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**Analysis of development using reverse genetics: Studies with the
UDP glucose pyrophosphorylase and a human rac protein kinase
homolog in *Dictyostelium discoideum***

Moon, Byoung Chon, Ph.D.

City University of New York, 1994

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A

**Analysis of Development Using Reverse Genetics: Studies
with the UDP Glucose Pyrophosphorylase and a Human rac
Protein kinase Homolog gene in *Dictyostelium discoideum***

by

Byoung Chon Moon

**A dissertation submitted to the Graduate Faculty in
Biology in partial fulfillment of the requirements for
the degree of Doctor of philosophy, The City University
of New York**

1994

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of philosophy.

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ABSTRACT**Analysis of Development Using Reverse Genetics in*****Dictyostelium discoideum*****by****Byoung Chon Moon****Adviser: Robert P. Dottin**

Using reverse genetics, the functions of the UDP glucose pyrophosphorylase (UDPGP) and a human rac protein kinase homolog gene have been characterized in development of *Dictyostelium*.

The enzyme UDP glucose pyrophosphorylase plays a central role in carbohydrate metabolism in many species. To analyze its function, we have created a null mutation by homologous recombination. The mutants grow normally but with no activity and develop with no obvious morphological defect. Nevertheless, enzyme activity appears at 15 hr into development. The disruptants have a slightly lower level of cellulose during culmination but the same as wild type organisms at the end development. Therefore, a second unknown UDPGP gene is expressed in late development. We cloned part of the second gene, UDPGP2, by taking advantage of the null mutant cell lines and using a novel polymerase chain reaction (PCR) strategy. The deduced amino acid sequence of part of UDPGP2 has significant homology with the UDPGP1 of *Dictyostelium* (46%) and slightly

greater (51%) homology with the UDPGP enzyme of potato tuber. In contrast with UDPGP1, UDPGP2 mRNA accumulates only late in development, suggesting its expression may be regulated by different signal transduction pathways.

We also isolated a serine/threonine protein kinase, DdK6 which has a pleckstrin homology (PH) domain which might be involved in signal transduction and promote protein-protein aggregation. The protein kinase is closely related to the Rac protein kinase family. A reverse genetic approach was also used to reveal the role of DdK6 gene. Null mutant cell lines were created by gene targeting through homologous recombination. The null mutants caused heterochronic development delaying formation of mounds and fruiting bodies. The expression of several stage specific genes also was retarded in the mutants. Cyclic AMP pulsing experiments suggest that the null mutant is deranged in a signalling pathway(s) via the cell surface cAMP receptor(s). Expression of the DdK6 gene in wild type cells is strictly regulated at early stages. The protein is localized in the nucleus. These data show that the DdK6 protein kinase might establish an early developmental program, through transient protein aggregation perhaps by the pleckstrin homology domain.

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While I have been traveling down a road to experience new horizons of life, I have met many great people who helped me to be more scientific than poet.ic

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Finally I want to appreciate financial assistance: this work was supported by National Institutes of Health Grant to Dr. Robert Dottin and an award to Hunter College, City University of New York, for the support of a Center for Study of Gene Structure and Function by National Institutes of Health-Research Centers in

Minority Institutions Program

Now I am ready to commence a new experience of my life by choosing a road probably less traveled by and to meet people for whom I will write another acknowledgement in future.

DEDICATION

부모님의 은혜에 감사드리며 이 논문으로 보답합니다.

This document is dedicated to my parents, Jong Ju Moon
and Jung Sik Hong who have been loving and caring me
for my whole life.

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ABBREVIATIONS

ALC	Anterior-Like Cells
ATP γ S	Adenosine 5'-O-(3'-thio)triphosphate
β ARK	β -adrenergic Receptor Kinase
cAMP	Adenosine 3',5'-cyclic monophosphate
cAR1	Cyclic AMP receptor 1
DAPI	4,6-diamidino-2-phenylindole
DAG	Diacyl glycerol
DIF	Differentiation Inducing factor
EB	Electroporation Buffer
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
IP3	Inositide 1,4,5-triphosphate
kD	Kilodalton
MAP	Multiple Antigenic Peptide
NLS	Nuclear Localization Signal
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PH	Pleckstrin Homology
PIP2	Phosphatidyl inositol 4,5-bisphosphate
PKA	Cyclic AMP dependent Protein Kinase
PBS	Phosphate buffered Saline
PDGF	Platelet-derived Growth Factor

PKC	Protein Kinase C
PLC	Phospholipase C
Pst	Prestalk
SDS	Sodium Dodecyl Sulfate
SH2	Src Homology 2
SH3	Src Homology 3
3BP-1	SH3 Binding Protein-1
TRITC	Tetramethyl Rhodamine Isothiocyanate
UDPG	Uridine Diphosphoglucose
UDPGP	Uridine Diphosphoglucose Pyrophosphorylase
XID	Murine-X-linked Immunodeficiency

GENERAL INTRODUCTION

The organism

Dictyostelium discoideum is a cellular slime mold. The name cellular slime mold distinguishes it from true slime molds which share with the cellular slime molds the common feature of a slime sheath at a certain stage in their life cycle. However the two forms are phylogenetically unrelated since the true slime molds are in the class Myxomycetes, having a multinucleate plasmodium stage resulting from cytoplasmic fusion. By contrast the cellular slime molds are in the class Eumycetozoa which form uninucleate pseudoplasmodia enclosed in a slime sheath without syncytium (Bonner, 1959).

Phylogenetically the cellular slime molds are classified in the kingdom Protista, the phylum Gymnomyxa, the subphylum of the Mycetozoa, the class Eumycetozoa, the subclass Dictyostelia, the order Dictyosteliida, the families Dictyosteliidae, the genera Dictyostelium, the species *Dictyostelium discoideum* (Olive, 1975). The cellular slime molds have branched off during the early stages of eukaryotic evolution. Recent comparison of the amino acid sequences of several proteins indicates that *Dictyostelium* is more closely related to mammals than is yeast, indicating *Dictyostelium* diverged from the line leading to metazoa at about the same time as the plant/animal divergence (Loomis and Smith, 1990)

Developmental Cycle

Dictyostelium is a social amoeba and an eucaryotic microorganism. The amoeba grow by binary fission in the presense of enough food sources. A few hours after starvation, a subset of the cells spontaneously begin to emit pulses of cAMP, a chemoattractant, at 6- to 10-minutes intervals (Konijin, 1975; Loomis, 1975). The surrounding cells respond by moving chemotactically toward an aggregation center made of the cells which have started pulsing cAMP and by relaying the cAMP signal that is synthesizing and secreting cAMP. About 10-12 hours after starvation, approximately 10^5 cells move together to form a tight multicellular aggregate, a tissue-like entity.

In the aggregate, the cells that arrive first at the aggregation center are elevated above the substratum by the cells that arrive later and a finger-like structure forms developing a tip at the top. The structure falls over on its side to transform into a pseudoplasmodium, or slug enclosed by a protein-carbohydrate sheath. Depending on the environmental conditions, the slug with the tip at a leading edge may migrate to search for more favorable conditions for culmination, responding positively to light, humidity and temperature. At the slug stage, two major cell types are differentiated - anterior prestalk cells and posterior

prespore cells.

Under favorable conditions, slugs enter into culmination. A stalk tube made of cellulose is formed just below the tip. The prestalk cells in the anterior region of a slug move into the stalk tube like a reversed fountain. They vacuolize to form terminally differentiated stalk cells. On the other hand, prespore cells located in the posterior part in the slug undergo differentiation to spore cells, which are lifted up from the support by the stalk cells growing down to the substratum. The anterior part contains two classes of prestalk cells. Prestalk (Pst) A cells occupy the front half of the prestalk zone surrounding prestalk (Pst) B cells which localize near the tip and enter the stalk tube first at culmination (Jermyn, et al., 1989). There are anterior-like cells (ALCs) which resemble prestalk cells but which are scattered throughout the prespore region (Sternfeld and David, 1981). This developmental cycle is completed in 24 hours, resulting in the formation of fruiting body which consists of a cap (encasing viable spores) on a stalk (non viable cells).

Advantages of Using Dictyostelium as an Experimental Model System

Dictyostelium is an excellent organism to study the molecular basis of developmental processes such as

signal transduction pathways or networks, cell differentiation and morphogenesis. Firstly, growth and development of the microorganism are almost mutually exclusive during its life cycle because development proceeds in the absence of nutrition, so that developmentally defective mutants can be isolated and maintained for further analysis without losing the capacity for growth. Secondly, the haploid genome with a complexity of 40,000 Kb facilitates isolation of mutant alleles without interference by non-defective alleles which would be found in diploid organisms (Devreotes, 1989). Thirdly, several reverse genetic tools are applicable to analyze the functions of genes in development using gene targeting by homologous recombination, antisense RNA and restriction enzyme - mediated insertion (REMI) to inactivate genes and isolate mutant organisms (Kuspa and Loomis, 1992). Finally signal transduction pathways leading to gene expression during development similar to those found in mammals. Chemotaxis and gene expression are mediated by hormone - like signaling molecules such as cAMP, DIF, ammonia and adenosine. Many of the genes encoding the molecules involved in signal transduction such as G protein, cAMP receptors, phospholipase C and adenylate cyclase have been well defined in *Dictyostelium* (Gerisch, 1987; Janssen and Van Haastert, 1987; Schaap et al., 1993).

Signal Transduction Pathways in *Dictyostelium*

Although *Dictyostelium* appears to have several different receptor-mediated signal transduction pathways, signal transduction by G-protein coupled receptors has been most extensively studied (Klein et al, 1988). Three cell - surface receptors, CAR1, CAR2, and CAR3, that bind extracellular cAMP have been cloned. They have seven putative transmembrane-spanning domains that share high homology and N-terminal domains and C-terminal domains that are divergent. Each receptor gene is expressed at different times in development (Klein et al, 1988). Null mutants of CAR1 arrest in early development and CAR3 disruptants proceed through early development but are detained at the tight mound stage (Sun et al., 1991; Sax III et al., 1993). Several genes encoding subunits of the heterotrimeric G proteins have been cloned, at least eight encode α subunits and one the β subunit. Targeted disruption of $G_{\alpha 2}$ or the β subunit gene blocked cells in aggregation (Pupillo et al., 1989; Hardwiger et al., 1991; Lilly et al., 1993). The analysis of a frigid A mutant in the gene encoding the $G_{\alpha 2}$ shows that the subunit is essential for chemotaxis toward cAMP and expression of many genes (Kesbeke et al 1987; Kumagai et al, 1989; Firtel et al, 1989). In vertebrates, G protein-mediated phosphoinositol turnover generates second messengers. Hydrolysis of phosphatidylinositol 4,5-

bisphosphate(PIP₂) yields diacylglycerol and inositol 1,4,5-triphosphate(IP₃). IP₃ releases calcium from a non-mitochondrial pool, while diacylglycerol (DAG) and calcium stimulate the calcium/phospholipid dependent protein kinase C(PKC) (Nishizuka,1988; Berridge and Irvine, 1989; Shears, 1989). *Dictyostelium* also shows similar pathways. The formation of IP₃ is stimulated by the receptor agonist cAMP and the G-protein agonist GTP- γ S, suggesting that G protein and receptor are involved in the inositolphosphate pathway (Van Haastert et al., 1991; Europe-Finner et al,1989), but at present we can not link specific G proteins to IP₃ pathways. DAG stimulates PKC, an activity recently identified in *Dictyostelium* (Luderus et al,1989). Treatment of cells permeabilized with saponin with DAG and IP₃ resulted in gene induction (Ginsberg and Kimmel, 1989), which suggests that PKC and/or calcium are involved in signal transduction pathways leading to induction of those genes.

UDP Glucose Pyrophosphorylase Expression During Development

Extracellular cAMP induces the expression of many genes by binding to cell surface receptors (Schaap and Vandriel,1985; Haribabu and Dottin,1986; Kimmel,1987; Oyama and Blumberg,1986). The results showed that

Dictyostelium can be used to study signal transduction. Many laboratories have been studying the signal transduction pathways that regulate gene expression during the development of *Dictyostelium discoideum*. Early genes encoding G proteins, cAMP receptors and other proteins are not expressed in vegetative cells, but pulses of cAMP can induce them by 2-3 hours after starvation (Gerisch, 1987; Kimmel, 1987; Mann et al., 1988; Klein et al., 1988; Mann and Firtel, 1989; Kumagai et al., 1989; Pupillo et al., 1989). Other genes such as discoidin have been shown to be repressed by repeated stimulation with cAMP (Mann et al., 1988; Mann and Firtel, 1987; Kimmel and Saxe, 1986; Kimmel, 1987). Late genes are inducible by pulses of cAMP and / or by higher constant levels of cAMP (Chisholm et al., 1984; Haribabu and Dottin, 1986; Haribabu et al., 1987). Adenylate cyclase might not be directly involved in the signal transduction pathways that generate second messengers in *Dictyostelium* because caffeine blocks activation of adenylate cyclase to deplete internal cAMP, but gene induction is normal (Kimmel, 1987; Haribabu et al., 1989). In addition, synag 7 mutants, in which the mutated gene is not known, fail to activate adenylate cyclase, but induce many genes in response to exogenous cAMP (Theibert and Devreotes, 1986; Mann et al., 1988). Two adenylate cyclase genes were cloned, one expressed during

development and the other during germination. Null mutation of the development-specific adenylate cyclase resulting failure to aggregate (Pitt et al, 1992). Although this result suggests that intracellular cAMP is required as a second messenger, this interpretation is confounded by the fact that if mutants defective in the development-specific adenylate cyclase are pulsed early in development with cAMP, they develop normally (Pitt et al., 1993). Since considerable evidence suggest that cAMP dependent protein kinase (PKA) is required for development (Mann and Firtel, 1991; Harwood et al., 1992a.b; Mann et al., 1992; Simon et al; 1992; Hopper et al., 1993). One interpretation of these results is that PKA may be activated by ligand other than cAMP in *Dictyostelium* (Schaap et al., 1993). LiCl can distinguish pathways leading to prespore and prestalk mRNA accumulation, because it inhibits prespore gene expression and promotes prestalk gene expression (Peters et al., 1989). Since lithium affects the inositol phosphate cycle, the results suggest that inositides might be second messengers. However mutation of a PLC gene by gene disruption does not affect development. Thus the role of inositides in the signal transduction pathways leading to development remains unclear.

We have been using a uridine diphosphoglucose pyrophosphorylase (UDPGP1) gene of *Dictyosotelium*

discoideum to elucidate the pathways that control gene expression. UDPGP has been known as a key enzyme for development. The enzyme catalyzes the production of UDP glucose which is involved in the synthesis of cellulose, trehalose, and mucopolysaccharides, which are necessary for the formation of normal fruiting bodies (Sussman and Osborn, 1964; Wright and Dahlberg, 1967) (Fig. 8). The enzyme activity increases several - fold during development (Newell and Sussman, 1969; Ashworth and Sussman, 1967). This laboratory isolated cDNA and genomic clones of the UDPGP1 gene (Fishel et al., 1985; Ragheb and Dottin, 1987). Expression of this gene is regulated at the transcriptional level during development. Its mRNA level declines 5 to 10 fold at 5 hours after the initiation of development, then it rises 5 to 15 fold by 8-10 hours. At 12 hours its expression is reduced two fold and eventually peaks at 20 hours (Haribabu et al., 1986; Pavlovic et al., 1988). Exogenous cAMP induces the UDPGP1 gene by activating cell surface receptors (Haribabu and Dottin, 1986). Two DNA *cis* acting elements have been identified as controlling UDPGP1 gene expression. One is required for the basal expression of the gene in the absence of cAMP and the other, a GC rich palindromic sequence, is required for its induction by cAMP (Pavlovic et al., 1989). Strains that lack UDPGP activity in *Dictyostelium* were isolated

by chemical mutagenesis and the mutants are unable to culminate (Dimond et al., 1976). However antisense expression of UDGP1 reduces UDGP1 mRNA but the cells develop normally (Haribabu et al, 1991). Therefore the necessity for UDGP activity and for the UDGP gene(s) in development remains unclear. This thesis addresses the question by using targeted gene disruption and homologous recombination to inactivate the UDGP1 gene.

The Roles of Protein Kinases

Many cellular processes such as motility, metabolism, growth, differentiation and signal transduction are regulated by reversible protein phosphorylation (Shenolikar, 1987; Edelman et al, 1987; Hunter, 1987). A variety of cell surface receptors undergo phosphorylation to modulate the propagation of signals. Phosphorylation of the β adrenergic receptors leads to receptor desensitization (Sibley, et al., 1986). In *Dictyostelium discoideum*, the cell surface receptor CAR1 is phosphorylated after ligand binding. Specific receptor kinases might be responsible for receptor phosphorylation and desensitization (Vaughan and Devreotes, 1989). A number of growth factor receptors contain intrinsic protein tyrosine kinases, and they can be activated by ligands to phosphorylate themselves and other substrates. Receptor activation might result in the

regulation of cell proliferation (Ullrich and Schlessinger, 1990 ; Cantly et al, 1991). In eukaryotes, Serine/ threonine protein kinases also play a role in many cellular events including cell cycle progression and gene expression (Pelech, et al 1990., Karin, 1991). As an example, transcription factor NFkB translocates from the cytoplasm into the nucleus after NFkB-IkB complex dissociate by phosphorylation of IkB (Zabel and Baeuerle, 1990 ; Ghosh and Baltimore, 1990). Also as mentioned earlier, PKA is critical for development in *Dictyostelium* because constitutive activation of PKA results in rapid development (Simon et al, 1992). To reveal the role of phosphorylation in signal transduction pathway, we cloned out several protein kinases by PCR based on homology of serine/threonine protein kinase genes with the view to using reverse genetics to determine their functions (Haribabu and Dottin, 1991). This thesis describes the inactivation of one serine/threonine kinase gene and analyzes the consequences of such mutation.

Reverse Genetics in *Dictyostelium*

Gene targeting by homologous recombination revolutionizes the analysis of gene function, regulation and gene therapy (Capecchi, 1989). A major problem is that homologous recombination in mammalian cells is rare.

The DNA preferentially integrates randomly at nonhomologous sites in the genome (Subramani, and Seaton, 1988). By contrast, in yeast homologous recombination occurs at high frequency (Orr-Weaver, et al., 1981). In *Dictyostelium* cells, disruption of myosin II heavy chain occurred with a frequency of 30% relative to illegitimate recombination and the mutants showed a defect in cytokinesis (DeLozanne and Spudich, 1987). Disruption may be achieved by insertion or by replacement depending on whether they occur by single or double crossovers. In *Dictyostelium* a single crossover may integrate one copy or multiple tandem repeats of exogenous DNA at the homologous locus to create null mutations. Integration events have been shown in other genes such as α -actinin (Witke et al., 1987), myosin II heavy chain (DeLozanne and Spudich 1987), contact site A (Harloff et al. 1989), Synexin (Doring et al, 1991) and cAMP binding protein (Bain and Tsang, 1991). Gene replacement by double crossing - over has also been reported in genes encoding the cAMP receptor (Sun and Devreotes, 1991), G proteins (Kumagai et al., 1991), Myosin I heavy chain (Jung and Hammer, 1990), myosin II heavy chain (Manstein, et al., 1989) and adenylate cyclase (Pitt et al, 1992). An alternative way to create null mutations is by expression of antisense RNA. The expression of an antisense myosin heavy chain gene

blocks morphogenesis in *Dictyostelium* (Knecht and Loomis, 1987). Inactivation of discoidin genes by antisense discoidin gene blocked formation of aggregation streams (Crowley et al. 1985). An esterase gene, D2 (Rubino et al., 1989) and cAMP receptor (Klein et al. 1988; Sun et al. 1990) have also been inactivated by antisense RNA.

