined intracellular signal transduction processes (Figure 4) (Janssens and Van Haastert, 1987; Newell, *et al.*, 1987; Firtel, *et al.*, 1989). Kinetic analysis has determined that there are two classes of cAMP cell surface receptors defined by a fast or slow rate of dissociation of cAMP (Janssens and Van Haastert, 1987). The interaction of cAMP with the fast dissociating receptors (R_A) transiently activates adenylyl cyclase resulting in the synthesis and secretion of a pulse of cAMP into the extracellular medium. The secreted cAMP binds to the cell surface receptors on a nearby cell, which in turn pulse cAMP hence relaying the signal away from the initial cAMP secreting cell. This relay ensures the outward propagation of a wave of cAMP (Gerisch, 1987; Janssens and Van Haastert, 1987). The slow dissociating class of receptors (R_B) are coupled to the chemotactic response. The chemotactic response involves the activation of guanylyl cyclase resulting in a transient 5 to 10 fold increase in intracellular cGMP levels and in a 2 to 3 fold increase in IP_3 levels suggesting an involvement of phospholipase C (Janssens and Van Haastert, 1987; Newell, *et al.*, 1987; Newell, *et al.*, 1988). Chemotaxis results in the inward movement of the cells towards an aggregation

Figure 4. Model of cAMP-mediated signal transduction pathways in *D. discoideum* cells. cAMP binds to two forms of the cell surface cAMP receptor, R_A and R_B, that activate the signal relay pathway and the chemotaxis pathway respectively. The signal relay pathway is linked to a yet to be identified G protein and GRP (an associated protein) which act to stimulate adenylyl cyclase (AdCy) activity and the susbsequent secretion of cAMP. The receptor mediated activation of the chemotactic pathway is linked via the Gα2 protein, resulting in an increase in IP_3 levels and the subsequent mobilization of Ca^{2+} stores and increases in cGMP levels due to an activation of guanylyl cyclase (GuCy). Both of these intracellular messengers are involved in actin and myosin polymerization, resulting in chemotaxis. Both pathways appear to be involved in the regulation of certain classes of genes. The dashed arrow indicates the requirment of the chemotactic pathway for the actvation of the adenylyl cyclase pathway. The *Frigid A* mutants are defective in the chemotactic pathway and the *Synag 7* mutants are defective in the signal relay pathway. See text for the specific details on the two pathways and the appropriate references. The Figure has been adapted from one appearing in a recent review (Van Haastert, 1990).


out
in
signal relay
synag7
Gi
GRP
Gs
R^A
?
?
AdCy
cAMP
function ?
gene expression
cAMP
DAG
PLC
IP_3
Ca^{2+}
actin
PDE
R^B
Gα2
?
polymerization
chemotaxis
fgdA
GuCy
cGMP
myosin
5'AMP
PDE
5'GMP

center. Both cAMP-mediated responses become adapted upon binding of cAMP in that they no longer respond to a cAMP signal (Janssens and Van Haastert, 1987; Small, *et al.*, 1987). Developmentally regulated cell surface and membrane bound forms of cAMP phosphodiesterase degrade the extracellular cAMP and the lowered cAMP concentration results in the return of the cAMP receptor to the active state so that it can then respond once more to cAMP (Janssens and Van Haastert, 1987; Kessin, 1988). The phosphorylation and dephosphorylation of the cAMP receptor is probably essential in the adaptation and de-adaptation responses, respectively (Knox, *et al.*, 1986; Vaughan and Devreotes, 1988). This overall process ensures the outward propagation of a pulsatile cAMP signal transmitted every 6-7 minutes, whereas the pulsatile chemotactic response results in the inward movement of the cells towards the aggregation center.

A gene encoding a cAMP receptor has been cloned and the deduced amino acid sequence suggests that it has seven transmembrane-spanning regions, a structural motif similar to other G protein linked receptors such as rhodopsin and the β-adrenergic receptor (Klein, *et al.*, 1988; Saxe, *et al.*, 1988). The C-terminal region of the cAMP receptor contains multiple stretches of serine residues which are the proposed sites of ligand-induced phosphorylation (Klein, *et al.*, 1987; Klein, *et al.*, 1988; Devreotes, 1989). The cAMP receptor is essential to development since *D. discoideum* cells expressing an antisense cAMP receptor mRNA do not enter the developmental program (Klein, *et al.*, 1988; Sun, *et al.*, 1990). Several genes have been isolated that share a high degree of sequence identity to the cAMP receptor and thus may encode other cAMP receptor proteins (Devreotes, 1989; Saxe, *et al.*, 1990).

Biochemical, genetic and molecular evidence indicate that both R_A and R_B classes of cAMP receptor are regulated by heterotrimeric G proteins (Van Haastert, *et al.*, 1986; Van Haastert, *et al.*, 1987b; Snaar-Jagalska, *et al.*, 1988). At

present it is not known whether the different classes of cAMP cell surface receptors are products of different genes and/or whether they are coupled to different G proteins. Several putative G protein α-subunits have been cloned from *D. discoideum* (Kumagai, *et al.*, 1989; Pupillo, *et al.*, 1989). The Gα2 protein is coupled to the slow dissociating receptors (R_B) that activate phospholipase C and guanylyl cyclase (Figure 4) (Firtel, *et al.*, 1989; Kumagai, *et al.*, 1989). Mutations in the *Frigid A* complementation group are within the Gα2 gene or result in reduced expression of Gα2 and result in strains that do not aggregate, do not respond chemotactically towards cAMP, and do not relay the cAMP signal (Coukell, *et al.*, 1983; Firtel, *et al.*, 1989; Kumagai, *et al.*, 1989). Since the *Frigid A* strains do not relay the cAMP signal, the Gα2 protein may also be involved in the activation of adenylyl cyclase (Coukell, *et al.*, 1983). However, since non-hydrolyzable analogues of GTP are able to stimulate adenylyl cyclase in isolated membranes from the *Frigid A* strains, the direct activation of the signal relay pathway appears to involve an additional as yet unidentified G protein (Kesbeke, *et al.*, 1988; Snaar-Jagalska, *et al.*, 1988; Firtel, *et al.*, 1989).

1.6.3. Control of gene expression in *D. discoideum*

In addition to being the chemoattractant during aggregation, cAMP regulates the expression of a number of genes throughout development. During early development a class of genes are induced in response to pulses of cAMP including genes that encode proteins essential in the aggregation process, such as the cAMP receptor, Gα2, D2 (a serine esterase) and gp80 (cell surface adhesion molecule) (Gerisch, 1987; Firtel, *et al.*, 1989). *Synag 7* mutants which are defective in the receptor-mediated activation of adenylyl cyclase show a normal expression of the cAMP pulse induced genes. These genes are not expressed in strains carrying a mutation in the *Frigid A* complementation group which are

defective in the chemotactic signal transduction pathway suggesting that cAMP pulse-induced gene expression is mediated through the slow dissociating cAMP receptors, and thus is tightly linked to chemotaxis (Figure 4) (Kimmel, 1987; Mann, *et al.*, 1988; Firtel, *et al.*, 1989; Ma and Siu, 1990). However, recent studies suggest that a novel cAMP receptor that is pharmacologically distinct from the cell surface chemotactic cAMP receptor is involved in gp80 regulation (Ma and Siu, 1990). It remains to be determined whether this novel cAMP receptor regulates the expression of other cAMP induced genes.

In addition to inducing gene expression early in development there appear to be at least two classes of genes that are inactivated during early development in response to pulses of cAMP. The K5 gene is induced shortly after the initiation of starvation and is repressed during aggregation in response to pulsatile cAMP (Mann and Firtel, 1987). It appears that the cAMP-mediated repression of this gene is also mediated through the chemotactic signal transduction pathway (Mann, *et al.*, 1988). In contrast, the M4-1 gene is expressed during vegetative growth and then repressed during aggregation in response to pulsatile cAMP (Kimmel and Carlisle, 1986). The repression of this gene appears to be mediated by the signal relay response pathway, requiring the activation of adenylyl cyclase (Kimmel, 1987).

During multicellular development cAMP is also the agent that regulates the expression of a number of prestalk and prespore enriched mRNAs. However, in contrast to the cAMP pulse-induced genes, the induction of post-aggregative mRNAs require the continuous presence of high concentrations of cAMP (Chung, *et al.*, 1981). Pharmacological studies with cAMP analogues show that exogenous cAMP regulates these genes by acting through proteins with the same specificity as the cell surface cAMP receptor (Schaap and Van Driel, 1985; Haribabu and Dottin, 1986; Oyama and Blumberg, 1986). The high

concentrations of cAMP which can induce post-aggregative gene expression provide conditions which adapt the receptor-mediated responses that regulate early gene expression. These observations suggest the possibility that post-aggregative gene expression is mediated through a cAMP cell surface receptor that is not sensitive to adaptation. The recent discovery of multiple cAMP receptor genes that are expressed during multicellular development now raises the question of which cell surface receptor transduces the signal (Devreotes, 1989; Saxe, *et al.*, 1990). Interestingly, these putative cAMP receptor genes encode proteins that do not contain potential phosphorylation sites and thus are presumably not subject to adaptation (Saxe, *et al.*, 1990).

Inhibition of the activation of adenylyl cyclase has no discernible effect on post-aggregative gene expression suggesting that intracellular messengers other than cAMP modulate the expression of these genes (Schaap, *et al.*, 1986; Kimmel, 1987). Binding of cAMP to the cell surface receptor results in the elevation of IP_3 levels and the subsequent mobilization of intracellular calcium (Europe-Finner and Newell, 1986; Europe-Finner and Newell, 1987; Newell, *et al.*, 1988; Van Haastert, *et al.*, 1989). Both prestalk and prespore genes can be induced by DAG and IP_3 suggesting that protein kinase C and/or calcium may be the second messengers that regulate the expression of these genes (Kimmel and Eisen, 1988; Ginsburg and Kimmel, 1989). However, lithium, which inhibits cAMP-stimulated IP_3 formation and various calcium antagonists have a more pronounced inhibitory effect on the expression of the prespore genes suggesting that different signal transduction systems are utilized for cAMP receptor-mediated accumulation of prestalk and prespore mRNAs (Schaap, *et al.*, 1986; Blumberg, *et al.*, 1988; Pavlovic, *et al.*, 1988; Van Lookeren Campagne, *et al.*, 1988; Blumberg, *et al.*, 1989; Peters, *et al.*, 1989). The mechanism by which these

potential second messengers modulate cAMP induced gene expression is not yet known.

1.6.4 *Ras*-related genes in *D. discoideum.*

Evidence for *ras* genes and their encoded products in *D. discoideum* has come from two independent laboratories. Firtel and co-workers isolated a gene (Dd*ras*) that was preferentially expressed in the prestalk cells during the pseudoplasmodial stage of development. DNA sequence analysis revealed that the deduced amino acid sequence of the Ddras protein has approximately 65% identity to the mammalian ras proteins (Reymond, *et al.*, 1984). The Dd*ras* gene encoded two mRNAs (0.9 and 1.2 kb) that were differentially expressed during *D. discoideum* development. The 1.2 kb mRNA was detected at low levels in vegetative cells and disappeared rapidly upon the initiation of development. At the end of aggregation both mRNAs were detected reaching maximal levels during the pseudoplasmodial stage of development (Reymond, *et al.*, 1984). The expression of the 1.2 kb mRNA was induced by cAMP, a property characteristic of other prestalk enriched genes (Mehdy, *et al.*, 1983). Southern blot analysis suggested that there was only a single *ras* gene in *D. discoideum.*

In previous studies from the Weeks' laboratory, a major protein of 23,000 M_r (p23) was specifically immunoprecipitated from *D. discoideum* cell free extracts using the *ras*-specific monoclonal antibody, Y13-259 (Pawson and Weeks, 1984; Pawson, *et al.*, 1985). The relative rates of p23 synthesis were maximal during vegetative growth and early development with a sharp decline thereafter. However there was a small burst of synthesis during the pseudoplasmodial stage of development (Pawson and Weeks, 1984; Pawson, *et al.*, 1985). There was no specific degradation of p23 during differentiation suggesting that the decrease in synthesis of the ras protein was responsible for the reduction of p23 levels

that occured (Weeks and Pawson, 1987). In addition to p23 a minor protein of 24,000 M_r (p24) was also immunoprecipitated with the Y13-259 antibody from cell extracts of both vegetative and pseudoplasmodial cells (Pawson, *et al.*, 1985; Weeks and Pawson, 1987). There was no evidence for a precursor-product relationship between p23 and p24 suggesting the possibility that they are distinct gene products (Weeks and Pawson, 1987). Both p23 and p24 appeared to be membrane associated like the mammalian ras proteins (Weeks, *et al.*, 1987).

The Dd*ras* gene was maximally expressed during the pseudoplasmodial stage of development, whereas in contrast, there was a maximum level of *in vivo* ras protein synthesis in vegetative and early differentiating cells. The aim of this thesis was to explain these discrepancies and to try to understand more about the role of the *ras* genes in *D. discoideum* development.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Materials

All restriction endonucleases and modifying enzymes were purchased from BRL (Burlington, Ont. Canada) and all other chemicals were from either BDH (Vancouver, B.C., Canada) or Sigma Chemical Company (St. Louis, Mo. USA) unless otherwise specified in the text. All X-ray film XRP or XAR-5 was from Kodak, Canada. The oligonucleotides used were synthesized chemically on an Applied Biosystems 380A DNA synthesizer by T. Atkinson (Department of Biochemistry, U.B.C.).

2.2 Growth and development of *D. discoideum*

2.2.1 Growth

D. discoideum wild type strains, V12-M2 and NC4 were maintained on nutrient agar (SM agar plates: 10 g glucose, 10 g neutralized bacteriological peptone (Oxoid), 1 g yeast extract (Oxoid), 1.0 g $MgSO_4 \cdot 7H_2O$, 1.55 g $NaH_2PO_4 \cdot H_2O$, 1.0 g K_2HPO_4 and 20 g bacto-agar (Difco) per liter of deionized H_2O) in association with *Enterobacter aerogenes*. To produce a confluent lawn of vegetative *D. discoideum* cells, spores were innoculated into a fresh culture of *E. aerogenes* and 0.3 ml aliquots were plated on SM agar plates and incubated at 22°C for approximately 48 hours.

Basic stock cultures of the axenic *D. discoideum* strain, Ax-2 were also maintained on lawns of *E. aerogenes*.. For axenic growth, spores were

innoculated into HL5 medium (15.4 g glucose, 14.3 neutralized bacteriological peptone (Oxoid), 7.0 g yeast extract (Oxoid), 0.96 g $Na_2HPO_4 \cdot 7H_2O$ and 0.49 g $K_2H_2PO_4$ per liter of deionized H_20, pH 6.8) and the cultures were shaken at 100 rev/min at 22°C (Watts and Ashworth, 1970). Exponentially growing cells at a density of 2-4 x 10^6 cells/ml were passaged by an approximately 100 fold dilution into fresh HL5 medium. Under these conditions cells grew with a generation time of 8-9 hours. The axenic cells were passaged for approximately 1 month at which point fresh spores from the stock plates were used to innoculate HL5 medium.

2.2.2 Synchronous growth

Ax-2 cells were grown in HL5 medium to stationary phase (1.8-2.0 x 10^7 cells/ml). After approximately 12-16 hours in stationary phase, the cells were harvested by centrifugation at 700xg and washed once in HL5 media. The cells were then diluted in fresh HL5 media to a cell density of 1 X 10^6 cells/ml and incubated under normal growth conditions (Soll, *et al.*, 1976; Jimenez, *et al.*, 1986). Cell number was monitored by haemocytometer counting.

2.2.3 Development

To initiate *in vivo* development of V12-M2 and NC4, the vegetative amoebae were separated from the bacteria by four low speed centrifugations (700xg for 3 minutes) using potassium phosphate buffer (KK_2 buffer: 0.45 g K_2HPO_4 and 2.4 g KH_2PO_4 per liter of deionized H_2O resulting in solution containing 20 mM phosphate at pH 6.0). The washed cells were resuspended in KK_2 buffer and 10^8 cells were plated onto 4.0 cm membrane filters (Millipore, Corp) resting on support pads saturated with KK_2 buffer. The cells were incubated in a humid chamber at 22°C for the times indicated in the text. In

some experiments Bonner's salts (10 mM NaCl, 10 mM KCl and 2.0 mM $CaCl_2$) (Bonner, 1947) was used instead of KK_2 buffer without any appreciable effect on development.

For development of the Ax-2 strain, cells were grown axenically to a cell density of 2-4 x 10^6 cells/ml and then harvested by centrifugation at 700xg for 3 minutes. The cells were then washed twice in Bonner's salts and 2 x 10^7 cells were plated on filters resting on support pads saturated with Bonner's salts.

2.2.4 Separation of prestalk and prespore cells

The separation of prestalk and prespore cells was performed using either the continous Percoll gradient method of Ratner and Borth (1983) or a modification of this method utilizing a Percoll step gradient (Kwong, *et al.*, 1988). For both methods, pseudoplasmodia of the V12-M2 strain were obtained by allowing vegetative cells to differentiate for 16 hours on the surface of 2% non-nutrient agar plates, containing 5% Bonner's salts (Bonner, 1947). The pseudoplasmodia were harvested and filtered through a fine nylon mesh to remove undifferentiated cells and the prestalk and prespore cells were isolated as described in detail elsewhere (Kwong, *et al.*, 1990).

2.2.5 Differentiation in shake suspension

To initiate *in vitro* differentiation in suspension, vegetative V12-M2 cells were separated from the bacteria as described above. Washed cells were suspended at 5 x 10^7 cells/ml in KK_2 buffer and subjected to gyratory shaking at 250 rev/min. For cAMP pulse conditions, cAMP was added manually to a final concentration of 25 nM every 5 minutes for the length of the experiment. For continuous cAMP conditions, cells were shaken in the presence of 500 μM cAMP with additional cAMP added to a concentration of 100 μM every hour.

Samples were collected at the times indicate in the appropriate Figure legends. All of these samples were prepared in collaboration with M. Khosla and are described elsewhere (Khosla, *et al.*, 1990).

2.3 Isolation of nucleic acids from *D. discoideum*

2.3.1 DNA

To isolate genomic DNA, Ax-2 cells were grown to a cell density of 8×10^6 cells/ml and then harvested as described above. The nuclei were isolated as described by Cocucci and Sussman (1970) and the genomic DNA was extracted as descibed by Maniatis *et al.*, (1982).

2.3.2 RNA

Total RNA was isolated by a modification of a previously published procedure (Birnboim, 1988). Briefly, approximately 10^8 cells were resuspended in 4 mls of RES-1 buffer (0.5 M LiCl, 1 M urea, 1% SDS, 20 mM sodium citrate, 2.5 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetra-acetic acid, pH 6.8) to lyse the cells. Proteinase K was added to a final concentration of 50 µg/ml and the mixture was incubated at 50°C for 30 minutes. The lysate was extracted twice with an equal volume of phenol/chloroform (1:1) and the aqueous phase was then ethanol precipitated. After ethanol precipitation, the pellet containing both RNA and DNA was resuspended in 500 µl of RES-1 buffer. The RNA was selectively precipitated by the addition of an equal volume of 5 M LiCl/95% ethanol (3/2) and the mixture was stored overnight at 4°C to allow complete precipitation. The supernatant was removed after centrifugation for 15 minutes in a microfuge and the pellet was washed several times with 75% ethanol to remove traces of LiCl. The extraction of total RNA from separated prestalk and

prespore cells was as described above except the volumes were reduced proportionally to the number of cells isolated. For some experiments polyadenylated RNA was isolated by oligo d(T) cellulose chromatography (Aviv and Leder, 1972) as described by Maniatis *et al.*, (1982). RNA pellets were resuspended in diethylpyrocarbonate treated water, and the concentration of RNA was determined by A_{260} spectrophotometric analysis.

2.4 Isolation of cDNA and genomic DNA clones

2.4.1 Isolation of the DdrasG cDNAs

To isolate the *ras*-related cDNAs a λgt10 cDNA library prepared from mRNA isolated from growing cells of *D. discoideum,* kindly donated by Drs S. Rosejohn and W. Rowekamp, was plated (200,000 plaques) using the bacterial strain NM514 and blotted onto nitrocellulose filters as described by Maniatis *et al.*, (1982). The filters were prehybridized for 2-4 hours at 37°C in 30% formamide, 5X Denhardt's solution (1X Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 5X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50mM sodium phosphate buffer, pH 6.5, 0.5% SDS and 250 μg/ml of denatured salmon sperm DNA. The filters were then hybridized for 16 hours at 37°C in a solution containing 30% formamide, 5X SSC, 1X Denhardt's solution, 20 mM sodium phosphate buffer, pH 6.5, 0.5% SDS and approximately 3 x 10^6 cpm/ml of Dd*ras*-c1 insert that had been labelled using [α-^{32}P]dCTP (3000 Ci/mmol; ICN Pharmaceuticals Inc.) the random primer method (Feinberg and Vogelstein, 1983). All DNA fragments used for labelling were isolated on DEAE membranes (Schleicher & Schuell, NA45) according to the manufacturer's recommendations. The labelled probes were separated from unincorporated nucleotides by passage through a Sephadex G-25 spun column

(Maniatis, *et al.*, 1982), heat-denatured for 5 minutes, quenched on ice and then added to the hybridization solution. The filters were washed twice (30 minutes per wash) in 30% formamide, 3X SSC, 0.1% SDS at 37°C and then used to expose X-ray film for 12 hours. The filters were then washed twice (30 minutes per wash) in 40% formamide, 2X SSC, 0.1% SDS at 37°C and used to re-expose X-ray film for an additional 12 hours. Approximately 100 plaques that hybridized after the initial washes but failed to or showed reduced signals after the second higher stringency washes were selected and plaque purified by two additional rounds of screening. The original screening of the library was done by Dr. G. Weeks in the laboratory of Dr. J.G. Williams (I.C.R.F. England) and the plaques were then brought back to U.B.C.. DNA was isolated from liquid lysates of 11 of the λgt10 recombinant phage using DEAE Sephacel (Pharmacia) chromatography (Helms, *et al.*, 1987). The purified DNA was digested with *Eco*R1, separated on an agarose gel and then transferred to a nitrocellulose membrane (see Southern analysis). The filter was hybridized with the Dd*ras*-c1 probe as described above. Three of the recombinant cDNA clones (Dd*ras*G-c3, Dd*ras*G-c11 and Dd*ras*G-c23) were selected for further analysis since they had the strongest hybridization signal under the low stringency conditions. Characterization and analysis of these clones is described in Chapter 4.

2.4.2 Isolation of the Dd*rap*1 cDNA clones

Following the identification of the Dd*ras*G cDNAs, 44 of the originally isolated plaques that hybridized to the Dd*ras*-c1 cDNA under low stringency conditions were analyzed to determine if they represented other *ras*-related genes. DNA was isolated from liquid phage lysates using DEAE sephacel chromatography (Helms, *et al.*, 1987) and digested with *Eco*RI. The resulting fragments were size fractionated by electrophoresis through 0.8% agarose gels

and transferred to nitrocellulose filters (see Southern blot analysis). The filters were prehybridized and hybridized with randomly primed Dd*ras*G-c3 insert as described above. The filters were then washed under low stringency conditions; two times 20 minutes in 2X SSC, 0.1% SDS at 50°C. After exposure to X-ray film for 12-16 hours the filters were then washed under high stringency conditions; two times 20 minutes in 0.1X SSC, 0.1% SDS and re-exposed to X-ray film. The filters were then reprobed with the Dd*ras*-c1 cDNA and washed using an identical protocol. One clone c51 hybridized to both the Dd*ras*G-c3 and Dd*ras*-c1 cDNAs under low but not under high stringency conditions and was selected for further study (a summer student, V. Suttorp helped with the analysis of the 44 recombinant phage). The c51 clone (Dd*rap*-c51) was used to isolate five additional Dd*rap*1 cDNA clones under the high stringency conditions from a λgt11 cDNA library, prepared from mRNA from *D. discoideum* cells at 3-4 hours of development (kindly provided by Dr. P. Devreotes).

2.4.3 Isolation of Dd*ras*G genomic clone

The isolation of the genomic clones was done in collaboration with Dr. J.G. Williams' laboratory (ICRF, England). Purified Dd*ras*G-c3 insert was sent to Dr. Williams where it was labelled and used to screen a genomic library (Pears, *et al.*, 1985) prepared from total nuclear DNA, partially cleaved with *Sau*3A to give an average size of 5kb and ligated into the *Bam*H1 site of the pBR322 plasmid derivative, pAT153 (Maniatis, *et al.*, 1982). The ampicillin resistant colonies were screened by the modified method of Grunstein and Hogness (1975) as described in Maniatis *et al.*, (1982). The genomic library was stored between nitrocellulose filters in the presence of glycerol at -70°C (J. Williams, personal communication). Ten regions of the filters that hybridized to the Dd*ras*G-c3 cDNA were cut out of the master storage filters and sent to us at U.B.C.. The

filters containing the potential Dd*ras*G genomic clones were diluted in LB medium (10 g Bacto-tryptone (Difco), 10 g NaCl and 5 g yeast extract (Difco) per liter of H_2O) and plated as single colonies on LB agar (LB plus 1.2% Bacto-agar (Difco)) containing 50 µg/ml of ampicillin and screened with the Dd*ras*G-c3 cDNA as above. Eight of the ten potential positives clones hybridized to the Dd*ras*G-c3 under high stringency conditions. Partial restriction maps of the eight clones were obtained by digestion with a wide range of enzymes and separation by agarose gel electrophoresis. The Dd*ras*G genomic clones were analyzed by Southern blot analysis (see Southern blot analysis) using the Dd*ras*G-c3 cDNA and an oligonucleotide (oligonucleotide a) complementary to the 5' end of the coding region of the Dd*ras*G gene (Figure 16). The oligonucleotide was end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP (7,000 Ci/mmole; ICN Pharmaceuticals Inc.) and used to hybridize the Southern blots containing the genomic clones using the conditions described by Maniatis *et al.*, (1982). The filters were washed four times (five minutes per wash) in 6X SSC, 0.1% SDS at 42°C and used to expose X-ray film for an appropriate period of time.

2.5 Sequence determination of DNA and RNA

2.5.1 DNA sequencing

DNA from the Dd*ras*G and Dd*rap*1 λgt10 and λgt11 recombinant phages was isolated from liquid lysates, digested with *Eco*RI and then subcloned into M13mp18 (Yanisch-Perron, *et al.*, 1985) in both orientations. Three restriction fragments of the Dd*ras*G genomic clone (Dd*ras*G-g5.2) were purified by preparative gel electrophoresis using DEAE membranes and subcloned into M13mp18 or the Bluescript vector (Stratagene, La Jolla, USA) (Figure 15). The

600 bp *Eco*RI fragment was cloned into M13mp18 in both orientations. The 2.2 kb *Acc* I and the 1.8 kb *Bgl*II/*Eco*RI fragments were cloned into the Bluescript vector. From each clone smaller subclones containing shorter inserts were generated using the exonuclease III (Boehringer Manheim, Montreal, Canada) unidirectional digestion technique (Henikoff, 1984). Subclones containing inserts that differed in size by approximately 250 bp were selected for sequencing.

Single stranded DNA was isolated from each of the M13mp18 subclones (Messing, 1983) and from the various Bluescript recombinant clones using the VCS-M13 helper phage (Stratagene, La Jolla, USA) following the manufacturer's protocol. Approximately 0.5-1.0 μg of purified single stranded DNA or denatured double stranded DNA was sequenced using modified T7 polymerase (Sequenase, United States Biochemical Corp, Ohio, USA) following the manufacturer's protocol with [α–^{35}S]dATP (1000 Ci/mmol; NEN Dupont, Boston, USA) using either the M13 universal primer or specific oligonucleotides from known sequences as primers. The sequencing mixtures were analyzed on 6% denaturing (7M urea) polyacrylamide gels , following electrophoresis the gels were dried and used to expose X-ray film for 24-48 hours. The sequences obtained were recorded, aligned and analyzed using either the DNA strider program or PC Gene with help from J. Hewitt, Dr. R. McMaster and Dr. D. Theilmann.