The Aim of this Dissertation

In this thesis, I have tried to analyze the functions of developmentally important genes and to elucidate signal transduction pathways that function in the development of *Dictyostelium*. The approach is to use cloned genes to create null mutations by homologous recombination or double crossovers leading to replacement of a normal gene with a gene containing deletion / insertion mutation. Part I describes this approach in examining the roles of UDP-glucose pyrophosphorylases in development. It shows that we have disrupted a UDP-glucose pyrophosphorylase 1 (UDPGP1) gene to isolate null mutants of the gene. The disruptant was used for cloning a second UDPGP gene by taking advantage of disruptant cell lines and using a novel polymerase chain reaction. Thus the organism contains at least two UDPGP genes not one as was suggested by experiments using a chemical mutagen. Part II demonstrates the molecular analysis and the function of a serine/threonine kinase, DdK6. This

protein contains a pleckstrin homology (PH) domain within the regulatory region. The study of the targeted null mutants of DdK6 gene shows that the kinase is required for normal development. PH domains might play an important role for intracellular signalling pathways.

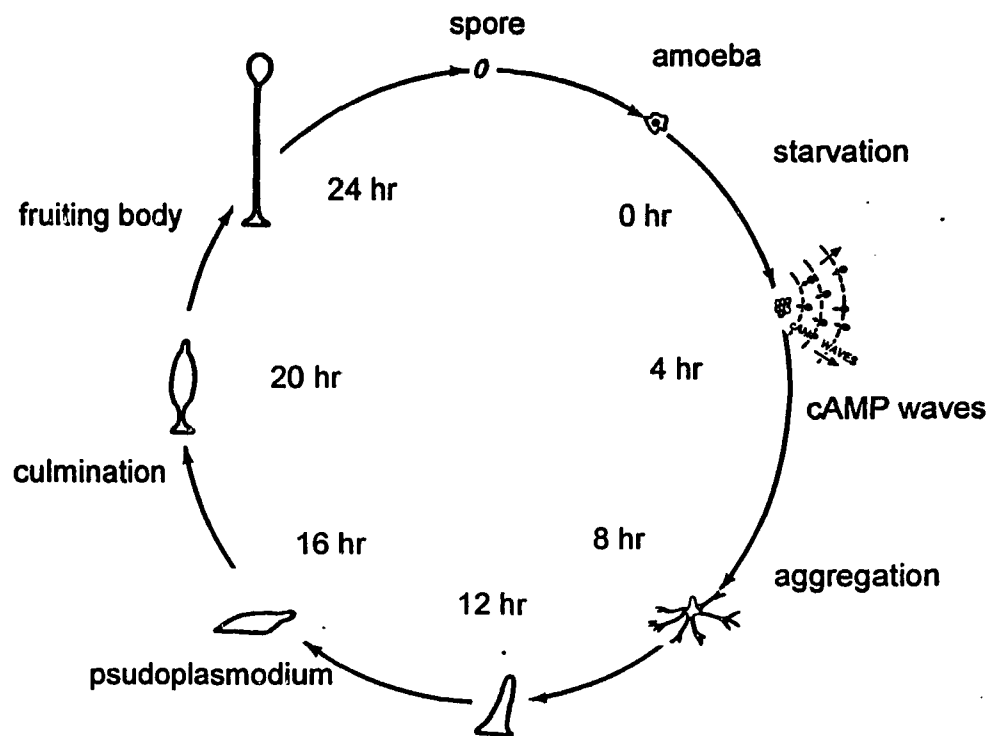


Fig. 1. The developmental cycle of *Dictyostelium discoideum*.

PART 1

**NULL MUTATION OF A UDP-GLUCOSE PYROPHOSPHORYLASE GENE
BY HOMOLOGOUS RECOMBINATION PROVIDES
EVIDENCE FOR A SECOND GENE IN *DICTYOSTELIUM DISCOIDEUM***

INTRODUCTION

UDP-glucose pyrophosphorylase (UDPGP) is a key enzyme for carbohydrate metabolism in many species. It catalyzes the production of UDP-glucose, which plays a central role as glycosyl donor in the synthesis of various carbohydrates, such as cellulose, trehalose and mucopolysaccharides, that are important food sources. In potato tuber, sucrose synthase breaks down sucrose to UDP-glucose and fructose, and the enzyme UDPGP degrades the UDP-glucose so formed to glucose-1-P which can cross the amyloplast envelope and form ADP-glucose for starch synthesis (Keepling *et al.*, 1988). In *Dictyostelium*, these carbohydrates are important for the formation of normal fruiting bodies (Sussman and Osborn, 1964; Wright and Dahlberg, 1967; Clegg and Filosa, 1961; Newell and Sussman, 1969; Gustafson, 1973). The accumulation of the enzyme activity is regulated during the developmental cycle. We had earlier isolated and characterized full-length of genomic DNA and cDNA clones of the UDPGP1 gene from *Dictyostelium* (Fishel *et al.*, 1985; Ragheb and Dottin, 1987) and shown that the UDPGP1 gene as well as the enzyme activity is also temporally regulated during development. Moreover, the expression of the UDPGP1 gene is controlled by exogenous cAMP through receptor-

mediated signal transduction pathways (Haribabu and Dottin, 1986; Pavlovic et al, 1989).

Mutants lacking UDPGP activity have been recovered by chemical mutagenesis with nitrosoguanidine and they are unable to complete development (Dimond et al., 1976). They were isolated at a frequency which suggested that *Dictyostelium* harbors only a single UDPGP gene and that it is essential for development. However, cells in which substantial antisense inactivation of the UDPGP1 gene product had occurred could nonetheless show complete development (Haribabu et al., 1991) suggesting that if the activity is essential, it must be made by more than one gene. Some heterogeneity in the enzyme had been suggested by earlier studies (Newell and Susman, 1969; Gustafson, 1973). In addition, *in vitro* translation of total cytoplasmic RNA from different developmental stages showed that two polypeptides could be immunoprecipitated with antiserum to UDPGP enzyme. Furthermore, the mRNA rescued by hybrid selection with the cloned UDPGP1 cDNA is translated to yield only the larger of the two immunoprecipitable polypeptides, suggesting two distinct mRNA species encode the immunoprecipitable polypeptides (Fishel et al., 1985).

In order to analyze further the expression of UDPGP in development and the heterogeneity of the UDPGP

enzyme, we have used gene replacement by homologous recombination to create a transgenic organism carrying a null mutation (deletion) in the UDPGP1 gene. The mutants showed no developmental defect, and contained the same amount of cellulose as did wild type cells at the end of development. Although no enzyme activity is detected in the null mutant cells in vegetative cells or early in development, some activity appears at 15 hrs, suggesting that a second gene exists. We used DNA from the mutant line deleted in part of the UDPGP1 gene to clone part of the second gene using a novel PCR strategy. The deduced amino acid sequence of the second gene, UDPGP2, is 46% identical to UDPGP1 of *Dictyostelium* and 51% identical to potato tuber UDPGP polypeptide. The accumulation of its mRNA parallels that of the enzyme activity and accounts for the appearance of the late isoenzyme.

MATERIALS AND METHODS

Construction of Vectors

Fig. 1 shows the construction of the vector, pTUPneo1 containing a mutated UDPGP1 gene with a neomycin cassette replacing some protein coding sequences. The vector pSPUneo contains 1.3 kbp of UDPGP1 cDNA but lacks a central 200 bp Bgl II fragment. The Bgl II ends were filled in with Klenow fragment and blunt end ligated to a neo cassette (Witke *et al.*, 1987) first cut with XbaI and also filled in. That neo cassette was removed from pSPU neo, by digestion with Eco RV. The plasmid pTUP1 containing a cloned UDPGP1 genomic fragment was digested with Eco RV to remove a central 1.7 kbp protein coding region and the remaining 7.7 kbp fragment was ligated to the neo cassette. The neo cassette has its own transcription termination site down stream and is transcribed in the same direction as the endogenous gene.

Growth and transformation of Dictyostelium D.

discoideum strain AX3 was grown axenically in HL5 medium to a density of 2×10^6 /ml or in association with *Klebsiella* bacteria on SM plates (Sussman, 1966). Transformation was performed by electroporation (Dynes and Firtel, 1989) with some modifications. Cells were grown axenically to 2×10^6 /ml and 5ml were collected

by centrifugation at 450 x g, and washed in 2 volumes of electroporation buffer EB, (1 mM sodium phosphate, pH 6.1 and 500 mM sucrose). The pellet was resuspended in 0.3 ml of EB. The cell suspension was mixed with 10 µg of DNA. The mixture was transferred to an electroporation cuvette (0.2 cm gap, Bio Rad) in an ice water bath. After 3 to 4 minutes, the cells were electroporated (1.2-1.5 KV, capacitance 3 uF, time constant 0.5-0.9 msec) with a Bio Rad eletroporation device. The cells were immediately returned to an ice water bath and left standing for 10 min. The cells were transferred with a Pasteur pipettes to a tissue culture petri dish (100mm) and then treated with 3 µl of healing solution (100 mM CaCl₂ and 100 mM MgCl₂). After 15 min, 12 ml of HL5 was added. After 24 hours, the medium was replaced with 2 volumes HL5 containing G418 (10 µg/ml) and 200-300 µl was plated in wells of a 96 well multititer plate to isolate individual clones. After 10 days, aliquots of the culture were plated on SM plates with bacteria to isolate individual clones for further analysis.

Preparation of genomic DNA and RNA *Dictyostelium*
genomic DNA was prepared by a rapid method (Knecht, et al., 1991). 10⁷ to 10⁸ cells were pelleted at 1000 rpm for 5 minutes in a microcentrifuge, suspended in 1 ml of nuclei buffer (25 mM Tris-HCl (pH 7.5), 5 mM

magnesium acetate, 0.5 mM EDTA, 5 % sucrose) and lysed with 200 μ l of 20% Triton X-100 on ice for 5 min. The nuclei were pelleted at 5000 rpm 5 min in a microfuge and pellets were resuspended in 300 μ l of Proteinase K buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 % SDS, 0.1 mg/ml of Proteinase K) and incubated at 65°C for 20 minutes. DNA was extracted with one volume of phenol-chloroform (1:1) and subsequently with one volume of chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of ethanol in the presence of 1.7 M ammonium acetate to avoid RNA contamination. The DNA was pelleted at 5000 rpm in a microfuge for 5 min and dried briefly under vacuum, and resuspended in 30 μ l of TE. Total cellular RNA was isolated from vegetative or developed cells as previously described (Alton and Lodish. 1977).

Southern blot and Northern blot Northern blots were prepared as previously described (Haribabu and Dottin, 1986). 10 μ g of RNA was electrophoresed in 1.5% formaldehyde-agarose gels and transferred to nylon membranes. DNA probes were prepared by nick translation. Southern analysis was performed as previously described (Maniatis, 1982). Digested DNAs were fractionated in 0.8% agarose gels and then transferred to nylon membranes with 10 x SSPE.

Prehybridization was done in hybridization buffer (6 x SSPE, 5 x Denhardt's solution, 0.5 % SDS, 100 ug/ml salmon sperm DNA, 50% formamide pH7.4) for 1-2 hour at 42°C. The blot was hybridized in hybridization buffer with denatured probes. DNA probes were prepared by random priming method (Feinberg and Vogelstein, 1983). Typically the specific activity was 1×10^9 cpm/ug.

Development of Dictyostelium Cells were grown axenically in HL5 medium at a density of 2×10^6 /ml, centrifuged at 450 x g and washed three times with PDF buffer (33mM sodium phosphate pH 6.2, 13.5 mM KCl, 5mM MgSO₄, 0.75 mM CaCl₂ and 0.3 % streptomycin sulfate). The washed cells were resuspended at density of 1.2×10^8 cells/ml in PDF and plated on Millipore filters (0.45 um pore size) at a density of 1×10^7 cells /cm² on 3 pads (Whatman #3) and developed at 22°C.

Assays for UDPGP Pyrophosphorylase and Cellulose The assays were performed as previously described (Dimond et al, 1976) with modifications. Cell extracts were prepared by lysing the cells at a density of 2×10^6 in 0.1 % NP40. The assay mixture contained the following: 1mM UDPGP, 2mM sodium pyrophosphate, 1.6mM NADP, 10 uM glucose- 1,6-diphosphate, 1mM EDTA, 4mM MgCl₂, 0.05 unit phospho-glucomutase, 0.14 units glucose-6-phosphate dehydrogenase, 85mM Tricine (pH7.6) and cell extract to a total volume of 0.5ml. The increase in

optical density at 340 nm was measured with Gilford recording spectrophotometer at 37° C. An assay mixture lacking sodium pyrophosphate was always applied to all enzyme assays as a negative control because sodium pyrophosphate is essential for this reaction and it rules out artifactual production of glucose-1-phosphate or intermediates in the reduction of NADP. One unit of activity is defined as the amount of enzyme necessary to produce 1 nmole of NADPH per minute at 37°C. Specific activity is defined as units of enzyme activity per mg of total cellular protein. Total cellular protein was measured by the Bio Rad kit using bovine serum albumin as a standard.

Cellulose was extracted first in a mixture of acetic acid (80 %) and nitric acid (10 %) reagent, and then colorimetrically determined as anthrone positive materials as previously described (Updegraff, 1969).

PCR *Dictyostelium* genomic DNA was prepared as previously described (Ragheb and Dottin, 1987). 1 µg of the genomic DNA was used in each PCR experiment containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001 % gelatin, 200µM each of dATP, dCTP, dGTP, and dTTP, 2.5 U of Taq polymerase, and 2 µM of each primer. Amplifications were done according to the following schedule using a Coy temp cycler: 94°C for 1 min, 45°C for 1 min and 72°C for 3 min for 35 cycles.

The primers used for PCR were two degenerate oligonucleotides based on to the amino acid sequences EWYPPG and GKEI/VF found in the domains conserved in both *Dictyostelium* UDPGP1 (Ragheb and Dottin, 1987) and potato tuber enzyme (Katsube. et al., 1990). The sequences of the primer were 5'-AGAAGCTTGA/GNTGGTAT/CCCA/TCCA/TGG -3' (forward ; F1) and 5'- GAGAATTCAAT/A/GAT/CA/GTAC/TTCC/TTTNCC -3' (reverse : R1). The amplified PCR products were cloned into pBluescript (Stratagene). The sizes of inserts in individual clones were analyzed by digestion with EcoR1 and HindIII electrophoresis, 3% Nusieve/ 1% Seakem (FMC) agarose gels , and staining with ethidium bromide.

Isolation and Analysis of Genomic Clones for the Second gene A λ ZAP library of *Dictyostelium* genomic DNA (kindly provided by Dr. Herbert Ennis) was screened with PCR-amplified DNA labelled by random priming (Feinberg and Vogelstein, 1983). Hybridization was performed in 5x SSC, 20mM Tris pH 7.5, 100 μ g/ml salmon sperm DNA (denatured), 0.1% SDS and 5x Denhart's solution at 50°C with nitrocellulose. Five clones appeared positive and one of them contained a 700 bp insert. To obtain the plasmid from the isolated phage clones, phagemid rescue was performed according to the directions of the manufacturer (Stratagene). The

insert was sequenced using the Sequenase system (United States Biochemical Corp.)

RESULTS

Generation of a Transgenic Organism with a Mutated UDPGP1 gene The RNA coding region of UDPGP1 contains 4 exons and 3 introns, and is 2 Kb long. We constructed a 6.4 Kbp fragment containing the UDPGP1 genomic locus in which 1.7 kb in the central region was replaced by a 2.1 kbp neo cassette (see Methods and Fig. 1). The modified UDPGP1 sequence with the neo cassette was liberated from the vector DNA by digesting with HincII and SacI and introduced into AX3 cells by electroporation. Transformants were selected in the presence of G418.

Genomic DNA from individual transformants was digested with EcoRI and subjected to Southern analysis to detect gene replacement. 10 of the 30 individual clones screened showed the patterns expected of fragments in which the altered gene had replaced the wild type UDPGP1 gene in the genome. One of the null mutant lines, $\Delta 604$, was analyzed further. Three different restriction digests of the genomic wild type and $\Delta 604$ DNA were probed with a 0.5 Kb NsiI-EcoRV fragment from the protein coding region (Fig.1B). EcoRI digestion shows 2.5 Kb and 0.85 Kb fragments with wild type DNA and 2.5Kb and 9.9 Kb with the null mutant, because the 0.85 Kb region containing one of

EcoR1 sites was deleted during construction of the modified gene. The HindIII digestion shows a 3.5Kb fragment with wild type DNA and a 3.7 Kb fragment with $\Delta 604$ DNA, because a different HindIII site was created by the neo cassette in the modified gene. Finally, NsiI digestion shows an 8.8 Kb fragment with wild type DNA but a 1.7Kb fragment with the $\Delta 604$, because the neo cassette creates a new NsiI site in the genome. Furthermore, from the data with EcoR1 and HindIII digestions, we can conclude that only a single copy of the transforming DNA fragment has replaced with the endogenous genomic locus in $\Delta 604$: we could not see any bands of the size (6.8 kb) of the transforming DNA fragment, which would have suggested that multiple copies were integrated in the genomic locus of the UDPGP1 gene (Fig 1B and 2). Nine of the 10 cell lines resulted from a single integration and replacement event. The other showed additional integration elsewhere in the genome. Similar transfection experiments were performed with the cDNA construct from pSPUneo but of 300 colonies screened, none showed evidence of disruption of the UDPGP1 gene. Thus, a large genomic fragment seems disproportionately more efficient than the cDNA sequence.

We have examined the expression of the UDPGP1 gene in the disruptant and wild type: Total cytoplasmic

RNAs were isolated from wild type, Ax3 and the disruptant $\Delta 604$ during vegetative growing and 15 hr and 20 hr post-starvation. The RNAs were fractionated and transferred onto nylon membrane. The RNA blots were hybridized with ^{32}P labeled full-length UDPGP1 cDNA. Wild type, Ax3 shows the 1.8 Kb UDPGP1 mRNA with expected expression patterns during development while the $\Delta 604$ cell line generates a 0.8 Kb with the same expression pattern as that of Ax3 (Fig. 3A). The reduced size of this RNA suggests that the Neo cassette inserted in the middle of the second exon of UDPGP1 caused production of a stable truncated RNA (Fig. 1B). Taken together, the Southern and northern data, show that in the $\Delta 604$ cell line the UDPGP1 gene is replaced with the altered deleted construct by double crossing over as indicated Fig. 1 B.

The Enzyme Activity of Mutant and Wild type Cells

The developmental profile of UDPGP enzyme activity in *Dictyostelium* has been well established (Ashworth and Sussman, 1967; Dimond et al., 1976). Fig. 4 shows the expected increase in activity that was previously observed during development for wild type AX3 cells. The enzyme activity declines slightly at 5 to 10 hr into development and then it rises at late development. It peaks at 20 hrs into development (Ashworth and Sussman, 1967; Fishel et al., 1985). The

Δ604 cell line does not show any enzyme activity in vegetative cells. Surprisingly, however, from 15 hr of development, enzyme activity appears and increases up to about one-third of the total specific activity of wild type cells. In addition, the morphogenesis of the transgenic organism is the same as the wild type. Previously, we reported that *in vitro* translation of total mRNA from wild type Ax3 and then immunoprecipitation with antiserum to UDPGP enzyme showed two polypeptides, 55 KD and 53 KD suggesting that there may be two UDPGP mRNA species. However, hybrid selection with cloned UDPGP1-cDNA rescued mRNA encoding only the higher molecular weight, *in vitro* translation product. The result suggested that the other lower molecular weight species was encoded by a second gene (Fishel et al., 1985; Haribabu and Dottin, 1986). Furthermore, antisense inactivation of UDPGP1 mRNA showed no visible effect on development of *Dictyostelium* (Haribabu, et al., 1991). These data suggested the existence of a second UDPGP gene. The new data in which UDPGP activity was observed late in development with the transgenic null mutant organisms show that another gene is expressed in development and that deletion of a single UDPGP gene does not affect development.

Expression of UDPGP1 and UDPGP2 RNA During Development

Figure 3B shows that the UDPGP1 mRNA is 2.4 Kb, larger than the UDPGP2 mRNA which is 2.1 kb. The accumulation of the UDPGP1 and UDPGP2 transcripts is consistent with the accumulation of UDPGP1 and UDPGP2 enzyme activities (Fig. 4). In the mutant, the UDPGP2 transcript is expressed between 8 hour and 16 hours in those cells (Fig. 3B) and could account for the appearance of the enzyme activity after 10 hour (Fig. 4). We also observed that the UDPGP1 and UDPGP2 RNA is expressed in both prestalk and prespore cell types (data not shown).

The UDPGP Null Mutant Makes Similar Amounts of Cellulose as Wild type Cellulose is almost undetectable in vegetative cells but the formation of a normal fruiting body in *Dictyostelium* requires cellulose as a major component of stalk and spore cells and comprises about 4% of dry weight in mature fruiting bodies, (Dimond et al., 1976; Loomis, 1975; Sussman and Osborn, 1964; Wright and Dahlberg, 1967; Clegg and Filosa, 1961). Although we could find no differences in the growth rates and morphogenesis between wild type and the mutant line, we investigated whether disruption of the UDPGP1 gene might affect cellulose synthesis during development. The total amount of cellulose in wild type Ax3 cells remains fairly low at 10 $\mu\text{g}/10^8$ cells up to 15 hr of development., and then rapidly increases to 80

µg per 10⁸ cells by 25 hr (Fig.5). The Δ604 cell line shows slightly less cellulose than wild type Ax3 during development, but in the fully developed transgenic organism it is normal (Fig. 5).

Strategies for cloning and analysis of the second gene by PCR A large number of gene sequences have been cloned by PCR, using amino acid sequence domains conserved in known gene families such as protein kinases (Haribabu and Dottin, 1991; Kamb *et al*, 1989) and the G protein family (Buck and Axel , 1991). A UDPGP gene from potato tuber has recently been cloned (Katsube *et al*; 1990) and it shows some amino acid sequence homology (42%) with the UDPGP1 gene of *Dictyostelium*. Although the UDPGP gene family may be very small, a similar strategy was attempted to clone out fragments of the second UDPGP gene in *Dictyostelium*. Amino acid sequence comparison between UDPGP1 of *Dictyostelium* (Ragheb and Dottin; 1987) and potato tuber UDPGP (Katsube *et al*; 1990) shows well conserved domains. Two regions were chosen to design degenerate forward and reverse primers, F1 and R1 (Fig. 7). The forward primer from F1 domain (EWYPPG) was 64-fold degenerate and included a HindIII restriction site at the 5' end. The reverse primer from the R1 domain (GKEYI/VF) was 192-fold degenerate and included in a EcoRI site at the 5' end (Fig.7). PCR amplification

with the two primers and template DNA from wild type cells should generate the products from both the UDPGP1 gene and the second gene UDPGP2 . But if the $\Delta 604$ genomic DNA is used as a template the reverse primer should not initiate a reaction with the UDPGP1 gene because that region was deleted during construction of the disruption vector (Fig 1B and 6A) . Thus the amplified PCR product should come only from the second gene, UDPGP2 (Fig. 6A) .

The genomic DNAs obtained from wild type AX3 and the mutant $\Delta 604$ were amplified using two primers. As expected, the PCR products from wild type and the mutant DNA generated 112 base pairs fragments (Fig. 6B, lane 2 and 4) . We expected that the PCR product from wild type would be a mixture amplified from both UDPGP1 and UDPGP2 while that of the disruptant would come solely from the second gene. To test this assumption, these two PCR products were digested with BglII because a BglII site occurs in the middle of the amplified region of the UDPGP1 gene (Ragheb and Dottin, 1987) and would most likely not also occur in the UDPGP2 gene. The assumption turned out to be true. The 112 bp PCR product from wild type DNA was reduced by more than 70% on digestion with BglII but the 112bp PCR product from $\Delta 604$ DNA was unaffected (Fig. 6. lanes 1 and 3) . The fragments from digestion of the PCR products made from

wild type DNA migrate at 54 and 58 bp, just above the primer dimers (Fig. 6, lane 1 and 3). Moreover roughly 1/5 of PCR product from wild type DNA was not sensitive to BglIII digestion and remained intact, suggesting that it originated from the second gene (Fig. 6 lane 3). The 112 bp PCR product from the mutant cell DNA, $\Delta 604$ was subcloned into a vector, and sequenced. The sequence in the PCR product amplified from mutant DNA $\Delta 604$ showed 59% and 68% identity to UDPGP1 and potato tuber UDPGP sequences respectively (Fig. 7). We synthesized a unique oligonucleotide from the *Dictyostelium* sequence between the primers to screen a genomic library for the second gene. Part of one genomic clone (700 bp) has been sequenced enough to compare the deduced amino acid sequence with other known UDPGP amino acid sequences (Fig. 7). The comparison showed 46% and 51% identity of UDPGP2 to UDPGP1 and potato tuber UDPGP respectively, suggesting that these 3 enzymes have a common evolutionary origin. Furthermore, in potato tuber UDPGP, 3 lysine residues (Lys-263, 329 and 367) are required for normal enzyme activity (Katsube et al., 1991): Lys-367 is conserved in potato tuber UDPGP and *Dictyostelium* UDPGP1 enzyme, but Lys-263 and Lys-329 are not (Ragheb and Dottin, 1987; Katsube et al., 1990; Katsube et al., 1991). However, lysine 329 in potato tuber UDPGP is

well conserved in UDPGP2 of *Dictyostelium* (Fig.7). We do not have the sequence information of UDPGP2 in the region corresponding to lysine-367 and lysine-263 in potato tuber UDPGP. These sequence data suggest the UDPGP2 protein of *Dictyostelium* is more closely related to the potato tuber UDPGP than UDPGP1 of *Dictyostelium*.

DISCUSSION

This report shows that UDPGP1 is not necessary for normal development and for making cellulose. Gene targeting by homologous recombination generated a null mutant of UDPGP1 gene which develops normally and makes normal amounts of cellulose. In the null mutant, enzyme activity appears only late in development, while UDPGP1 is expressed at varying levels at all stages including vegetative cells. The data strongly suggest that *Dictyostelium* has at least two different structural genes for UDPGP, UDPGP1 and UDPGP2 and that the enzyme activity in the null mutant arises from the transcription of UDPGP2. Several lines of evidence show that the late appearance of the enzyme activity in the $\Delta 604$ cell line is unlikely to arise from the enzyme activity of UDPGP1. First, we deleted a region containing 269 amino acids of the total 511 amino acids as well as the 3' untranslated region. Second, in the studies of the potato tuber UDPGP enzyme, there are five lysyl residues at or near the active site of the enzyme (Kazuta et. al., 1991 a b). One of them, Lys 367, which is conserved in UDPGP1 of *Dictyostelium* has been shown to be essential for the UDPGP activity from the study of site-directed mutants (Katsube et al, 1991). The region including Lys 367 is deleted from the locus of

UDPGP1 in the null mutant. Third, we could not detect any residual enzyme activity in UDPGP1 disruptants in vegetative cells up to 10 hour of development (Fig. 4). Thus all of the activity in vegetative cells is provided by UDPGP1. What is a function of UDPGP in development of *Dictyostelium*. The enzyme catalyzes the synthesis of UDPG which is a precursor for the biosynthesis of many polysaccharides found in differentiated cells (Asworth and Sussman, 1967). Fig. 8. shows that the pathways in which UDP-glucose is known to be involved during development of *Dictyostelium*. The metabolic pathways for synthesis of many carbohydrates require UDP- glucose as a precursor of all the carbohydrates, suggesting that UDPGP might be essential for normal development. Clearly it is not essential for growth since null mutants in UDPGP1 continue to grow. The high level of enzyme activity may be due to the general elevation of expression in many genes when the cells are grown axenically. Vegetative cells grown on bacteria is shown lower level of UDPGP activity. The activity increases markedly late in development when polysaccharide synthesis is demanding UDPG. Thus UDPGP is one of developmentally regulated enzymes (Newell and Sussman, 1969). If, as seems likely, UDPGP is essential for development presumably UDPGP2 is sufficient to supply

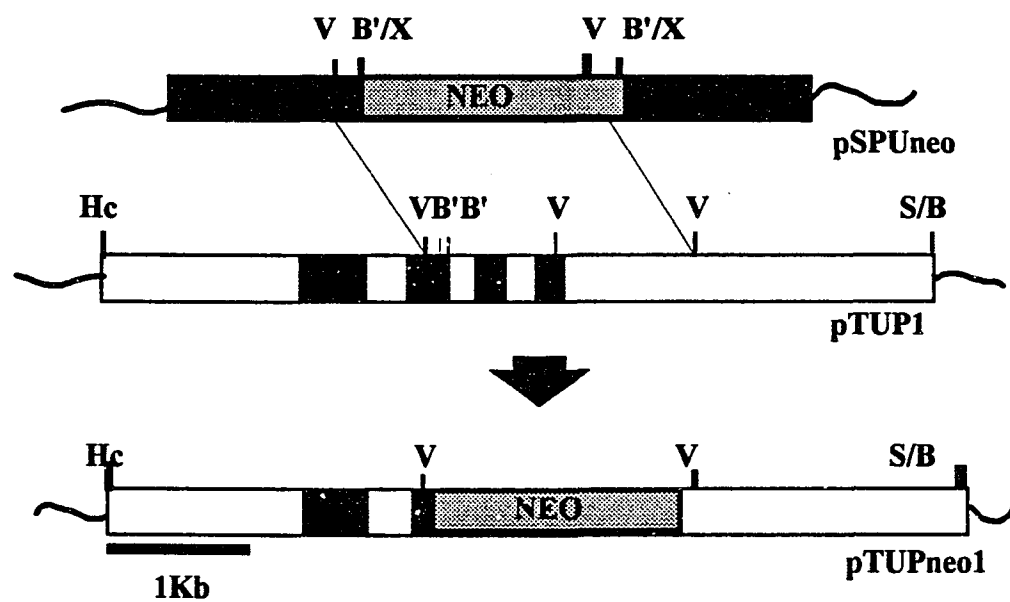
that activity. The result support previous suggestions from experiments with antisense RNA and with hybrid selection that another UDPGP gene is present and shows a strategy for isolating such genes in organisms that contain multiple genes. Earlier suggestions that a single gene might exist and that it is essential are probably due to multiple mutations arising from nitrosoguanidine mutagenesis which was performed at a time when gene disruption was not possible. We and others have shown that in *Dictyostelium* many genes including the UDPGP1 gene are regulated by extracellular cAMP acting on cell surface receptors activating unknown second messenger(s). (Haribabu and Dottin, 1986). Two DNA elements have been defined as affecting the transcription of the UDPGP1 gene, one is required for basal expression and the other, which contains a GC-rich palindromic sequence, is required for the induction by cAMP (Pavlovic, et al, 1989). We observed two major DNA binding complexes with the regulatory regions of the UDPGP1 promoter (Bodduluri, S., and Dottin, R.P. unpublished data). Other developmentally regulated *Dictyostelium* genes such as CP1 and α -Fucosidase are regulated by extracellular cAMP and have *cis* elements that control their expression in response to cAMP (Datta and Firtel, 1988; Pears and Williams, 1987; May et al,

1989). However these sequences show little similarity to the GC-rich palindromic sequence of UDPGP1 or to each other. UDPGP1 mRNA is made in vegetative cells and induced in development (Fig.3A; Fishel *et al*, 1985), while UDPGP2 mRNA is expressed exclusively during development, suggesting that the two genes might contain different *cis* elements and /or use

The disproportionate frequency of integration of genomic DNA compared to cDNA suggests that the larger piece of homologous DNA increases the efficiency of homologous replacement and that the extragenic regions in genomic DNA may be more efficient than the RNA coding regions. In addition, one copy of the neomycin gene appears to be enough for cells to survive in the selection conditions used in these experiments. In mammalian cells, the frequency of homologous replacement is usually low and very sensitive to the extent of homology between the exogenous DNA and the endogenous cognate sequence (Thomas and Capecchi, 1987). Therefore these data suggest that gene targeting events by double crossing over can be efficiently performed in *Dictyostelium* with a considerable size of genomic fragment containing a single copy of the neomycin gene.

Fig. 1. Construction of a null mutation by homologous recombination. (A) Construction of the replacement vector pTUP1 contains a 6.8 Kb genomic fragment of UDPGP1 of *Dictyostelium*. pSPUneo is a vector containing the 5' and 3' ends of the UDPGP1 cDNA and the neo cassette. It was digested with EcoRV to remove the neo cassette, which was cloned into the EcoRV sites of pTUP1 to generate the replacement vector pTUPneo1 as shown. (B) Targeted disruption of UDPGP1 locus by the replacement vector pTUPneo1. After homologous pairing between the linearized targeting vector and the genomic UDPGP1 locus, homologous recombination in the regions labelled "X" replaces the endogenous UDPGP1 sequence with an exogenous sequence containing the neo cassette and a deletion of the UDPGP1 gene. The cell lines produced by this recombination event will be resistant to G418. Solid box, exons; open box, intron and flanking region. Restriction sites: V, EcoRV; Hc, Hinc II; N, NsiI; H, HindIII; S, SacI; B, BamHI; R, EcoRI, B', BglII.

A.



B.

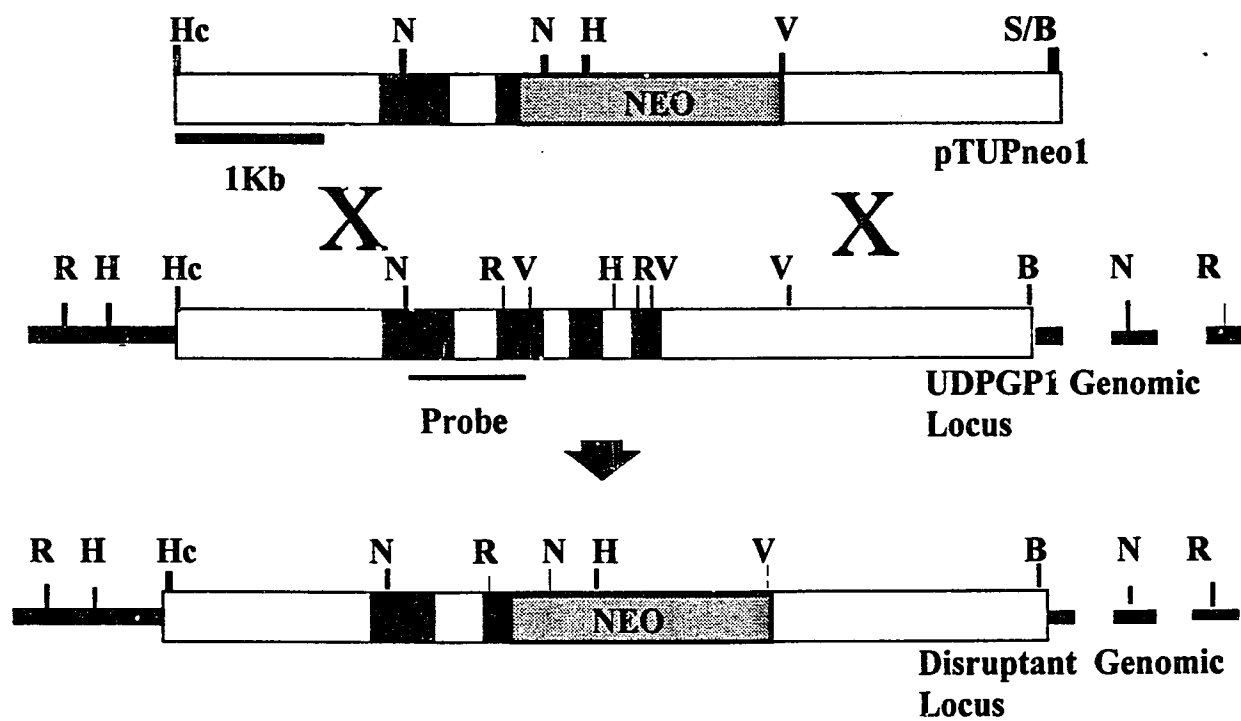


Fig. 2. Demonstration by Southern blot of homologous recombination at the UDPGP1 locus. Ax3 refers to DNA from the parental, wild type AX3. Δ604 refers to DNA from the disruptant Δ604 cell line. The DNAs were digested with the restriction enzymes indicated above each lane. A part of UDPGP1 coding region digested with NsiI and EcoRV was used as a probe for Southern analysis (Fig. 1B).

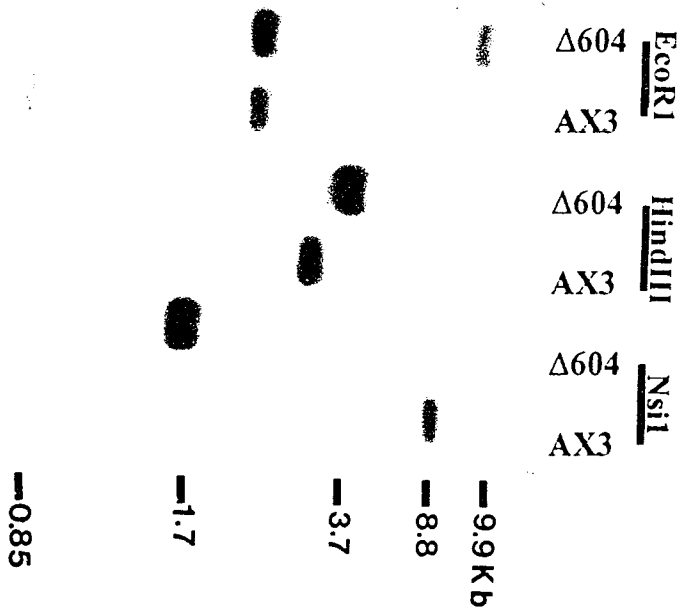


Fig. 3 A. Northern blot analysis of RNA from transgenic organism. Wild type Ax3 cells and the $\Delta 604$ cells were grown and allowed to develop into transgenic organisms as described in materials and methods. Total cellular RNA was isolated from cells at different developmental stages. Lane 0 hr contains RNA from vegetative cells. Lane 15 hr and lane 20 hr contain RNA from cells developed on filters for 15 hr and 20 hr, respectively. RNA was hybridized with ^{32}P -labelled cDNA from UDPGP1. Fig.3B. Northern analysis of UDPGP1 and UDPGP2 mRNA. Each lane contains 20 ug of total cellular RNA isolated from cells at the indicated times of development. The size of transcripts was calibrated by an RNA marker (Gibco BRL).