2.5.2 RNA sequencing

The 5' end of the coding region of the Dd*rap*1 gene was obtained by sequencing the mRNA directly by a dideoxy RNA sequencing technique (Geliebter, *et al.*, 1986; Geliebter, 1988). Briefly, total RNA (10 μg) isolated from vegetative *D. discoideum* cells was annealed with 50 ng of end-labelled oligonucleotide complementary to nucleotides 84-107 (5'-

ATACCTTGAACAAATTGCACAGTC-3') (Figure 22) of the Dd*rap*1 gene at 45°C. The mixture was sequenced using 5 units of avian myeloblastosis virus (AMV) reverse transcriptase at 45°C and then analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. The 24 base oligonucleotide used for RNA sequence analysis was separated from incomplete products by electrophoresis on a 15% denaturing polyacrylamide gel, located by UV-shadowing and extracted from the gel by the crush and soak method (Atkinson and Smith, 1984).

2.6 Primer extension analysis

To map the sites of transcription initiation for Dd*ras*G, two oligonucleotide primers were synthesized that were complementary to nucleotides +1 to +30 (oligonucleotide a; 5'-ACCAACAATAACTAATTTGTATTCTGTCAT-3') and -19 to +4 (oligonucleotide b; 5'-TCATTTTTTTAAATTAAGATCTG-3') of the Dd*ras*G transcription unit (Figure 16). Approximately 100,000-300,000 cpm of end-labeled primer was incubated with 2 µg of mRNA isolated from vegetative, aggregative or pseudoplasmodial cells in a 5 µl reaction mixture containing 0.4 M NaCl, 40 mM PIPES (pH 6.8) and 1 mM EDTA for 90 minutes at 50°C. The annealed primer reaction mixture was diluted 1:10 into primer extension buffer (50 mM Tris-HCl pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 1 mM DTT, 1 unit/µl RNasin (Promega, Madison, Wi. USA) and 50 µg/ml actinomycin D (Sigma, St. Louis, USA) and was extended with either 5 units of AMV reverse transcriptase or with 100 units of modified murine leukemia virus reverse transcriptase (Superscript) per 50 µl reaction for 60 minutes at 42°C (Tsang, *et al.*, 1982), and the extension products were separated by electrophoresis on 6% denaturing polyacrylamide gels.

2.7 Southern and Northern blot analyses

2.7.1 Southern blots

D. discoideum genomic DNA was digested to completion with various enzymes according to the manufacturer's specifications and size fractionated by agarose gel electrophoresis in 1X TBE buffer (0.089 M Tris, 0.089 M boric acid and 2 mM EDTA). Following electrophoresis the gels were denatured by soaking in 1.5 M NaCl, 0.5 M NaOH for 30 minutes, followed by neutralization in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5 for 30 minutes and then transferred to nitrocellulose membranes (Schleicher and Schuell, 0.45 μm) overnight in 20X SSC by passive diffusion (Maniatis, *et al.*, 1982). The filters were baked for 2 hours at 80°C and then prehybridized and hybridized using the conditions described under clone isolation. After hybridization for approximately 16 hours with the desired probe the filters were washed twice (5 minutes per wash) in 2X SSC, 0.1% SDS at room temperature and then washed twice (20 minutes per wash) in 2X SSC, 0.1% SDS at 50°C to provide low stringency conditions. After the filters were used to expose X-ray film, they were washed under high stringency conditions, twice (20 minutes per wash) in 0.1X SSC, 0.1% SDS at 65°C and were then used to re-expose X-ray film. To reprobe the Southern blot filters they were washed for 20 minutes in 50 mM NaOH and then neutralized with several changes of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl. The filters were then prehybridized and hybridized with the desired probe as described above.

2.7.2 Northern blots

RNA samples for Northern blot analysis were adjusted to 50% formamide, 40 mM 3-(N-Morpholino)propanesulfonic acid pH 7.0, 10 mM sodium acetate, 1 mM EDTA and 6% formaldehyde, heat denatured for 10 minutes at 65°C and

then size fractionated on 1.25% formaldehyde-agarose gels (Lehrach, *et al.*, 1977). For visualization, 1 µl of a 1 mg/ml stock solution of ethidium bromide was added to each RNA sample before heating. The gels were washed several times in deionized water and then transferred to nitrocellulose membranes overnight in the presence of 20X SSC. The filters were prehybridized and hybridized using the conditions described under clone isolation, except that the prehybridization and hybridization buffers also contained 30 µg/ml polyadenylic acid. The filters were then washed under the desired conditions as described for the Southern blots. Before reprobing, Northern blots were washed twice in 30% formamide, 0.1X SSC, 0.1% SDS at 65°C. To determine whether the Northern blots contained equal amounts of RNA they were probed with the IG7 cDNA, which detects a mRNA expressed constitutively during *D. discoideum* differentiation (Jermyn, *et al.*, 1987).

2.8 *In vitro* translation and immunoprecipitation

Samples of polyadenylated mRNA (0.5 µg) were translated by mRNA dependent rabbit reticulocyte lysate prepared by the method of Pelham and Jackson (Pelham and Jackson, 1976). The ^{35}S-methionine translation products were immunoprecipitated with the *ras*-specific monoclonal antibody, Y13-259 (Furth, *et al.*, 1982), and the precipitates electrophoresed on a 10% SDS-polyacrylamide gel as described in detail elsewhere (Pawson, *et al.*, 1985).

2.9 Preparation of antisera with predetermined specificity to variable regions of the Ddras, DdrasG and Ddrap1 proteins

Three peptides were synthesized that corresponded to the deduced C-terminal variable regions of the Ddras, DdrasG and Ddrap1 proteins (Figures 11 and 24) by the peptide synthesis group at the University of Victoria. These peptides ($Ddras^{170-184}$, $DdrasG^{172-186}$ and $Ddrap1^{169-183}$) were coupled to the carrier protein keyhole limpet hemocyanin (KLH) through the cysteine residue of the peptides using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Biochemicals) (Lerner, *et al.*, 1981). Briefly, 5 mg of each peptide was coupled to 3 mg of KLH-MBS in phosphate buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 2.17 g $Na_2HPO_4 \cdot 7H_2O$, per liter of H_2O, pH 7.5) by stirring at room temperature for 3 hours. The mixture was passed through Sephadex-G75 and eluted with PBS to remove unreacted peptide, KLH recovery was monitored by A_{280} and the peak fractions were pooled.

New Zealand White rabbits (9-10 weeks old) were injected subcutaneously with 0.5 mgs of each of the peptide-KLH conjugate mixed 1:1 with complete Freund's adjuvant. Each rabbit received additional subcutaneous injections using 0.5 mgs of the appropriate peptide conjugate mixed 1:1 with incomplete Freund's adjuvant on days 14 and 28 (Lerner, *et al.*, 1981). Serum was collected from each of the rabbits 10 days after the innoculation on day 28. The antisera from the three rabbits were tested for their specificity by a standard enzyme-linked immunosorbent assay using microtiter plates coated with each of the peptides (10 ng) (Bizub, *et al.*, 1987). The plates were incubated with 2-fold serial dilutions of each of the serum samples and the amount of bound antibody was determined with alkaline phosphatase labelled goat anti-rabbit IgG antibody (Bio-Rad Laboratories, Mississauga, Canada), using disodium *p*-nitrophenyl

phosphate (Sigma, St. Louis, USA) as a substrate. Once it was determined that the rabbits had developed an immune response to each of the peptides they were boosted for a final time on day 56 by subcutaneous injections as described above and the rabbits were sacrificed on day 70-72. The serum was collected and stored at -70°C.

The peptide specific antibodies were purified from the antisera by peptide affinity chromatography according to the manufacturer's protocol. The Ddras$^{170-184}$, DdrasG$^{172-186}$ and Ddrap1$^{169-183}$ specific peptides (4 mg) were each coupled to 2 mls of activated thiol-sepharose 4B (Pharmacia, Montreal, Canada) in PBS pH 7.2 for 48 hours at room temperature with gentle agitation. Each of the peptide conjugated resins were passed through Bio-Rad disposable columns and the eluate was monitored at A_{210} to determine the amount of bound peptide. Approximately 80% of each of the peptides bound to the resin. The columns were precycled with 5 bed volumes of 0.1 M glycine pH 2.5 and re-equilibrated with 5 bed volumes of PBS pH 7.2. The three rabbit antisera (anti-Ddras$^{170-184}$, anti-DdrasG$^{172-186}$ and anti-Ddrap1$^{169-183}$) were passed through a 0.22 μm filter and then added to its respective peptide column. The columns were capped, rocked gently for 4 hours at room temperature and then washed with 10 bed volumes of PBS pH 7.2. The bound antibodies were eluted with 0.1 M glycine pH 2.5, 0.15 M NaCl and collected in 0.5 ml fractions. Each of the fractions was neutralized immediately by the addition of 50 μl of 1 M Tris-HCl pH 8.0. The fractions were monitored at A_{280} and the peak fractions were pooled and dialyzed against PBS pH 7.2 overnight at 4°C.

2.10 Preparation of antisera directed to Ddras, DdrasG and Ddrap1 glutathione S-transferase fusion proteins

A set of pGEX based vectors (Smith and Johnson, 1988) were designed that expressed the Dd*ras*, Dd*ras*$^{60-187}$, Dd*ras*G and Dd*rap*1 cDNAs as glutathione *S*-transferase (GST) fusion proteins in *E. coli*. Each of the cDNAs were cloned in the appropriate reading frame into the pGEX vectors as shown in Figure 28 and the correct constructions were verified by restriction endonuclease mapping and sequence analysis of the fusion junction (in collaboration with R. Pachal). The GST-Ddras$^{60-187}$, GST-DdrasG and GST-Ddrap1 fusion proteins were purified by a modification of a previously published procedure (Koland, *et al.*, 1990). Cultures containing *E. coli* cells harbouring each of the pGEX-Dd*ras*$^{60-187}$ pGEX-Dd*ras*G and pGEX-Dd*rap*1 vectors were grown to late log-phase in 500 mls of LB medium supplemented with 100 µg/ml of ampicillin at 37°C. Fusion protein expression was induced by the addition of IPTG to a final concentration of 1 mM. The pGEX-DdrasG and pGEX-Ddrap1 expressing cells were harvested by centrifugation at 4,500xg for 10 minutes 2 hours later. The pGEX-Ddras$^{60-187}$ expressing cells were harvested after a 4 hour incubation at room temperature following induction, since the GST-Ddras fusion protein was found to be more stable under these conditions (R. Pachal, unpublished observations). The cells were resuspended in 10 mls of buffer A (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 300 mM NaCl, 5 mM DTT, 1 mM PMSF and 1 mg/ml lysozyme) and incubated for 10 minutes on ice. Triton X-100 was added to a final concentration of 1%, the suspension sonicated for 15 seconds and then incubated on ice for an additional 5 minutes. The suspension was then centrifuged for 15 minutes at 15,000xg. The high speed supernatant was then mixed with 2 ml of reduced glutathione-agarose (Sigma, St. Louis, USA) in buffer B (20 mM Hepes pH 7.4, 25mM NaCl,

0.05% Triton X-100, 10% gylcerol (v/v)) and rocked gently for 1 hour at 4°C. The mixture was collected in a Bio-Rad 10 ml disposable column and washed with 5 mls of buffer B. The recombinant proteins were eluted with 5 succesive 0.5 ml volumes of buffer B supplemented with 5 mM reduced-glutathione (Sigma) and fractions containing significant protein concentrations were pooled and frozen at -70°C. The pooled fractions contained predominantly the recombinant fusion proteins as assayed by SDS-polyacrylamide gel electrophoresis (PAGE).

Approximately 100 µg of each of the three recombinant GST fusion proteins was mixed 1:1 with complete Freund's adjuvant and injected into New Zealand White rabbits subcutaneously. The injection schedule was identical to the peptide conjugate immunizations except complete Freund's adjuvant was used during each injection. The antibodies directed to the GST portion of the fusion protein were adsorbed from each of the antisera by incubation with purified GST coupled to glutathione-agarose. Similarily antibodies that recognized more than one of the recombinant proteins were removed by adsorption with the appropriate recombinant proteins coupled to glutathione agarose. Briefly, the GST-Ddras$^{60-187}$ antisera (10 mls) was incubated with glutathione-agarose beads coupled to the GST, GST-DdrasG and GST-Ddrap1 fusion proteins (1 ml of each) and rocked gently overnight at 4°C. Similar protocols were used for the GST-DdrasG and Ddrap1 antisera. The various antisera were then collected after centrifugation at 700xg and stored at -70°C.

2.11 Immunoblot analysis of the Ddras, DdrasG and Ddrap1 specific antisera

The specificity of the various polyclonal antisera was determined by immunoblot analysis with the recombinant GST fusion proteins. Crude lysates of *E. coli* cultures carrying the recombinant plasmids, pGEX-Dd*ras*, pGEX-

Dd*ras*G, pGEX-Dd*rap*1 and the parental pGEX plasmid were prepared after induction for 60 minutes with 1 mM IPTG at 37°C. The bacterial cells were harvested and the samples were heated at 90°C for 3 minutes in SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10 mM Tris-HCl (pH 6.8), 10% glycerol [vol/vol], 0.001% Bromophenol blue) and then subjected to electrophoresis using the SDS-polyacrylamide, discontinous buffer system described by Laemmli (1970). The vertical gels contained a 4.5% polyacrylamide stacking gel and usually a 10% resolving gel, prepared from a stock solution of 29.2% wt/vol of acrylamide and 0.8% wt/vol of *N-N'*-bis-methylene acrylamide. The separating gel buffer consisted of 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS and the stacking gel buffer contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. Both the separating and stacking gels were polymerized by the addition of 0.1% ammonium persulfate and 0.01% *N,N,N',N'*-tetamethyl-ethylenediamine. The electrophoresis buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. The solubilized protein samples were subjected to electrophoresis at 100 volts through the stacking gel and 130 volts through the separating gel. Prestained molecular weight standards (Bio-Rad Laboratories, Mississauga, Canada) were also electrophoresed for molecular weight determinations. After electrophoresis, the separated proteins were electroblotted onto nitrocellulose (Schleicher & Schuell, 0.45 μm) or Immobilon-P membranes (Millipore, Mississauga, Canada) for 1 hour at 90 volts in prechilled transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris) (Towbin, *et al.*, 1979).

The filters were reacted with the rabbit polyclonal antisera using the following protocols. The filters were blocked in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.5% Tween 20 and either 10% fetal calf serum or 5% non-fat dry milk for at least 1 hour at room temperature. The filters were then rinsed in wash buffer (TBS, 0.5% Tween 20) and then incubated with the desired

antibody solution (see appropriate Figure legends for dilutions) in TBS containing 0.5% Tween 20 and either 1% fetal calf serum or 0.5% non-fat dry milk for 1 hour at room temperature or for 16 hours at 4°C. The blots were then washed 4 times for 5 minutes each in wash buffer. Two detection systems were used. The washed blots were then incubated with an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Bio-Rad) diluted at 1:3000 in TBS/0.5% Tween 20 for 1 hour at room temperature. The blots were then washed as described previously and then developed for alkaline phosphatase activity in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 0.16 mg/ml 5'bromo-4'chloro-3'indoyl phosphate and 0.33 mg/ml Nitro Blue Tetrazolium for approximately 5-15 minutes. Alternatively, the washed blots were incubated with a horseradish peroxidase donkey anti-rabbit secondary antibody diluted to 1:30,000 in TBS containing 0.5% Tween 20 and 1% non-fat dry milk for 1 hour at room temperature. The blots were then washed as before and developed with the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Oakville, Canada) according to the manufacturer's specifications. The blots were then exposed to X-ray film for 1 minute. The detection method used is indicated in the appropriate figure legends.

CHAPTER 3

3.0 EVIDENCE FOR ADDITIONAL *RAS*-RELATED GENES IN *D. DISCOIDEUM*

3.1 Introduction

As stated in the general introduction Southern blot analysis of genomic DNA indicated that *D. discoideum* possessed a single *ras* gene, Dd*ras*. The Dd*ras* gene was predominantly expressed in pseudoplasmodial cells, although a small amount of Dd*ras* mRNA was also detected in vegetative cells (Reymond, *et al.*, 1984). In contrast, the maximal levels of ras protein was found in vegetative and early developing cells (Pawson and Weeks, 1984; Pawson, *et al.*, 1985; Weeks and Pawson, 1987). One explanation is that the vegetative Dd*ras* mRNA is extremely unstable but efficiently translated. Alternatively the high rates of ras protein synthesis in vegetative cells might be an artifact of the *in vivo* labelling technique. However, it is possible that like most other eukaryotic organisms, *D. discoideum* has multiple *ras* genes.

The experiments described in this chapter suggest the possibility that there are additional *ras*-related genes within the *D discoideum* genome that could account for the differences in the expression of the Dd*ras* mRNAs and ras protein synthesis during development.

3.2 Results

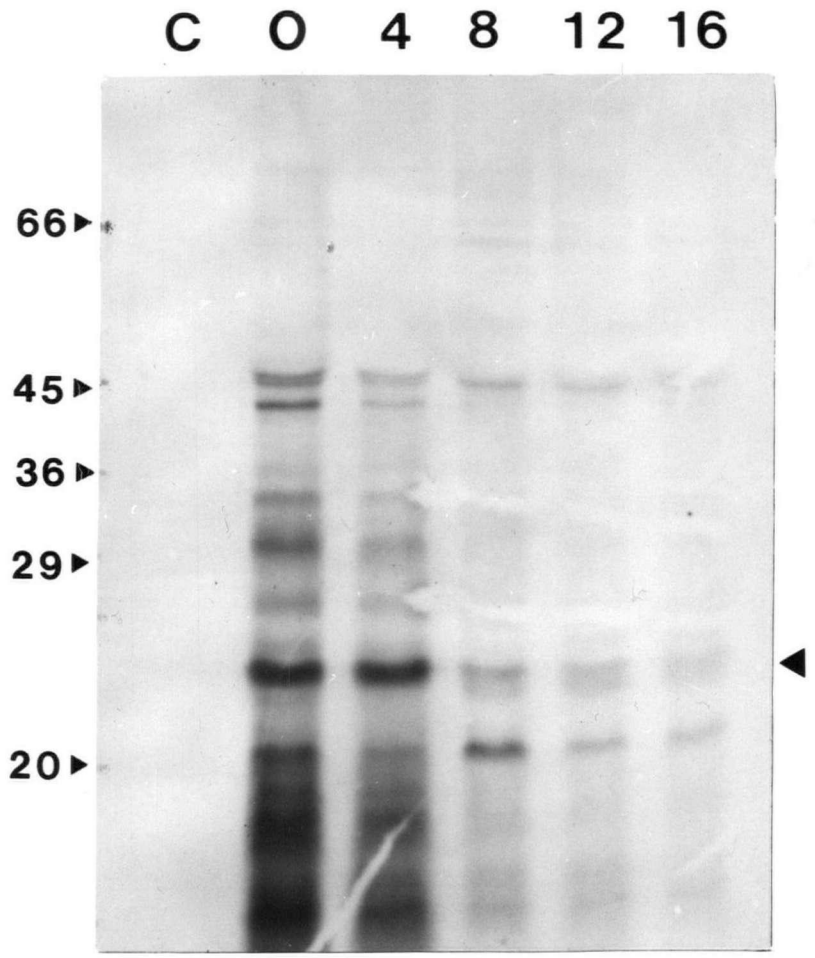
3.2.1 *In vitro* translation of *D. discoideum* mRNA from various stages of development

To address the concern that the high rates of ras protein synthesis were artifacts of the labelling procedure, polyadenylated mRNA was isolated from vegetative cells and from cells at various stages of development. The resulting mRNA was *in vitro* translated using the rabbit reticulocyte lysate system (Pelham and Jackson, 1976) and the resulting translation products were immunoprecipitated with the Y13-259 monoclonal antibody (Furth, *et al.*, 1982). Considerably more ras protein was translated from vegetative and early developmental mRNA relative to that from the subsequent stages of differentiation (Figure 5). These results more closely resemble the developmental pattern of the *in vivo* ras protein synthesis, however, the marked increase in ras protein synthesis during the pseudoplasmodial stage was more pronounced *in vivo* than *in vitro*. These results suggest that either the mRNA detected by the Dd*ras* cDNA (Dd*ras*-c1) is translated more efficiently in vegetative cells than in pseudoplasmodial stage cells, or that the vegetative and early developing cells contain an additional mRNA species expressed from a different *ras*-related gene.

3.2.2 Analysis of *ras* mRNA using the Dd*ras* cDNA under conditions of high and low stringency

Samples from the same preparations of mRNA used in the *in vitro* translation study were subjected to Northern blot analysis to determine if the Dd*ras*-c1 cDNA could detect an additional *ras* mRNA species from vegetative

Figure 5. *In vitro* translation of mRNA isolated from *D.discoideum* V12-M2 cells at various stages of development. Messenger RNA (0.5 μg) from the indicated developmental times (hours) was translated in a messenger-dependent rabbit reticulocyte lysate. The synthesized products were immunoprecipitated with the ras specific monoclonal antibody, Y13-259, and the immunoprecipitates were analyzed by electrophoresis using a 10% polyacrylamide gel. Lane C represents the products of *in vitro* translation in the absence of mRNA. The gel was treated with En^{3}Hance (NEN), dried and used to expose X-ray film for 5 days. Molecular weight standards (kd) are indicated by the small arrows and the large arrow indicates the position of the ras protein (p23/p24).



cells under low stringency conditions. When Northern blots containing mRNA isolated from various stages during development were hybridized with the Dd*ras*-c1 probe under conditions of high stringency (Figure 6a), the results confirmed the previously published analysis of Reymond *et al.*, (1984). However, when the same Northern blot filter was probed under low stringency conditions considerably more vegetative mRNA was detected, but there was no substantial increase in the amount of pseudoplasmodial mRNA detected (Figure 6b). The background signal on the blots remained relatively low and no other bands were apparent at low stringency. Since the relative intensities of the pseudoplasmodial mRNAs were not significantly altered under the low stringency conditions, the increased hybridization to the vegetative messages suggested the presence of a specific *ras* mRNA(s) in vegetative cells that is not encoded by the previously characterized Dd*ras* gene.

3.2.3 Analysis of genomic DNA using Dd*ras*-c1 under conditions of low stringency

Since additional *ras* mRNA species were detected in RNA samples isolated from vegetative cells using Dd*ras*-c1 under low stringency conditions, a Southern blot analysis of *D. discoideum* DNA should reveal evidence for additional *ras* genes if probed under the appropriate low stringency conditions. A Southern blot of *D. discoideum* genomic DNA, that had been digested with the restriction endonucleases *Eco*RI or *Hind*III was probed with Dd*ras*-c1 cDNA. Under high stringency conditions the Dd*ras*-c1 cDNA probe detected a genomic fragment of 5.5 kb when the DNA was digested with *Eco*R1 and a 7.0 kb fragment when digested with *Hind*III. These bands corresponded to those described by Reymond *et al.*, (1984). However, under low stringency conditions

Figure 6. Northern blot analysis of Dd*ras* mRNA expression during *D. discoideum* development. Samples (5 μg) of the same mRNA used in the experiment described in Figure 5 were separated on a 1.25% formaldehyde-agarose gel, transferred to nitrocellulose and then probed with randomly labelled Dd*ras*-c1 cDNA. The filter was washed under (A) high stringency conditions and (B) low stringency conditions (see Materials and Methods). The arrows indicate the positions and approximate sizes (kb) of the two mRNA species. The Dd*ras*-c1 cDNA was kindly provided by Drs. C. Reymond and R. Firtel.

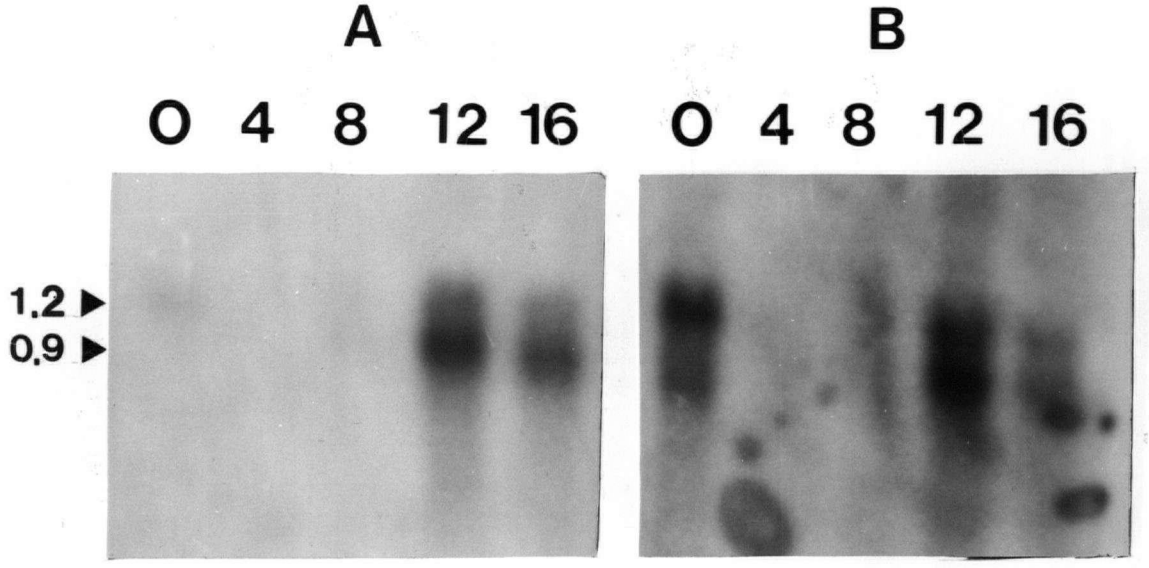
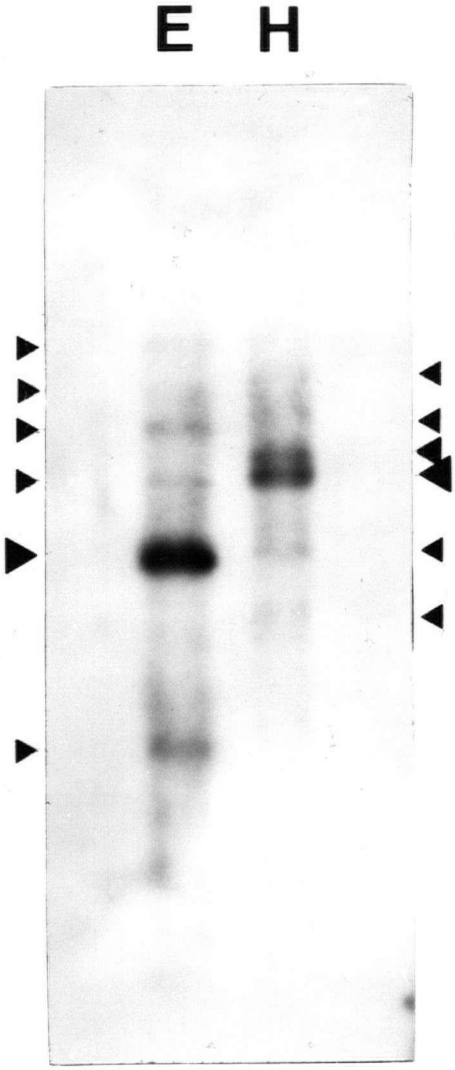


Figure 7. Southern blot analysis with the Dd*ras* cDNA. Genomic DNA (5 µg), from strain Ax-2, was digested with either *Eco*RI (lane E) or *Hind*III (lane H), size-fractionated by electrophoresis on a 0.8% agarose gel and then transferred to nitrocellulose. The filter was probed under low stringency conditions with labelled Dd*ras*-c1. The large arrows indicate the positions of strongly hybridizing fragments that were also detected under high stringency conditions. The small arrows indicate the positions of weakly hybridizing fragments.



several additional weakly hybridizing fragments were detected, suggesting the existence of multiple *ras*-related sequences in *D. discoideum* (Figure 7).