A.

AX3

Δ604

0 15 20 0 15 20 TIME (hr)



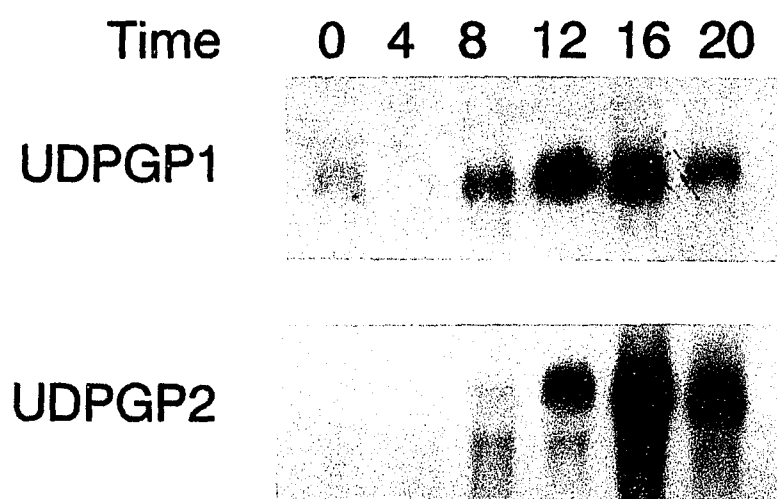
B.

Fig. 4. The enzyme activity of UDPG pyrophosphorylase during development of *Dictyostelium discoideum*. The open circle is Ax3, wild type and the filled triangle is the cell line Δ604 containing the disrupted UDGP1 gene.

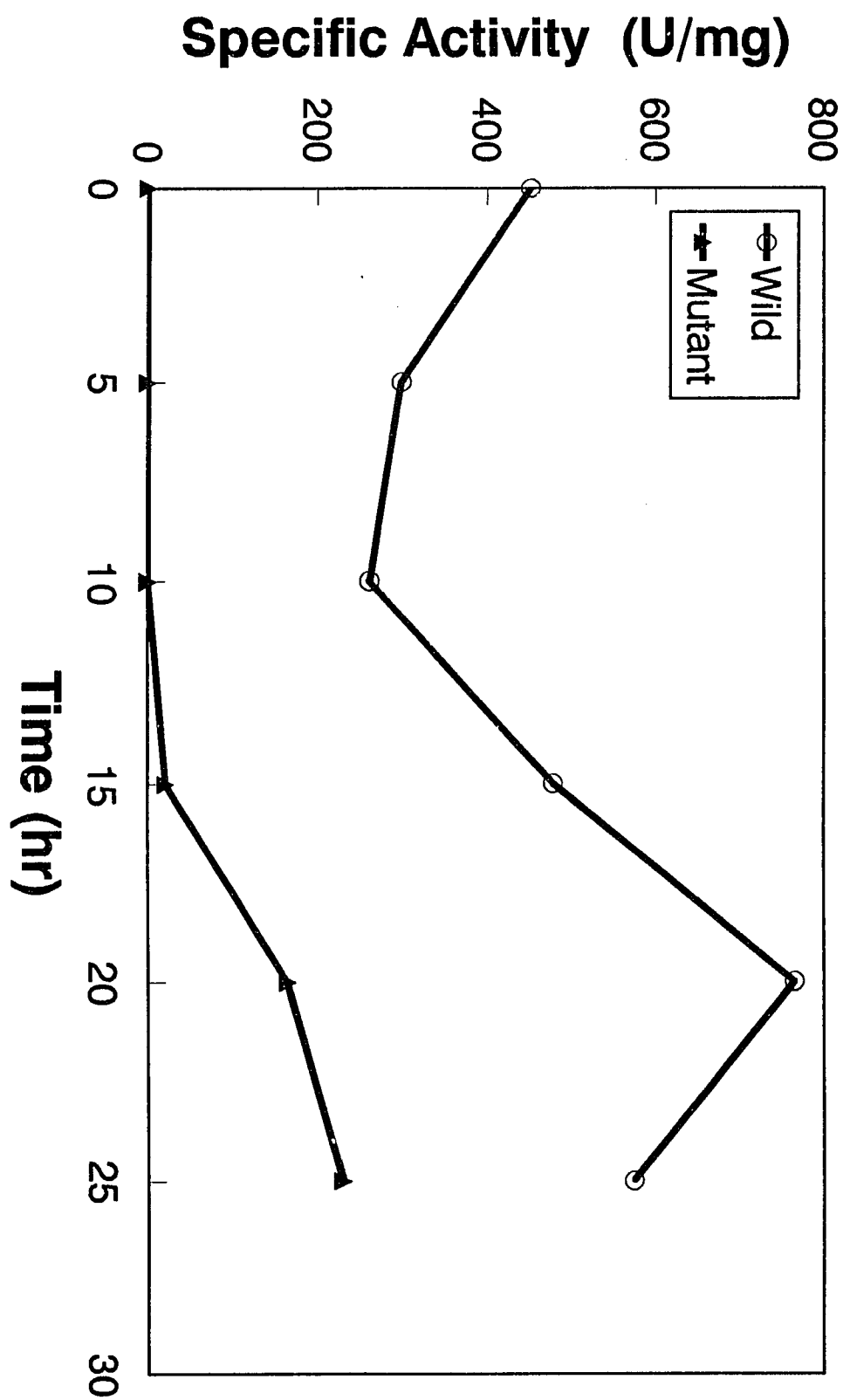


Fig. 5. Cellulose concentration from wild type Ax3 and the cell line $\Delta 604$. Ax3 and $\Delta 604$ cell lines were developed on filters and 10^8 cell were collected for determination of cellulose concentration as described in materials and methods.

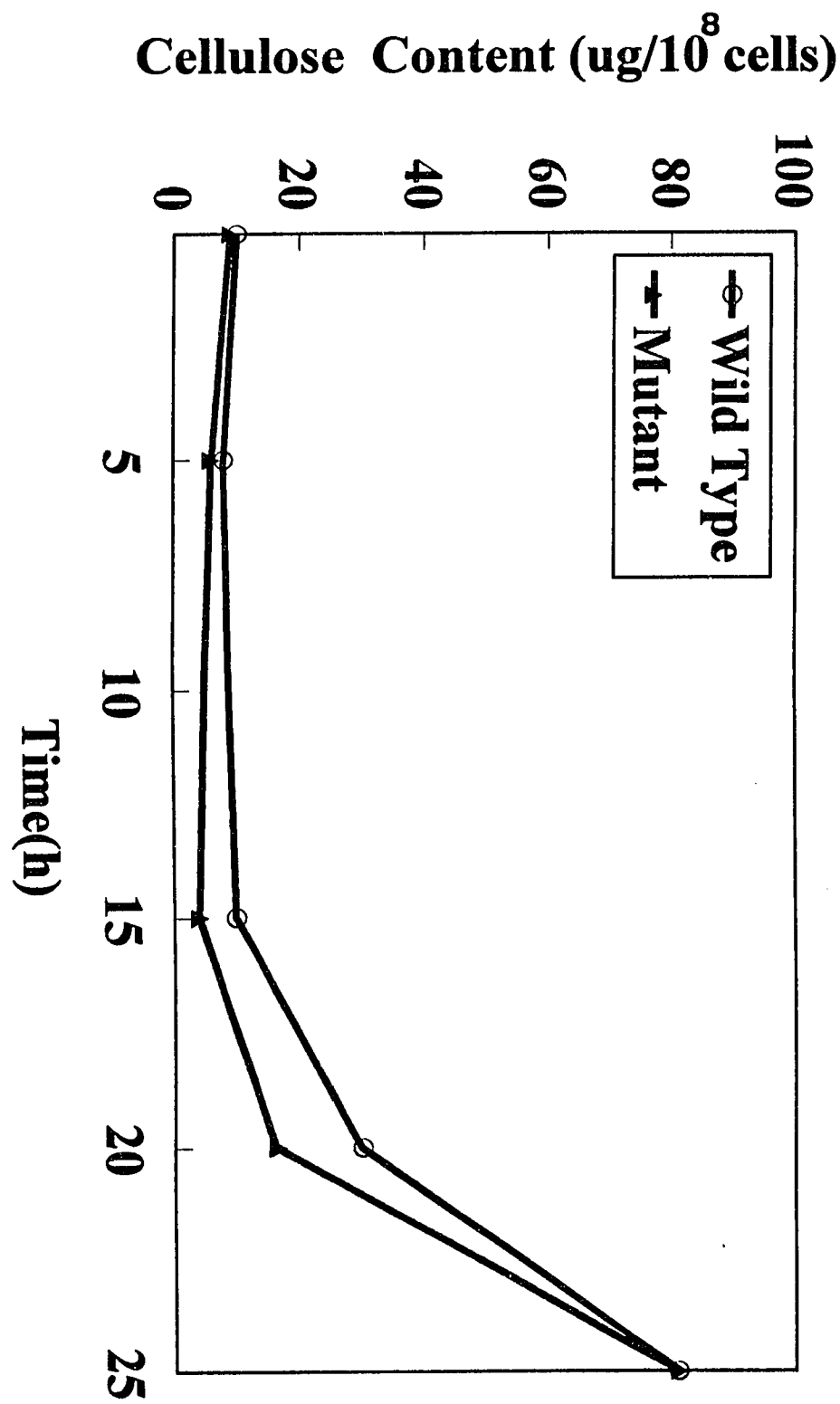


Fig. 6. Cloning strategy of UDPGP2 by PCR. (A) The presumed genomic locus of UDPGP1 and UDPGP2 in the replacement cell line $\Delta 604$ and wild type. The disrupted UDPGP1 gene in the $\Delta 604$ mutant line lacks UDPGP1 sequences from the EcoRV site in the second exon to the EcoRV site in 3'end downstream region and contains the neo cassette (see Fig. 1A). Two primers (F1 and R1) were derived from domains conserved in both the recently isolated UDPGP gene of potato tuber and UDPGP1 gene of *Dictyostelium*. The primer binding site of the R1 was chosen because it is missing in the UDPGP1 gene and replaced by the neo cassette. (B) PCR products from both primer F1 and R1 and genomic DNA templates from Ax3 wild type cells or the mutant cell line $\Delta 604$. The samples were run on 3 % Nusieve/ 1% Seakem agarose gel and stained with ethidium bromide. Lanes 1 and 2 are from reactions with the genomic DNA of the mutant $\Delta 604$ cell line. Lanes 3 and 4 are from reactions with the genomic DNA of the Ax3, wild type. Lanes 1 and 3 contain DNA digested with Bgl II. The two arrows indicate the positions of untreated samples and the BglII products. Lane M contains molecular weight marker.

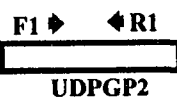
A.

DISRUPTANT



↓

NO PRODUCT

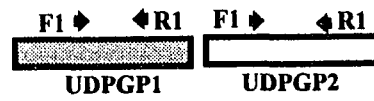


↓

112 bp



WILD TYPE



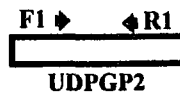
PCR

↓

112 bp



BglII



↓

112 bp



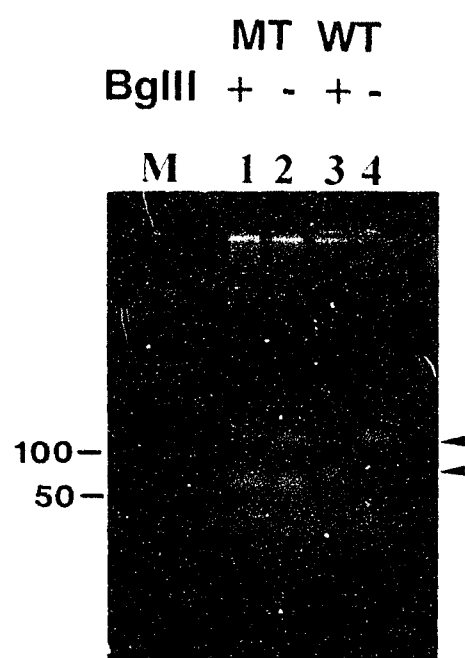
B.

Fig. 7. Amino acid sequence comparisons of UDPGP2, UDPGP1 and potato tuber UDPGP using GAP program (UWGCG package; Devereux *et al.*, 1984) was used. Upper lines (U2) are the sequence of the UDPGP2 of *Dictyostelium*. Middle lines (U1) are for the UDPGP1 of *Dictyostelium*. Lower lines (PU) are the sequence of potato tuber UDPGP. Identical residues are shaded. The two primers (F1 and R1) used in the PCR amplifications are shown as dotted on the tops. Lysine 329 on the UDPGP of potato tuber is marked with an asterisk.

U2 --- LILIKKLVKSF.....EKYKYSDVKIHC F ---
 U1 170 LVIMNSYKTHNETNKVIEKYKTHKVS IKTF 199
 PU 137 LILMNSFNTHDDTLKIVEKYANSNIDIHTE 166

U2 --- NESRFPRI LKD..NLMP.VPDKLFGSDSGW ---
 U1 200 QQSMEPKMYKDTINLVP.KPNTPMNPK.EW 229
 PU 167 NOSQYPR LVTEDFAPLPCKGNSGKD...GW 196

U2 --- YPPGHGDVFFALQNSGLLETLIN EGKEYIF ---
 U1 230 YPPGSGDIFERSLORSGLIDEFLAAGKEYIF 259
 PU 197 YPPGHGDVFFPSLMNSGKLDALLAKGKEYVF 226

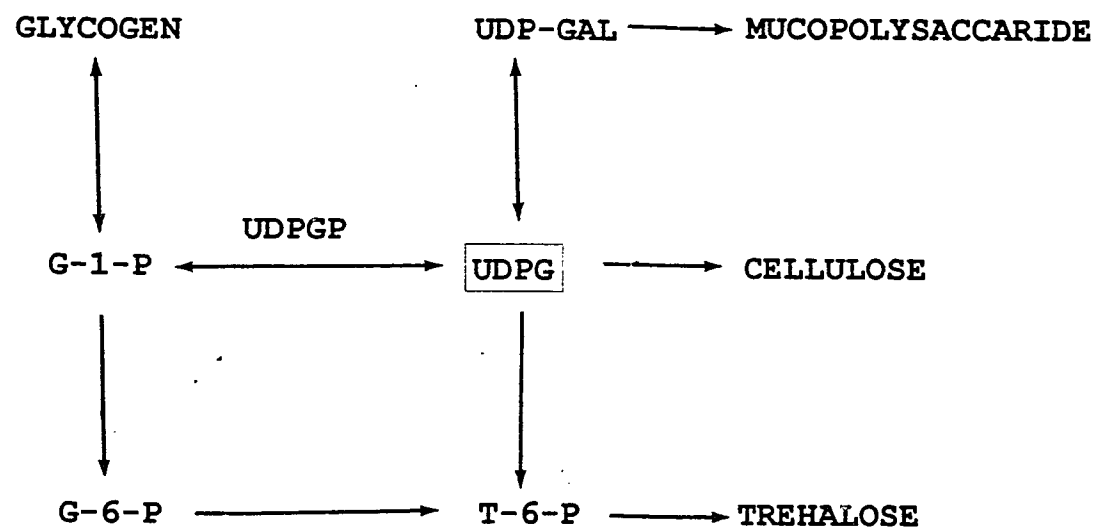
U2 --- KVENTNNI WVN LKAMDRI LKQNL LDDMDIT ---
 U1 359 KLWNTNNI WVN LKSVSNLIKEDKL.DLDWI 388
 PU 298 KI ENTNNL WVNLSAIKRLVEADAL.KMETI 327

U2 --- INPKVADGKNITQLRLAAGAAI ---
 U1 389 VNYPLENHKAMVQLETPAGMGI 410
 PU 328 PNPKEVDGKVKVLQLETAAGAAI 349

*

Fig.8. Biosynthetic pathways involving UDP-glucose which is produced by UDPG pyrophosphorylase (UDPGP). UDPG, UDP- glucose; UDPGP, UDP- glucose pyrophosphorylase; G-1-P, Glucose-1-phosphate; UDP-GAL, UDP galactose; G-6-P, glucose-6-phosphate; T-6-P, trehalose-6-phosphate.

Fig. 8



PART II

**TARGETED DISRUPTION IN *Dictyostelium* OF A HUMAN RAC
PROTEIN KINASE HOMOLOG GENE ENCODING A PLECKSTRIN
HOMOLOGY DOMAIN CAUSES DERANGED DEVELOPMENT.**

INTRODUCTION

Dictyostelium discoideum grows on bacteria or in axenic medium as unicellular amoebae by binary fission. When their food source is depleted, the amoebae aggregate in group of approximately 10^5 cells and undergo differentiation and a well-defined, synchronous morphogenesis culminating in the formation of a mature fruiting body made of two major cell types, spore and stalk cells by 24 hours (Reviewed in Loomis, 1975). The multicellular developmental process is mediated through many signaling molecules, several of which have been identified and is controlled by the differential expression of numerous genes. During early development extracellular cAMP acts as a chemoattractant to cause unicellular amoebae to aggregate (Konijin et al., 1967). In addition, extracellular cAMP regulates the expression of several cell type specific genes by binding to cell-surface cAMP receptors which act through heterotrimeric guanine nucleotide-binding proteins (Schaap and Van driel, 1985; Haribabu and Dottin, 1986; Devreotes, 1989; Firtel, 1991; Haribabu et al., 1991a; Schaap et al., 1993). But the intracellular signaling pathways that lead to gene expression for differentiation and morphogenesis are still poorly understood.

Some signal transduction pathways are mediated by transient protein-protein aggregations through one or more modular domains such as SH2 and SH3. The SH2 domain recognizes phosphorylated tyrosine residues in specific amino acid sequences (Pawson and Schlessinger, 1993; Birge and Hanafusa, 1993). The Abl SH3 domain binds to a short proline-rich motif, PXXPPPYXP, in the SH3 binding protein-1 (3BP-1). The short motif appears to be sufficient for the binding (Cicchetti et al., 1992; Ren et al., 1993). In the activation of Ras proteins, the SH3 domain of Grb2, which contains two SH2 domains flanking a SH3 domain, is bound to the proline-rich carboxyl-terminal tail of SOS protein. Upon stimulation, the tyrosine kinases such as EGF and PDGF receptors become autophosphorylated and the Grb2-SOS complex binds to the autophosphorylated tyrosine through its SH2 domains, allowing SOS to activate Ras proteins at the plasma membrane (Egan et al., 1993; Rozakis-Adcock et al., 1993; Li et al., 1993; Gale et al., 1993). Recently another domain, a pleckstrin homology domain or PH domain, has been found in several signaling molecules including ras-GAP, ras-GRF, SOS, PLC- γ 1, β ARK kinase and the Rac family of protein serine/threonine kinases (Mayer et al., 1993; Haslam et al., 1993; Musacchio et al., 1993). Two PH domains are found in pleckstrin which is a target of

phosphorylation by PKC in platelets (Tyers et al., 1988). Pleckstrin contains 2 PH domains at each end, suggesting that it like Grb2 might act as a molecular adaptor. Mutation of the PH domain in β ARK blocks its ability to bind to the $\beta\gamma$ subunit of G proteins and thereby blocks membrane translocation (Koch et al., 1993; Touhara et al., 1994). These results suggest that the PH domain mediate protein-protein interactions involved in signal transduction.

Many cellular processes such as motility, metabolism, growth and differentiation are regulated by reversible protein phosphorylation (Shenolikar, 1987; Edelman et al, 1987; Hunter, 1987). The phosphorylation of transcription factors is also a major mechanism to regulate gene expression (Hunter and Karin, 1992). Several protein kinases exist in *Dictyostelium* and many must play important roles in development (Haribabu and Dottin, 1991b). Stimulation of cell surface cAMP receptors results in their phosphorylation presumably by receptor kinases (Vaughan and Devreotes, 1989). The protein kinase A (PKA) activity of *Dictyostelium* is critical for development because constitutive activation of PKA results in rapid development (Simon et al, 1992). Selective expression of a dominant-negative inhibitor derived from the regulatory subunit

of *Dictyostelium* PKA in prestalk cells or prespore cells arrests culmination and prevents spore differentiation, respectively (Harwood et al., 1992; Hopper et al., 1993). But the roles of kinases in the regulation of gene expression in development of *Dictyostelium* are still poorly understood. In order to reveal the role of phosphorylation in the development of *Dictyostelium*, we cloned several protein kinases fragments by PCR based on homology of serine/threonine protein kinases (Haribabu and Dottin, 1991b).

Here we describe the molecular cloning, sequencing and characterization of a serine/threonine kinase, DdK6, which encodes 444 amino acids and encodes a 51 kD protein. DdK6 kinase shows 50% identity to the Rac family of protein serine/threonine kinases (Bellacosa et al., 1991; Jones et al., 1991) and encodes a pleckstrin homology domain (Mayer et al., 1993; Haslam et al., 1993). The mRNA is detected in small amounts in vegetative cells and increases early in development between 2 and 4 hours. To analyze the role of DdK6 in development, we created null mutations by gene targeting using homologous recombination. The mutants appear to be deranged in their development, delaying by more than 8 hours the formation of fruiting bodies and showing heterogeneous populations

of developing structures with abnormal phenotypes; some arrested at mound and tip stages and others formed fruiting bodies with thick, short stalks. Furthermore the expression of developmentally regulated genes was also delayed in the null mutants. Peptide specific antibodies to the C terminal region detect a unique protein but those against the PH domain detect multiple species on western blots. This result suggests that other PH domains are detectable by the N terminal anti peptide antibody. The mRNA levels increase markedly early in development and decline by 4 hours but the protein is stable and based on immunofluorescence staining is localized in the nucleus. These findings suggest that the DdK6 kinase is necessary for normal development and the PH domain in the regulatory region of DdK6 gene might be shared with PH domains in other proteins and play an important role as a module for protein-protein interactions in signaling pathways.

MATERIALS AND METHODS

Growth and Transformation of *Dictyostelium D. discoideum* strain AX3 was grown axenically in HL5 medium to a density of 2×10^6 /ml or in association with *Klebsiella* bacteria on SM plates (Sussman, 1966). Transformation was performed by electroporation (Dynes and Firtel, 1989) with some modifications. Cells were grown axenically to 2×10^6 /ml and 5ml were collected by centrifugation at $450 \times g$, and washed in 2 volumes of electroporation buffer (EB: 1 mM sodium phosphate, pH 6.1 and 500 mM sucrose). The pellet was resuspended in 0.3 ml of EB on ice. The cell suspension was mixed with 10 μ g of DNA in TE (10 mM Tris, 0.1 mM EDTA, pH 8). The mixture was transferred to a electroporation cuvette (0.2 cm gap, Bio Rad) in an ice water bath. After 5 minutes, the cells were electroporated (1.2-1.5 KV, capacitance 3 μ F, time constant 0.5-0.9 msec) with a Bio Rad eletroporation device. The cells were immediately returned to ice water bath and left standing for 10 min. They were transferred with a Pasteur pipette to a tissue culture petri dish (100mm) and then treated with 3 μ l of healing solution (100 mM CaCl₂ and 100 mM MgCl₂). After 15 min, 12 ml of HL5 was added. After 24 hours, the medium was replaced with 2 volumes HL5 containing G418 (10 μ g/ml) and 200-

300 μ l was plated in wells of a 96 well multititer plate to isolate individual clones. After 10 days aliquots of the culture were plated on SM plates with bacteria to isolate individual clones for further analysis.

Development of Dictyostelium Cells were grown axenically in HL5 medium at a density of 2×10^6 /ml, centrifuged at $450 \times g$ and washed three times with PDF buffer (33mM sodium phosphate pH 6.2, 13.5 mM KCl, 5mM MgSO₄, 0.75 mM CaCl₂ and 0.3 % streptomycin sulfate). The washed cells were resuspended at density of 1.0×10^8 cells/ml in PDF and plated on Millipore filters (0.45 μ m pore size) on 3 pads (Whatman #3) at a density of 6.5×10^6 cells /cm₂ for development at 22°C.

Construction of Vectors p2.9G (Skehel , 1993) contains a partial Sau3A genomic fragment (3.6 Kb) containing the DdK6 gene inserted in the Bam HI site of pAT153 vector (Twigg and Sherratt, 1980). A 4.0 Kb fragment was liberated from p2.9G by digestion with both BamHI and SalI, and subcloned into the BamHI and SalI sites of pBluescript (3.0 Kb) to construct pBK6 (7.0 Kb). to build the disruption vector, pBK6neo (9.1 Kb), the pBK6 was digested with HindIII and the ends were filled in with Klenow fragment and the blunt

end - ligated to a neo cassette (2.1 Kb), (Witke et al., 1987) first cut with XbaI and also filled in.

Preparation of Genomic DNA *Dictyostelium* genomic DNA was prepared by a rapid method (Knecht, et al., 1991). 10^7 to 10^8 cells were pelleted at 1000 rpm for 5 minutes in a microcentrifuge, suspended in 1 ml of nuclei buffer (25 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 0,5 mM EDTA, 5 % sucrose) and lysed with 200 μ l of 20% Triton X-100 on ice for 5 min. The nuclei were pelleted at 5000 rpm, 5 min in a microfuge, and the pellets were resuspended in 300 μ l of proteinase K buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 % SDS, 0.1 mg/ml of proteinase K) and incubated at 65°C for 20 minutes. DNA was extracted with one volume of phenol-chloroform (1:1) and subsequently with one volume of chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of ethanol in the presence of 1.7 M ammonium acetate to avoid RNA contamination. The DNA was pelleted at 5000 rpm in a microfuge for 5 min and dried briefly under vacuum, and resuspended in 30 μ l of TE. Total cellular RNA was isolated from vegetative or developed cells as previously described (Alton and Lodish. 1977).

Southern blot and Northern blot The procedures were

prepared as previously described (Haribabu and Dottin, 1986). 20 µg of RNA was electrophoresed in 1.5% formaldehyde-agarose gels and transferred to nylon membranes. DNA probes were prepared by the nick translation. Southern analysis was performed as previously described (Maniatis, 1982). Digested DNAs were fractionated in 0.8% agarose gels and then transferred to nylon membranes with 10 x SSPE. Prehybridization was done in hybridization buffer (6 x SSPE, 5 x Denhardt's solution, 0.5 % SDS, 100 µg/ml salmon sperm DNA, 50% formamide pH7.4) for 1-2 hour at 42°C. The blot was hybridized in hybridization buffer (same as prehybridization buffer except including 10% dextran sulfate) with denatured probes. DNA probes were prepared by random priming method (Feinberg and Vogelstein, 1983). Typically the specific activity was 1×10^9 cpm/ug.

Multiple Antigenic Peptide (MAP) Antibodies Two oligopeptides (K6N and K6C) were synthesized using MAP resin on Applied Biosystems' peptide synthesizer. The resin contains a 7 lysine core matrix from which peptides can be synthesized. The K6N oligopeptide contained 15 residues derived from the 53th to 67th amino acids in the NH₂-terminal region of DdK6 gene while K6C peptide had 11 residues from the 423th to 433th

amino acids in the carboxyl terminus of DdK6 gene. The synthesized peptides were desalted with a G25 Sepharose column (0.6 cm diameter, 6 cm height, 8 ml bed volume). The peptide sample (20mg/ml) was loaded in 1 ml and eluted with H₂O. The concentration of peptide in the fractions was measured at OD 205 nm or 215 nm. The fractions showing high absorbance were pooled and lyophilized. The amino acid sequences were confirmed by peptide sequencing at New York Institute for Basic Research by Dr. James Styles. NZW rabbits were immunized by subcutaneous injection by HRP, INC., Denver, PA. with 1 mg MAPs in complete Freund's adjuvant on day 0, boosted with two injections of 500 ug MAPs in incomplete Freund's adjuvant on day 10 and 33 and bled on day 40.

Western Blot. Cells were resuspended in water and then divided into two aliquots. One aliquot was treated with an equal volume of SDS sample buffer (2X: 125 mM Tris, pH 6.8, 4 % SDS and 20 % glycerol) and bromophenol blue for further analysis. The other was treated with SDS sample buffer without bromophenol blue for the Lowry protein assay (the BioRad DC protein assay kit). Cell lysates (5 or 20 ug protein) were loaded into polyacrylamide gels (15 %), then electrophoresed with the Mini-V8.10 gel apparatus

(BRL) at 220 volts for 1.5 hour. The gels were transferred onto nitrocellulose membranes (Hybond, ECL). The ECL detection system was used for detecting antigen(s) reacting with the antiserum (1:1500 dilution) which was used without purification, following the manufacture's instructions. Preimmune serum was used for control experiments.

Immunofluorescence For cell preparation, AX3 cells (1×10^6) were plated on a polylysine coated coverslip in a well (24 well plate), allowed to adhere for 20 min and then rinsed 2X with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 , pH~7.3) before fixation. For fixation, PBS-rinsed cells were incubated with a solution of methanol-acetone (1:1) or ethanol-acetone (1:1) for 5 min. at room temperature. Alternatively a 4% solution of paraformaldehyde in PBS was used to fix the cells for 5 min at room temperature followed by permeabilization of the cell with Hepes-Triton X-100 for 3 min. Cell were rinsed 3X in PBS before immunodetection. For antibody binding, the fixed cells were preincubated for 30 min at 37° C in PBS containing 3%BSA and 1% normal serum from the animal used to prepare secondary antibody, rinsed 3X with PBS containing 1% BSA and 0.1% Tween-20, incubated for 1h at 37° C in a humidified chamber with a dilution of

anti K6C-terminal rabbit serum (1:30). To confirm the specificity of the binding preimmune serum was used in control reaction. To detect the specifically bound antibody, fluorescein-isothiocyanate (FITC) donkey anti-rabbit antibody (1:50 dilution, Jackson ImmunoResearch, INC, West Grove, PA) was used. Rhodaminated (TRITC) phalloidin (Sigma) was added to stain F-actin filaments and 4,6-diamidino-2-phenylindole (DAPI, Sigma) was added to stain DNA. The reactions were incubated at 37° C in a humidified chamber for 30 min. The samples were prepared for mounting and photography by rinsing 3X in PBS containing 1% BSA and mounting in Gel/Mount (Biomed, Foster City, CA). The preparations were examined with a Zeiss microscope.

Cyclic AMP Pulsing Experiment. *D. discoideum* AX3 (a parental strain) and 104-10 cell line (a disruptant of DdK6) were used in these studies. Cells were grown axenically in HL5 to a density of 3×10^6 /ml, harvested by centrifugation, washed twice in cold MES-PDF (8 mM 2-(N-morpholino)ethanesulfonic acid, 2 mM KCL, 0.6 mM MgSO₄, pH 6.5), and then suspended at a density of 5×10^6 /ml in MES-PDF containing 0.03% streptomycin sulfate. The suspension was distributed into two flasks (100 ml in a 500 ml Erlenmeyer flask) and shaken at 220 rpm on a gyratory shaker for 1 hour. One flask was

pulsed with cAMP (200 μ M) to yield a final concentration of approximately 100 nM every 6 min using a Gilson peristaltic pump connected to a power supply regulated by a computerized timer (Chronotrol, San Diego, CA). Every 4 hour, 4 ml of cells was removed for RNA isolation. At 12 hour, the input concentration of cAMP was adjusted to 149 μ M from 200 μ M to keep the final concentration of cAMP at 100 nM. The other flask was pulsed with buffer (MES-PDF). Cell samples were harvested every 4 hour up to 16 hour and then RNA was extracted.

RESULTS

Molecular Analysis of a Protein Kinase cDNA.

We have identified members of a protein kinase multigene family of *Dictyostelium* using PCR to amplify short sequences from highly degenerate primers (Haribabu and Dottin, 1991). The fragments were cloned and sequenced and at least six different kinase sequences could be deduced from the cloned fragments (Haribabu and Dottin, 1991). Screening a cDNA library with one of them yielded K6A cDNA. This clone was sequenced by Bodduluri Haribabu and Benzamin Ortiz in this Lab and found to be almost identical to a cDNA clone being sequenced by Sandrinne Bozo in Michel Veron's Lab and a genomic clone from Jeff Williams Lab (Skehel, Ph.D. thesis, 1993). Fig. 1 shows the nucleotide sequence and the deduced amino acid of the DdK6 gene. The data are from the three Labs and are rebuilt for completeness. It contains a single large open reading frame 1332 base-pair (bp) from nucleotides 274-1605. The translation of the longest open reading frame predicts a protein with a molecular weight of 51 kD. A FASTA search of current data bases revealed homology only to protein kinases. The genomic sequence deduced by Skehel has an intron (105 bp)

between the cDNA nucleotides 123 and 124 (see Fig. 1). We found only a few discrepancies between the cDNA and genomic sequence at position 859, 865, and 1071 but they did not result in attenuation of the reading frames. They were checked and corrected by resequencing the genomic fragment in these region in this lab. However, we found one nucleotide was omitted at position 1415, resulting in a frame shift of the amino acid sequence downstream of the base pair. The deduced amino acid of the genomic DNA sequence terminates at 414, 30 residues earlier than our sequence deduced from cDNA which extends to 444 residues (Fig 2B). The highest degree of homology (50 % identity in the kinase domain) is found with members of the Rac family of serine/threonine protein kinases (Fig. 3) (Bellacosa et al., 1991; Jones et al., 1991).

The DdK6 protein contains all conserved sequence motifs in the protein kinase family (Taylor, 1989; Taylor et al., 1992): Gly¹²⁷-Lys-Gly-Ser-Phe-Gly¹³² (G⁵⁰TGSFG⁵⁵), a glycine-rich region is a part of ATP binding domain; the Lys¹⁴⁹ (K⁷²), Asp²⁴³ (D¹⁶⁶), Asn²⁴⁸ (N¹⁷¹) implicated in ATP binding and catalytic activity; and Asp²⁶¹-Phe-Gly²⁶³ (DFG¹⁸⁴⁻¹⁸⁶) and Ala²⁸⁷-Pro-Glu²⁸⁹ (APE²⁰⁶⁻²⁰⁸), diagnostic sequences of a protein kinase catalytic domain. The amino acid sequences in parentheses are derived from bovine cAMP-dependent kinase (Taylor,

1989). The consensus DLKPEN²⁴³⁻²⁴⁸ and GTPEYLAPE²⁸¹⁻²⁸⁹ strongly suggest serine/threonine specificity of DdK6 gene (Hanks and Quinn, 1991).

DdK6 is a Human Rac Kinase Homolog in *Dictyostelium*.

DdK6 shows the highest identity to human rac protein kinase (50 % identity in kinase domain) according to recent gene bank search. Human rac kinase showed a considerable degree of similarity to PKC and cAMP-dependent protein kinase and the murine homolog of rac kinase appears to be a retroviral oncogene which causes T cell lymphoma in rodents (Jones et al., 1991; Bellacosa et al., 1991). Recently it has been reported that the amino terminus of Rac kinase contains a pleckstrin homology (PH) domain (Mayer et al., 1993; Haslam et al., 1993) which is derived from repeat amino acid sequences of about 100 residues in length of pleckstrin, a major protein kinase C substrate of platelets (Tyers et al., 1988). A number of proteins important in signal transduction or cytoskeletal function have been shown to contain a PH domain which is thought to be a module for protein-protein interactions similar to SH2 and SH3 domains (Mayer et al., 1993; Haslam et al., 1993). Some of them are shown in Fig 4A. Analysis using FASTA with the PH domain

(amino acid 4 to 98) of the DdK6 gene identified several PH domain-containing proteins including pleckstrin. The sequence comparison with known PH domains shows that all consensus sequences are conserved in the PH domain of the DdK6 protein. (Fig.4). PH family sequences share only 21-25% identity, but the similarities are statistically highly significant (Mayer et al., 1993) and the sequence and position of the consensus amino acids are highly diagnostic. To test the statistical significance of similarities in PH domains, I used the SEQALIGN program package (Clark, K.L., D.C. Teller, and G.R. Reek, personal communication; Mayer et al, 1993) using the scoring matrix of Henikoff and Henikoff (1992) to weight conservative amino acid substitutes. Table 1 summarizes the z values for the similarity scores of PH domains in many proteins. For example, the PH domain of DdK6 showed very strong significance of relationships with the Pleckstrin N ($z > 12$) and Rac ($z > 8$). Interestingly, the PH domains of DdK6 and Rac are more closely related to pleckstrin C than to pleckstrin N. By contrast, PH domains from GRF and Dynamin resemble more closely pleckstrin N.

Developmental Regulation of Expression of DdK6 Gene and Genomic Southern Analysis.

Figure 6 shows the time course of expression of the DdK6 gene. RNA isolated from cells developed on filters for the indicated times was analysed by Northern blots which were hybridized with a 3'end cDNA fragment of 800 bp in size. A 2.1 kb mRNA is expressed with a peak at 2 hours and then quickly declined. The mRNA is present in small amounts in vegetative cells and not detected later than 4 hour in development. RNA from cells developed on bacterial lawns also showed identical patterns of hybridization, but RNA from growing stage on bacterial lawn expressed much less than RNA from axenically growing cells (Skehel, Ph.D. thesis, 1993). Genomic Southern analysis was performed by digesting aliquots of genomic DNA with several restriction enzymes in turn and hybridized with a cDNA probe missing 82bp in the end of the amino terminus. The pattern was what could be predicted from the restriction sites in the DdK6 gene, indicating that DdK6 is a single copy gene. For example, digestion with HindIII, which is contained in the gene, gives only two bands, whereas EcoRI and others, which do not cut within the gene, give a single band (Fig. 5A). Double digestion and analysis with either the full-length cDNA probe or the 3'end probe allow us to construct a partial restriction map of the genomic DNA (Fig. 5B).