3.3 Discussion

The results described above suggest the possibilty that the high level of ras protein synthesis observed in vegetative cells is due to a *ras* gene, distinct from Dd*ras* and might explain why Reymond *et al.*, (1984) found the expression of Dd*ras* mRNA to be so variable in vegetative cells. When Reymond *et al.*, (1984) probed Southern blots of *D. discoideum* genomic DNA with Dd*ras*-c1 under high stringency conditions only single genomic fragments were observed with enzymes that did not cut within the portion of the gene complementary to the probe. When the Southern blots were probed with a 3.2 kb genomic clone that contained the Dd*ras* gene the same hybridizing fragments were observed plus a number of weakly hybridizing fragments (Reymond, *et al.*, 1984). The authors suggested that these weakly hybridizing bands could be attributed to a sequence in the genomic clone that has sequence similarity to a repeated sequence in the *D. discoideum* genome. This possibility was reasonable since the non-coding regions of *D. discoideum* genes contain long stretches of adenine and thymine nucleotide residues (Kimmel and Firtel, 1983). However, when the Dd*ras* cDNA was used to probe Southern blots under low stringency conditions at least five weakly hybridizing bands were detected in addition to the cognate gene (Figure 7). These weakly hybridizing bands could represent *ras* genes that have a sufficiently divergent nucleotide sequence to prevent hybridization under high stringency conditions or alternatively, represent less related members of the *ras* gene superfamily that have been isolated from other organisms (Chardin, 1988).

Since the Dd*ras*-c1 cDNA corresponds to the more divergent C-terminal half of the ras-related proteins, it is more likely that the additional fragments represent highly related *ras* genes. In addition, vegetative cells contain a mRNA that was efficiently translated into ras protein specifically immunoprecipitated by the *ras* specific monoclonal antibody Y13-259 (Figure 5). The fact that the Y13-259 monoclonal antibody is very specific for ras proteins (Furth, *et al.*, 1982) further suggests that that at least one of the weakly hybridizing genomic fragments represents a highly related *ras* gene.

CHAPTER 4

4.0 ISOLATION AND CHARACTERIZATION OF A SECOND *RAS* GENE FROM *D. DISCOIDEUM*

4.1 Introduction

The data presented in the previous chapter suggested that there was at least one additional *ras* gene in *D. discoideum* that could be detected by the Dd*ras* cDNA under low stringency conditions. In this chapter the isolation and characterization of cDNAs corresponding to a second *ras* gene from *D. discoideum* is described.

4.2 Results

4.2.1 Isolation of *ras*-related cDNAs

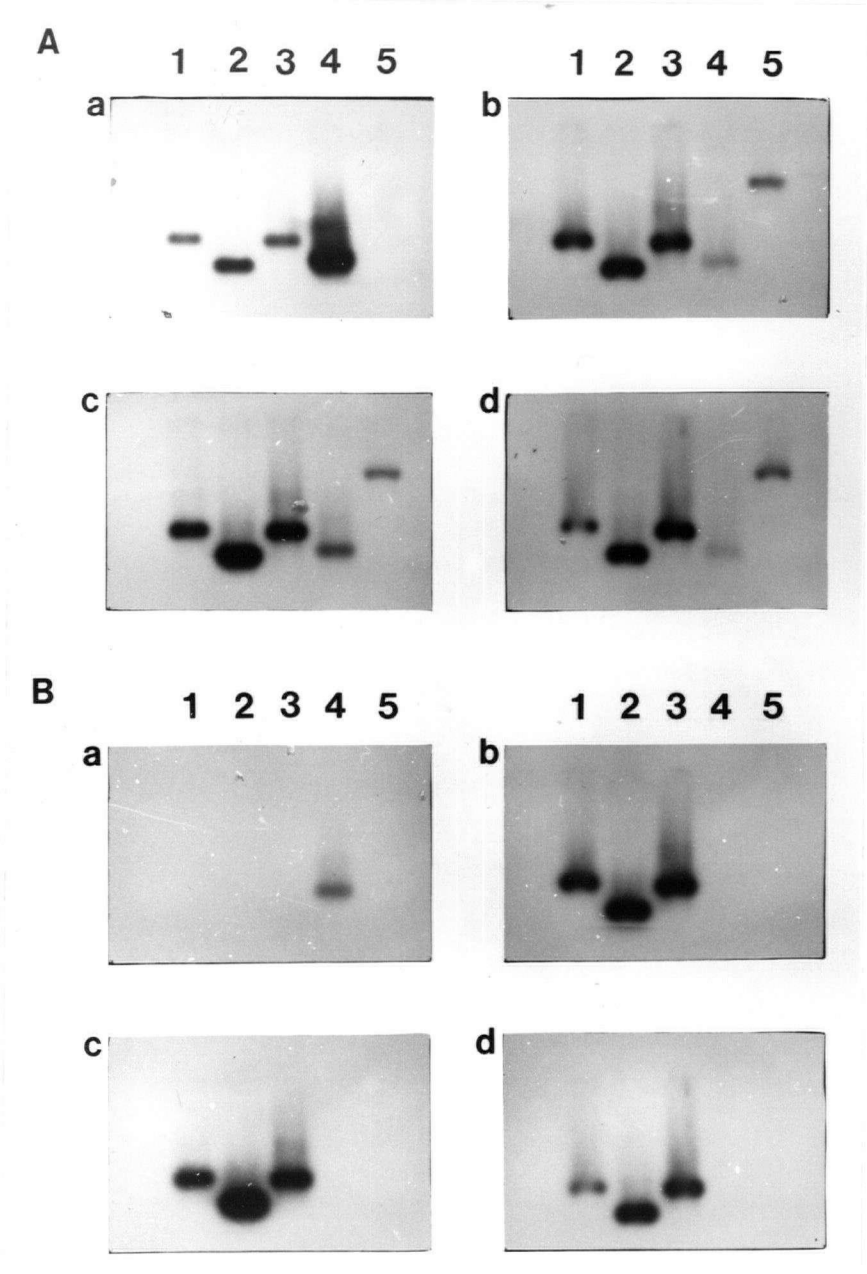
Since the Y13-259 monoclonal antibody is very specific for the ras proteins (Furth, *et al.*, 1982), this antibody could have been used to screen a cDNA expression library constructed from vegetative cell mRNA. However, although the antibody immunoprecipitated the ras proteins from *D. discoideum* cell-free extracts (Pawson and Weeks, 1984; Pawson, *et al.*, 1985), many additional proteins were also detected (Figure 5). The antibody also detected many cross-reacting proteins when used to probe Western blots of vegetative cell lysates. A similar result was obtained when the Y13-259 antibody was used to probe *S. cerevisiae* cell lysates by Western blot analysis (R. Deschenes personal communication). These results indicated that the Y13-259 antibody would have been insufficiently specific to screen a cDNA expression library.

The fact that the Dd*ras* cDNA detected additional bands on genomic Southern blots and a large amount of *ras* message in vegetative cells under low, but not under high stringency conditions suggested an alternative screening procedure. Recombinant cDNA clones isolated from a cDNA library made from vegetative cell mRNA that hybridized at low stringency but not under high stringency conditions should represent additional *ras*-related genes.

This screening procedure was initiated by Dr. G. Weeks while he was visiting Dr. J. G. Williams' laboratory (Imperial Cancer Research Fund, England). A λgt10 cDNA library prepared from mRNA isolated from growing cells of *D. discoideum*, (kindly provided by Drs. S. Rosejohn and W. Rowekamp, Heidelberg, Germany) was screened with the Dd*ras*-c1 clone under low stringency conditions. Approximately 100 clones were isolated that hybridized when the filters were washed under low stringency conditions but failed or showed a reduced hybridization signal when washed under high stringency conditions (see Materials and Methods).

These λgt10 isolates were then brought back to U.B.C. where they were plaque purified and rescreened with the Dd*ras*-c1 clone under low stringency conditions. Several recombinant phage with the strongest hybridization signals were selected. DNA from several of the recombinant phage and the Dd*ras*-c1 cDNA clone were digested with *Eco*RI, separated on an agarose gel and then transferred to a nitrocellulose membrane. The resulting filter was then hybridized with the Dd*ras*-c1 clone under both low and high stringency conditions. The Dd*ras*-c1 clone hybridized under both low and high stringency conditions, as expected, whereas the λgt10 recombinant clones only hybridized under low stringency conditions. Three of the λgt10 recombinant phage (c3, c11 and c23) were selected for further analyses.

Figure 8. Cross-hybridization between the c3, c11 and c23 cDNA clones and the Dd*ras*-c1 cDNA. The c3, c11, c23, Dd*ras*-c1 and yeast *RAS1* cDNA inserts were isolated and equal concentrations (25 ng) were size-fractionated by electrophoresis on agarose gels (lanes 1-5, respectively). The DNA was transferred to nitrocellulose membranes, and the filters were hybridized with either randomly labelled (a) Dd*ras*-c1, (b) c3, (c) c11 or (d) c23 cDNAs. (A) The filters were washed under low stringency conditions and used to expose X-ray film for 1 hour. (B) The filters were then washed under high stringency conditions and used to expose X-ray film for 4 hours (see Materials and Methods for the wash conditions).

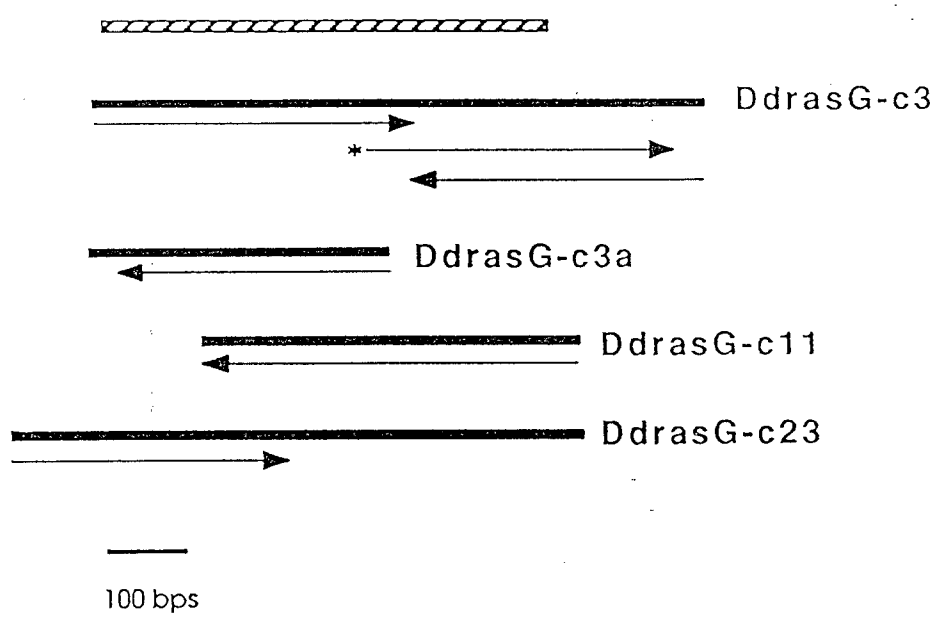


To address the question of whether the c3, c11 and c23 cDNA clones represented the same gene, the ability of the individual clones to cross-hybridize to each other was tested. Four Southern blots containing equal concentrations of the c3, c11, c23, Dd*ras*-c1 and Yeast *RAS1* cDNAs were hybridized with either the c3 clone, c11 clone, c23 clone or Dd*ras*-c1 clone (Figure 8). The blots were washed both under low stringency (Figure 8A) and under high stringency conditions (Figure 8B). With the exception of the yeast *RAS1* cDNA all of the clones hybridized under low stringency conditions irrespective of the cDNA that they were probed with (Figure 8A) confirming the previous results and indicating that they were closely related genes. The c3, c11 and c23 cDNAs hybridized to each other even under high stringency conditions (Figure 8B) suggesting that they were derived from the same mRNA and thus represented the same gene. Interestingly, only the c3, c11 and c23 detected the yeast *RAS1* cDNA providing further evidence that they represented an additional *ras* gene (Figure 8A).

4.2.2 Sequence analysis of the c3, c11 and c23 cDNA clones

Purified DNA from the three recombinant phages was subcloned into the M13 phage and the sequence was determined by the dideoxy chain termination method (Sanger, *et al.*, 1977) using modified T7 polymerase (Sequenase, United States Biochemical, Cleveland, Ohio). Sequence analysis revealed that all three clones were derived from the same gene confirming the cross-hybridization data. The strategy used to derive the sequence of the entire coding region of the gene is shown in Figure 9. The c3 cDNA was digested with *Hind*III yielding a 500 bp fragment which was then subcloned into M13mp18 to allow the complete sequencing of both strands. The nucleotide sequences of the three clones were

Figure 9. Sequencing strategy for Dd*ras*G using three independent cDNA clones, Dd*ras*G-c3, Dd*ras*G-c11 and Dd*ras*G-c23. Dd*ras*G-c3a is a subcloned fragment of Dd*ras*G-c3 obtained after cleavage at a *Hind*III restriction site. Arrowed lines indicate the direction and length of the portions of each clone that was sequenced. The hatched bar represents the single open reading frame within the Dd*ras*G gene. The region complementary to the synthetic oligonucleotide (nucleotides 300-320) that was used for confirmation of the nucleotide sequence is marked with an asterisk.



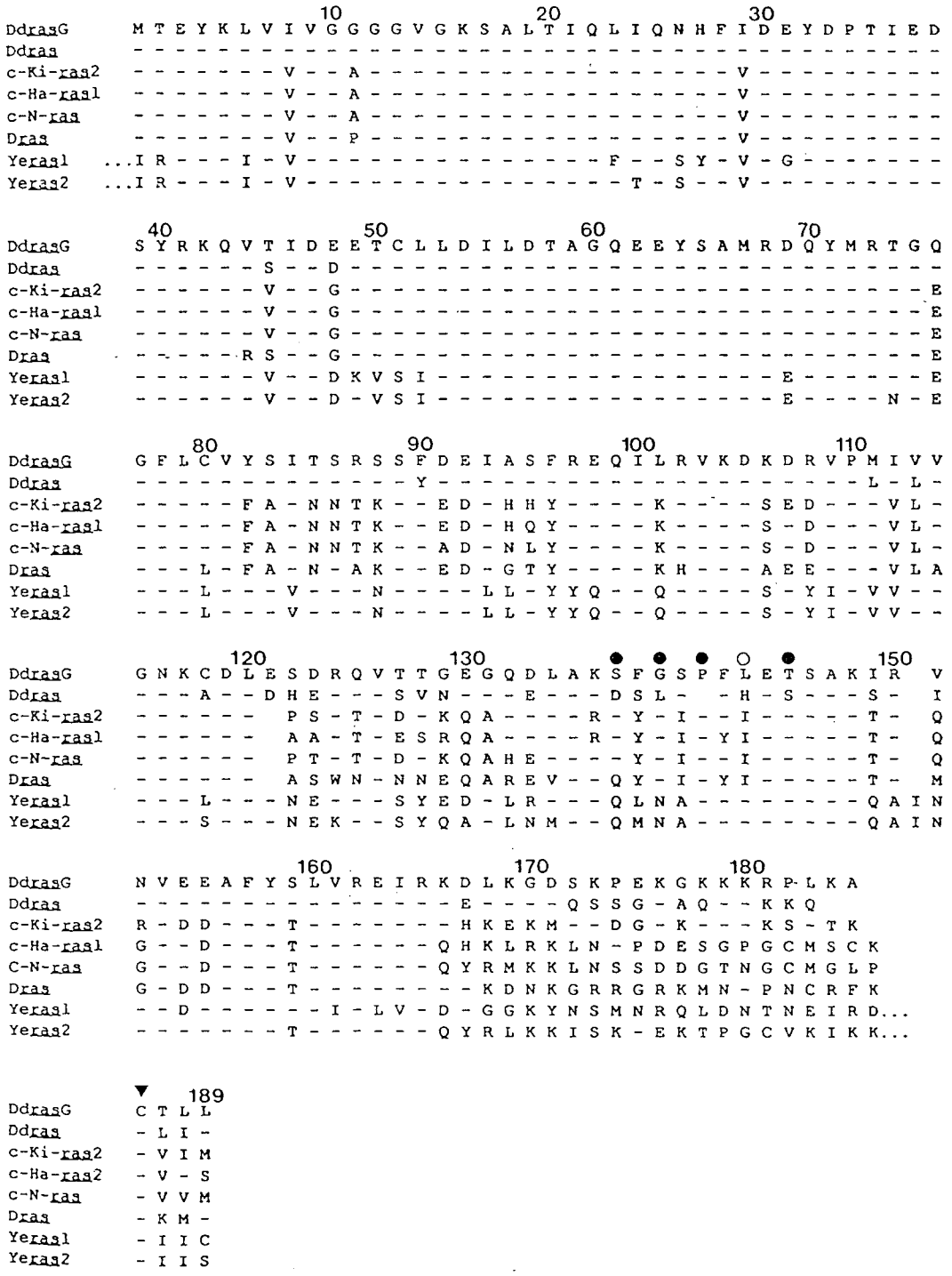
aligned to yield a total sequence of 907 nucleotides. The overlapping sequences of the three clones were identical suggesting that there were no sequence anomalies during the synthesis of the cDNAs. There was a single open reading frame of 570 bps and the approximately 800 bp c3 cDNA clone contained the entire coding region of the gene. The AUG intiating codon is preceded by a stretch of six adenine nucleotides which is common for many *D. discoideum* genes (Kimmel and Firtel, 1983). The coding region terminates with a UAA codon, the most preferred stop codon used in *D. discoideum* genes (Sharp and Devine, 1989). The 3' end of the c3 cDNA contained a long stretch of adenine nucleotides which could potentially represent the poly A tail, although non-coding regions of *D. discoideum* genes often contain long homopolymer stretches of adenine nucleotides.

The nucleotide sequence of the coding region of the isolated cDNAs is shown in Figure 10 along with the previously published sequence of Dd*ras*. There are numerous nucleotide differences along the entire length of the gene, suggesting that the two cDNAs were not derived from the alternative splicing of a single *ras* gene. The deduced amino acid sequence of the newly isolated gene is presented in Figure 11 and compared to the amino acid sequences of other ras proteins. The high degree of sequence conservation in comparison with other ras proteins established that the new gene is a novel *ras* gene, the second isolated from *D. discoideum*. This new gene was designated Dd*ras*G for reasons that will become evident later in this chapter. The protein encoded by the Dd*ras*G gene is 82% conserved relative to the Dd*ras* encoded product. The DdrasG protein shows a slightly higher degree of identity with the human ras proteins than does the Ddras protein (Figure 11, Table 2). The Ddras protein is 65% conserved relative to the three human ras proteins whereas DdrasG is 69% conserved.

Figure 10. Nucleotide sequence of the Dd*ras*G coding region and comparison with that of the one previously published for Dd*ras* (Reymond, *et al.*, 1984). The nucleotides are numbered relative to the start of the coding sequence. Differences in nucleotides between the two genes are denoted with an asterisk. Spaces have been introduced into the Dd*ras* sequence to maximize alignment. The Dd*ras*G sequence has been deposited in the EMBL/GenBank data base (accession no. J04160).

```
DdrasG ATGACAGAATACAAATTAGTTATTGTTGGTGGTGGTGGTGTCGGTAAAAGTGCCTTAACC 60
       ATGACAGAATATAAATTAGTTATTGTAGGTGGTGGTGGTGTTGGTAAAAGTGCATTAACA 60

DdrasG ATTCAATTAATCCAAAACCATTTCATTGATGAATACGATCCAACTATCGAAGATTCATAC 120
       ATTCAATTAATTCAAAATCATTTTATTGATGAATATGATCCAACAATTGAAGATAGTTAT 120

DdrasG AGAAAACAAGTTACCATTGATGAAGAAACTTGTTTATTAGATATTTTAGATACTGCTGGT 180
       CGTAAACAAGTTTCAATTGATGATGAAACTTGTTTATTAGATATTTTAGATACTGCAGGT 180

DdrasG CAAGAGGAATACTCTGCAATGAGAGACCAATATATGAGAACTGGTCAAGGTTTCCTTTGT 240
       CAAGAGGAATATAGTGCAATGAGAGATCAATATATGAGAACTGGTCAAGGATTTTTATGT 240

DdrasG GTCTACTCTATCACTTCAAGATCATCATTTGATGAAATTGCATCATTCCGTGAACAAATT 300
       GTTTATTCAATTACATCAAGATCATCATATGATGAAATTGCATCATTTAGAGAACAAATT 300

DdrasG CTTAGAGTTAAGGATAAGGATAGAGTACCAATGATTGTCGTTGGTAACAAATGCGATTTG 360
       CTAAGAGTTAAAGACAAAGATAGAGTACCATTGATTTTGGTTGGTAATAAAGCAGATTTG 360

DdrasG GAATCTGATCGTCAAGTCACAACTGGTGAAGGTCAAGATTTAGCAAAATCCTTCGGTAGT 420
       GATCATGAACGTCAAGTTAGTGTAAATGAAGGTCAAGAACTTGCAAAGGATTCATTGTCC 420

DdrasG CCATTCCTTGAAACCTCTGCCAAGATTCGTGTCAACGTTGAGGAAGCTTTCTATTCACTC 480
       ---TTTCATGAGTCATCTGCTAAAAGTAGAATTAATGTTGAAGAGGCATTTTACTCTTTA 477

DdrasG GTACGTGAAATCAGAAAAGACCTCAAGGGTGACTCTAAACCAGAAAAAGGCAAGAAGAAG 540
       GTTCGTGAAATTAGAAAAGAACTAAAAGGTGATCAATCAAGTGGCAAAGCTCAAAAAAAG 537

DdrasG AGACCATTAAAAGCTTGTACTCTTTTATAA                                570
       AAAAAACAA------TGTTTAATTTTATAA                                561
```

Figure 11. Comparison of the derived amino acid sequence of the DdrasG protein with the sequences of the other *D. discoideum* ras protein (Ddras), the three human ras proteins (Ki-ras2, Ha-ras1 and N-ras), a *Drosophila* ras protein (Dras), and the yeast RAS proteins (Yeras1 andYeras2). The DdrasG protein is numbered and the other ras proteins are aligned relative to it, with gaps inserted to maximize identity. A dash indicates identity with the DdrasG protein and only the amino acids that differ are indicated. The two series of dots represent the positions of additional residues present in the yeast RAS proteins. The triangle indicates the conserved cysteine residue, which is post-translationally modified. The circles in the region 137-145 indicate amino acids conserved between the DdrasG protein and the human ras proteins but differ in the Ddras protein. Solid circles indicate identity, while the open circle indicates chemical similarity. The sequence of the *ras* gene products have been taken from the following publications (Capon, *et al.*, 1983; McGrath, *et al.*, 1983; Neuman-Silberberg, *et al.*, 1984; Powers, *et al.*, 1984; Reymond, *et al.*, 1984). Amino acid are identified by the single letter code as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

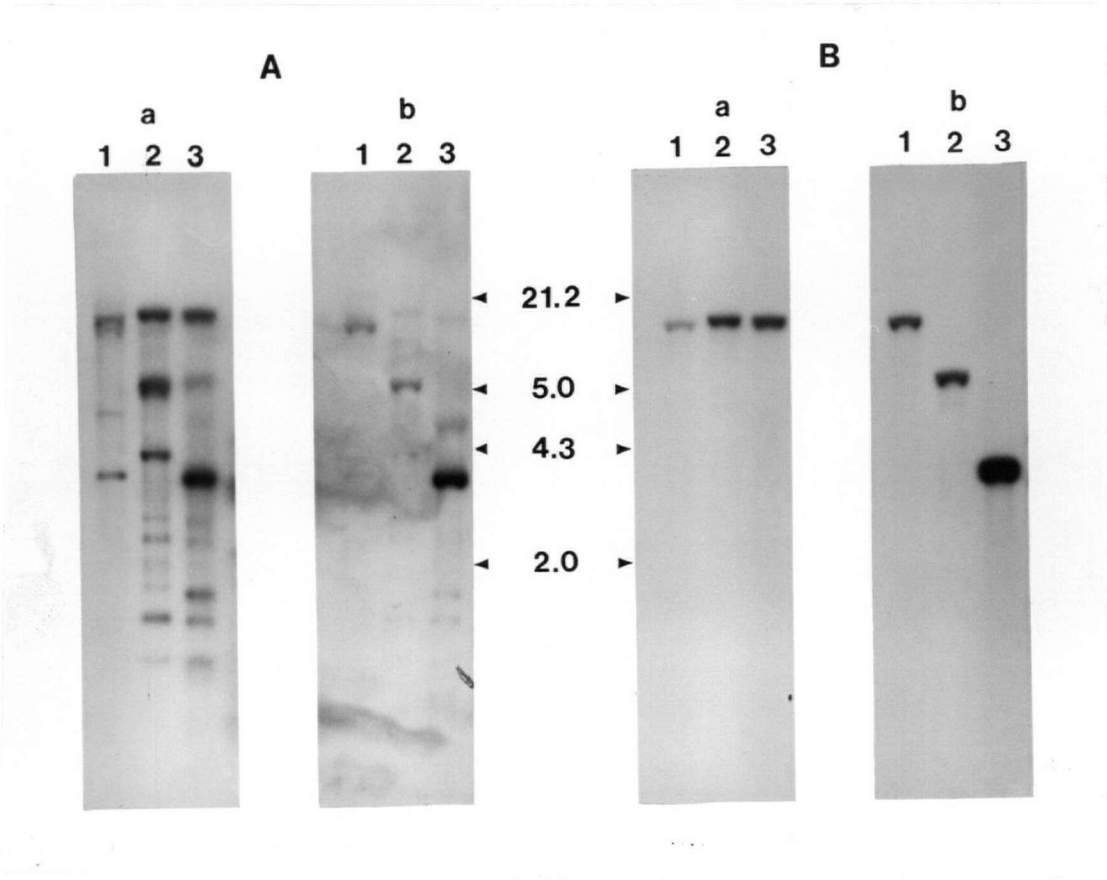

10 20 30
DdrasG M T E Y K L V I V G G G G V G K S A L T I Q L I Q N H F I D E Y D P T I E D
Ddras -
c-Ki-ras2 - - - - - - - V - - A - - - - - - - - - - - - - - - - V - - - - - - - - -
c-Ha-ras1 - - - - - - - V - - A - - - - - - - - - - - - - - - - V - - - - - - - - -
c-N-ras - - - - - - - V - - A - - - - - - - - - - - - - - - - V - - - - - - - - -
Dras - - - - - - - V - - P - - - - - - - - - - - - - - - - V - - - - - - - - -
Yeras1 ...I R - - - I - V - - - - - - - - - - - - - - F - - S Y - V - G - - - - - - -
Yeras2 ...I R - - - I - V - - - - - - - - - - - - - - T - S - - V - - - - - - - - -
40 50 60 70
DdrasG S Y R K Q V T I D E E T C L L D I L D T A G Q E E Y S A M R D Q Y M R T G Q
Ddras - - - - - - S - - D -
c-Ki-ras2 - - - - - - V - - G - E
c-Ha-ras1 - - - - - - V - - G - E
c-N-ras - - - - - - V - - G - E
Dras - - - - - - R S - - G - E
Yeras1 - - - - - - V - - D K V S I - - - - - - - - - - - - - - - - E - - - - - - E
Yeras2 - - - - - - V - - D - V S I - - - - - - - - - - - - - - - - E - - - - N - E
80 90 100 110
DdrasG G F L C V Y S I T S R S S F D E I A S F R E Q I L R V K D K D R V P M I V V
Ddras - - - - - - - - - - - - - - Y - L - L -
c-Ki-ras2 - - - - - F A - N N T K - - E D - H H Y - - - - K - - - - S E D - - - V L -
c-Ha-ras1 - - - - - F A - N N T K - - E D - H Q Y - - - - K - - - - S - D - - - V L -
c-N-ras - - - - - F A - N N T K - - A D - N L Y - - - - K - - - - S - D - - - V L -
Dras - - - L - F A - N - A K - - E D - G T Y - - - - K H - - - A E E - - - V L A
Yeras1 - - - L - - - V - - - N - - - - - L L - Y Y Q - - Q - - - - S - Y I - V V - -
Yeras2 - - - L - - - V - - - N - - - - - L L - Y Y Q - - Q - - - - S - Y I - V V - -
120 130 150
DdrasG G N K C D L E S D R Q V T T G E G Q D L A K S F G S P F L E T S A K I R V
Ddras - - - A - - D H E - - - S V N - - - E - - - D S L - - H - S - - - S - I
c-Ki-ras2 - - - - - - - P S - T - D - K Q A - - - - R - Y - I - - I - - - - - T - Q
c-Ha-ras1 - - - - - - - A A - T - E S R Q A - - - - R - Y - I - Y I - - - - - T - Q
c-N-ras - - - - - - - P T - T - D - K Q A H E - - - - Y - I - - I - - - - - T - Q
Dras - - - - - - - A S W N - N N E Q A R E V - - Q Y - I - Y I - - - - - T - M
Yeras1 - - - L - - - N E - - - S Y E D - L R - - - Q L N A - - - - - - - - Q A I N
Yeras2 - - - S - - - N E K - - S Y Q A - L N M - - Q M N A - - - - - - - - Q A I N
160 170 180
DdrasG N V E E A F Y S L V R E I R K D L K G D S K P E K G K K K R P L K A
Ddras - - - - - - - - - - - - - - E - - - - Q S S G - A Q - - K K Q
c-Ki-ras2 R - D D - - - T - - - - - - - - H K E K M - - D G - K - - - K S - T K
c-Ha-ras1 G - - D - - - T - - - - - - - Q H K L R K L N - P D E S G P G C M S C K
C-N-ras G - - D - - - T - - - - - - - Q Y R M K K L N S S D D G T N G C M G L P
Dras G - D D - - - T - - - - - - - - K D N K G R R G R K M N - P N C R F K
Yeras1 - - D - - - - - - I - L V - D - G G K Y N S M N R Q L D N T N E I R D...
Yeras2 - - - - - - - T - - - - - - Q Y R L K K I S K - E K T P G C V K I K K...
▼ 189
DdrasG C T L L
Ddras - L I -
c-Ki-ras2 - V I M
c-Ha-ras2 - V - S
c-N-ras - V V M
Dras - K M -
Yeras1 - I I C
Yeras2 - I I S

Both of the *D. discoideum* ras proteins show a higher degree of amino acid conservation with the human ras proteins than with the two yeast ras proteins (Figure 11, Table 2). Over the first 81 amino acids DdrasG differed from Ddras, H-ras1 and yeast RAS2 by only 2, 6, and 16 amino acids respectively, thus confirming the strong evolutionary conservation of the N-terminal region of all ras proteins (Figure 11). The C-terminal region of the ras proteins are more highly diverged and the DdrasG and Ddras proteins are only 59% identical over the last 100 amino acids. As would be expected, this is a significantly higher degree of conservation than is observed when the sequence is compared to that of ras proteins from other organisms. The DdrasG protein contains the characteristic cysteine residue four amino acids from the extreme C-terminus which in the mammalian and yeast ras proteins is involved in anchoring the proteins to the inner surface of the plasma membrane (Figure 11).