Targeted Disruption of the DdK6 Gene

To analyse the function of DdK6 in development and in signal transduction, we created DdK6 null cell lines by gene targeting. A disruption vector pBK6neo contains a 3.7 Kb genomic fragment interrupted at the HindIII site of the coding region by insertion of a 2.1 Kb neo cassette (Fig. 7 and see Materials and Methods). A linearized disruption vector was transformed into AX3, wild type cells by electroporation, and clonal transformants were selected in medium with G418 (10 ug/ml). Genomic DNAs from individual transformants were digested with HindIII, subjected to Southern blots and hybridized with a 3' end probe of DdK6 cDNA. 4 of 27 individual clones showed a 5.3 Kb band which is the size predicted to result from a double crossover event. By contrast DNAs from wild type AX3 cells yielded a 4.5 Kb band (data not shown). One of the *ddk6*⁻ cell lines (104-10) was analyzed further. Three different restriction enzyme digestions of the genomic DNA from wild type and *ddk6*⁻ cells were probed with the 3' end probe. NsiI digestion showed that a 4.5 kb in *ddk6*⁻ DNA is shorter than 6.0 Kb in wild type DNA because the inserted neo cassette creates a NsiI site. HincII digestion yielded a 2.9 Kb fragment in wild type DNA and a 5.0 Kb in *ddk6*⁻ DNA because the 2.1 Kb of neo

cassette is added. Finally, HindIII digestion showed a 4.5 Kb fragment with wild type DNA but a 5.3 Kb fragment with the mutant DNA because the original HindIII site is lost at the point where the neo cassette is inserted and the next HindIII site in the neo gene is 0.8 Kb away (Fig. 7 and 8). Taken together, these data show that a single copy of the transforming DNA fragment has replaced the endogenous DNA fragment by a double cross over.

To provide additional evidence of the gene disruption we have examined the expression of the Ddk6 gene in *ddk6⁻* cells and wild type. Total cytoplasmic RNAs were isolated from wild type and the *ddk6⁻* cells at 2 hour. Northern blots of the RNAs were hybridized with the 3' end probe of the Ddk6 cDNA. Wild type RNA shows about 2.3 Kb RNA while *ddk6⁻* cells generates a 1.3 Kb mRNA (Fig 9A). We believe that the shorter mRNA is transcribed from an internal promoter, probably near the actin 15 promoter that transcribes the neo gene. The actin 15 promoter for expression of neo cassette is inserted in the opposite direction with respect to the transcription of the endogenous promoter. However the DNA containing the actin 15 promoter is probably responsible for transcribing the 1.3 Kb mRNA, because the actin 15 promoter is able to transcribe bidirectionally (Knecht et al., 1993). We

performed a western blot with proteins prepared from control and *ddk6⁻* cells. In the control cells, the K6C antibody, directed to the C terminal region sequence, recognized a 51 Kd protein, which is consistent with the calculated size of DdK6 protein. By contrast, the antibody did not detect any proteins isolated from *ddk6⁻* cells, suggesting that the shorter mRNA might not be translated or might generate a protein fragment with a different reading frame (Fig.9B).

Development of DdK6 Null Mutants is Deranged

The developmental morphology of the *ddk6⁻* cells was examined by developing them on Millipore filters. Fig 10 shows developmental phenotypes of two different *ddk6⁻* cell lines. Wild type cells aggregated at 8 hour and began to form mature culminants by 20 hour, whereas *ddk6⁻* cells were slightly delayed in forming aggregates and remained as aggregates or finger-like structure as late as 20 hours. By 12 hour of development, wild type cells formed finger-like structures, but *ddk6⁻* cells still remained at aggregation stages up to 20 hour of development (Fig 10). At 24 hour of development, wild type cells completed culmination to form spores and stalks while *ddk6⁻* cells formed small and thin finger-like

structures sprouting from tight mounds (data not shown). The developmental morphology of *ddk6⁻* cells at 36 hour of development showed heterogeneous populations: some of them completed culmination forming stalks and spores similar to wild type, while others showed abnormal phenotypes with short and thick stalks. Still others arrested at early developmental stages including mound and tip stages (Fig. 10). We also have observed the same phenotypes for *ddk6⁻* cells developing on a lawn of *Klebsiella aerogenus* (data not shown). The degree of retardation of development and abnormality are different for each mutant and even between batches of the same mutant. Spores from wild type and *ddk6⁻* were counted under a microscope and plated on bacterial lawns to test viability. We observed no significant difference in the viability of spores from wild type and mutant cell lines (data not shown).

The Expression of Developmentally regulated genes is delayed.

To examine the relation between morphological phenotypes and gene expression, we have examined the expression of developmentally regulated marker genes in *ddk6⁻* cells. The PL3 gene is not present in

undifferentiated cells but is expressed in prespore-specific cell types in multicellular stages (Barklis and Lodish, 1983). In wild type cells, the PL3 gene was expressed at between 12 and 16 hour in development and then its expression quickly declined, but *ddk6⁻* cells started to express PL3 gene at 20 hour and its expression increased up to 24 hour of development, indicating the expression of PL3 gene was delayed by about 8 hours in *ddk6⁻* cells (Fig. 11, PL3 panel). Cyclic AMP receptor (*cAR1*) is maximally expressed during the early stages of development, peaking at 4 hours (Saxe et al., 1991). Fig. 11 (*cAR1* panel) shows a similar pattern of expression of *cAR1* mRNA. It peaked at 4 hours in wild type but in *ddk6⁻* cells, *cAR1* transcript was expressed a lower level at 4 hour, and peaked at 16 hours of development when the cells had already formed mound and then declined. A discoidin 1 gene is expressed at moderately high levels in axenically grown vegetative cells of wild type, but its transcription is reduced early in the developmental cycle in the response to exogenous cAMP (Williams et al., 1978; Berger and Clark, 1983; Haribabu et al., 1987). Fig. 11 shows that wild type cells expressed the discoidin 1 transcript at maximum level in vegetative cells and it declined to zero by 4 hours of development. However *ddk6⁻* cells express little

discoidin mRNA between 0 and 4 hours and showed a maximum level of expression at 8 hour of development (Fig. 11, Disc panel). These data show that the marker genes were expressed in a heterochronic manner.

Effect of cAMP Pulses on Gene Expression in *ddk6*⁻ Cells

Pulsing competent amoebae with nanomolar levels of cAMP in a shaking culture is an efficient way to study cAMP receptor mediated signal transduction pathway(s) (Haribabu and Dottin, 1986; Mann and Firtel, 1987). We examined the effects of cAMP on the expression of several genes by pulsing cAMP every 6 min. In wild type cells, the *cAR1* mRNA was present through out the period of pulses and reached a maximum at 12 hour. In contrast, *ddk6*⁻ cells showed no detectable RNA up to 8 hour, start the expression of *cAR1* at 12 hour (Fig. 12, *cAR1* panel). Discoidin 1 mRNA expression was detectable between 0 and 4 hour and then disappeared in wild type cells, while in *ddk6*⁻ cells, it was detectable by 8 hour (Fig. 12, Disc panel). RNA of *DdK6* was detectable between 0 and 4 hours in the wild type, but in the mutant, the RNA was detected at 0 hour, but maximally expressed between 8 and 12 hours

(Fig. 12, DdK6 panel). These data suggest that cell surface cAMP receptor - mediated signal transduction is also deranged in ddk6- cells.

DdK6 Protein is localized in the Nucleus.

Fig 14. shows that indirect immunofluorescence with the C-terminal antibody (K6C) localized the DdK6 in the nucleus (K6C panel) (Mauro rabino mainly performed this work in this lab). DAPI staining of nuclear DNA overlapped with the antibody staining (Fig. 14. DAPI and K6C panels). Preimmune serum did not detect any proteins (Fig. 14, pre panel). The K6C antibody in western blots recognizes a specific protein of DdK6 (Fig13.A), and it did not detect any protein in the null cell line of DdK6 (Fig.9B), indicating that the antibody is specific for the DdK6. Several nuclear proteins have been shown to contain a minimal nuclear localization signal (NLS) sequence, Lys-Arg/Lys-X-Arg/Lys that is homologous to the SV40 T antigen sequence (Chelsky et al., 1989). Although there are similar sequences related to this, the predicted amino acid sequence of DdK6 does not contain the minimal NLS.

Peptide Antibody from the PH Domain Recognizes Many

Proteins.

We made two polyclonal peptide antibodies, K6N and K6C, derived from the N terminus (PH domain) and C terminus, respectively (see Materials and Methods for details). Immunoblot of cell lysates from wild type with the K6N antibody showed a smear of bands at all stages in development. It suggests that many proteins in the cell lysates react with the antibody (Fig. 13B). Since the MAP peptide was not attached to a carrier to make antiserum, it is unlikely that the antiserum contained non specific species caused by a carrier. Moreover when an antibody was prepared against a protein made by expressing the Ddk6 cDNA sequence in *E.Coli*, a similar smear pattern was observed. By contrast K6C antibody recognizes a single band of 51 kD. These data suggest that the K6N peptide derived from PH domain is eliciting antibodies that react with many proteins, perhaps because these proteins also contain similar domains while the C terminal domain is unique. As a control, K6C antibody did not detect any protein in the null cell line of Ddk6 (Fig.9B). Although the expression of Ddk6 protein peaks at 2 hour, the protein is detectable throughout development (Fig.13A).

DISCUSSION

This report describes the molecular cloning and functional analysis of a serine/threonine kinase, DdK6 that is required for normal development of *Dictyostelium*. The DdK6 gene shows all features of a typical serine/threonine kinase and identity (50%) to Rac protein kinase family. The amino terminal domain of DdK6 contains a PH domain which is found in many signaling molecules and is likely to be involved in signal transduction pathways. The DdK6 is a single copy gene and its mRNA is found between 2 and 4 hour in development of *Dictyostelium*, but DdK6 protein is detectable through out development. We created DdK6 null mutants by homologous recombination. The DdK6 null mutants delayed development with abnormal phenotypes and retarded the expressions of stage-specific genes.

The gene DdK6 appears to be a putative serine/threonine kinase belonging to the Rac kinase family. An intriguing feature of the DdK6 kinase is a PH domain located in the NH₂-terminal side. The PH domain was found for the first time in the major protein kinase C substrate of platelets, pleckstrin (Tyler et al., 1988), and was subsequently noted as a putative modular domain present in diverse signaling

proteins to mediate protein associations like SH2 and SH3 domains (Mayer et al., 1993; Haslam et al., 1993). PH-containing proteins include ras-GAP, a GTPase-activating protein (Trahey et al., 1988); ras-GRF (Shou et al., 1992), a guanine-nucleotide-exchanging factor for Ras and Rho, mouse SOS1 and Drosophila SOS (Bowtel et al., 1992; Simon et al., 1991), which are guanine-nucleotide-exchange factors for Ras. All of the previous proteins interact with Ras proteins, suggesting roles in signaling pathways.

In addition to the DdK6 gene, other members of the Rac kinase family including the Akt and the NrK6 also contain a PH domain at the same position in a NH-terminal side as DdK6 gene (Haslam et al., 1993), but the function(s) of the PH domain are unknown. The Akt protein is a proto oncogene product, was thought to contain a SH2-like domain in the regulatory region, suggesting a role of a functional link between tyrosine and serine-threonine phosphorylation pathways (Bellacosa et al., 1991). The proposed SH2-like domain in the Akt gene however is unlikely to be a SH2 domain as judged by the crystal structure of the src SH2 domain. Instead, it turns out to be a PH domain (Waksman et al., 1992; Haslam et al., 1993; Musacchio et al., 1993). The PH domains are present not only in

the Rac kinase family, but also in other kinases including the β adrenergic receptor kinase (β ARK) and the Human agammaglobulinemia tyrosine kinase (Atk) (Musacchio et al., 1993). The β ARK has a PH domain at the carboxyl-terminal side which can bind to the $\beta\gamma$ subunits of heterotrimeric G proteins *in vitro*. A synthetic peptide in the region of the PH domain can inhibit the binding. Truncated PH domains from the β ARK kinase neither bind to the $\beta\gamma$ subunit nor translocate the kinase to membrane, implying that the PH domain is required for the β ARK to recognize the $\beta\gamma$ complex and then to phosphorylate the β adrenergic receptor leading to the desensitization of the receptor (Inglese et al., 1992; Koch et al., 1993; Musacchio et al., 1993; Touhara et al., 1994). The murine X-linked immunodeficiency (XID) is caused by the mutation of a single conserved residue within the PH domain of the Btk, a cytoplasmic tyrosine kinase, suggesting that the change interferes with normal B cell signaling pathways mediated by Btk protein interactions with other proteins (Rawlings et al., 1993). In addition, loss of Atk, the human homologue of Btk, also results in a human immunodeficiency (Vetrie et al., 1993).

Dictyostelium development is characterized by

an orderly expression of spatial- and temporal-specific genes and responses to specific signals for normal differentiation and morphogenesis (Loomis 1982). The DdK6 null mutant results in alterations of the time course for the expression of the stage-specific genes and abnormalities of morphogenesis in development, indicating the DdK6 gene plays a critical role in development of *Dictyostelium*. The expression of the DdK6 gene in wild type cells is strictly regulated with a very narrow window, a peak at 2 hour in development, and seems to be stimulated upon starvation. Furthermore all so far tested-developmentally regulated genes including PL3, CAR1, discoidin and DdK6 gene tested so far delayed expression and/or induction in the DdK6 null mutants. Experiments involving addition of cAMP in pulses to cells shaking in suspension mimick pulsing in vivo. The cells showed heterochronic expression of the marker genes in the DdK6 null mutants, suggesting that signal transduction pathways might be deranged in ddk6-cells. These findings suggest that DdK6 gene may be involved in establishing or maintaining an orderly developmental program at early stage.

Because the DdK6 gene has a PH domain which might play a role in signal transduction pathways by being a

module for protein-protein interactions, one tempting speculation is that DdK6 might be one of the intracellular signaling molecules to receive and transmit starvation signals to target proteins in development of *Dictyostelium*. The hypothetical pathway for starvation signals might require expression of early genes including the DdK6 gene itself for establishing a developmental program. A substrate for the DdK6 could be a nuclear transcription factor since the kinase is localized in the nucleus and its defect causes abnormal expression such as delay for the most of gene expression we tested. A signal from the cytoplasm can be perhaps received through the PH domain of DdK6 to control the kinase activity. However, the understanding of protein associations that are regulated by a PH domain and the substrates of the kinase will require further analyses including the identification of targets of a PH domain-containing protein, and a mutational analysis within a PH domain region to define its essential regions.

Fig. 1. Composite Nucleotide Sequence and Derived Amino Acid Sequence of DdK6. The cDNA sequence was performed mainly by Benzamin Ortiz and Bodduluri Haribabu in this Lab and by Sandrinne Buzo in michel verons Lab. The genomic sequence wass done by Paul Skehel in Jeff Williams Lab as part of his PhD thesis. The nucleotide sequences that were different between the cDNA and genomic DNA are changed as a result of sequence comparison and sequencing. They are an extra T at 859 and an absence of A at 865 in Skehel's sequence. Removing T at 859 and insertion of A at 865 change deduced amino acids from YLG to ILD, and at 1071, G in our sequence to C changes deduced amino acid Q to E. For omitting A at 1415, see Fig. 2 and text. The first 22 amino acids in the N terminal region are from Skehel's work since the cDNA lacked this sequence. An intron (105 bp) is located between nucleotide 123 and 124 and is indicated by an arrow. The amino acid residues characteristic of protein kinases are in boldface and underlined.

TTTATATATTGGGGGAGGTGTGTGCTTTTTTTTTTAAAAATAAATAATGAATAAATTTA 60
 GAAATAATAAAATTTAAAAATAAATAAACTATTATATATATGAATTATATAATTTAA
 CAAATAAAAAAAAAAATAATAATAGAAAAAAAAATTAATAATAATAATAA 180
 ATAAATAAATAAATAAAAAAAAAAATAGTAATAATAATAATAATAATAAATAAAT
 AATAATTTAAAAAAAAAAAAAAAAAAAAATGTCAACAGCACCAATTAAACATGAA 300
 M S T A P I K H E 9
 GGTTCCTCACTAAAGAAGGTGGTGGTTTCAAAGTTGGAAAAAGAGATGGTTCATTCTC
 G F L T K E G G G F K S W K K R W F I L
 AAAGGTGGTGATTTAAGTTATTATAAAACAAAAGGTGAACCTGTACCATTAGGAGTTATT 420
 K G G D L S Y Y K T K G E L V P L G V I 49
 CATTTAAATACATCAGGTCATATTAATAATCTGATCGTAAGAAAAGAGTTAATGGATTT
 H L N T S G H I K N S D R K K R V N G F
 GAAGTACAAACACCATCACGTACATATTTCTTATGTTTACAGAGACAGAGGAAGAACGTGCA 540
 E V Q T P S R T Y F L C S E T E E E R A 89
 AAATGGATAGAGATATTAATTAATGAAAGAGAAATTATTATTGAATGGTGGTAACAACCA
 K W I E I L I N E R E L L N G G K Q P
 AAGAAATCGGAAAAGGTAGGAGTTGCAGATTTTGAATTATTGAATTTAGTTGGTAAAGGT 660
 K K S E K V G V A D F E L L N L V G K G 129
 AGTTTTGGTAAAGTTATTCAAGTTAGAAAGAAAGATACTGGTGAAGTGTATGCAATGAAA
 S F G K V I Q V R K K D T G E V Y A M K
 GTTTTATCAAAGAACATATCGTAGAGCATAACGAAGTCGAACATACATTGAGTGAGCGT 780
 V L S K K H I V E H N E V E H T L S E R 169
 AATATTCTTCAAAGATCAATCACCCATTTTTGGTTAATCTCAACTACAGTTTTCAAACA
 N I L Q K I N H P F L V N L N Y S F Q T
 GAGGATAAGCTTTACTTTATCTTGGATTATGTTAATGGTGGTGAGTTATTCTATCATCTT 900
 E D L Y F I L D Y V N G G E L F Y H L 209
 CAAAAGGACAAAAAGTTTACAGAGGATCGTGCCGTTATTATGGCGCAGAGATCGTATTG
 Q K D K K F T E D R V R Y Y G A E I V I
 GCATTGGAACATTTACATTTGTCTGGGTGTCTATAGAGATTTGAAACCAGAGAATTTA 1020
 A L E H L H L S G V I Y R D L K P E N L 249
 CTACTCACCAACGAGGGTCACATTTGCATGACCGATTTCGGTCTTTGCAAGAGGGTCTA
 L L T N E G H I C M T D F G L C K E G L
 TTGACACCAACCGACAAAACCTGGTACTTTCTGTGGTACTCTGAATATTTAGCACCCGAA 1140
 L T P T D K T G T F C G T P E Y L A P E 289
 GACTTCAAGGCAATGGTTATGGTAAACAAGTGGATTTGGTGGAGTTTGGTTCTCTCCTC
 V L Q G N G Y G K Q V D W W S F G S L L
 TATGAAATGCTCACTGGTTTACCACCATTTCTACAATCAAGACGTCCAAGAGATGTATCGT 1260
 Y E M L T G L P P F Y N Q D V Q E M Y R 329
 AAGATCATGATGGAGAAATTATCTTCCCACATTTCAATTTCTCCAGATGCTCGTTCCTC
 K I M M E K L S F P H F I S P D A R S L
 TTGGAACAACCTTTGGAAAGAGATCCTGAAAAAGACTTGCCGATCCAAATCTTATTA 1380
 L E Q L L E R D P E K R L A D P N L I K 369
 AGACATCCTTTCTTCCGTTCCATCGATTGGGAACAATTATTCCAAAAGAATATTCCACCA
 R H P F F R S I D W E Q L F Q K N I P P
 CCATTCAATCCAAATGTTAAAGGTCTGCTGATACCTCTCAAATTGATCCAGTTTTCACT 1500
 P F I P N V K G S A D T S Q I D P V F T 409
 GATGAAGCTCCTTTAACTATGGCTGGTGAATGTGCTTTAAATCCGCAACAACAAAA
 D E A P S L T M A G E C A L N P Q Q Q K
 GATTTTGAAGGATTTACATATGTCGCTGAATCTGAACATTTAAGATAAATTTAATAAATG 1620
 D F E G F T Y V A E S E H L R * 444
 ATTTTAAATAATAAAAAAAAAAAAAA

Fig. 2. Sequence Comparison of DdK6 gene between ours and Skehel's.