4.2.3 Southern blot analysis of the Dd*ras*G gene

To re-evaluate the number of *ras* genes in *D. discoideum*, Southern blots of genomic DNA, digested with enzymes that did not cleave within the coding region of either the Dd*ras* and Dd*ras*G genes, were probed with both the Dd*ras*-c1 and Dd*ras*G-c3 cDNAs. Under high stringency conditions the cDNAs detected single, unique fragments indicating that they had been derived from different genes that are present in a single copy within the *D. discoideum* haploid genome (Figure 12B). The Dd*ras* gene detected a 3.2 kb *Eco*RI-*Bgl*II genomic restriction fragment as previously published (Reymond, *et al.*, 1984), whereas the Dd*ras*G gene detected an approximately 8.0 kb *Eco*RI-*Bgl*II genomic restriction fragment. When hybridized under low stringency conditions the Dd*ras* and Dd*ras*G cDNA clones hybridized to their cognate genes, cross-

Figure 12. Southern blot analysis of the *D. discoideum ras* genes. Genomic DNA (5 μg) from the Ax-2 strain was digested with *Bgl*II (lane 1), *Eco*RI (lane 2) and *Bgl*II/*Eco*RI (lane 3), electrophoresed on a 0.8% agarose gel, and then transferred to nitrocellulose. The filter was probed with labelled Dd*ras*G-c3 (a) or Dd*ras*-c1 (b) inserts and the filters were washed under low stringency conditions (A) and then under high stringency conditions (B). The filters were used to expose X-ray film for 12 hours. Molecular sizes (kb) are shown in the middle.

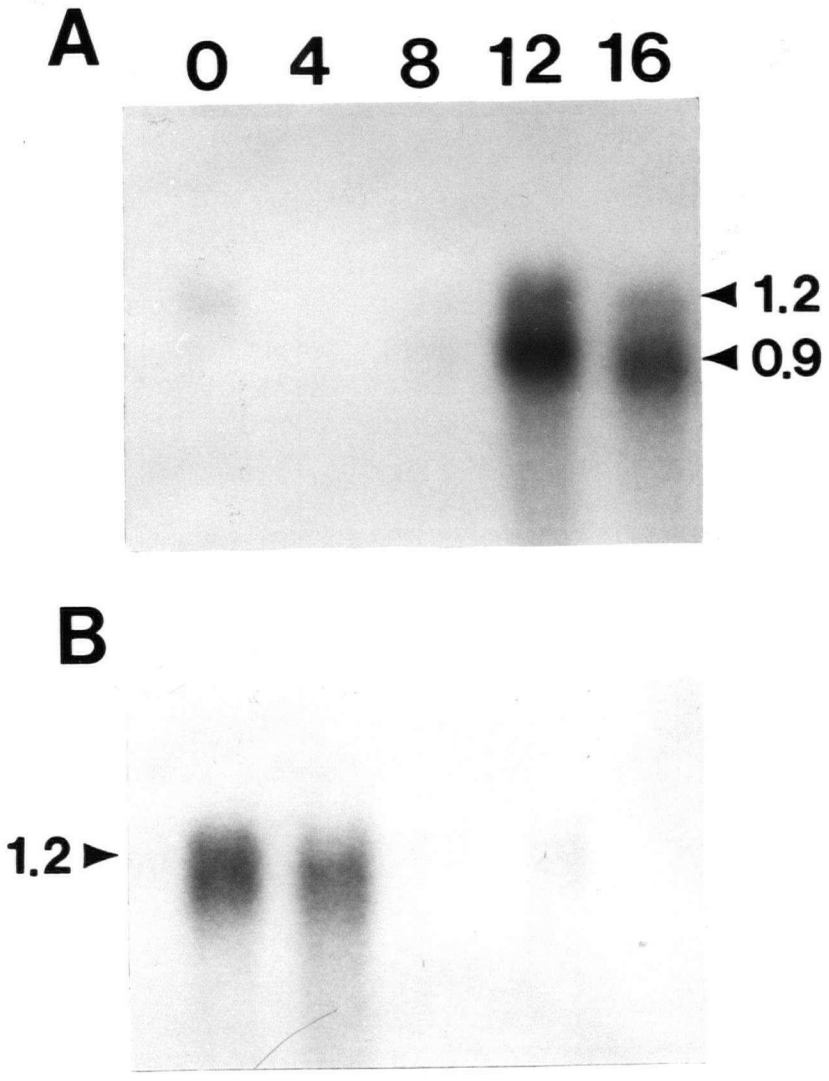


hybridized to one another, and also hybridized weakly to a number of other fragments (Figure 12A). These weakly hybridizing bands observed under low stringency conditions may represent other *ras* genes and/or *D. discoideum* homologues of the *ras* gene superfamily.

4.2.4. Developmental expression of Dd*ras*G

To examine the developmental expression of the Dd*ras*G gene, V12-M2 cells grown on bacteria were plated on filters to initiate development. Filters were harvested at 0, 4, 8, 12, 16 and 20 hours after the onset of development and polyadenylated RNA was isolated. The RNA samples were size fractionated on formaldehyde-agarose gels, transferred to nitrocellulose membranes and then probed with the Dd*ras*G-c3 or Dd*ras*-c1 clones. As shown in Figure 13A two transcripts of approximately 1.2 and 0.9 kb were detected with the Dd*ras*-c1 probe under the high stringency conditions used in the Southern analysis. Both mRNAs start to accumulate subsequent to multicellular aggregation (8 hours) reaching maximal levels during the pseudoplasmodial stage of development (12-14 hours). When the same RNA samples were analyzed with the Dd*ras*G cDNA a single mRNA of approximately 1.2 kbs was observed (Figure 13B). The Dd*ras*G transcript was expressed during vegetative growth (0 hours) and during early development (4 hours), with an abrupt drop in transcript levels thereafter. The developmental expression accounts for the gene designation Dd*ras*G (*Dictyostelium discoideum ras* gene expressed during Growth). Depending on the stringency of the hybridization conditions there was a low level of cross-hybridization of the Dd*ras* and Dd*ras*G genes, which was not surprising given their high degree of sequence identity. Reymond *et al.*, (1984) suggested that the Dd*ras* gene was expressed in vegetative cells on the basis of their Northern blot analysis. In addition, they reported the vegetative mRNA levels as being highly

Figure 13. Northern blot analysis of Dd*ras* and Dd*ras*G gene expression during *D. discoideum* growth and development. Poly(A)+ RNA (5 μg) from each of the indicated times after the onset of differentiation (hours) was separated on a 1.25% formaldehyde-agarose gel, transferred to nitrocellulose, and probed with Dd*ras*-c1 (A) or Dd*ras*G-c3 (B). The filters were washed under high stringency conditions and used to expose X-ray film for 16 hours. The approximate sizes (kb) of the mRNAs are indicated next to the arrowheads.



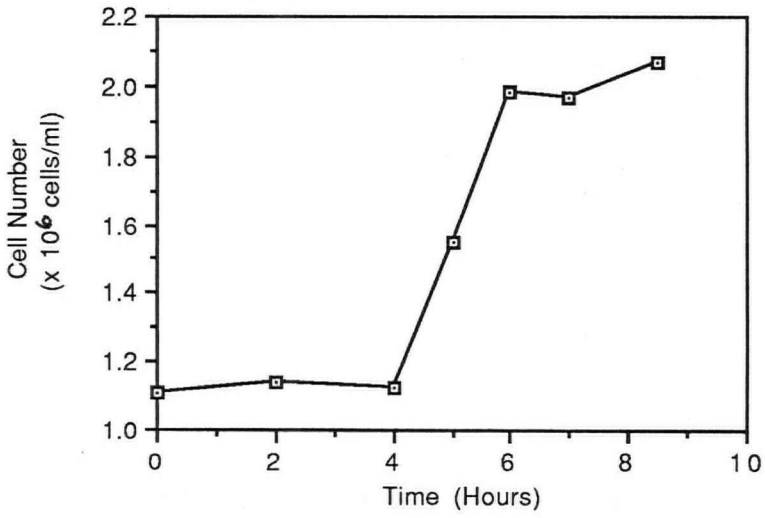
variable. From the result presented in Figure 13 the vegetative *ras*-specific mRNA observed by Reymond et al., (1984) was probably derived from the Dd*ras*G gene. Such variability could have been the result of inconsistent levels of cross-hybridization due to slight changes in probe length and hybridization conditions, which is consistent with this interpretation.

4.2.5. Cell cycle expression of Dd*ras*G

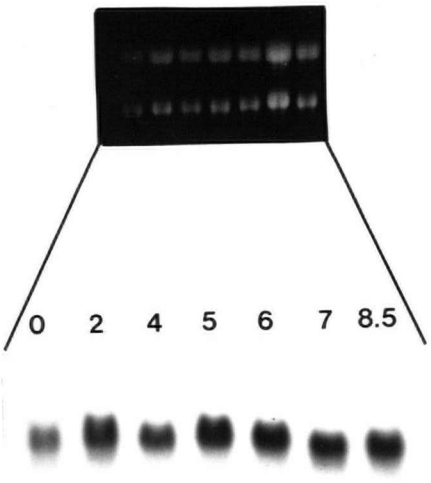
Amoebae of the axenic strains of *D. discoideum* grow and divide in nutrient media in the absence of bacteria with a doubling time of approximately 8-9 hours (Watts and Ashworth, 1970). To determine whether Dd*ras*G gene expression was cell cycle regulated the axenic strain, Ax-2, was grown under conditions which induce synchronous cell growth. The Ax-2 cells were grown in axenic medium until they reached stationary phase (2.0×10^7 cells/ml). The cells were maintained in the growth media at stationary phase for an additional 14 hours at which time they were diluted in fresh axenic medium to a cell density of 1×10^6 cells/ml. Samples were collected and counted at 0, 2, 4, 5, 6, 7, and 8 hours after the dilution and total RNA was extracted. After dilution in fresh media, there was a 4-5 hour lag before the cells divided as was shown previously (Figure 14A) (Soll, *et al.*, 1976; Jimenez, *et al.*, 1986). Northern blots of the RNA samples were probed with the Dd*ras*G cDNA under high stringency conditions. Although the ethidium bromide stained formaldehyde gel shown in Figure 14B reveals that different amounts of each RNA sample was loaded it is clear that the level of Dd*ras*G mRNA did not alter significantly throughout the cell cycle (Figure 14). In agreement with the interpretation that the Dd*ras* gene was not expressed during vegetative growth the Dd*ras*-c1 cDNA did not detect any mRNA when used to probe the Northern blot presented in Figure 14 (data not shown).

Figure 14. The expression of Dd*ras*G and the *ras*-related gene, Dd*rap*1 during synchronous cell growth. (A) Ax-2 cells were grown in axenic media to stationary phase and then diluted to a cell density of 1.1 x 10^6 cells/ml (0 hours) and the cell number was determined at the times indicated (hours) by haemocytometer counting. The cell number plotted represents the average from two cell counts. (B and C) Total RNA was isolated from cells at the indicated times (hours) and subjected to Northern blot analysis. The filter was hybridized with either (B) Dd*ras*G-c3 or (C) Dd*rap*1-c51 under high stringency conditions. The panel above each the Northern blots (B and C) shows the ethidium bromide stained gels before transfer to nitrocellulose. After removal of the hybridized probe, the filters were hybridized with Dd*ras*-c1 but no mRNA was detected.

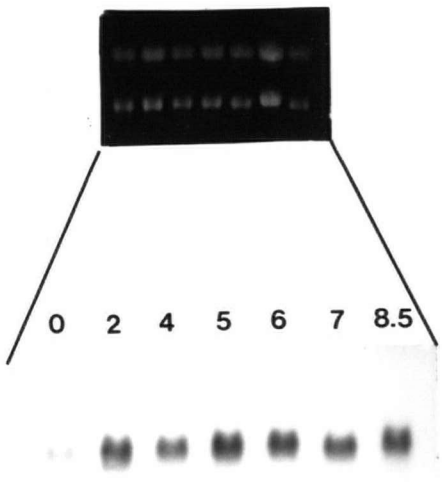
A.


Cell Number
(x 10⁶ cells/ml)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
2
4
6
8
10
Time (Hours)

B


0 2 4 5 6 7 8.5

C


0 2 4 5 6 7 8.5

4.3 Discussion

Evidence has been presented for the existence of a second *D. discoideum ras* gene, Dd*ras*G. Most other eukaryotic organisms possess more than one *ras* gene (Barbacid, 1987) and therefore *D. discoideum* is no exception. Both the Ddras and DdrasG proteins have features that are characteristic of other ras proteins (Figure 11). The amino half of the two proteins are over 90% conserved but the carboxyl region is variable with the exception of the tetrapeptide sequence at the extreme terminus. Both proteins contain the four highly conserved domains (amino acids 10-16, 57-62, 116-119, 143-147) that in mammalian ras proteins are associated with guanine nucleotide binding and GTPase activity (Figure 11) (Feig, *et al.*, 1986; Willumsen, *et al.*, 1986; Clanton, *et al.*, 1987; Feig, *et al.*, 1987). When the Dd*ras* and Dd*ras*G cDNAs were expressed in a bacterial expression system the two proteins were able to bind guanine nucleotides specifically (R. Pachal, unpublished observations) and thus it is not unreasonable to assume that the endogenous proteins also bind guanine nucleotides. In addition the *D. discoideum* ras proteins contain the effector domain (amino acids 32-40) which in mammalian proteins is believed to interact with the GTPase activating molecule (GAP) which modulates the GTPase activity of the ras proteins (Adari, *et al.*, 1988; Cales, *et al.*, 1988). The presence of the effector domain suggests the possible existence of a GAP-like molecule in *D. discoideum.*

The mammalian ras proteins are attached to the inner surface of the plasma membrane. In order for the ras proteins to attach to the plasma membrane there is a series of post-translational modifications which involve the conserved tetrapeptide sequence at the C-terminus (described in the General Introduction). Both the vegetative and pseudoplasmodial proteins are attached to the plasma membrane and appear to be acylated (Weeks, *et al.*, 1987). Both

the Ddras and DdrasG proteins have the conserved tetrapeptide sequence at the C-terminus which may be required for similar post-translational modifications. However, neither the Ddras or DdrasG proteins contain a cysteine residue in a position analogous to the one in the mammalian H-ras and N-ras proteins which is palmityolyated (Hancock, *et al.*, 1989). They do however, contain a stretch of basic amino acids similar to that found in the K-*ras* gene product and this may be sufficient for membrane localization (Hancock, *et al.*, 1990).

The Dd*ras*G and Dd*ras* genes are present as single copies within the *D. discoideum* haploid genome. The two genes cross-hybridize with each other under low stringency (Figures 8 and 12) presumably because of the high degree of nucleotide sequence identity (Figure 10). The Dd*ras*G gene cross-hybridized more efficiently with Dd*ras* and with other weakly hybridizing fragments on Southern blots (Figure 12). This can be explained by the fact that the Dd*ras*G cDNA encodes the complete protein and therefore includes the more conserved 5' end of the coding region of the gene, whereas the Dd*ras* cDNA does not start until nucleotide 180 of the coding sequence and the 3' sequences are more divergent (Figures 10 and 11).

Clearly, the Dd*ras* and Dd*ras*G genes show quite distinct patterns of expression. The Dd*ras* gene was only expressed subsequent to multicellular aggregation, while the Dd*ras*G gene was expressed during growth and early development. The ras proteins encoded by the two genes are identical between amino acids 63-73, the region recognized by the Y13-259 monoclonal antibody (Sigal, *et al.*, 1986). Using this antibody in *D. discoideum* cell-free extracts, ras protein was detected during growth and its concentration was found to decline during development (Weeks and Pawson, 1987). The rate of ras protein synthesis also declined during early aggregation but increased transiently during the pseudoplasmodial formation (Pawson and Weeks, 1984; Pawson, *et al.*,

1985). The combined expression of the Dd*ras* and Dd*ras*G genes could account for this protein accumulation pattern, but this does not preclude the possibility that there are other *ras*-related genes that encode immunologically cross-reacting proteins.

Although other eukaryotic organisms possess more than one *ras* gene, *D. discoideum* is thus far unique in expressing two genes at distinct stages of development. The Dd*ras* gene was expressed at the pseudoplasmodial stage of development as two mRNA species of 1.2 and 0.9 kb which are enriched in the prestalk cell population (Figure 13) (Reymond, *et al.*, 1984). Induction of the 1.2 kb mRNA occurs precociously under *in vitro* conditions in response to the addition of cAMP. In contrast, the Dd*ras*G gene was expressed as a single mRNA species of 1.2 kb during growth and early development (Figure 13). The amount of Dd*ras*G mRNA increased approximately 2 fold during the first 2-3 hours of development and then declined, reaching negligible levels by the aggregation stage of development (Khosla, *et al.*, 1990). The decline in Dd*ras*G mRNA level required a developmentally-regulated gene product since it does not occur when the cells are differentiated in the presence of the protein synthesis inhibitor, cycloheximide. During differentiation in shake suspension Dd*ras*G mRNA levels did not decline, unless the cells were treated with pulses of cAMP (Khosla, *et al.*, 1990). These results suggested that the decline in Dd*ras*G mRNA level during aggregation is a response of the cAMP relay system to pulses of cAMP. Consistent with this conclusion was the finding that the decrease in the Dd*ras*G mRNA levels did not occur when the pulses of cAMP were applied in the presence of caffeine, an inhibitor of the cAMP signal relay response (Khosla, *et al.*, 1990). The differential response of the Dd*ras* and Dd*ras*G genes to cAMP would ensure that the two genes are not expressed at the same time during development.

The differential expression of the two *ras* genes during *D. discoideum* development may reflect a requirement for the different ras proteins during growth and development. The DdrasG protein is slightly more conserved to the human ras protein than is the Ddras protein (Figure 11, Table 2). There is a region between residues 137 to 145 where the two *D. discodieum* ras proteins are diverged and where the DdrasG protein has a higher degree of identity with the proteins encoded by the human *ras* genes. Structure/function studies have shown that residues 143 to 147 form part of the guanine-base binding site of the mammalian ras proteins, and mutations in this region reduce the binding affinity for GTP (McGrath, *et al.*, 1984; McCormick, *et al.*, 1985; de Vos, *et al.*, 1988). The amino acids adjoining this loop might, therefore, influence the precise function of the ras proteins. It is possible that this region is more diverged between the DdrasG and Ddras proteins because the two proteins regulate different signalling pathways; the DdrasG protein having a role in cell growth and the Ddras protein having a role in cellular differentiation. However, it is possible that as is the case in yeast the two *D. discoideum ras* genes perform identical functions during both growth and development.

The expression of the Dd*ras*G gene did not appear to be regulated during the cell cycle (Figure 14B). Recently Whitbread and Katz have isolated a *D. discoideum* mutant that requires a factor for vegetative growth (unpublished observations). When this mutant was grown under the non-permissive conditions and then treated with the purified growth factor to induce cellular proliferation the Dd*ras*G mRNA levels were not altered at any time after the addition of the growth factor (Whitbread, Katz and Robbins, unpublished observations). Although the Dd*ras*G mRNA levels were not affected during the cell cycle or during the transition from quiescence to proliferation, it does not

preclude the possibility that the DdrasG protein is involved in control of cell growth.

The mammalian *ras* proteins appear to play a role in regulating cellular proliferation. The microinjection of the Y13-259 monoclonal antibody blocks the mitogenic response of NIH3T3 cells to serum stimulation (Mulcahy, *et al.*, 1985). The injection of the antibody at different stages of the cell cycle revealed that the ras proteins were required just prior to the intiation of the S phase after which point cells are committed to complete the cell cycle (Yu, *et al.*, 1990). In addition *trans*-dominant ras-inhibitory mutant proteins (a change from Ser-17 to Asn-17 in the H-ras protein) with a preferential affinity for GDP have been found to block the mitogenic response of NIH 3T3 cells to serum stimulation (Feig and Cooper, 1988). The mammalian ras proteins appear to have a general role in growth control since the microinjection of the antibodies or the dominant inhibitory H-ras Asn-17 mutant protein block the mitogenic stimulation of a wide variety of growth factors (Cai, *et al.*, 1990; Yu *et al.*, 1990).

CHAPTER 5

5.0 ISOLATION AND CHARACTERIZATION OF THE GENOMIC SEQUENCES CORRESPONDING TO THE Dd*ras*G GENE

5.1 Introduction

In view of the fact that *ras* genes appear to encode essential gene products, the mechanisms controlling their expression are likely to be important. The yeast *RAS*1 and *RAS*2 genes are essential for cell growth (Kataoka, *et al.*, 1984). The two genes are differentially expressed under a wide variety of culture conditions, but conditions that result in low amounts of the *RAS* gene products do not allow cell growth (Breviario, *et al.*, 1986; Breviario, *et al.*, 1988). It has also been shown that the experimental induction of elevated levels of ras proteins can result in the transformation of certain mammalian cell types (Chang, *et al.*, 1982a).

The differential response of the *D. discoideum* Dd*ras* and Dd*ras*G genes to cAMP would ensure that the two genes are not expressed at the same time during development. To begin to understand the molecular mechanisms controlling *ras* gene expression in *D. discoideum* it was important to analyze the genomic structure of the two genes.

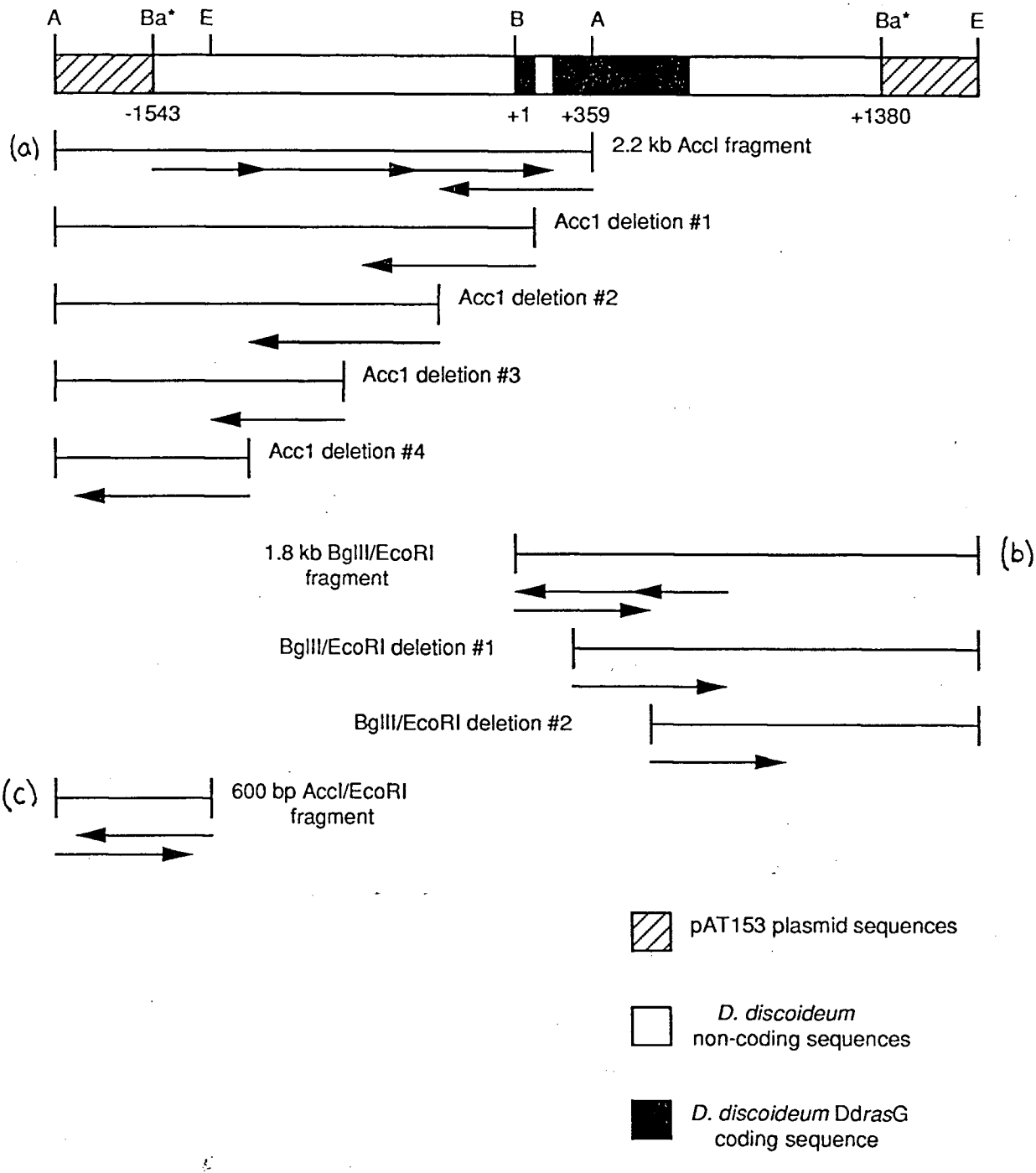
5.2 Results

5.2.1 Isolation and sequencing of the Dd*ras*G genomic sequences

The Southern blot analysis of *D. discoideum* genomic DNA indicated that the Dd*ras*G gene was present in a single copy within the genome (Figure 12). In order to isolate the Dd*ras*G genomic sequences, purified insert from the Dd*ras*G-c3 cDNA clone was used to screen a genomic library containing *D. discoideum* Ax-2 nuclear DNA that was partially digested with *Sau*3A to give an average size of 5 kb (see Materials and Methods). Eight positive clones were isolated, plasmid DNA was prepared and was digested with a number of restriction enzymes. The DNA was size fractionated on agarose gels and analyzed by Southern blot analysis. The Southern blot of the Dd*ras*G genomic clones was hybridized with an oligonucleotide derived from the 5' end of the coding region of the gene to determine which of the genomic clones contained the 5' end of the Dd*ras*G gene. Only one of the eight clones, Dd*ras*G-g5.2 hybridized to both the cDNA and the oligonucleotide and was selected for further analyses. A partial restriction map (Figure 15) of the genomic clone was consistent with the Southern blot analysis of the Dd*ras*G gene suggesting that genomic fragment was not rearranged, a problem that has been encountered previously when *D. discoideum* genomic sequences are cloned.

The strategy used to determine the nucleotide sequence of the Dd*ras*G genomic clone is outlined in Figure 15. The clone was digested into three restriction fragments and subcloned into either M13mp18 or Bluescript (Stratagene, La Jolla, USA). Two sets of unidirectional deletion series were performed on the subclones which contained the 2.2 kb*Acc*1 fragment and the 1.8 kb *Eco*R1/*Bgl*II fragment in order to sequence the 5' and 3' flanking regions of the gene respectively (Figure 15). The sequence was determined on single

Figure 15. Partial restriction map and sequencing strategy for the Dd*ras*G genomic clone, Dd*ras*G-g5.2. The Dd*ras*G-g5.2 genomic clone was isolated from a genomic library that had been constructed with *Sau*3A partially digested *D. discoideum* nuclear DNA ligated into the *Bam*HI site of pAT153 (see Materials and Methods). The box at the top of the Figure is a schematic representation of the Dd*ras*G-g5.2 clone. The black boxes represent translated sequences, open boxes represent non-coding sequences and the hatched boxes are the pAT153 plasmid sequences. Only restriction sites that are relevant to the construction of the various subclones are shown (Abbreviations: A, *Acc*I; E, *Eco*RI; B, *Bgl*II). The *Bam*H1/*Sau*3A (Ba*) represents the cloning junction between the plasmid and *D. discoideum* sequences. The numbers below the boxed lines represent the nucleotide positions of enzyme sites, +1 indicates the location of the first nucleotide of the Dd*ras*G coding sequence. Three subclones designated by the lines between the vertical bars, (a, b, c) were constructed; the 2.2 kb *Acc*I fragment and the 1.8 kb *Bgl*II/*Eco*RI fragment were cloned into Bluescript and the 600 bp *Acc*I/*Eco*RI fragment was cloned into M13mp18 in both orientations. Two series of unidirectional deletions were generated using exonuclease III for the 2.2 *Acc*I fragment and the 1.8 *Bgl*II/*Eco*RI fragment. The size and direction of the deletions are represented by the remainder of the lines between the vertical bars. The arrowed lines indicate the direction and length of the portions of each clone that was sequenced. Lines with multiple arrowheads indicate sequence determinations using oligonucleotides complementary to determined sequences.


A
Ba*
E
B
A
Ba*
E
-1543
+1
+359
+1380
(a)
2.2 kb AccI fragment
Acc1 deletion #1
Acc1 deletion #2
Acc1 deletion #3
Acc1 deletion #4
1.8 kb BglII/EcoRI fragment
(b)
BglII/EcoRI deletion #1
BglII/EcoRI deletion #2
(c)
600 bp AccI/EcoRI fragment
pAT153 plasmid sequences
D. discoideum non-coding sequences
D. discoideum DdrasG coding sequence

stranded DNA or denatured double stranded DNA by the dideoxy chain termination method (Sanger, *et al.*, 1977) using modified T7 polymerase (Sequenase, United States Biochemical Corp, Ohio, USA).

The nucleotide sequence of the Dd*ras*G genomic clone is shown in Figure 16 . The genomic clone isolated contained 1.6 kb of 5' upstream sequences presumably containing the promoter and regulatory sequences. The non-coding sequences contained over 85% adenine/thymine nucleotide residues which is consistent with all of the intervening sequences thus far sequenced in *D. discoideum*. Within the coding region of the Dd*ras*G gene there is a single 121 bp intron. Although *D. discoideum* introns are much shorter than most other eukaryotic introns they possess the conserved GT and AG dinucleotides at the extreme 5' and 3' boundaries respectively.

5.2.2 Determination of the 5' end of the DdrasG mRNA

The Dd*ras*G gene was transcribed as a single 1.2 kb mRNA during vegetative growth and early development (Figure 13). Two oligonucleotides complementary to a portion of the deduced Dd*ras*G mRNA were used in primer extension analysis to determine the site(s) of transcription initiation. The oligonucleotide (b) sequence that was chosen was sufficiently divergent from the Dd*ras* gene to prevent the extension of any Dd*ras* mRNA present in the sample. Three extension products of similar size were consistently obtained when vegetative mRNA from V12-M2 cells was used (Figure 17). When these products were analyzed next to a set of dideoxy chain termination reactions performed using the same oligonucleotide and a DNA template encompassing the 5' flanking sequences of the Dd*ras*G gene, the sites of transcription initiation could be located. The three primer extension products were mapped to 219, 220 and 234 bps upstream from the AUG initiating codon (Figure 16).

Figure 16. Nucleotide sequence of the Dd*ras*G genomic clone, including 5' and 3' flanking sequences. The 5', 3' and intervening nucleotide sequences are designated by small letters and the coding region of the Dd*ras*G is in capital letters. The numbering of the nucleotides starts with the first nucleotide of the coding region. The boxed sequences represent potential regulatory sequences that are described in the text. A potential polyadenylation signal is indicated by a double underline. The arrowheads indicate transcription initiation sites for the Dd*ras*G mRNA as determined by primer extension analysis using two oligonucleotide primers (a and b). The location of the oligonucleotide primers within the Dd*ras*G genomic clone are underlined and designated.

```
   10      |   20      |   30      |   40      |   50      |   60

atcgcttaac tgaaaaaaca gcgtagtatg  aaatcggaaa atctttttgt tttttatcat   -1480

aattgtcaac aattattttt catttgaaat  attttgccca catccgtgtg attgaaaaaa   -1420

aaaaaaataa taaaaatgag ggaatatttt  tgggagttta taaatcttgg taattgataa   -1360

tatttttttt gattattata ataaacaaga  aatcgtgggg gagagaattc tgaattttta   -1300

gtataagtta tttgaaaaaa aaaaaaaata  aaaaataaaa caaaaaaaat aaaataaaat   -1240

aaaataaaaa tctttggatt tgtctgtaat  ccttttttaaa ataaaactac gtagttttat   -1180

tgccaactcg cattgtttat ttatttattt  ttttttttt ttttttttcta cttaaatttt   -1120

ttttttttg tttggattta ttttttgttt  ttttttttat tcttctattt ttttacactt   -1060

tattatataa attttttttt ttttatttt  attttttttt ttttttctaa gtaatcaaac   -1000

aaagatttta catttttttt ttattgaatg  aatttcgaat aaaaaaaaaa aagatatttt   -940

attaaatttg tctatttaat tttttgtata  aaaaaaaata ataaaaaatt ttcgaaaaaa   -880

aaaaaatata aaaaattttt tcaaagattg  agtggtgcaa aaatagattt tctaaaacta   -820

tttaaacttt ttttttttt ttttttattat  tatcaattgt acaaataaaa acaaaaataa   -760

aaaacaaaaa aacaaataat ttaaaatgta  tattattatt tatcattaat ttcaatatct   -700

ttaaaagtat gtaggaactt ttttttttt  catacttata catttaaaaa aataaaaata   -640

aaaataaaaa tttcaaaaat aaaaagtttt  aaatttattt ataaataaaa tatatattta   -580

aattatttat ttataaataa aaataataaa  atttttattt gtataaagaa aaaaaaaaaa   -520

aaataaaaaa aaaaataaag tagtgataaa  aaaaaaataa aaaaaaaata aaaaaaaata   -460

tatattcaaa agtcattttt tgtccataac  ttttttttt ttgccacaaa aaaatctaca   -400
```

Figure 16 (cont). Nucleotide sequence of the Dd*ras*G genomic clone.

```
catgcgttaa ctgccactgg aggatacatg  aacattataa taaataacgg tttaaaaaat   -340
ctatattttt caatttttt ttatttttt   tatttttat ttttttttt ttatttttt    -280
ttttttttca aaaaattttt taaaaaaact  ttttttttt tttttattt ttttcttttc   -220
cacacacaca aaacaacaat atcactacat  tttatttatt attataaaat ccgcattttt  -160
tggcattcgc aacacccccct aatcgttttt  ttatataatt tttaaatttt atataattaa  -100
actgtaatat attaacaacc cacacaaaaa  aaaaaaaaaa aaccaaatca aattataaaa  -40
cccacacata tttatatata acagatctta  atttaaaaaa ATGACAGAAT ACAAATTAGT   20
TATTGTTGGT GGTGGTGGTG TCGGTAAAAG  TGCCTTAACC ATTCAATTAA TCCAAgtatg   80
tattatattt taaaaataaa aaaagataaa  taaaataaaa aaaattatca caagataaga   140
aagaaattaa ataaaatatt aatttttttt  ttttttttt tttattatta aaaaagAACC   200
ATTTCATTGA TGAATACGAT CCAACTATCG  AAGATTCATA CAGAAAACAA GTTACCATTG   260
ATGAAGAAAC TTGTTTATTA GATATTTTAG  ATACTGCTGG TCAAGAGGAA TACTCTGCAA   320
TGAGAGACCA ATATATGAGA ACTGGTCAAG  GTTTCCTTTG TGTCTACTCT ATCACTTCAA   400
GATCATCATT TGATGAAATT GCATCATTCC  GTGAACAAAT TCTTAGAGTT AAGGATAAGG   460
ATAGAGTACC AATGATTGTC GTTGGTAACA  AATGCGATTT GGAATCTGAT CGTCAAGTCA   520
CAACTGGTGA AGGTCAAGAT TTAGCAAAAT  CCTTCGGTAG TCCATTCCTT GAAACCTCTG   600
CCAAGATTCG TGTCAACGTT GAGGAAGCTT  TCTATTCACT CGTACGTGAA ATCAGAAAAG   660
ACCTCAAGGG TGACTCTAAA CCAGAAAAAG  GCAAGAAGAA GAGACCATTA AAAGCTTGTA   720
CTCTTTTATA Aacaaacttt ttctcaccaa  aaaaaaaaaa aaaaaacccg attataaaaa   800
aaaaaaaata ataatttatt aaacatttta  gagagtaaat tagaaataat aattcaacta   860
attttttaa taaattatct ttatttattt  tttaaaaggg tcaaaaaaaa aaaaaaaaaa   920
aaatcaaatc agatcaat                                                938
```

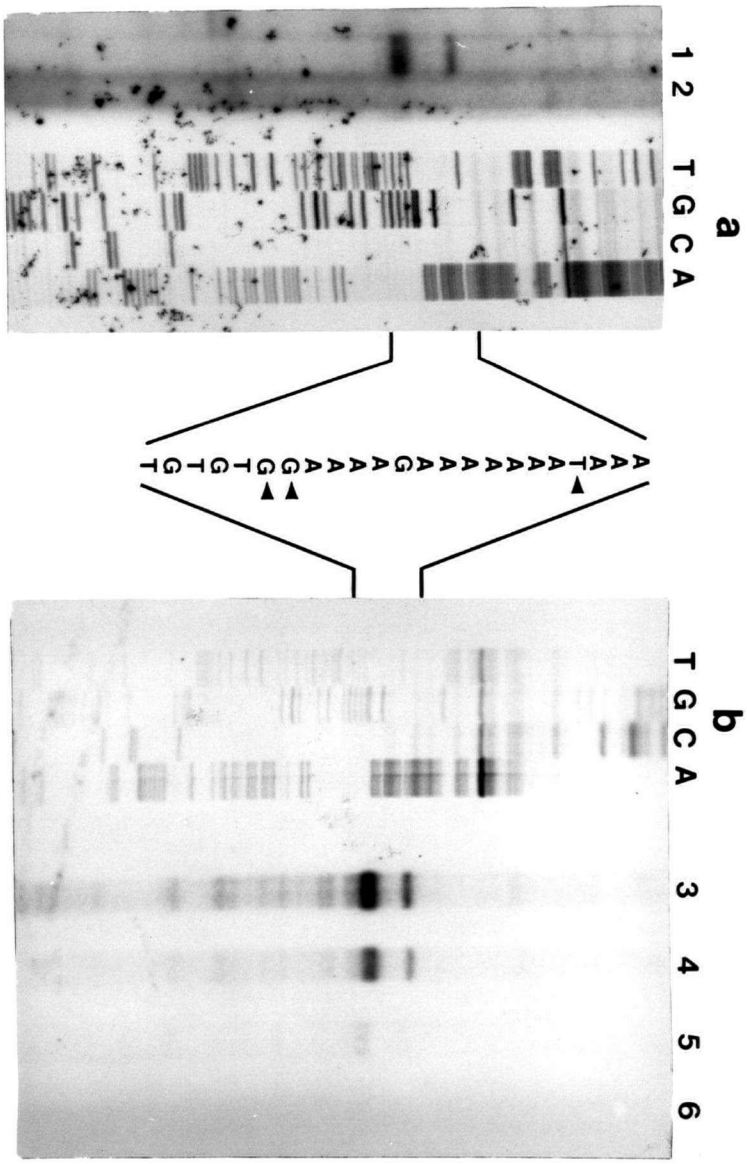
10 | 20 | 30 | 40 | 50 | 60

The same three extension products were observed when vegetative mRNA from Ax-2 cells was used in the analysis (Figure 17). In addition, three extension products mapped to the same positions within the Dd*ras*G genomic clone when a second downstream oligonucleotide (a) was used as a primer (Figure 17). These results confirmed that transcription of the Dd*ras*G mRNA initiates at three distinct sites. In agreement with the developmental expression of the Dd*ras*G gene (Figure 13) no extension products were observed when mRNA isolated from pseudoplasmodial stage cells was used as template for the primer extension reactions (Figure 17).

The 5' non-coding region of the Dd*ras*G mRNA appears to be colinear with the genomic sequence. The Dd*ras*G-c23 cDNA clone contained 137 bps of 5' non-coding sequence that was identical to that of the genomic clone. Within the remaining 97 bps of 5' non-coding sequence, there were no GT or AG dinucleotides which would indicate intron boundaries. These results suggest that the entire 234 bps of the 5' non-coding sequence of the Dd*ras*G mRNA is completely colinear with the genomic clone.

The 3' end of the Dd*ras*G transcript was not mapped in this study. There is a potential polyadenylation recognition sequence in the 3' non-coding region, but it remains to be determined if it is utilized. The Dd*ras*G-c3 cDNA clone contained a long stretch of adenine nucleotides 170 bps downstream from the TAA termination codon, but these sequences are colinear with the genomic clone suggesting that the end of the transcript is further downstream.

Figure 17. Determination of the Dd*ras*G transcription initiation sites by primer extension analysis. Two oligonucleotide primers (a) and (b) that were complementary to overlapping sequences of the Dd*ras*G gene (Figure 16) were end-labelled, annealed with mRNA (2μg) and extended with AMV reverse transcriptase. The (a) oligonucleotide primer was used to extend V12-M2 mRNA isolated from (lane 1) vegetative and (lane 2) pseudoplasmodial cells. Primer extension reactions with oligonucleotide (b) were carried out with (lane 3) mRNA from V12-M2 vegetative cells, (lane 4) mRNA from Ax-2 vegetative cells, (lane 5) mRNA from V12-M2 aggregating cells and (lane 6) mRNA from V12-M2 pseudoplasmodial cells. The Dd*ras*G-g5.2 (2.2 *Acc*I) genomic subclone (see Figure 15) was sequenced (T,G,C,A) with either the (a) oligonucleotide or (b) oligonucleotide as primers. Part of the nucleotide sequence of the Dd*ras*G sequence is shown, the arrowheads mark the sites of transcription initiation. The more intense primer extended product is actually two products that can be distinguished by a shorter exposure.


a
1 2
T G C A
b
T G C A
3 4 5 6

5.3 Discussion

A genomic clone was isolated that contained the Dd*ras*G gene. Comparison of the genomic sequence with that of the Dd*ras*G cDNA indicated that the Dd*ras*G gene contains a single intron (Figure 16), whereas the highly related Dd*ras* gene contains three introns (Reymond, *et al.*, 1984). The location of the first intron in Dd*ras* corresponds to the exact position of the single intron in Dd*ras*G. However, the sequence and size of the introns are not strictly conserved. The conserved position of the intron suggests that the gene duplication event resulting in the Dd*ras* and Dd*ras*G genes probably occurred after the insertion of the first intron. The other two introns might have been added to the Dd*ras* gene or deleted from the Dd*ras*G gene depending on the structure of the ancestral gene.

As is the case for many eukaryotic genes that are transcribed by RNA polymerase II, *D. discoideum* genes usually contain a conserved sequence, the TATA box located 25-35 bps upstream from the site of transcription initiation. In addition, *D. discoideum* promoters usually contain a stretch of thymine residues located just downstream from the TATA box (Kimmel and Firtel, 1983). The Dd*ras*G gene does contain a stretch of thymine residues upstream from the initiation of transcription but does not contain the TATA box sequences (Figure 16). Interestingly, the promoter regions of the mammalian K-*ras*, N-*ras* and H-*ras* genes also lack the characteristic TATA box (McGrath, *et al.*, 1983; Hall and Brown, 1985; Ishii, *et al.*, 1985). In addition the mammalian *ras* genes possess multiple transcription initiation sites and multiple copies of the hexanucleotide sequence CCGCCC (GC box) or its inverted complement, that have been shown to bind the transcription factor SP1 (Ishii, *et al.*, 1986; Damante, *et al.*, 1987; Hoffman, *et al.*, 1987; Honkawa, *et al.*, 1987). It has been

suggested that these sequences are involved in the regulation of growth control genes and they are required for the transcription of the H-*ras* gene (Ishii, *et al.*, 1986).

Primer extension analysis demonstrated that the Dd*ras*G gene contains multiple transcription intiation sites (Figure 17). There is also a fairly GC rich region located approximately 150 bps upstream from the transcription initiation sites, however, it does not contain the GC box consensus sequence. These sequences may be required for the regulation of Dd*ras*G gene expression, however, a transcription factor analogous to SP1 has not yet been identified in *D. discoideum.* Regulatory sequences have also been found in the first (Spandidos and Pintzas, 1988) and fourth (Cohen and Levinson, 1988; Cohen, *et al.*, 1989) introns of the H-*ras* gene. It will be interesting to determine whether there are sequences within the introns that play a role in the transcriptional regulation of the Dd*ras* and Dd*ras*G genes.

Our laboratory has demonstrated that the levels of Dd*ras*G mRNA decline in response to pulses of cAMP (Khosla, *et al.*, 1990). Molecular analysis has identified the *cis*-acting sequences required for the induction of a number of cAMP regulated *D. discoideum* genes (Pears and Williams, 1987; Datta and Firtel, 1988; Pears and Williams, 1988; Pavlovic, *et al.*, 1989), as well as a developmentally regulated trans-acting factor that controls the expression of the cAMP-inducible early prestalk genes (Hjorth, *et al.*, 1989; Hjorth, *et al.*, 1990). However, little information is known about the molecular requirements for the down regulation in response to cAMP. Recently Vauti *et al.*, (Vauti, *et al.*, 1990) have used a promoter analysis vector to define a 79 bp element within the discoidin Iγ promoter that is necessary for the down regulation of gene expression in response to cAMP. It does not appear that the Dd*ras*G promoter has this 79 bp element but a sequence comparison is very difficult because of the

high content of adenine and thymine nucleotides within the 5' flanking sequences and it is not known which portions of the sequence within the 79 bp element are required for the proper regulation. More genes that are negatively regulated in response to cAMP will need to be analyzed to determine if there are conserved regulatory sequences.

Sequences responsible for the regulation and expression of a number of *D. discoideum* genes have been delineated to within the first 1 kb of 5' flanking sequences. Previous results indicated that 550 bps of the 5' flanking region of the Dd*ras* gene confers the proper temporal and spatial regulation when fused to a reporter gene (Reymond, *et al.*, 1985). With the isolation of the Dd*ras*G genomic clone that contains over 1 kb of 5' upstream sequences it should be possible to define the sequence elements required for the proper regulation of the Dd*ras*G gene. The Dd*ras*G promoter and putative regulatory sequences are now being used to express an activated Dd*ras*G gene in *D. discoideum* to examine the effect on the developmental phenotype (R. Thiery, studies in progress).

CHAPTER 6

6.0 ABERRANT EXPRESSION OF THE Dd*ras*G GENE IN THE AXENIC STRAIN, AX-2

6.1 Introduction

Dd*ras*G mRNA levels decline in response to pulses of cAMP (Khosla, *et al.*, 1990) in a manner similar to that of the previously described M4-1 gene (Kimmel and Carlisle, 1986; Kimmel, 1987). In contrast, Dd*ras* gene expression is induced by cAMP (Reymond, *et al.*, 1984; Reymond, *et al.*, 1985). The differential expression of the two *ras* genes to cAMP would ensure that they are not expressed at the same time during normal development. The two ras proteins may fulfill different functions; Dd*ras*G having a regulatory role in cellular proliferation and Dd*ras* a role in signal transduction at the pseudoplasmodial stage of development. However, since the predicted amino acid sequences of the two proteins are very similar (Figure 11), they might compete for the same regulatory molecule(s) and their simultaneous presence during development might be detrimental to the differentiation process.

A DNA-mediated transformation system has been used to introduce multiple copies of particular genes into the *D. discoideum* axenic strains (Nellen, *et al.*, 1984; Nellen and Firtel, 1985; Early and Wlliams, 1987). One way to investigate if the continued expression of the Dd*ras*G gene might impair development would be to transform *D. discoideum* with the Dd*ras*G gene under the control of a constitutive promoter. However, during preliminary experiments it was found that Dd*ras*G mRNA levels did not decline during the development of the

axenic strain, Ax-2, providing a situation in which both *D. discoideum ras* genes were expressed at the same time during development.

6.2 Results

6.2.1 Expression of Dd*ras*G in different *D. discoideum* strains

In the *D. discoideum* wild type strain, V12-M2, the Dd*ras*G gene was expressed in vegetative cells and then declined to negligible levels by the aggregation stage of development (6-8 hours in strain V12-M2) (Figure 13). Before the onset of this decline there was a transient increase in the expression during the first 2-3 hours of development (Khosla, *et al.*, 1990). In contrast, in the axenic strain Ax-2, there was a gradual increase in expression over the first 8 hours of development. The levels of Dd*ras*G mRNA then declined slightly but remained at least at vegetative levels throughout the remainder of development (Figure 18). This result suggested the possibility that Dd*ras*G developmental expression is different for the two wild type strains, V12-M2 and NC4, the parental strain of Ax-2. The developmental expression of the Dd*ras*G gene was therefore examined in the NC4 wild type strain and it was found that its expression was analogous to that observed in the V12-M2 strain (Figure 18). The transient increase and subsequent decline of the Dd*ras*G mRNA levels in the NC4 strain were slightly delayed relative to that observed with the strain V12-M2, but this probably reflects the more rapid development of the latter strain. Thus the aberrant expression appeared to be unique to the Ax-2 strain. Since the Ax-2 strain used in these studies had been maintained for a number of years in the Weeks' laboratory, an Ax-2 strain was obtained from Dr. B. Coukell (York University, Toronto). The new Ax-2 strain exhibited the same abnormal developmental expression of Dd*ras*G.

Figure 18. The expression of Dd*ras*G mRNA during the development of the *D. discoideum* strains NC4 and Ax-2. Total RNA (20 µg) from each of the indicated times after the onset of differentiation (hours) of both NC4 and AX2 was separated on 1.25% formaldehyde-agarose gels, transferred to nitrocellulose, and probed with Dd*ras*G-c3. The filters were washed under high stringency conditions (see Materials and Methods) and used to expose X-ray film for 16 hours.

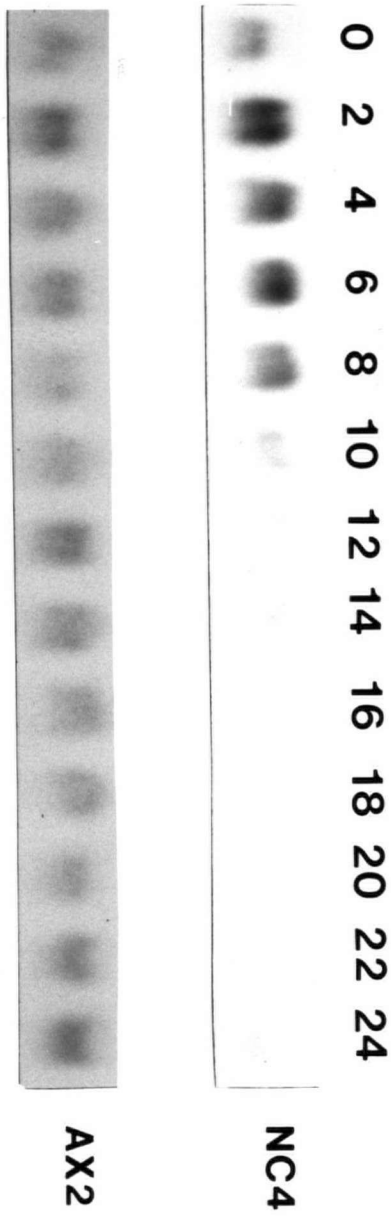
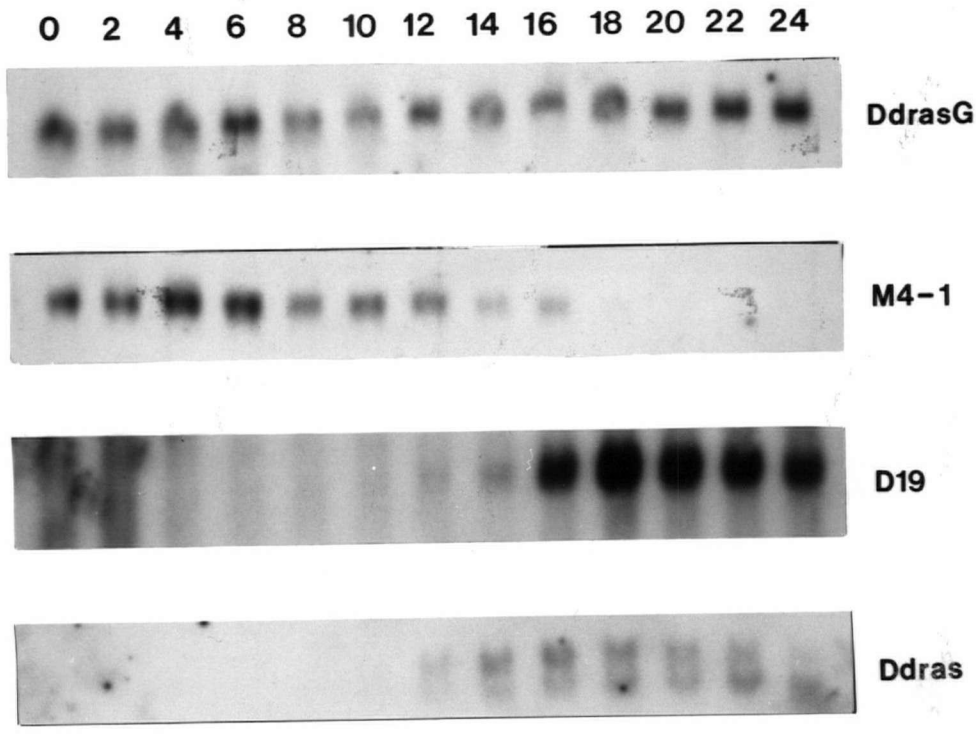


Figure 19. The expression of Dd*ras*G, M4-1, D19 and Dd*ras* mRNAs during development of strain Ax-2. A Northern blot of the Ax-2 RNA samples described in Figure 18 was probed with the Dd*ras*G, M4-1, D19 and Dd*ras* cDNA clones as indicated and washed under high stringency conditions. The same Northern blot filter was used for each of the hybridizations. The M4-1 and D19 cDNAs were kindly provided by Dr A. Kimmel and Dr. H. Lodish respectively.