A cDNA sequence of DdK6 was obtained from Jeff Williams (Skehel, 1993) and compared with our sequence using the GAP program within GCG package. Fig 2A displays the region that show an important discrepancy between the two nucleotide sequences at nucleotide 1415 (see Fig. 1 legend). Fig 2B shows comparison of deduced amino acid sequences corresponding to the nucleotide sequence of A. The nucleotides and amino acids that are different are in bold.

A.

```

OUR  TGGGAACAATTATTCCAAAAGAATATTCCACCACCATTTCATTCCAAATGT 1457
      ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
SKE  TGGGAAC.ATTATTCCAAAAGAATATTCCACCACCATTTCATTCCAAATGT

OUR  TAAAGGTTCTGCTGATACCTCTCAAATTGATCCAGTTTTCCTGATGAAG 1507
      ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
SKE  TAAAGGTTCTGCTGATACCTCTCAAATTGATCCAGTTTTCCTGATGAAG

OUR  CTCCTTCTTTAACTATGGCTGGTGAATGTGCTTTAAATCCGCAACAACAA 1557
      ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
SKE  CTCCTTCTTTAACTATGGCTGGTGAATGTGCTTTAAATCCGCAACAACAA

OUR  AAAGATTTTGAAGGATTTACATATGTCGCTGAATCTGAACATTTAAGATA 1607
      ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
SKE  AAAGATTTTGAAGGATTTACATATGTCGCTGAATCTGAACATTTAAGATA

OUR  AATTTAATAAATGATTTTAAAATAA
      ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
SKE  AATTTAATAAATGATTTTAAAATAA

```

B.

```

OUR 379 WEQLFQKNIPPPFIPNVKGSADTSQIDPVFTDEAPSLTM
      ||
SKE   WEHYSKRIFHHHSFQMLKVLLIPLKLIQFSLMKLLL* 414

OUR 418 AGECALNPQQQKDFEGFTYVAESEHLR* 444

```

Fig 3. Alignment of kinase domain from DdK6 with Human rac protein kinase (Jones et al., 1991). The alignment was generated using GAP program. Solid lines are identical residues and double and single dots show conservations.

Fig. 4. Alignment of PH domain Sequences.

Consensus residues that are defined by Mayer et al., (1993) are shaded and indicated below the alignment. Numbers in parentheses indicate the position of the first amino acid in alignment; dashes indicate gaps. Plus and minus signs, basic and acidic residues, respectively; \$, aromatic residues; *, hydrophobic residues. PleN and PleC are the N-terminal and C-terminal homology domain of pleckstrin respectively. References: Pleckstrin, Tyler et al., 1988; GAP, Trahey et al., 1988; Rac, Jones et al., 1991; Grb 7, Margolis, et al., 1992; Dynamin (Dyna), Obar et al., 1990.

```

DdK6 (4 ) APIKHEGELTKEGGGFK----SWKKRWFIKGGD---LSYYKT
PleC (243) GVITKQGCLLKQGHRR-K---NWKVRKFILREDPA-YLHYYDP
PleN (3 ) PKRIREGYLVK-KGSVFN---TWKPMWVVLLEDG---IEFYKK
Rac (4 ) VAIVKEGWLHKRGE----YIKTWRPRYFLI-KNDGTEIGYKER
GAP (473) KNIVKKGILLKKKGK--K---RWKNLYFILEGSDAQLIYFSE
Dyna (518) ILVIRKGWLTINNIGIMK--GGSKEYWEVLTAEEN---LSWYKD
Grb7 (227) SFPEIQGELQLRGSGRGSGRKLWKRFFCFELRRS---GLYYSTK
Consensus      ++ G$ L K G          W+ +$***      *$$$

```

```

DdK6 KGE----LVPLGVHINTSGMIKNSDRKK-----RVNGFEVQT
PleC AGA----EDPLGATHLRGVVTSVESNSNGRKSEE----ENLFEIIT
PleN KSD----NSEKGMIPKLGSTLTSPQDF-----GKRMEVEKI
Rac PQDVDQREAPLNNEFSVAQCQIMKTERP-----RPNTETIIRC
GAP KRA----TKKGLIDLSVCSVYVVDHSLFG-----RPNCFQIVV
Dyna DEE----KEKKYMLSVDNKKLRDVEKGEMS-----SKHIFALEN
Grb7 GTS----KDERHLOVADVNESNVYVVTQGRKLYG---MPTDFEGCV
Cons      P G** * * *          N*F * *

```

```

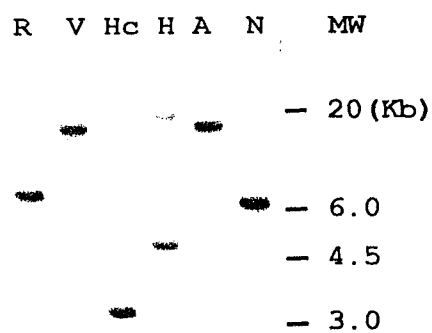
DdK6 PSR-----TYFLCSETBEERAKWIEILINE
PleC ADEVH-----YFLOAATPKERTEWIKAIQMA
PleN TTTKQQ-----DHFFQAAFLEERDAWVRDINKA
Rac LQWTTVIERT-----HHVETPEEREETTAIQTV
GAP QHFSEE-----HYIEYFAGETPROAEDWMKGLQAF
Dyna TEQ-----RNVYKDYRQLELACETQEEVDSWKASELRA
Grb7 KPNKLR-----NGHKGLHIECSEDEQSRTCWLAARLE
Cons      $ * *      --+--W* ** *

```

Fig.5. Genomic Southern Analysis and Physical Map of DdK6 Locus.

Genomic DNAs were digested with the restriction enzymes indicated and analyzed on Southern blots probing with a cDNA of DdK6 gene missing 82bp in the amino terminal end (A). Numbers on the left indicate size in kb. Partial restriction maps of the DdK6 locus are shown in B. Solid box represents the coding region. E, EcoRI; H, HindIII; V, EcoRV; Hc, HincII; A, AvaII; N, NdeI.

A.



B.

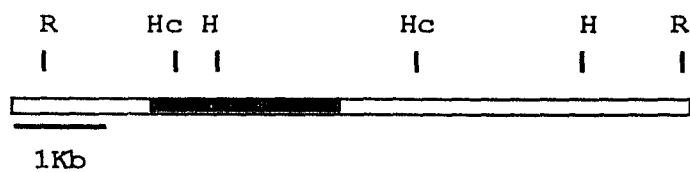


Fig. 6 . Developmental Regulation of DdK6 Gene.

RNA samples isolated from wild type (AX3 cells) and mutant (ddk6⁻ cells) developed on filters and harvested at the indicated times were hybridized with a labeled 3' end probe. Each lane indicates developmental times. WT, wild type; MT, mutant.

Time (hrs) 0 2 4 8 12 16 20 24

DdK6



Fig. 7. Disruption of the DdK6 Gene by Gene Targeting.
Scheme for the replacement of the normal DdK6 gene with
a DdK6-neo fragment by homologous recombination.
Following homologous pairing between the linearized
vector, pBK6neo and the genomic DdK6 locus, a
recombination event replaces the endogenous DdK6
sequence with an exogenous sequence altered by the Neo
cassette.
Solid box, genomic sequence; H, HindIII; N, NsiI; Hc,
HincII.

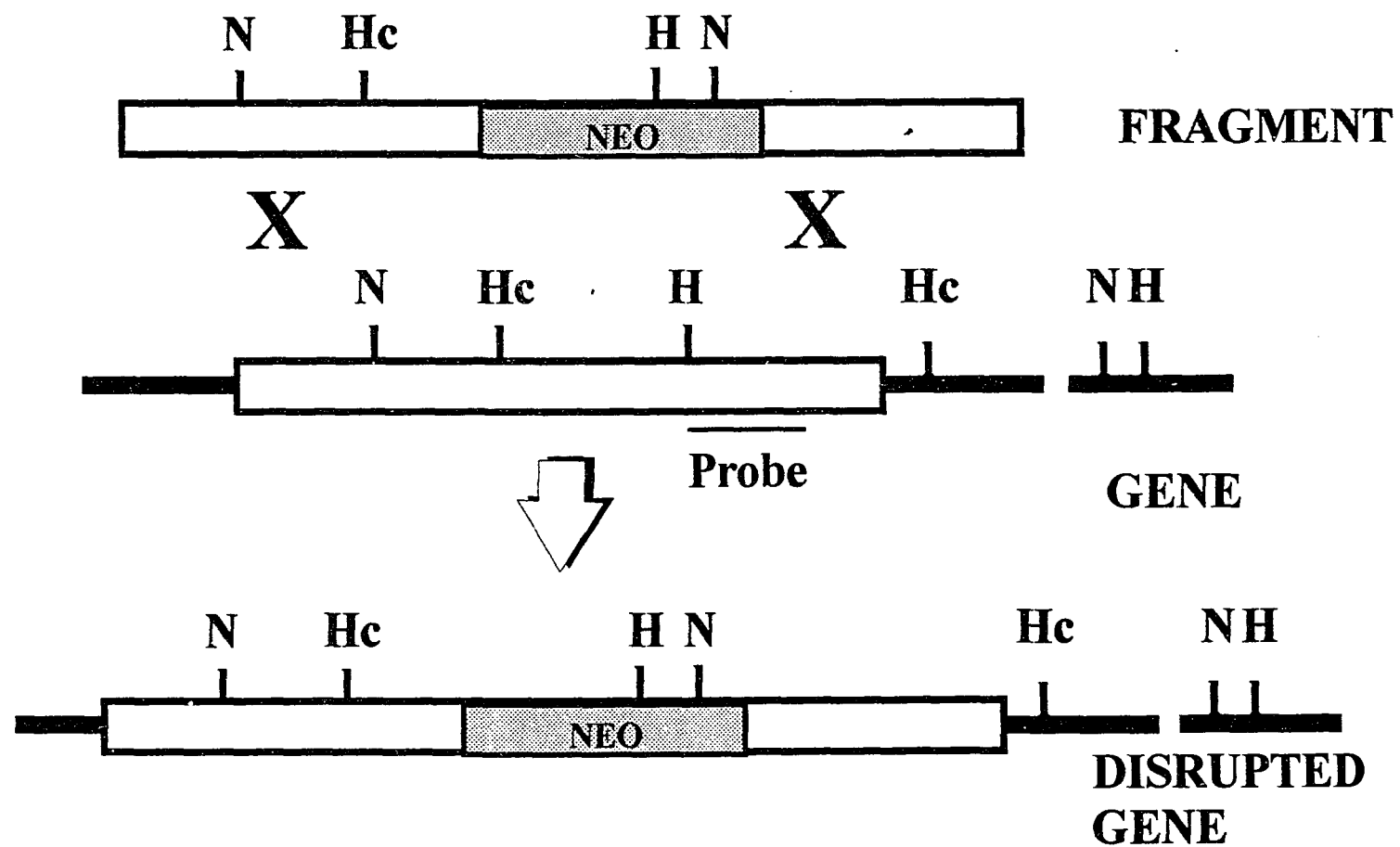


Fig. 8. Southern Blot Analysis of genomic DNA to demonstrate gene targeting.

DNAs were digested with indicated enzymes and probed with 3' end fragment of Ddk6 gene. W refers to DNA from the parental wild type AX3 cells. M refers to DNA from mutant *ddk6⁻* cells.

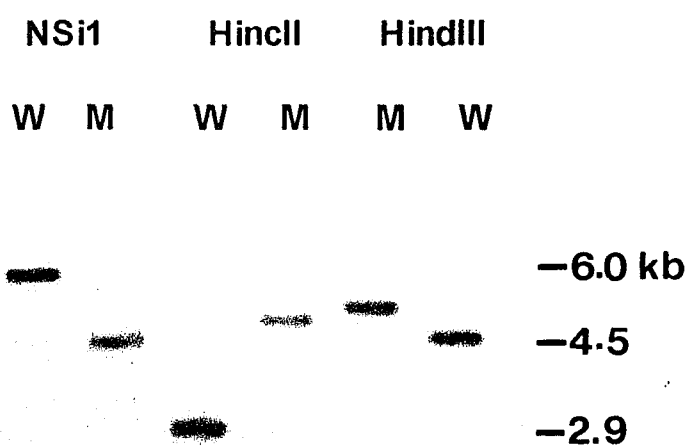
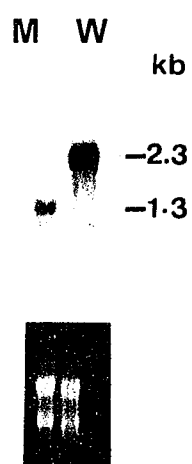


Fig. 9. Northern and Immuno Blot Analysis of a DdK6 Disruptant

A: RNAs were isolated from wild type and disruptant cells which were developed for 2 hours on Millipore filters and subjected to Northern blot analysis, probing with the 3'end probe of DdK6 gene. The sizes were determined by a standard RNA marker (Gibco BRL).

B: Cell lysates were prepared from wild and mutant cells after 2 hour of development. 20 ug total protein were separated on polyacrylamide gel. The gel was transferred onto nitrocellulose membrane (Hybond) which was subjected to western blot analysis with K6C antibody (1:1500 dilution) using ECL system. W, wild type; M, mutant. E, *Ecoli* cell lysates expressing cDNA of DdK6.

A.



B.

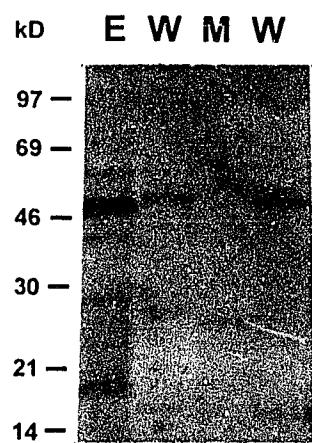


**Fig. 10. Developmental Phenotypes of Wild type and DdK6
Disruptant**

Vegetatively growing cells of wild type and disruptant were washed and plated at density of 6.5×10^6 cells/cm₂ on Millipore filters and photographs were taken at the times indicated using a Olympus microscope (15X magnification, Kodak black and white film, ASA 100) .

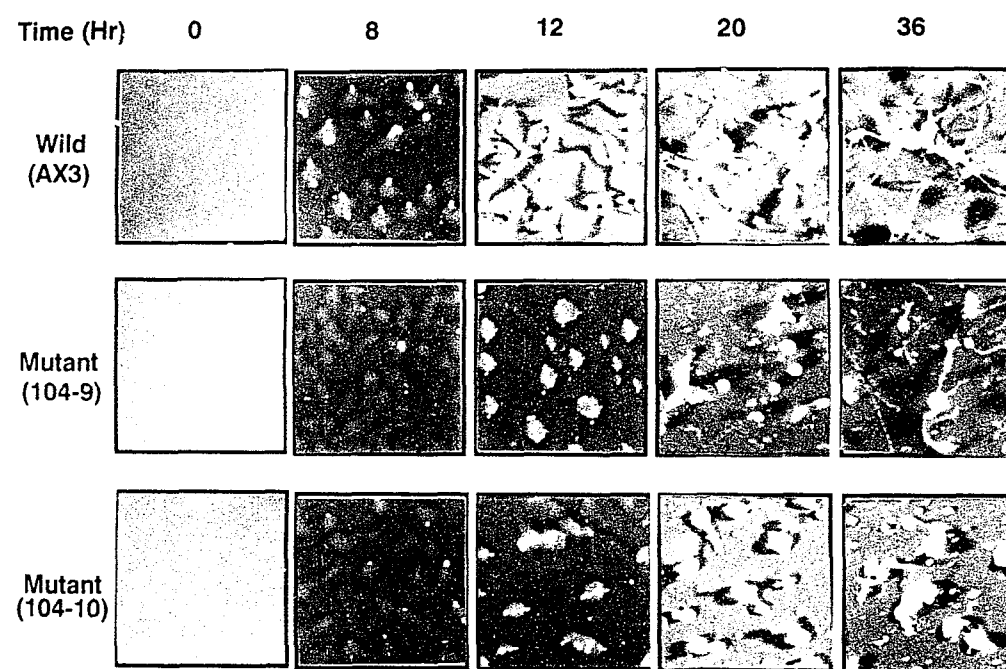


Fig. 11. Expression of Developmentally Regulated Genes in Wild Type and DdK6 Disruptant.

Northern blot analysis of total cellular RNA were hybridized with cAR1, Dicoidin 1 and PL3 cDNA probes. Each lane contains 20 ug of total cellular RNA isolated from cells harvested at the indicated times.