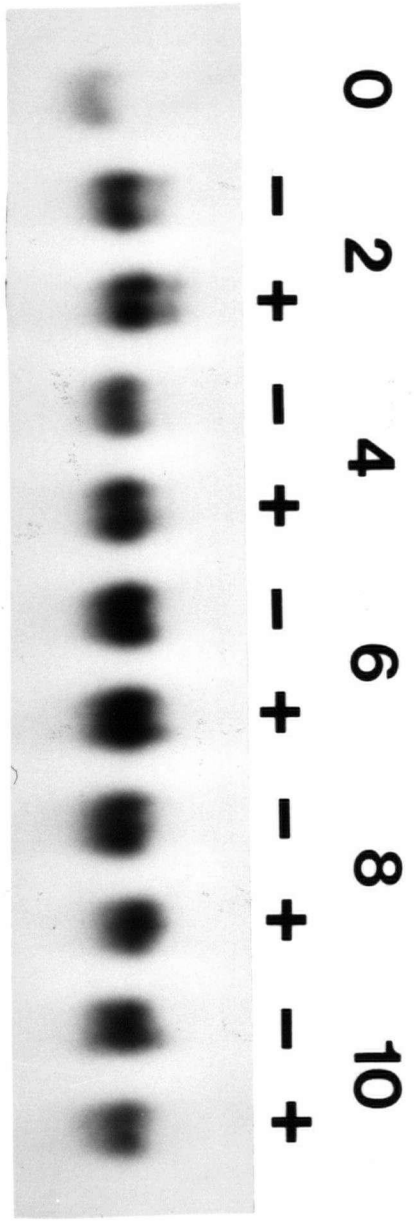


The expression of other developmentally regulated genes was analyzed to determine if the abnormal gene expression in Ax-2 was confined to Dd*ras*G. The expression of D19 and Dd*ras*, two genes which are expressed during the post-aggregative development was normal (Figure 19) (Reymond, *et al.*, 1984; Early, *et al.*, 1988). More significantly, the expression of M4-1, a gene that like Dd*ras*G is repressed by pulses of cAMP was also normal (Figure 19) (Kimmel and Carlisle, 1986; Kimmel, 1987).

6.2.2 The effects of cAMP on Dd*ras*G expression in Ax-2

The decline in Dd*ras*G mRNA levels in the V12-M2 strain was shown to be mediated by pulsatile cAMP (Khosla, *et al.*, 1990). The defective expression of this gene in the Ax-2 strain might be due to either the generation of cAMP pulses or in the response to cAMP. To test these possibilities Ax-2 cells were shaken in the presence or absence of pulses of cAMP under the same conditions in which the Dd*ras*G mRNA levels declined in the wild type strain, V12-M2 (Khosla, *et al.*, 1990). The levels of Dd*ras*G mRNA were not significantly altered in response to the pulses of cAMP (Figure 20), suggesting that the defect in Dd*ras*G expression was due to a defect in some aspect of the cAMP response rather than in the generation of the cAMP signal. There did appear to be an increase in the levels of Dd*ras*G mRNA in shake suspension relative to vegetative cells however the significance of this observation remains to be determined (Figure 20).

Figure 20. The effect of cAMP pulses on the expression of Dd*ras*G in shake suspension cultures of Ax-2. Ax-2 cells were grown axenically to a cell density of 2×10^6 cells/ml, washed and resuspended in KK_2 buffer, pH 6.0 at a density of 5×10^6 cells/ml. Suspensions were shaken at 250 rev/min in the absence (-) or presence (+) of pulses of cAMP to a final concentration of 25 nM. Pulses of cAMP were applied manually every 5 minutes. Total RNA was extracted at the times indicated (hours) and subjected to Northern blot analysis using the Dd*ras*G-c3 cDNA as a probe.



6.3 Discussion

The experiments described in this chapter were performed to address the question of whether the continued expression of the Dd*ras*G gene throughout development demonstrated an aberrant phenotype. It was found that Dd*ras*G was expressed throughout development in the axenic strain, Ax-2. This abnormal expression appeared to be unique to the Dd*ras*G gene since the expression of other developmentally regulated genes was normal (Figure 19). The aberrant expression of the Dd*ras*G gene did not have a major deleterious effect on differentiation. Although the Dd*ras*G mRNA was present throughout development, the presence of the DdrasG protein has yet to be analyzed. The only unusual developmental phenotype of the Ax-2 strain was a smaller aggregate size when compared to the wild type strain plated at the same cell density. In addition the development of the Ax-2 strain was aberrant at higher cell densities. It is premature to conclude that these abnormal developmental phenotypes are due to the abnormal expression of Dd*ras*G, since the axenic strains probably have multiple mutations (Cox, *et al.*, 1990). The converse experiment has been performed with the Dd*ras* gene. When another axenic strain, Ax-3 was transformed with multiple copies of the Dd*ras* gene, Dd*ras* mRNA was detected in vegetative cells and there was no adverse effects on growth or early development (Reymond, *et al.*, 1985; Reymond, *et al.*, 1986). These experiments suggest that the simultaneous presence of the Dd*ras* and Dd*ras*G gene products does not have an adverse effect on either growth or differentiation.

It is possible that the level of the DdrasG protein expressed in pseudoplasmodial cells was insufficient to effect the differentiation process. Strains are now being developed that dramatically overexpress the DdrasG

protein by transforming the axenic strains with multiple copies of the Dd*ras*G gene under the control of a constitutive promoter (R. Thiery, studies in progress).

The decline in Dd*ras*G mRNA levels in response to pulses of cAMP is at least superficially similar to the repression of the M4-1 gene (Kimmel and Carlisle, 1986). Furthermore, neither gene is repressed by pulses of cAMP in the presence of caffeine (Kimmel, 1987; Khosla, *et al.*, 1990), a known inhibitor of the cAMP relay system (Brenner and Thoms, 1984). This suggests that in both cases repression requires a transient increase in the intracellular cAMP concentration or some other component involved in this branch of the signal relay system. However, while the expression of the M4-1 was repressed normally in the axenic strain Ax-2, Dd*ras*G mRNA levels did not decline during development. In addition Dd*ras*G mRNA levels did not decline when cells were developed in the presence of pulses of cAMP. These results imply that the molecular mechanisms involved in the down regulation in response to cAMP are different for the two genes. At present, it is not possible to determine whether the defect in Dd*ras*G expression in Ax-2 is in the initial response to cAMP or further downstream from the signal. Since the response of the two genes to pulses of cAMP is otherwise so similar, the latter alternative is favoured with different downstream regulatory molecules responsible for the repression of transcription of the two genes. The *Synag7* mutant strains that are defective in adenylyl cyclase activation (see General Introduction) may be useful in elucidating the molecular mechanisms responsible for the decline in Dd*ras*G mRNA levels in response to cAMP.

CHAPTER 7

7.0 ISOLATION AND CHARACTERIZATION OF A MEMBER OF THE *RAS* GENE SUPERFAMILY, Dd*rap*1, FROM *D. DISCOIDEUM*

7.1 Introduction

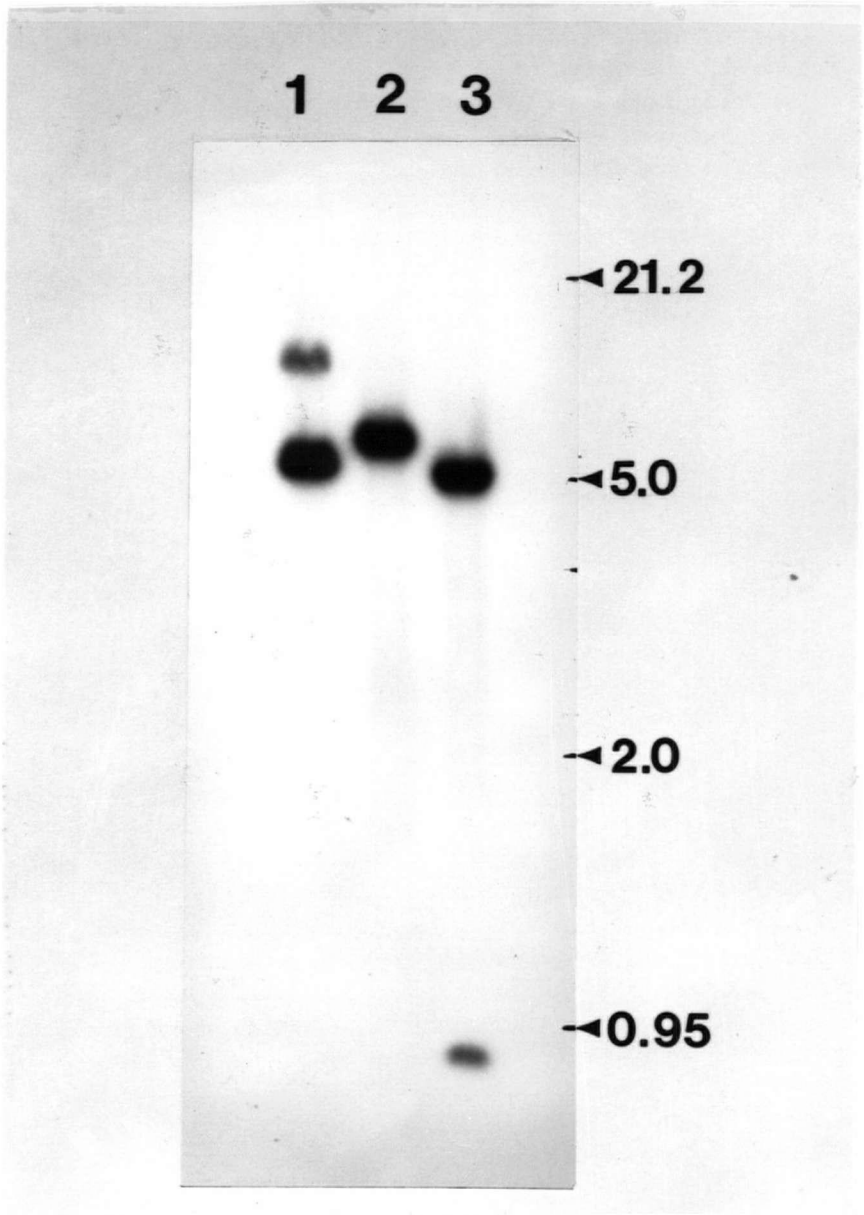
When Southern blots of *D. discoideum* genomic DNA were hybridized with the Dd*ras* and Dd*ras*G cDNAs under conditions of low stringency, the cDNAs hybridized to both genes and also hybridized to a number of other genomic fragments (Figure 12). These results suggested the possiblity that as in other eukaryotic organisms, the *D. discoideum* genome contains homologues of the *ras* gene superfamily. In this chapter the isolation and characterization of a *D. discoideum* gene that is highly conserved relative to the human *rap* genes is described.

7.2 Results

7.2.1 Isolation of cDNA clones related to the *ras* gene superfamily

A large number of clones were isolated from the vegetative λgt10 cDNA library that hybridized weakly to the Dd*ras*-c1 probe under low stringency conditions (see Chapter 4). These clones were then hybridized with the Dd*ras*G-c3 cDNA under conditions of both low and high stringency. One clone, c51, was selected since it hybridized to both Dd*ras*-c1 and Dd*ras*G-c3 under low stringency conditions but failed to hybridize to either under high stringency conditions. This clone was used to isolate five additional clones under high stringency

Figure 21. Southern blot analysis of the *D. discoideum* Dd*rap*1 gene. Genomic DNA (5 μg) isolated from Ax-2 cells was digested with *Bgl*II (lane 1), *Eco*RI (lane 2) and *Bgl*II/*Eco*RI (lane 3), electrophoresed on a 0.8% agarose gel and then transferred to nitrocellulose. The filter was hybridized with the Dd*rap*1-c51 cDNA and washed using the previously described high stringency conditions. Molecular weight sizes (kb) are shown on the right.



conditions from a λgt11 cDNA library, prepared from mRNA isolated from cells at 3-4 hours of development (kindly provided by Dr. P. Devreotes). When the isolated cDNA clones were used to probe a Southern blot of *D. discoideum* genomic DNA they all hybridized to the same genomic fragments indicating that they were derived from the same gene. The Southern blot analysis suggested that the c51 clone was present in a single copy within the haploid genome (Figure 21). The genomic fragments that hybridized to the isolated cDNA clones corresponded to one of the weakly hybridizing fragments that had been previously observed when the Dd*ras* and Dd*ras*G cDNA clones were used under low stringency conditions (Figure 12). Two genomic fragments hybridized to the c51 cDNA clone when the genomic DNA was digested with *Bgl*II or with *Eco*RI/*Bgl*II (Figure 21 lanes 1 and 3) because there is a *Bgl*II restriction site within the gene.

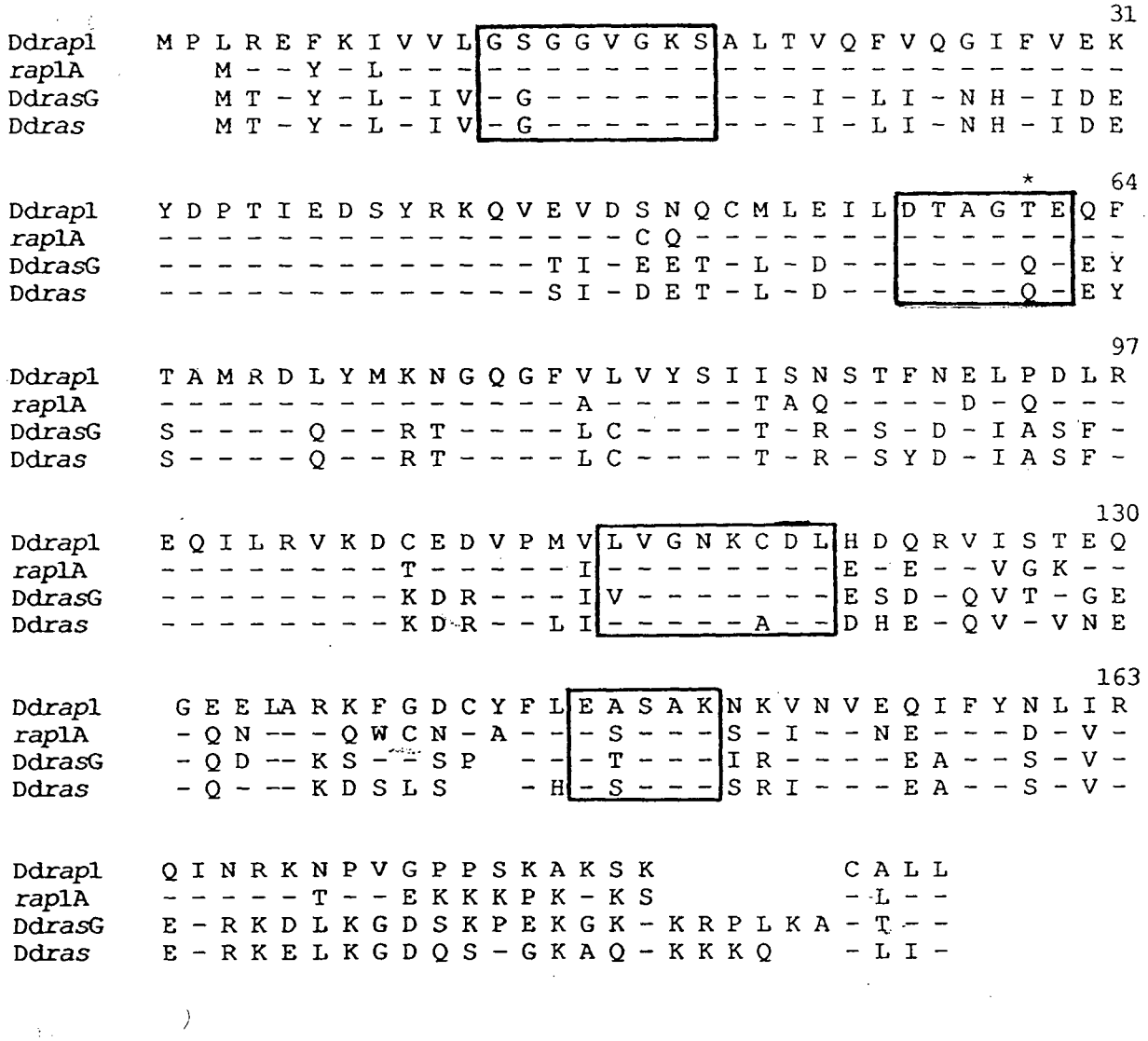
7.2.2 Sequence analysis of the *ras*-related cDNA clones

DNA from the six cDNA clones was subcloned into M13mp18 in both orientations and single stranded DNA from each was sequenced by the dideoxy chain termination method. Sequence analysis confirmed that all six cDNA clones were derived from the same mRNA and thus represented the same gene. In order to completely sequence both strands of the c51 clone it was further subcloned by digesting the DNA with *Eco*RI and *Bgl*II and the resulting fragments were then subcloned into M13mp18 and M13mp19 that had been digested with *Eco*RI and *Bam*H1. The recombinant clones did not contain an initiating methionine, terminating at or before an internal *Eco*R1 site that was probably not protected during the synthesis of the cDNAs. The remaining 5' sequence was obtained by sequencing RNA isolated from vegetative cells with a synthetic oligonucleotide that was complementary to the 5' end of the

Figure 22. Nucleotide and derived amino acid sequences of the Dd*rap*1 gene. The numbers on the left indicate nucleotide positions in the sequence and the numbers on the right indicate the amino acid positions. The synthetic nucleotide that was used to complete the 5' sequence is complementary to nucleotides 89 to 107. The amino acids are represented by the single letter code (see figure 11). The Dd*rap*1 sequence has been deposited in the EMBL/GenBank data base (accession no. X54291).

```
   1  GAATATATATTATAAACCAGATGCCTCTTAGAGAATTCAAAATCGTCGTTTTAGGTTCAG
                          M  P  L  R  E  F  K  I  V  V  L  G  S  G   14

  61  GTGGTGTAGGTAAATCTGCTTTGACTGTGCAATTTGTTCAAGGTATTTTTGTTGAAAAGT
        G  V  G  K  S  A  L  T  V  Q  F  V  Q  G  I  F  V  E  K  Y   34

 121  ACGATCCAACCATCGAAGATTCCTACAGAAAACAAGTCGAAGTTGATAGCAATCAATGCA
        D  P  T  I  E  D  S  Y  R  K  Q  V  E  V  D  S  N  Q  C  M   54

 181  TGTTAGAAATTTTAGATACAGCTGGTACTGAACAATTTACTGCAATGAGAGATCTTTACA
        L  E  I  L  D  T  A  G  T  E  Q  F  T  A  M  R  D  L  Y  M   74

 241  TGAAAAATGGTCAAGGTTTTGTTTTAGTATATTCAATCATTTCAAACTCCACTTTTAACG
        K  N  G  Q  G  F  V  L  V  Y  S  I  I  S  N  S  T  F  N  E   94

 301  AGTTACCAGATCTCCGTGAACAAATTCTCAGAGTTAAGGATTGTGAAGATGTTCCAATGG
        L  P  D  L  R  E  Q  I  L  R  V  K  D  C  E  D  V  P  M  V  114

 361  TTCTTGTTGGTAACAAATGCGATCTCCACGACCAACGTGTTATTAGCACAGAACAAGGTG
        L  V  G  N  K  C  D  L  H  D  Q  R  V  I  S  T  E  Q  G  E  134

 421  AAGAACTCGCTCGTAAATTTGGTGATTGTTACTTTTTAGAAGCATCTGCCAAGAATAAAG
        E  L  A  R  K  F  G  D  C  Y  F  L  E  A  S  A  K  N  K  V  154

 481  TTAATGTTGAACAAATTTTCTATAACTTAATCCGTCAAATCAACCGTAAAAACCCAGTTG
        N  V  E  Q  I  F  Y  N  L  I  R  Q  I  N  R  K  N  P  V  G  174

 541  GTCCACCAAGCAAAGCTAAATCAAAATGTGCTTTATTGTAAACAATCCATCAACTCTCCA
        P  P  S  K  A  K  S  K  C  A  L  L                        187

 601  ACACCCTTCCATACTCACCCACCCATTTCAAATGTAACAATTGAAAAACAGAAAAAAAAA

 661  AAAAAAAAAAACAGAAAAAAAAAAA
```

Figure 23. Comparison of the derived amino acid sequence of the *D. discoideum* rap protein (Ddrap1) with the predicted sequences of the Ddras (Reymond, *et al.*, 1984), DdrasG (this thesis) and the human rap1A proteins (Pizon, *et al.*, 1988a). The sequences are aligned relative to Ddrap1 but the numbers correspond to the amino acid positions in the human rap1A sequence. A dash indicates identity with the Ddrap1 and only amino acids that differ are indicated by the single letter amino acid code (Figure 11). Gaps have been inserted to maximize the identity between the sequences. The four guanine nucleotide binding domains that are characteristic for the *ras* protein superfamily (Chardin, 1988; Sanders, 1990) have been boxed and the variant threonine residue at position 61 is marked by an asterisk.


31
Ddrap1 M P L R E F K I V V L G S G G V G K S A L T V Q F V Q G I F V E K
rap1A M - - Y - L -
DdrasG M T - Y - L - I V - G - - - - - - - - - I - L I - N H - I D E
Ddras M T - Y - L - I V - G - - - - - - - - - I - L I - N H - I D E
64
Ddrap1 Y D P T I E D S Y R K Q V E V D S N Q C M L E I L D T A G T E Q F
rap1A - - - - - - - - - - - - - - - - C Q - - - - - - - - - - - - - -
DdrasG - - - - - - - - - - - - - - T I - E E T - L - D - - - - - - Q - E Y
Ddras - - - - - - - - - - - - - - S I - D E T - L - D - - - - - - Q - E Y
97
Ddrap1 T A M R D L Y M K N G Q G F V L V Y S I I S N S T F N E L P D L R
rap1A - - - - - - - - - - - - - - - A - - - - - T A Q - - - - D - Q - - -
DdrasG S - - - - Q - - R T - - - - L C - - - - T - R - S - D - I A S F -
Ddras S - - - - Q - - R T - - - - L C - - - - T - R - S Y D - I A S F -
130
Ddrap1 E Q I L R V K D C E D V P M V L V G N K C D L H D Q R V I S T E Q
rap1A - - - - - - - - T - - - - - I - - - - - - - - - E - E - - V G K - -
DdrasG - - - - - - - - K D R - - - I V - - - - - - - - E S D - Q V T - G E
Ddras - - - - - - - - K D R - - L I - - - - - A - - D H E - Q V - V N E
163
Ddrap1 G E E LA R K F G D C Y F L E A S A K N K V N V E Q I F Y N L I R
rap1A - Q N -- - Q W C N - A - - - S - - - S - I - - N E - - - D - V -
DdrasG - Q D -- K S - - S P - - - T - - - I R - - - - E A - - S - V -
Ddras - Q - -- K D S L S - H - S - - - S R I - - - E A - - S - V -
Ddrap1 Q I N R K N P V G P P S K A K S K C A L L
rap1A - - - - - T - - E K K K P K - K S - L - -
DdrasG E - R K D L K G D S K P E K G K - K R P L K A - T - -
Ddras E - R K E L K G D Q S - G K A Q - K K K Q - L I -

incomplete cDNA. The nucleotide sequence and the deduced amino acid sequence from the single open reading frame are shown in Figure 22. The sequence presented does not contain the entire 5' non-coding region of the gene because the RNA sequencing could not be resolved past the indicated end. Recently a cDNA clone from a vegetative library has been isolated that extends past the initiating methionine and its sequence confirms the one obtained by sequencing the RNA (J. Daniel, P. Rebstein, unpublished observations).

The deduced amino acid sequence contains the four domains that are common to the ras family of proteins, but there are considerable differences relative to the two *D. discoideum* ras proteins (Figure 23). The Ddras and DdrasG proteins are only 52% and 55% identical to the predicted amino acid sequence, respectively, whereas the the human rap1A protein is 76% identical. These results suggest that the isolated gene represents the *D. discoideum* homologue for the human rap protein and thus the gene has been designated Dd*rap*1.

7.2.3 Developmental expression of the Dd*rap*1 gene

To determine the developmental expression of the Dd*rap*1 gene, Northern blots of RNA isolated from various times during *D. discoideum* development were hybridized with the Dd*rap*1-c51 cDNA clone. A complex pattern of Dd*rap*1 specific mRNA expression was observed (Figures 24 and 25). The amount of mRNA that hybridized to the Dd*rap*1 cDNA changed dramatically during development reaching maximal levels at the aggregation stage (8-10 hours) and during culmination (18-22 hours). During vegetative growth and early development a single 1.1 kb transcript was observed, but late in aggregation there were two transcripts of 1.0 and 1.3 kb (Figures 24 and 25).

Figure 24. Expression of Dd*ras*G, Dd*ras* and Dd*rap*1 during *D. Discoideum* development. Messenger RNA (5 μg) isolated from V12-M2 cells at the indicated time points after the onset of differentiation was subjected to Northern blot analysis as described previously. The filter was hybridized, as indicated, with the Dd*ras*G-c3, Dd*ras*-c1 and Dd*rap*1-c51 cDNA probes under high stringency conditions (see Materials and Methods).

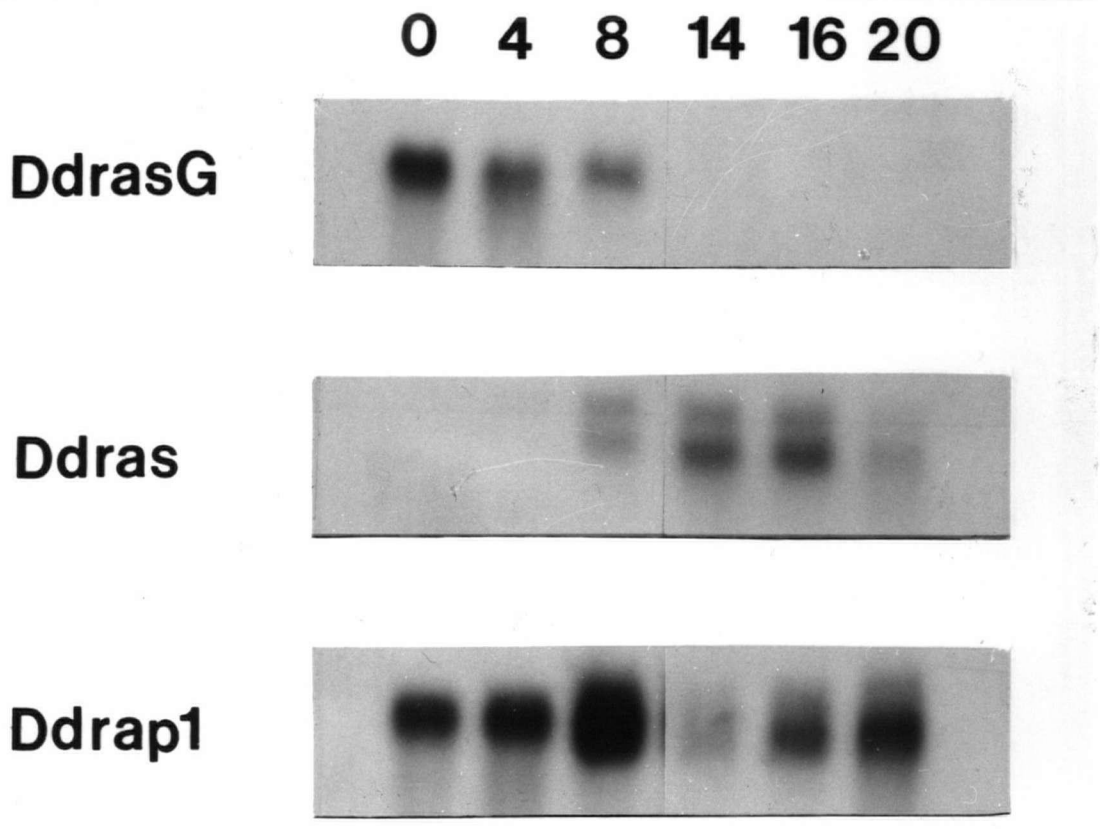


Figure 25. Developmental expression of Dd*rap*1 during the development of *D. discoideum* strain, V12-M2. Total RNA (20 μg) isolated at the indicated times (hours) from two independent experiments was subjected to Northern blot analysis and hybridized with the Dd*rap*1-c51 cDNA probe using high stringency wash conditions. 0 hour and 18 hour samples were electrophoresed side by side to allow direct size comparison. The single 0 hour mRNA was approximately 1.1 kb and the two 18 hour mRNAs were approximately 1.0 and 1.3 kb.

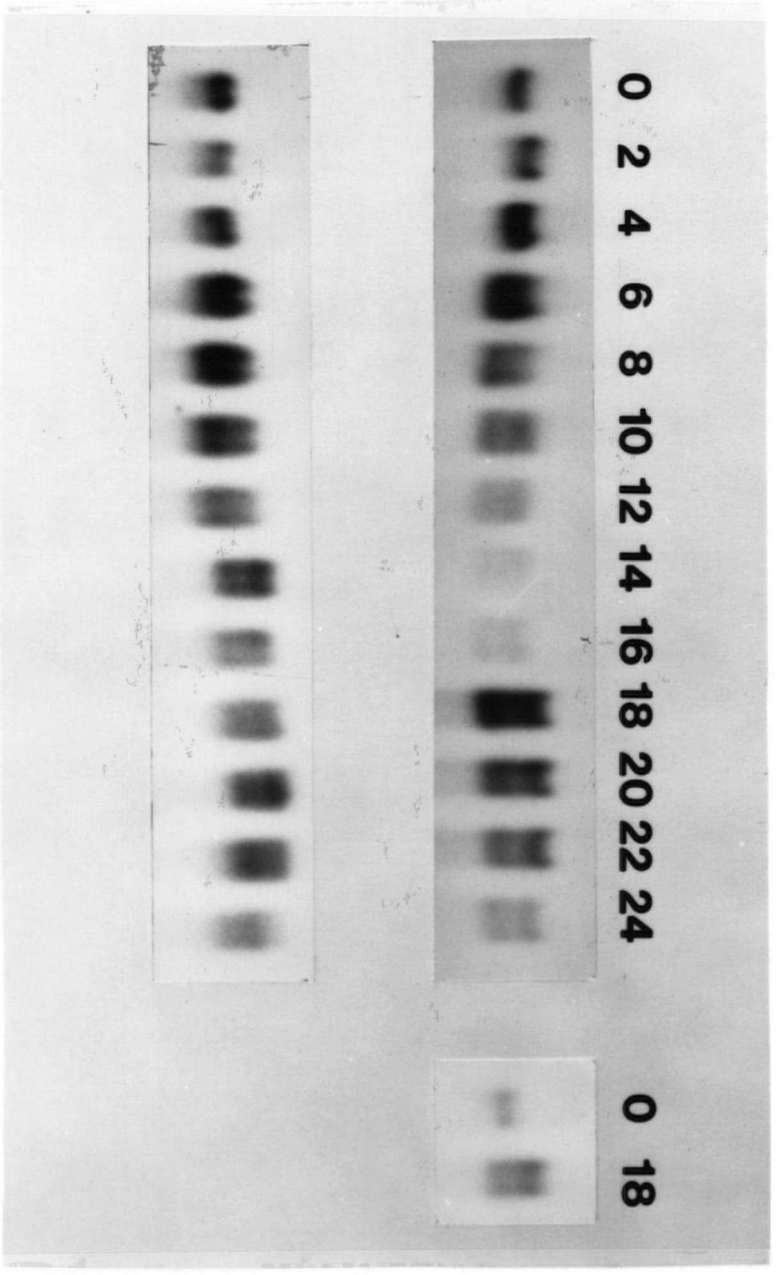
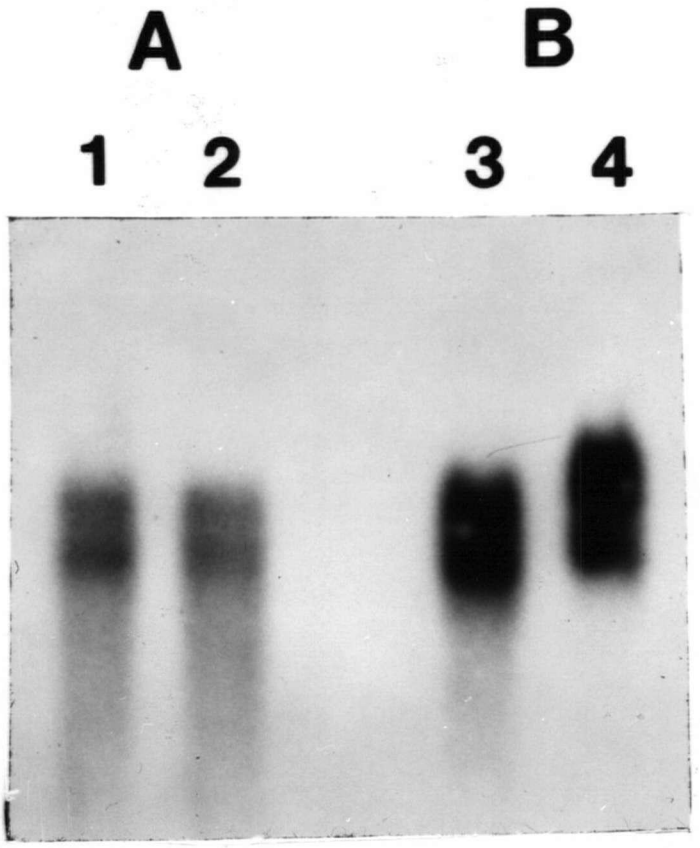


Figure 26. Expression of Dd*rap*1 in prestalk and prespore cells. Pseudoplasmodia of the V12-M2 strain were obtained by differentiation on 2% non-nutrient agar plates. The prestalk and prespore cells were separated using either (A) continuous Percoll gradients or (B) a Percoll step gradient (see Materials and Methods). Total RNA was extracted from the isolated prestalk cells (lanes 1 and 3) and prespore cells (lanes 2 and 4). RNA (20 µg) and was subjected to Northern blot analysis and hybridized with the Dd*rap*1-c51 cDNA clone as a probe.



Both transcripts declined in quantity during pseudoplasmodial formation and then increased during culmination. There did not appear to be an enrichment of Dd*rap*1 mRNA in prestalk or prespore cells (Figure 26).

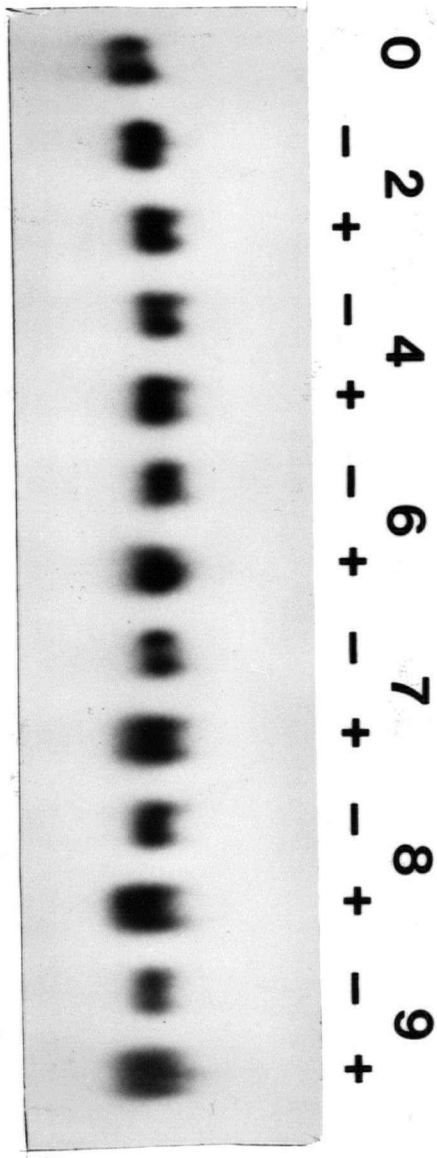
All three mRNAs appear to be derived from the Dd*rap*1 gene since they all hybridized to the cDNA under high stringency conditions, but the possibility that the Northern blot hybridizations represent the sum of more than one highly related *rap* gene can not be totally ruled out. The three transcripts might be generated from the same gene by the use of alternate promoters, differential splicing or modifications of the 3' terminus of the Dd*rap*1 mRNA. Low stringency Southern blots with the Dd*rap*1 cDNA revealed an additional band which may represent a second *rap* gene or an additional member of the *ras* gene superfamily.

7.2.4 Control of Dd*rap*1 expression

The Dd*rap*1 gene was expressed as a single 1.1 kb transcript in vegetative cells. The expression of the Dd*rap*1 gene during vegetative growth was not apparently affected by the position of the cells within the cell cycle, as was the case for the Dd*ras*G mRNA (Figure 14).

In order to investigate the factors involved in the expression of the Dd*rap*1 mRNAs during *D. discoideum* development, cells were shaken in suspension in the absence of nutrients and RNA samples were isolated at various times (in collaboration with M. Khosla). When shake suspension cultures were incubated in the presence of continuous cAMP, the 1.3 kb mRNA was expressed at 8 hours of development (Figure 27). It is interesting to note that the Dd*ras* 1.2 kb mRNA, but not the 0.9 kb Dd*ras* mRNA was induced in response to cAMP, suggesting that the 1.0 and 1.1 kb Dd*rap*1 mRNAs are not under cAMP regulation. These results suggest the possibility that the 1.0 and 1.1 kb mRNAs

Figure 27. The effect of cAMP on the expression of Dd*rap*1 in shake suspension cultures of strain V12-M2. V12-M2 cells were separated from the bacteria and resuspended in KK_2 buffer, pH 6.0 to a density of 5×10^6 cells/ml; and shaken at 250 rev/min in the absence (-) or presence (+) of 500 μM cAMP with additional cAMP added to a concentration of 100 μM every hour. Total RNA was extracted at the times indicated (hours) and subjected to Northern blot analysis (20μg) using the Dd*rap*1 -c51 cDNA as a probe.



are actually the same transcripts and the size difference that is observed late in development could be due to a change in the length of the poly A tail. Alterations in the length of the poly A tails of certain mRNAs have been reported during *D. discoideum* development, and these may result in a change in mRNA stability (Margolskee and Lodish, 1980; Shapiro, *et al.*, 1988), or in the initiation of protein synthesis (Palatnik, *et al.*, 1984; Manrow, *et al.*, 1988). The significance of the alteration in Dd*rap*1 transcript size during development remains to be elucidated.

7.3 Discussion

A cDNA clone, Dd*rap*1, was isolated from *D. discoideum* that hybridized to the Dd*ras* and Dd*ras*G cDNAs under low but not under high stringency conditions. The predicted amino acid sequence of the Ddrap1 protein has a high degree of identity with the *Drosophila* Dras3 protein (Schejter and Shilo, 1985) and the human rap proteins (Kawata, *et al.*, 1988; Pizon, *et al.*, 1988a; Pizon, *et al.*, 1988b; Kitayama, *et al.*, 1989; Farrell, *et al.*, 1990; Ohmstede, *et al.*, 1990). Despite the wide evolutionary divergance the Ddrap1 protein shares 60% and 76% amino acid identities with the Dras3 and human rap1A proteins respectively (Figure 23, Table 2). The conservation of the rap proteins between humans and *D. discoideum,* is even higher than that between the ras proteins of these species, suggesting that the rap proteins are an evolutionarily conserved branch of the ras-related family and thus may perform an essential function(s) in all eukaryotic organisms.

Despite an otherwise high level of sequence conservation within the N-terminal portion, the *D. discoideum* protein differs from the human rap proteins at the extreme amino terminus in that it is two amino acids longer. To

date there have been four *rap* homologues isolated in human cells (rap1A also called Krev-1 or smg 21, rap1B, rap2A and *rap*2B) which all share a high degree of overall sequence conservation and the same initiation sequence (Kawata, *et al.*, 1988; Pizon, *et al.*, 1988a; Pizon, *et al.*, 1988b; Kitayama, *et al.*, 1989; Farrell, *et al.*, 1990; Ohmstede, *et al.*, 1990). Although only a single *rap* gene has been identified thus far in *D. discoideum* low stringency Southern blots suggest the possibility that there may be an additional *rap* gene or a highly related sequence.

The rap and ras proteins share several structural properties. They have molecular weights of approximately 21,000 daltons and have similar biochemical properties (Kawata, *et al.*, 1988; Quilliam, *et al.*, 1990). The rap proteins contain the four non-contiguous domains that are involved in binding of guanine nucleotides to the ras proteins (Figure 23). The conservation of the amino acid sequences within these four domains as well as their identical spatial distribution within the rap proteins suggest that these proteins are also able to bind guanine nucleotides. Recently, Quilliam *et al.*, (1990) have demonstrated that the guanine nucleotide binding properties of the human rap1A protein are similar to the human ras proteins. In addition when the *D. discoideum* Dd*rap*1 gene was expressed in a bacterial expression system, it was also able to bind guanine nucleotides (R. Pachal, unpublished observations). Within one of the four domains associated with guanine nucleotide binding, the human, *Drosophila* and *D. discoideum rap* genes encode a threonine at position 61, instead of the customary glutamine found in all other ras-related proteins (Figure 23). The rap proteins are the first members of the ras-related proteins to exhibit such a change. This difference is of major interest since virtually any amino acid substitution at position 61 in the human H-ras protein is correlated with the ability to transform cell lines (Der, *et al.*, 1986).

In addition to the guanine nucleotide binding domains, the putative effector region encompassing amino acids 32-40 is strictly conserved within the ras and rap proteins (Figure 23). This region has been shown to be essential for biological activity of the ras oncoproteins and is the site within the protein that interacts with GAP (Sigal, *et al.*, 1986; Willumsen, *et al.*, 1986; Trahey and McCormick, 1987; Stone, *et al.*, 1988; Trahey, *et al.*, 1988).

The rap proteins also have the consensus Cys-A-A-X tetrapeptide sequence at their C-terminus and may therefore undergo similar post-translational modifications in order to be attached to the inner surface of the plasma membrane (see General Introduction) (Figure 23). The human rap proteins were found to be abundant in human platelet membranes (Ohmori, *et al.*, 1989) and in the particulate fractions of a wide range of other tissues (Kim, *et al.*, 1990). The rap1(A and B) proteins are released from platelet membranes after phosphorylation by cAMP-dependent protein kinase (Hoshijima, *et al.*, 1988; Nagata, *et al.*, 1989; Siess, *et al.*, 1990). This putative regulatory event has not been observed for any of the ras proteins although the K-ras protein has a potential phosphorylation site close to the carboxyl end of the protein.

The Dd*rap*1 gene exhibits a complex pattern of gene expression during *D. discoideum* development (Figures 24 and 25). The level of Dd*rap*1 mRNA changed dramatically during development reaching maximal levels at the aggregation stage, declining through the pseudoplasmodial stage and then peaking again during culmination. There were no other examples of this type of developmental regulation when it was first observed but recently another *D. discoideum* gene , 5G was reported to exhibit a similar biphasic developmental expression (Corney, *et al.*, 1990). It remains to be determined whether 5G is a *ras*-related gene. When the developmental expression of the Dd*rap*1 gene was compared to that of the two *D. discoideum ras* genes, Dd*ras*G and Dd*ras* an

interesting relationship was apparent (Figure 24). The maximum levels of Dd*rap*1 specific mRNA were detected during *D. discoideum* developmental at stages where the levels of the Dd*ras*G and Dd*ras* mRNAs were declining. Although there is no direct evidence for the function of the Ddrap1 protein in *D. discoideum*, the reciprocal nature of Dd*rap*1 gene expression with respect to the two *ras* genes suggests the possibility that the *ras* and *rap* gene products have antagonistic roles. Furthermore, the biphasic pattern of expression suggests the possibility that the Ddrap1 protein antagonizes both the Dd*ras*G and Dd*ras* gene products.

Although a direct antagonistic interaction between the *ras* and *rap* gene products has been suggested (Kitayama, *et al.*, 1989; Kitayama, *et al.*, 1990; Zhang, *et al.*, 1990b), the precise function of the rap proteins in mammalian cells remains unknown. Overexpression of the human *rap*1A (K-*rev*1) gene induced morphological reversion of a cell line transformed by an activated *ras* gene (Kitayama, *et al.*, 1989). The reverted cell line still expressed the activated *ras* gene suggesting that the *rap* gene product might interfere with some aspect of the ras pathway. The region encompassing the effector domain is required for rap suppression suggesting the possibility that the rap protein competes with the ras oncoprotein for GAP and blocks downstream signalling (Frech, *et al.*, 1990; Hata, *et al.*, 1990; Zhang, *et al.*, 1990b). The rap protein can interact with the ras specific GAP but its GTPase activity is not stimulated (Frech, *et al.*, 1990; Hata, *et al.*, 1990), due to the presence of the threonine residue at position 61 of the rap1A protein (Hart and Marshall, 1990). Although this provides a molecular mechanism to account for the ability of the rap proteins to revert ras tranformed cells, it is probably an oversimplification because rap specific GAP proteins have been identified that can stimulate rap GTPase activity (Kikuchi, *et al.*, 1989;

Ueda, *et al.*, 1989). It is possible, therefore, that the ras and rap proteins interact with different target molecules which modify mutually antagonistic pathways.

A specialized function of the human rap proteins has recently been suggested since the rap1 proteins were found in the plasma membrane of neutrophils in association with the molecular complex for superoxide generation (Quinn, *et al.*, 1989). However it remains to be determined whether the rap proteins are involved in the assembly of this complex upon stimulation of the neutrophils.

CHAPTER 8

8.0 DETECTION OF THE Ddras, DdrasG AND Ddrap1 ENCODED PRODUCTS

8.1 Introduction

Although the precise roles for the members of the ras gene superfamily remain unknown it appears that the gene products perform diverse functions (Downward, 1990; Hall, 1990). To evaluate the specific function of each of the *D. discoideum* ras and ras-related proteins it is essential to discriminate between each of the closely related gene products. The Y13-259 monoclonal antibody recognition sequence has been mapped to a highly conserved domain of the human ras proteins encompassing amino acids 63-73 (Sigal, *et al.*, 1986). This domain is conserved in both the Ddras and DdrasG proteins but not the Ddrap1 protein (Figure 23), suggesting that the ras proteins immunoprecipitated from *D. discoideum* cell free extracts could have been products from either of the two *ras* genes. Since the Y13-259 antibody is not able to discriminate between the Ddras and DdrasG proteins it is important to generate specifc antisera to recognize each of the encoded products.

Although the amino acid sequences of three of the *D. discoideum* ras-related proteins, Dd*ras*, Dd*ras*G and Dd*rap*1 are highly conserved, there are two short variable regions (Figure 23). The most significant differences among the proteins are confined to a short stretch of amino acids located just upstream from the C-terminus. Two procedures were used to generate a series of antisera which react uniquely with the Dd*ras* , Dd*ras*G and Dd*rap*1 gene products. The antisera were obtained by immunizations with either synthetic peptides corresponding to the

unique C-terminal amino acid sequences or with recombinant proteins purified from bacteria expressing each of the three genes.

8.2 Results

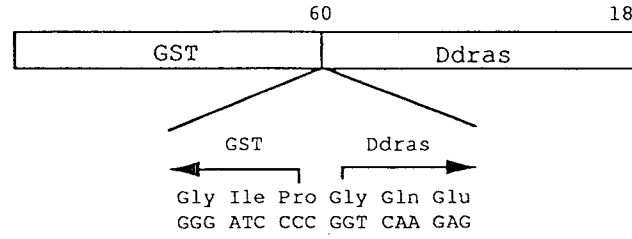
8.2.1 Preparation of antisera which recognize peptides corresponding to the Ddras, DdrasG and Ddrap1 variable regions

Peptides based on nucleic acid sequence analysis have been successfully used in the development of antibodies with predetermined specificity (Lerner, 1982). This strategy has been used to generate antisera that interact specifically with the effector region of the ras proteins (Rey, *et al.*, 1989) and to generate antisera unique for the human H-*ras* and K-*ras* gene products (Srivastava, *et al.*, 1985; Bizub, *et al.*, 1987). Figure 23 shows the deduced amino acid sequences of the Ddras, DdrasG and Ddrap1 proteins. The synthetic peptides which were used corresponded to 15 amino acids within the variable domain of each of the proteins, Ddras$^{170\text{-}184}$, DdrasG$^{172\text{-}186}$ and Ddrap1$^{169\text{-}183}$. The individual peptides were coupled to the carrier protein, keyhole limpet hemocyanin through the C-terminal cysteine residue of all three peptides and used to immunize rabbits. The preparation and purification of each of the antisera is described under Materials and Methods. The antisera obtained from the rabbits were titrated in an enzyme linked immunosorbent assay using the purified peptides as the antigen. Each of the antisera was specific for the corresponding peptide used in the immunizations.

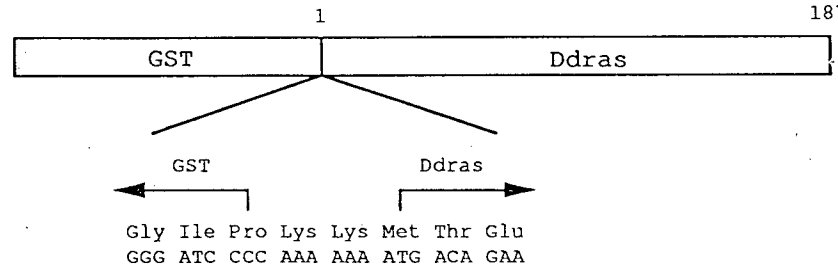
The antisera were tested for reactivity with extracts from bacteria that had expressed each of the Dd*ras*, Dd*ras*G and Dd*rap*1 cDNAs as fusion proteins. The three cDNAs were incorporated into the pGEX plasmid expression vectors, which include the coding sequences of the *Schistosoma japonicun* glutathione S-

Figure 28. Schematic structures of the Dd*ras*, Dd*ras*G and Dd*rap*1 sequences expressed as GST fusion proteins in *E. coli*. The boxes represent the GST recombinant proteins that were constructed, the numbers correspond to the amino acids of the Ddras, DdrasG and Ddrap1 proteins that were expressed. (A) The pGEX-Dd*ras*$^{60\text{-}187}$ construction expresses a fusion protein encoding amino acids 60-187 of the Dd*ras* protein; an approximately 800 bp *Pst*1/*Eco*RI Dd*ras* genomic fragment was isolated, blunt ends were generated by digesting with mung bean nuclease (Maniatis, *et al.*, 1982) and ligated into the *Sma*I site of pGEX-3X (Smith and Johnson, 1988). (B) The pGEX-Dd*ras* plasmid was constructed by R. Pachal. A *Dra*I fragment containing the entire Dd*ras* coding region was ligated into the *Sma*I site of pGEX-2T. (C) The pGEX-Dd*ras*G plasmid which expressed the entire Dd*ras*G coding sequence was also constructed by R. Pachal. A 700 bp *Dra*I fragment was isolated from the Dd*ras*G-c3 cDNA and subcloned into the *Sma*I site of pGEX-2T. (D) The pGEX-Dd*rap*1 construction encodes amino acids 6 to 187. The Dd*rap*1-c51 cDNA was subcloned into the *Eco*RI site of pGEX-1N. The nucleotide sequences and corresponding amino acids at the junction of the recombinant pGEX constructions are shown. The various constructions were confirmed by restriction mapping and the plasmid junctions were verified by sequence analysis (R. Pachal).

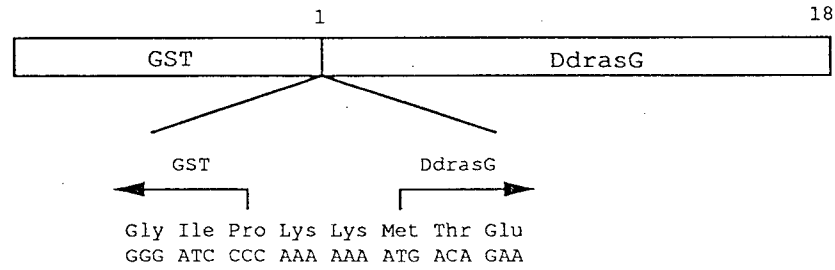
A.pGEX-Ddras$^{60-187}$



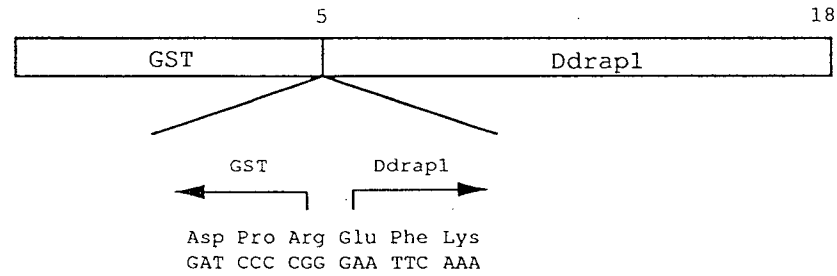
B.pGEX-Ddras



C.pGEX-DdrasG



D.pGEX-Ddrap1



transferase (GST) gene under control of the *tac* promoter (Smith and Johnson, 1988). The cDNAs were fused to the GST gene in the correct reading frame (Figure 28) which upon induction with IPTG synthesized fusion proteins consisting of the 27.5 kd GST domain and either a Ddras, DdrasG or Ddrap1 domain. The Ddras$^{170-184}$ antiserum only detected the GST-Ddras recombinant protein (approximately 48 kd), the DdrasG$^{172-186}$ antiserum only detected the GST-DdrasG recombinant protein (approximately 49 kd) and the Ddrap1$^{169-183}$ antiserum only recognized the GST-Ddrap1 recombinant protein (approximately 47 kd) when used in an immunoblot analysis (Figure 29). Although the antisera generated were specific for each of the recombinant proteins they were very weak reactions and did not detect any specific proteins when immunoblots of *D. discoideum* cell extracts were incubated with the various antisera.

8.2.2 Preparation of antisera to the Ddras, DdrasG and Ddrap1 recombinant proteins

A second approach was used to obtain antisera specific for each of the *D. discoideum* ras-related proteins. The GST-Ddras (amino acids 60-187), GST-DdrasG and GST-Ddrap1 fusion proteins were purified to near homogeneity by glutathione-agarose affinity chromatography (see Materials and Methods). Each of the purified recombinant proteins was used to immunize rabbits (see Materials and Methods). The antisera obtained from the rabbits were tested for reactivity with each of the recombinant proteins by immunoblot analysis. The antisera cross-reacted with the GST protein, each of the recombinant proteins and a few other bacterial proteins. The cross-reacting antibodies within each of the antisera were removed by absorption with purified recombinant fusion proteins coupled to glutathione-agarose. The antisera generated to the GST-Ddras$^{60-187}$ fusion

Figure 29. Immunoblot analysis with the antisera generated to peptides corresponding to the variable regions of the Ddras, DdrasG and Ddrap1 proteins. Crude lysates (25 μg) of *E. coli* cultures containing the GST-Ddrap1 (lanes 1), GST-DdrasG (lanes 2), GST-Ddras (lanes 3) and GST (lanes 4) proteins were subjected to SDS-PAGE, and then transferred to nitrocellulose membranes (see Materials and Methods). The blots were incubated with 1/100 dilutions of: (A) $Ddras^{170-184}$ antisera, (B) $DdrasG^{172-186}$ antisera and (C) $Ddrap1^{169-183}$ antisera. The blots were then incubated with goat anti-rabbit antibody conjugated to alkaline phosphatase and bound antibody was detected using BCIP/NBT (see Materials and Methods). The arrowheads on the right indicate the size and position of the molecular weight standards (kd).

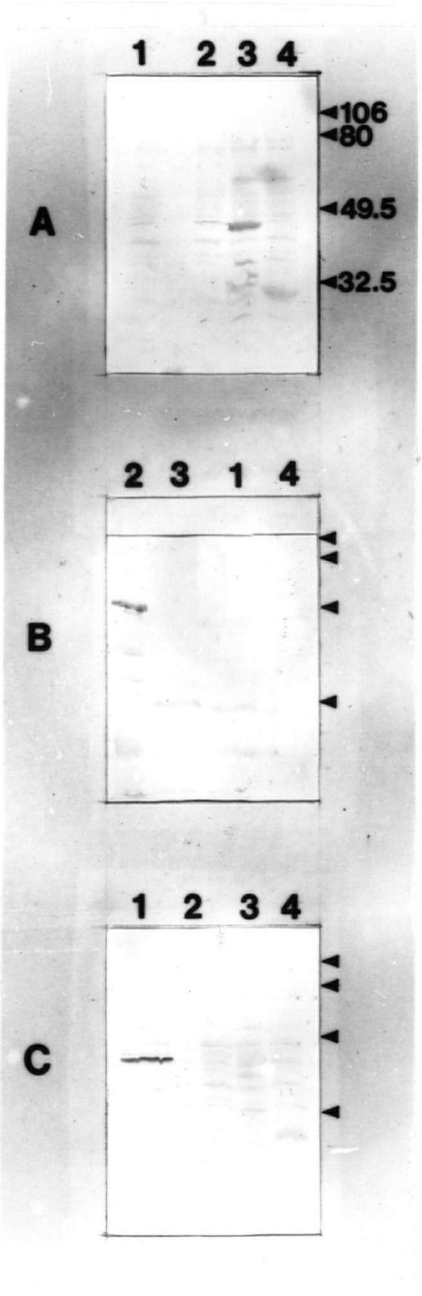
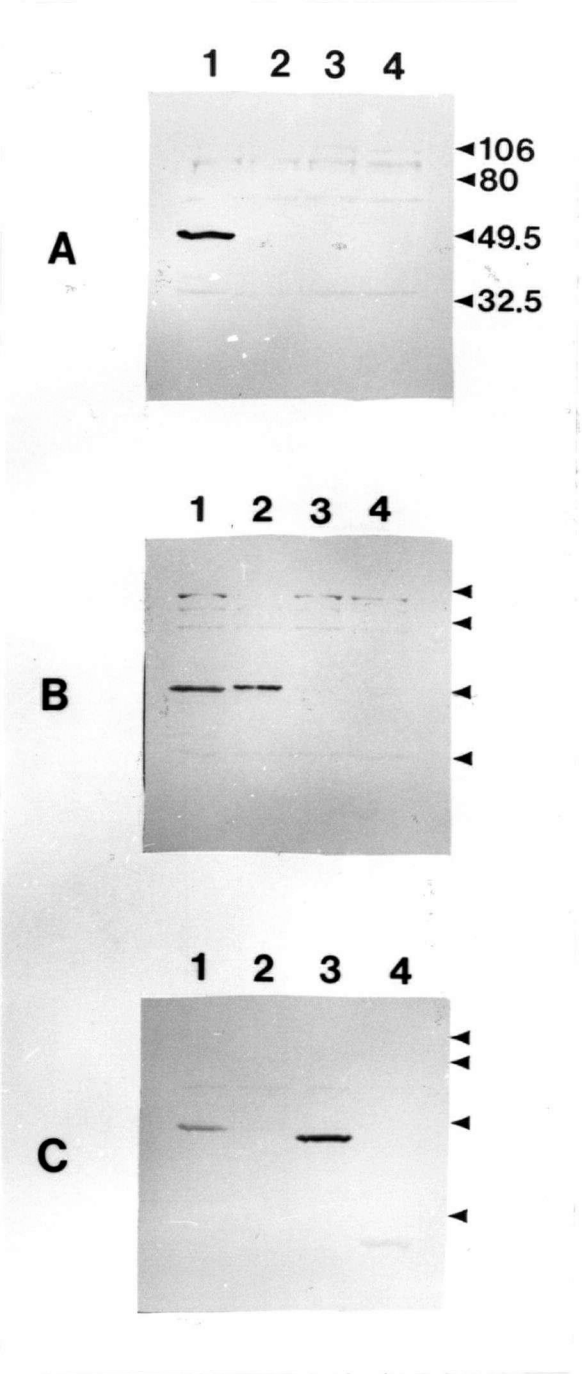

1 2 3 4
106
80
49.5
32.5
A
2 3 1 4
B
1 2 3 4
C

Figure 30. Immunoblot analysis with the antisera generated to the Ddras, DdrasG and Ddrap1 bacterial recombinant proteins. Crude lysates (25 μg) of *E. coli* cultures containing the GST-Ddras (lanes 1), GST-DdrasG (lanes 2), GST-Ddrap1 (lanes 3) and GST (lanes 4) proteins were subjected to SDS-PAGE, and transferred to nitrocellulose membranes (see Materials and Methods). The blots were incubated with 1/5000 dilutions of: (A) GST-Ddras$^{60-187}$ antisera, (B) GST-DdrasG antisera and (C) GST-Ddrap1 antisera. They were then incubated with donkey anti-rabbit antibody conjugated to horseradish peroxidase and bound antibody was detected using the ECL detection system (see Materials and Methods). The arrowheads indicate the size and position of the molecular weight standards (kd).


1 2 3 4
A
106
80
49.5
32.5
1 2 3 4
B
1 2 3 4
C

protein was incubated with both the GST-DdrasG and GST-Ddrap1 recombinant proteins. Similarly, the GST-DdrasG antisera was incubated with the GST-Ddras and GST-Ddrap1 recombinant proteins and the Ddrap1 antisera was incubated with both the GST-Ddras and GST-DdrasG fusion proteins. The purified antisera was then used to probe blots of bacterial extracts containing each of the recombinant fusion proteins. As can be seen in Figure 30 the Ddras$^{60-187}$ antisera specifically recognized the GST-Ddras fusion protein. The DdrasG antisera recognized the GST-DdrasG recombinant protein as well as the GST-Ddras recombinant protein (Figure 30). The Ddrap1 antisera detected the GST-Ddrap1 recombinant protein and weakly detected the GST-Ddras and GST recombinant (27.5 kd) proteins (Figure 30). The DdrasG antisera cross-reacted with the Ddras recombinant protein suggesting that the antibodies that recognized a common epitope in the two proteins were not removed during the adsorption with the recombinant proteins. This antisera will be adsorbed again with the GST-Ddras fusion protein to try to remove more of the cross-reacting antibodies (M. Khosla, studies in progress). Each of the antisera reacted with a few additional bacterial proteins that were common to all samples. This was probably due to contaminating bacterial proteins in the purified recombinant protein preparation used for the immunizations. These antibodies are sensitive enough to detect their corresponding proteins in *D. discoideum* extracts by immunoblot analysis (Robbins and Khosla, studies in progress).

8.3 Discussion

A series of polyclonal antibodies have been prepared that recognize the Ddras, DdrasG and Ddrap1 proteins. To maximize selectivity, rabbits were immunized with peptides corresponding to the C-terminal sequences, the regions of the

proteins with the highest variability. The purified antibodies were specific for each of the proteins when assayed by immunoblot analysis with bacterial lysates that expressed the Dd*ras*, Dd*ras*G and Dd*rap*1 cDNAs (Figure 29). However, the antisera were insufficiently sensitive to detect the corresponding proteins from *D. discoideum* cell extracts, increasing the antibody concentrations only resulted in an increased background on the immunoblots. It is possible that the antibodies cannot recognize the endogenous proteins since the peptides corresponded to the region of the proteins that may be post-translationally modified, whereas the bacterial recombinant proteins would not be expected to undergo the processing events.

Antibodies were also prepared against purified recombinant proteins. The Dd*ras*, Dd*ras*G and Dd*rap*1 cDNAs were expressed in *E. coli* as proteins fused to the bacterial GST protein. To generate specific antibodies for each of the gene products, each antiserum was incubated with the other recombinant proteins to remove antibodies that recognize common epitopes (see Results). The antisera were characterized in terms of their specificity of reaction with each of the recombinant proteins (Figure 30). Each of the antisera detected the appropriate recombinant protein used for immunization. In addition, the DdrasG antiserum also cross-reacted with the GST-Ddras recombinant protein and the Ddrap1 antiserum weakly detected the Ddras recombinant protein. The Ddras antiserum was raised to a GST fusion protein that only contained amino acids 60-187 of the Ddras protein, a region that is more highly diverged between the Ddras and DdrasG proteins (Figure 11). This might explain the higher specificity of the Ddras antiserum.

The recognition of the mammalian ras proteins by specific antibodies has been a useful tool to characterize the biochemical properties and functional domains of the ras proteins as well as the subcellular localization (Barbacid, 1987). In

addition, antibodies have been used to assess ras protein levels in normal and malignant tissues (Hand, *et al.*, 1984; Bizub, *et al.*, 1987). The antibodies generated in this study are specific for each of the Ddras, DdrasG and Ddrap1 encoded products and should be useful for determining some of the biochemical and functional properties of the ras and rap proteins in *D. discoideum.*

Recently a DNA-mediated transformation system for *D. discoideum* has been developed and has been used to assess the biological function of a number of gene products (Nellen, *et al.*, 1984; Nellen and Firtel, 1985; Early and Wlliams, 1987). *D. discoideum* transformant strains that have altered expression of the Dd*ras*G and Dd*rap*1 genes are being developed to determine their effect on growth and development (M. Khosla, R. Thiery and P. Rebstein, studies in progress). The specific Ddras, DdrasG and Ddrap1 antisera will be essential to define the levels of the respective proteins within the various transformants. In addition the antibodies may be used to select transformants that are overexpressing or have a reduced expression of either of the Ddras, DdrasG or Ddrap1 proteins, a procedure that has been used successfully to isolate α-actinin and gp80 transformants (Wallraff, *et al.*, 1986; Witke, *et al.*, 1987; Faix, *et al.*, 1990). The ability to discriminate between each of the Dd*ras*, Dd*ras*G and Dd*rap*1 encoded products should be useful in defining their specific roles during growth and differentiation of *D. discodieum.*

CHAPTER 9

9.0 GENERAL DISCUSSION

9.1 Correlation between ras protein levels and *ras* gene expression during *D. dsicoideum* development

At the outset of this work a single *ras* gene, Dd*ras*, had been isolated from the cellular slime mould, *Dictyostelium discoideum*. The Dd*ras* gene was maximally expressed during the pseudoplasmodial stage of development (Reymond, *et al.*, 1984). In contrast maximal rates of *in vivo* ras protein synthesis were observed in vegetative and early developing cells although there was a small burst of synthesis in pseudoplasmodial cells (Pawson and Weeks, 1984; Pawson, *et al.*, 1985; Weeks and Pawson, 1987). In agreement with these results a polyclonal antiserum raised to a fusion protein of the *E. coli* β-galactosidase and part of the amino acid sequence of the Ddras protein recognized proteins in vegetative cells and throughout aggregation with protein levels gradually diminishing late in development (Reymond, *et al.*, 1984).

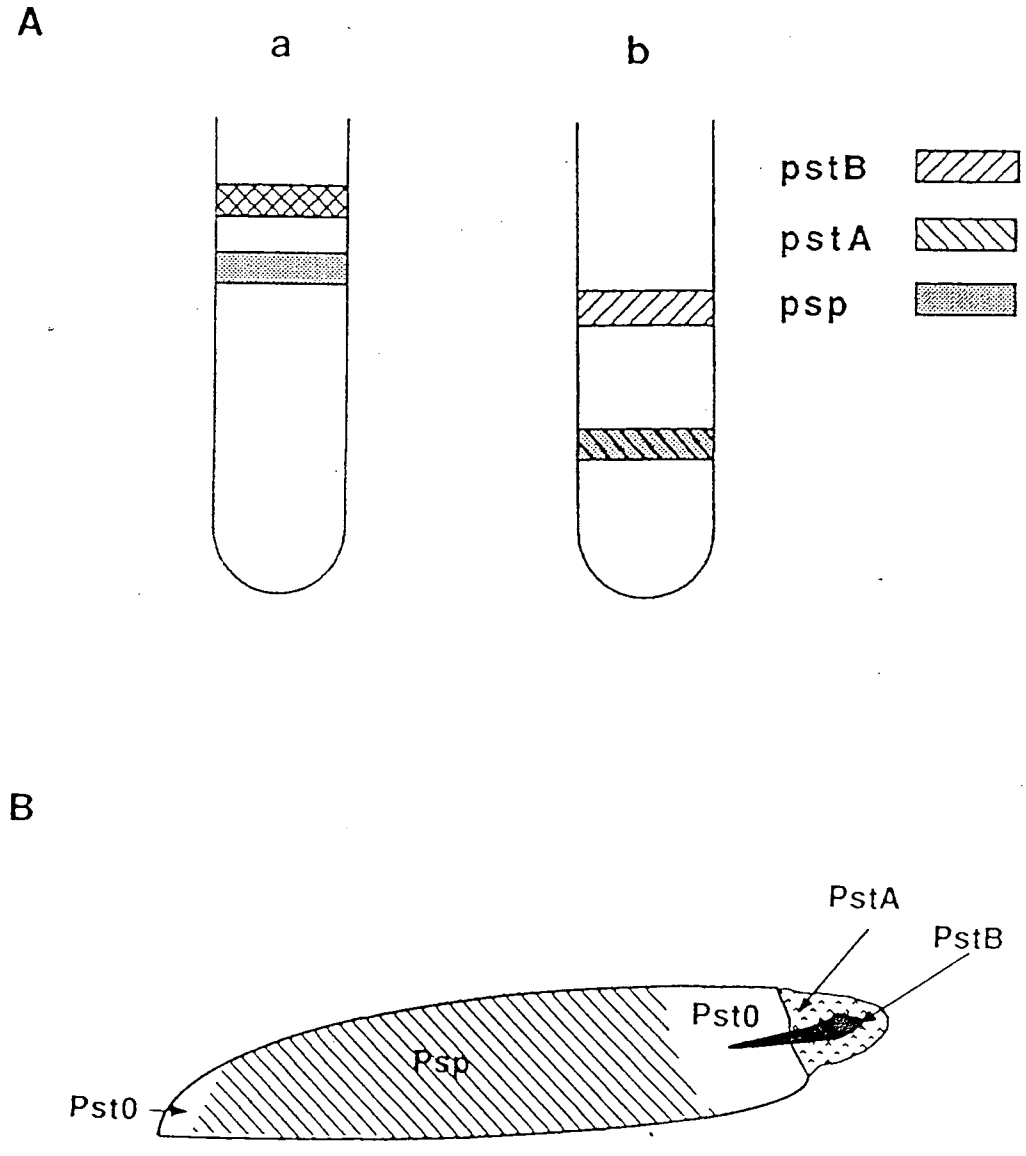
The isolation of second *ras* gene, Dd*ras*G, explains the discrepancies between the expression of the Dd*ras* mRNA and the ras protein synthesis observed during *D. discoideum* development. In the wild type strains the Dd*ras*G gene was only expressed during vegetative growth and early development, whereas the Dd*ras* gene was maximally expressed at the pseudoplasmodial stage of development (Figures 13 and 24). The Dd*ras* and Dd*ras*G gene products are highly conserved and both contain the recognition sequence of the *ras* specific monoclonal antibody, Y13-259 (Figure 11). Therefore, the combined expression of the Dd*ras*G and Dd*ras* genes can account for the changes in the ras protein

synthesis that are observed during the differentiation process (Pawson and Weeks, 1984; Pawson, *et al.*, 1985), although this does not preclude the possibility that there may be additional *ras*-related sequences that remain to be discovered. Furthermore, the high degree of sequence similarities between the two proteins suggests that the polyclonal antisera directed against the Ddras protein would also detect the DdrasG protein and thus explain the observations of Reymond *et al.*, (1984).

9.2 Cell-type specific expression of the Dd*ras* gene

It was initially shown that the Dd*ras* mRNA was enriched in the prestalk cell population (Reymond, *et al.*, 1984) and this result has subsequently been confirmed (Jermyn, *et al.*, 1987). In contrast, ras protein synthesis only occured in the prespore cell population (Weeks and Pawson, 1987). In these latter experiments the prestalk and prespore cells were separated by a modification of the Ratner and Borth (1981) Percoll gradient technique. It was subsequently found that the modified procedure yielded a prespore fraction contaminated with a subpopulation of prestalk cells (Kwong, *et al.*, 1990) (Figure 31). This contaminating subpopulation of prestalk cells expressed the prestalk specific gene pDd63 and Dd*ras* but does not express the prestalk specific gene, pDd56, suggesting that the Dd*ras* gene is expressed in a subset of the prestalk cells (Kwong, *et al.*, 1990). In keeping with the nomenclature established by Williams and co-workers the higher density fraction of the prestalk cells expressing the Ddras and pDd63 genes are the pstA cells and the low density prestalk cells that express both pDd63 and pDd56 are the pstB cells (Figure 31) (Jermyn, *et al.*, 1989; Williams, *et al.*, 1989). Using *D. discoideum* cells that express a Dd*ras*/*lac*Z fusion the pattern of Dd*ras* expression could be localized to a subset of prestalk

Figure 31. Heterogeneity of prestalk cells in the pseudoplasmodium. (A) A summary of the Percoll gradient results showing the location of the pstA and pstB cells in (a) continous Percoll gradients and (b) in the Percoll step gradients. (B) The location of the pstA cells and pstB cells within the migrating pseudoplasmodium. The pstA cells express the pDd63 and Dd*ras* mRNAs, pstB cells express pDd56 mRNA and the pst0 cells located in the zone between the prestalk region and the prespore region do not express either the pDd56 or pDd63 genes. Part of the Figure, (B) was adapted from Jermyn and Williams (1989).



cells within the prestalk zone of the pseudoplasmodium (Esch and Firtel, personal communication), thus confirming the cell separation results.

This is not the first evidence for heterogeneity within the prestalk cell population. It has been shown recently that different areas of the prestalk region express different genes (Gomer, *et al.*, 1986) and that the prestalk cells can be functionally divided on their basis for requirements for *in vitro* stalk cell formation (Kwong, *et al.*, 1988). The expression of the Dd*ras* gene in a subset of prestalk cells provides further evidence for the heterogeneity within the prestalk zone of the developing pseudoplasmodium. However, the morphological significance of the heterogeneity within the prestalk region remains to be determined.

9.3 Evolutionary conservation of the ras and rap proteins

The ras protein superfamily is highly conserved throughout eukaryotic evolution, suggesting that they encode essential functions in all eukaryotes. The *D. discoideum* Ddras and DdrasG proteins show a higher degree of sequence identity to the human ras proteins identity than the yeast proteins do (Table 2). In addition the Ddrap1 protein has 76% identity to the human rap1 proteins whereas the yeast homologue, RSR1 (Bender and Pringle, 1989) shows a much lower sequence similarity (Table 2). Comparison of the amino acid sequences of the ras, rap, and other evolutionary conserved proteins indicate that *D. discoideum* is more closely related to mammals than is yeast and that *D. discoideum* diverged from the line leading to metazoa at about the same time of plant and animal divergence (Loomis and Smith, 1990). This is in contrast to the previous analysis based on the nucleotide sequence of 18S rRNA, which indicated that *D. discoideum* represented one of the earliest branches from the

TABLE 2.

Percentage of amino acid conservation within the ras and rap protein families

A.

Percentage of amino acid identity between the ras protein family

	H-ras	Dras1	Ddras	DdrasG	ScRAS1[a]	ScRAS2[a]
H-ras	100	75	65	69	60	60
Dras1		100	61	63	54	57
Ddras			100	82	59	61
DdrasG				100	60	61
ScRAS1					100	79
ScRAS2						100

[a] percentage identity was calculated over the 189 amino acid domain common to the ras protein family and does not include the domain unique for the Sc proteins

Amino acid identities were calculated using the sequences shown in figure 11.

B.

Percentage of amino acid identity beween the rap protein family

	rap1A	rap2A	Dras3	Ddrap1	ScRSR1
rap1A	100	61	67	76	46
rap2A		100	50	55	48
Dras3			100	60	51
Ddrap1				100	46
ScRSR1					100

Amino acid identities were calculated from the sequences presented in Pizon *et el.*, (1988), Bender and Pringle (1989) and figure 23.

Abbreviations: D, *Drosophila melanogaster;* Dd, *Dictyostelium discoideum;* Sc, *Saccharomyces cerevisiae.*

mainstream of eukaryotic evolution (McCarroll, *et al.*, 1983). It has been suggested that nucleotide sequence comparisons are misleading in *D. discoideum* because of the unusually high adenine/thymine nucleotide content within the genome and that the comparison of amino acid sequences are a more reliable indicator of phylogenetic comparisons (Loomis and Smith, 1990).

The main function of the ras proteins in *S. cerevisiae* is to control GTP-stimulated adenylyl cyclase activity (Broek, *et al.*, 1985; Toda, *et al.*, 1985). Even though the human ras proteins can complement *S. cerevisiae* ras function (Broek, *et al.*, 1985; DeFeo-Jones, *et al.*, 1985; Kataoka, *et al.*, 1985) it does not appear that the endogenous function of mammalian ras proteins is to regulate adenylyl cyclase activity (Beckner, *et al.*, 1985). The possibility that *D. discoideum* is evolutionary closer to higher eukaryotes than yeast argues strongly in favour of examining the physiological functions of the ras and ras-related proteins in *D. discoideum.*

9.4 *Ras* and *ras*-related gene function in *D. discoideum*

The expression of the *RAS* genes in *S. cerevisiae* is essential (Toda, *et al.*, 1985) and it has been proposed that *ras* gene expression in mammalian cells might also be essential. There are also indications as to the essential nature of *ras* gene expression in *D. discoideum.* Transformation of *D. discoideum* cells with the Dd*ras* (Reymond, *et al.*, 1985) or Dd*ras*G (M. Khosla, studies in progress) genes in the antisense orientations appears to be lethal, but it remains to be determined if the expression of either one or both of the genes is required for cell viability.

The Dd*ras* gene product does not appear to regulate adenylyl cyclase activity or intracellular cAMP levels in *D. dsicoideum,* but there is evidence to suggest

that it is involved in cAMP transmembrane signal transduction. In *D. discoideum*, cAMP signal transduction involves two pathways, one controlling the the signal relay response and one controlling the chemotactic response (Figure 4) (see General Introduction). *D. discoideum* transformants that overexpress an activated Dd*ras* gene (amino acid 12 changed from glycine to threonine) display an aberrant morphogenesis leading to multiple tipped aggregates that do not form the final fruiting bodies (Reymond, *et al.*, 1986). It was found that the activity of the adenylyl cyclase relay pathway was unaffected in the Dd*ras*^{Thr12} transformants but the ability to produce cGMP in response to chemotactic stimuli was reduced, presumably due to an enhanced desensitization, suggesting that the aberrant phenotype may be due to impaired regulation of the chemotactic response (Van Haastert, *et al.*, 1987a). The Ddras^{Thr12} transformants have a reduction in the number of cAMP receptors suggesting that the Ddras protein is involved in the down-regulation of the cAMP receptor (Luderus, *et al.*, 1988). There is evidence that guanylyl cyclase regulation in *D. discoideum* is coupled to the inositol pathway (see General Introduction). The Dd*ras*^{Thr12} transformants show a three to five fold increase in the basal levels of IP_3 and inositol hexakisphosphate (IP_6) (Europe-Finner, *et al.*, 1988). These results suggest the possibility that the Dd*ras* gene product regulates the activation of an as yet undetected phospholipase C, which may in turn regulate the desensitization of guanylyl cyclase. However, it was recently proposed that the increase in IP_3 levels can be explained by an increased conversion of phosphatidyl inositol (PtdIns) to phosphatidyl inositol phosphate, as a result of the regulation of PtdIns kinase activity (Van der Kaay, *et al.*, 1990). An increase in PtdIns kinase activity has been observed in *ras*-transformed fibroblasts (Huang, *et al.*, 1988). However, an increased *in vitro* activity of PtdIns

kinase was not detected in extracts of the Ddras^{Thr12} transformants (Van der Kaay, *et al.*, 1990).

It should be noted that in the above studies the effects of the activated Dd*ras* gene product on signal transduction were performed on early aggregation stage cells. Since the Dd*ras* gene is not expressed at this stage (Figures 13,19 and 24), the activated Ddras protein might be interfering with some other G protein rather than the normal Ddras protein. This argument suggests the possibility that the Ddras protein is not involved in cAMP mediated signal transduction during aggregation. However, since the Dd*ras* gene is expressed in prestalk cells at the pseudoplasmodial stage, it is conceivable that the gene product is involved with a specific cAMP receptor that has specialized function in prestalk cells. The existence of cAMP receptor genes that are preferentially expressed at the pseudoplasmodial stage of development has recently been reported (Saxe, *et al.*, 1990).

The Dd*ras*G and Dd*ras* genes are expressed at different times during *D. discoideum* development (Figures 13 and 24) and thus the two proteins might perform different functions: DdrasG having a role in cellular proliferation and Ddras a role in signal transduction at the multicellular stage of development. Dd*ras*G expression is repressed by cAMP, whereas the Dd*ras* gene is induced by cAMP, a differential regulation that would ensure that the two gene products were not expressed at the same time during development. It was found that the Dd*ras*G gene was expressed throughout development in the axenic strain Ax-2, providing a situation in which both *ras* genes were expressed at the pseudoplasmodial stage of development (Figure 18). Since the axenic strain develops normally, it does not appear that the continued expression of the Dd*ras*G gene has a deleterious effect on development.

The DdrasG protein is probably linked to an as yet, unidentified signal transduction pathway during cell growth and/or early development of *D. discoideum*. Two signal transduction pathways in which DdrasG could potentially interact have been identified in vegetative cells. Vegetative amoebae chemotactically respond to folic acid which is secreted by the bacterial food source (Janssens and Van Haastert, 1987; Kessin, 1988). Upon the initiation of development the cells become less responsive to folic acid and gain responsiveness to cAMP. The signal transduction pathways involved in the responses to cAMP and folic acid have a number of similarities but they involve different cell surface receptors and distinct G proteins (De Wit and Van Haastert, 1985; Kesbeke, *et al.*, 1990). If the Dd*ras* gene product is involved in the down regulation of a specific cAMP receptor it is possible that the DdrasG protein is coupled to the folic acid signal transduction pathway. Mutants have been isolated that do not respond chemotactically to folic acid but respond normally to cAMP (Segall, *et al.*, 1988) and they may be important in addressing the possible association of the DdrasG protein with the folic acid signal transduction pathway. However, failure to respond to folic acid is not a lethal event and the possible involvement of the DdrasG protein in this pathway is not consistent with the idea that the ras gene products are essential.

The other possibility is that the DdrasG protein is involved in transduction of mitogenic signals controlling cellular proliferation. The mitogenic signals that control cellular proliferation in *D. discoideum* remain largely unknown. However, the isolation of a specific growth factor has been recently reported and this growth factor may transduce a signal through the DdrasG protein to stimulate cellular proliferation (Whitbread and Katz, personal communication).

The function of the Ddrap1 protein in *D. discoideum* is also unknown. One of the interesting features of the human rap1A protein is that it can suppress the

transformed phenotype associated with an activated *ras* gene in NIH 3T3 cells (Kitayama, *et al.*, 1989; Kitayama, *et al.*, 1990; Zhang, *et al.*, 1990b). The Dd*rap*1 gene is maximally expressed during the developmental stages where the levels of the Dd*ras*G and Dd*ras* messages are declining (Figure 24). The reciprocal nature of Dd*rap*1 gene expression with respect to that of the two *ras* genes suggests the possibility that the *ras* and *rap* gene products in *D. discoideum* also have antagonistic roles. Furthermore, the biphasic pattern of Dd*rap*1 expression suggests the possibility that the Ddrap1 protein antagonizes both the Dd*ras* and Dd*ras*G gene products. It will be interesting to determine if the mutant phenotype of the activated Dd*ras* transformants will be suppressed by overexpression of the Dd*rap*1 gene.

In addition to Dd*ras*G, Dd*ras* and Dd*rap*1 two other *ras*-related genes, SAS1 and SAS2 have thus far been identified in *D. discoideum* (Saxe and Kimmel, 1988; Saxe and Kimmel, 1990). The encoded products of the SAS1 and SAS2 genes have similar sequences to those of the yeast YPT and SEC4 genes, which have been shown to be involved in GTP stimulated intracellular protein transport (Salmien and Novick, 1987; Goud, *et al.*, 1988; Segev, *et al.*, 1988), and the *D. discoideum* gene products may perform analogous functions.

Although a great deal is known about the three-dimensional structure and biochemical properties of the ras proteins the molecular mechanisms by which they control cellular proliferation and cellular differentiation remain to be determined. The elucidation of the function of the *D. discoideum* ras and ras-related proteins during growth and differentiation will hopefully provide an increased understanding into the role of the *ras* gene superfamily in normal and malignant mammalian cells.

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