Time (hrs) 0 2 4 8 12 16 20 24

WT

cAR1

MT

WT

Disc

MT

WT

PL3

MT

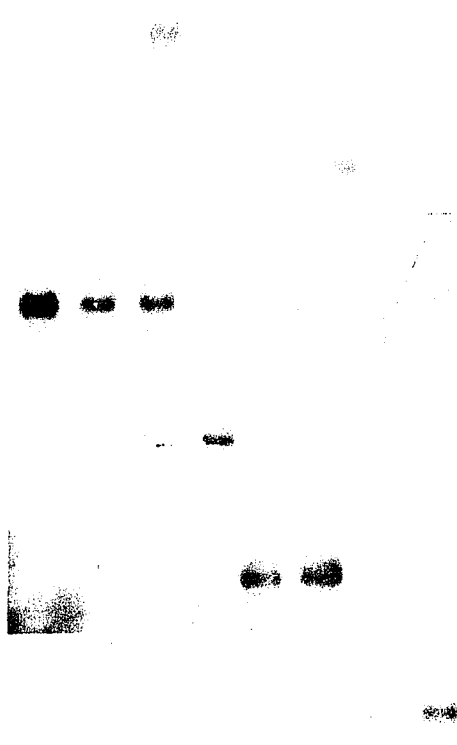


**Fig 12. Effect of Exogenous cAMP Pulses of the
Expression of Marker Genes.**

Ax3 and ddk6- cells were washed and allowed to develop in suspension as described in Materials and Methods. Beginning at 1 hour of development, the cells were treated with pulses of cAMP to give final concentration of 100 nM, and then collected samples at the given times. Total RNA was analysed by northern blot as described. The cDNA was used as a probe for cAR1 and discoidin mRNA while 500 bp of N-terminal sequence was used for Ddk6 mRNA. B indicates samples from buffer (MES-PDF) pulsing; cAMP lanes are samples from cAMP pulsing.

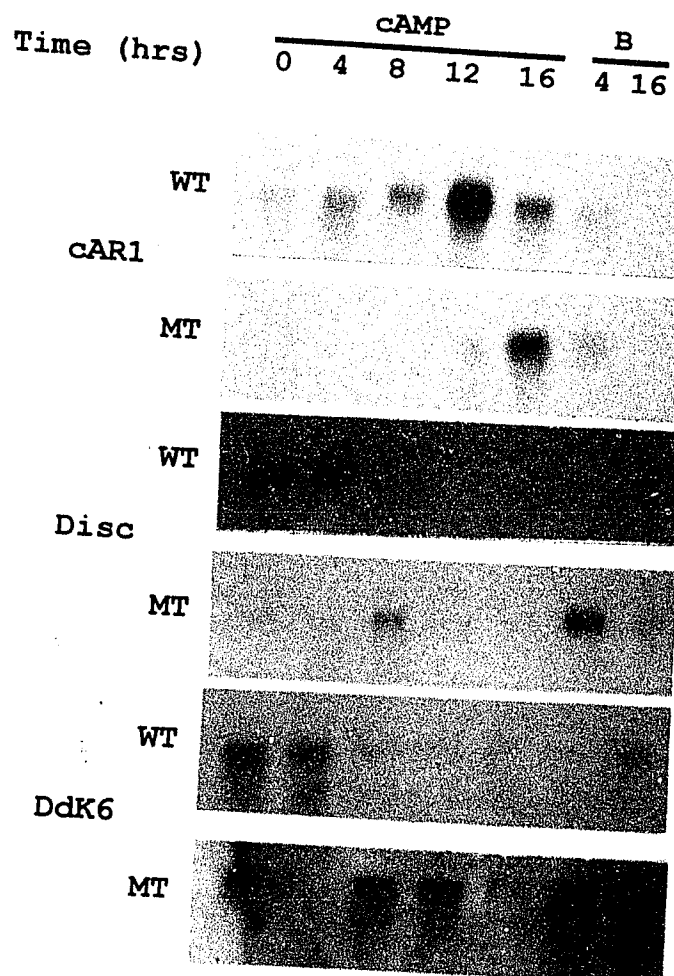
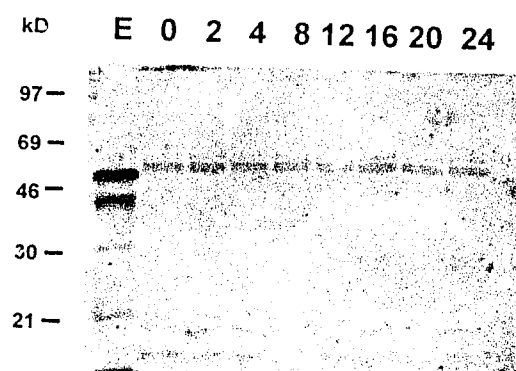


Fig 13. Western Blot with K6C and K6N Antibodies

Cell lysates were prepared at the given times during development. 20 ug of total proteins were separated on polyacrylamide gel. The gels were transferred onto nitrocellulose membranes, and then incubated with K6C (A) and K6N (B) antibody using ECL detection system. E, *E coli* lysates expressing cDNA of DdK6.

A.



B.

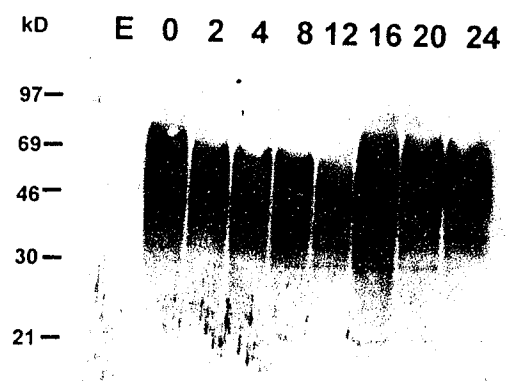


Fig. 14. Indirect immunofluorescence to localize DdK6 protein. Cells were stained with an anti-rabbit antibody specific for the C-terminal domain of DdK6 (K6C), DAPI for staining DNA (DAPI), phalloidin for F-actin (PHD) and preimmune serum for a control (Pre), then followed by fluorescein conjugated secondary antibody.

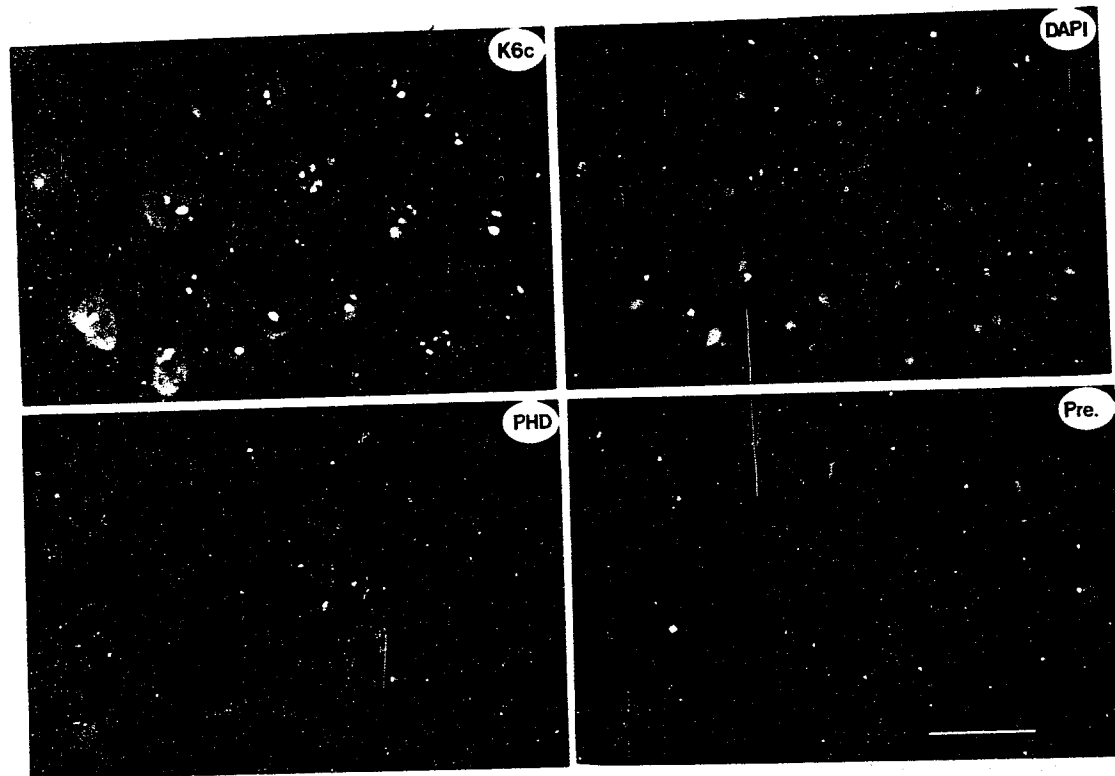


Table 1. Statistical significance (z value) of the similarity scores of PH domains. Pairwise similarity scores were determined and evaluated using SEQLINE program package (Clark, K.L., D.C. Teller, and G.R. Reeck, personal communication). To determine z value, first, an interesting sequence was aligned to and compared with a query sequence to generate an alignment score. Second, the query sequence was compared with each of randomly shuffled versions (100) of the interesting sequence to obtain 100 alignment scores. The z values were calculated by: $z = (\text{alignment score} - \text{mean of alignment scores from random sequences} / \text{standard deviation of random scores})$. z value is : $0 < z < 3$, very weak significance; $3 < z < 5$, weak significance; $5 < z < 10$, good evidence of structural similarity but the alignment may be erroneous; $z > 10$, very strong evidence of structural similarity and the alignment has structural meaning. Other groups also suggested that they used the following guidelines for z value to be biologically significant: $z > 3$, possibly significant; $z > 6$, probably significant; $z > 10$, significant (Lipman and Pearson, 1985; Pearson, 1990). References: 3bp2, Cicchetti et al., 1992; unc-104, Otsuka et al., 1991; Bem3p, A. Bender, unpublished data (Mayer et al., 1993) and for others, see the legend of Fig. 4.

Table 1.**Statistical Significance (z value) of the PH domains in various proteins**

	DdK6	Sec7	PleC	PleN	Rac	GAP	GRF	3BP2	Dyna	Grb7	Bem3
Sec7	14										
PleC	12.1	14.2									
PleN	10	12.6	13.5								
Rac	8	9.1	13.6	10.8							
GAP	6.4	15.1	12.9	8.5	10.3						
GRF	5.8	10.7	8.1	13.6	4.9	6.7					
3BP2	9.9	16.2	7.2	9.7	5.1	12.1	8.6				
Dyna	5.3	8.9	2.3	8.4	4.4	3.9	6.1	3.6			
Grb7	8.9	8.2	4.5	7	3	5.9	3.6	4.8	3.5		
Bem3	4	5.4	6.6	4.6	4.9	6.5	8	3.9	2.4	7.4	
unc	6.4	8.9	5	5.6	6.8	6.4	6.6	5.6	3.2	3	3.1

GENERAL CONCLUSION

In this thesis, I have exploited using gene targeting by homologous recombination to investigate the functions of developmentally important genes, the UDP glucose pyrophosphorylase and DdK6, a human rac protein kinase homolog gene. The experiments provided insights of signal transduction pathways in development of *Dictyostelium*. This reverse genetic approach seemed especially worthwhile to analyze the functions of the genes because the efficiency of gene targeting events is relatively high enough to create null mutants. Moreover, recent advanced molecular techniques such as the polymerase chain reaction (PCR) and the easy construction of libraries facilitate readily cloning genes without knowing their functions.

In part I, we have created a null mutation in the UDGP1 gene by homologous recombination using a modified genomic fragment to transfect *Dictyostelium* cells. We observed the high efficiency of targeted events by double crossing over (30 %). The resulting cell lines harboring a disrupted UDGP1 gene grow normally but with no activity and develop with no obvious morphological defect. Nevertheless, enzyme activity appears at 15 hr into development and rises to a maximum that is only 4-fold lower than in wild type

cells. The transgenic organisms have a slightly lower level of cellulose during culmination but the same as wild type organisms at the end development. These data suggested that at least one other unknown UDPGP gene is expressed in late development and perhaps it fully meets the requirements of the enzyme activity for development in the absence of UDPGP1 gene. We cloned part of a second gene, UDPGP2, by taking advantage of the null mutant cell lines and using PCR strategy. The deduced amino acid sequence of part of UDPGP2 has significant homology with the UDPGP1 of *Dictyostelium* (46%) and slightly greater (51%) homology with the UDPGP enzyme of potato tuber. Although we believe that the appearance of enzyme activity at the late stage of development in the UDPGP1 null mutant arises from UDPGP2 that we cloned, more than two genes may exist. This possibility can be investigated by knocking out the second gene or both first and second genes. In contrast with UDPGP1, UDPGP2 mRNA accumulates only late in development. Therefore, mutation by homologous recombination shows that at least two UDPGP1 genes exist and they are regulated in by different mechanisms. The two known structural genes of UDPGP in *Dictyostelium* are expressed at different developmental stages, and may be regulated by different signal transduction pathways. In previous

experiments, I had tried to do disruption with a cDNA 1.5 Kb long. Those experiments generated no disruptants. Since a long genomic clone was very efficient, either the additional sequences were highly recombinogenic because of their length or because of their sequence. Extragenic sequences of *Dictyostelium* are highly AT rich.

The previous work (part I) showed that gene targeting by homologous recombination is a very efficient tool to create null mutants and analyze the functions of genes. In part II, I used the reverse genetic approach to reveal the role of a serine/threonine protein kinase (DdK6) which we isolated. The protein kinase is closely related to the Rac protein kinase family (50 % identity in kinase domain) which is a serine/ threonine kinase and contains an almost identical PH domain which was recently identified as a module likely to be involved in signal transduction and to promote protein-protein aggregation in the N terminal putative regulatory region. A retroviral oncogene, akt, which causes a rodent T cell lymphoma appears to be the murine homolog of rac kinase. Thus one of the functions of Rac kinases seems to be involved in cell proliferation, perhaps by a functional link between tyrosine and

serine/threonine phosphorylation pathways in response to growth factors. Null mutant cell lines were created at a high efficiency by gene targeting through homologous recombination. The null mutants caused heterochronic development of *Dictyostelium* delaying formation of mounds and fruiting bodies. Relatively few structures completed development. The expression of several stage specific genes also was retarded in the mutants. Furthermore, expression of genes stimulated by pulses of cAMP in the null mutant is delayed compared to wild type, suggesting that the null mutant is deranged in a signalling pathway(s) via the cell surface cAMP receptor(s). Expression of the DdK6 gene in wild type cells is strictly regulated, rising to a maximum at 2 hour after starvation and quickly declining soon after in development. The protein is present at a high level throughout development. Immunofluorescence data show that the protein is localized in the nucleus as discrete spots. These data shows the DdK6 protein kinase might be one of signaling molecules to receive and/ or transmit early signals, probably starvation signal, for the establishment of the developmental program, through transient protein aggregation by the PH domain. The fact that antiserum to the PH domain detects many proteins suggest that they share PH domains - a

hypothesis that can be tested from cloning expression
libraries with the antiserum and sequencing the
clones.

REFERENCES

General introduction

Ashworth, J.M.. and Sussman M. (1969). The appearance and disappearance of UDPG Pyrophosphorylase during development of the cellular slime mold *Dictyostelium*. J. Biol. chem. 242, 1696-1700.

Bain, G. and Tsang, A. (1991). Disruption of the gene encoding the p34/31 polypeptides affects growth and development of *Dictyostelium discoideum*. Mol Gen genet. 226, 59-64.

Berridge, M.J. and Irvine, R.F. (1989). Inositol phosphates and cell signaling. Nature, 341, 197-205.

Bonner, J.T. (1959). The cellular slime molds. Princeton University Press, Princeton, New Jersey.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991). Oncogenes and singnal transduction. Cell 64, 281-302.

Capecchi, M.R. (1989). Altering the genome by homologous recombination . Science 244, 1288-1292.

Chisholm, R.L., Barklis, E. and Lodish, H.F. (1984). Mechanism of sequential induction of cell-type specific mRNA in *Dictyostelium* differentiation. Nature 310, 67-69.

Crowley, T.E., Nellen, W. Gomer, R.A. Firtel, R.A. (1985). Phenocopy of Discoidin 1-minus mutants by antisense transformation in *Dictyostelium*. Cell 43, 633-641.

De Lozanne, A. and Spudich, J.A. (1987) Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination . Science. 236, 1086-1091.

Devreotes, P.N. (1989). *Dictyostelium discoideum*: A model system for cell-cell interactions during development. Science 245, 1054-1058.

Dimond, R.L., Farnsworth, P.A. and Loomis, W.F. (1976). Isolation and characterization of mutations affecting UDPG pyrophosphorylase activity in *Dictyostelium*. Dev. Biol. 50, 169-181.

Edelman, A.M., Blumenthale, D.K. and Krebs, E.G. (1987). Protein serine threonine kinases. *Annu. Rev. Biochem.* 56, 567-613.

Europe-Finner, G.N., Gammon, B., Wood, C.A. and Newell PC (1989). Inositol tri- and poly- phosphate formation during chemotaxis of *Dictyostelium*. *J. Cell Sci.* 93, 585-592.

Firtel, A., Van Haastert, J.M., Kimmel, A.R., and Devreotes, P.N. (1989). G protein linked signal transduction pathway in development: *Dictyostelium* as an experimental system. *Cell*, 58, 235-239.

Fishel, B.R., Ragheb, J.A., Rajkovic, A., Haribabu, B., Schweinfest, C.W. and Dottin, R.P. (1985). Molecular cloning of a cDNA complementary to a UDP glucose pyrophosphorylase mRNA of *Dictyostelium discoideum*. *Dev. Biol.* 110, 369-381.

Gerisch, G. (1987). Cyclic AMP and other signals controlling cell development and differentiation in *Dictyostelium*. *Annu. Rev. Biochem.* 56, 853-879.

Ghosh, S. and Baltimore, D. (1990). Activation in vitro NFkB by phosphorylation of its inhibitor Ikb, 344, 678-682.

Ginsberg, G., and Kimmel, A.R. (1989). Inositol triphosphate and diacyl glycerol can differentially modulate gene expression in *Dictyostelium*. *Proc Natl Acad Sci USA.* 86, 9332-9336.

Hardwiger, J.A., and Firtel, R.A. (1991). Analysis of G α 4, a G-protein subunit required for multicellular development in *Dictyostelium*. *Genes & Dev.* 6, 38-49.

Harloff, C., Gerisch, G. and Noegel, A.A. (1989). Selective elimination of the contact site A protein of *Dictyostelium discoideum* by gene disruption. *Gene & Dev.* 3, 2011-2019.

Haribabu B. and Dottin R.P. (1986). Pharmacological characterization of cAMP receptors mediating gene regulation in *Dictyostelium*. *Mol. Cell. Biol.* 6, 2402-2408.

Haribabu, B., Ragheb, J.A. and Dottin, R.D. (1987). A transmembrane signal transduction model for cAMP mediated gene regulation in *Dictyostelium discoideum*. In Davidson, E.H. and Firtel, R.A. (eds): 'Molecular approaches to developmental biology' New York. Alan R. Liss, Inc., 385-

399.

Haribabu, B. and Dottin, R.P. (1991) Identification of a protein kinase multigene family of *Dictyostelium discoideum*: Molecular cloning and expression of a cDNA encoding a developmentally regulated protein kinase. Proc. Natl. Acad. Sci. USA 88, 1115-1119.

Harwood, A.J., Hopper, N.A., Simon, M.N., Bouzid, s., Veron, M. and Williams, J.G. (1992a). Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. Dev. Biol. 149, 90-99.

Harwood, A.J., Hopper, N.A., Simon, M.N., Driscoll, D.M., Veron, M. and Williams, J.G. (1992b). Culmination in *Dictyostelium* is regulated by cAMP-dependent protein kinase. Cell. 69, 615-624.

Hopper, N.A., Harwood, A.J., Bouzid, S., Veron., M. and Williams, J. G. (1993). Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP dependent protein kinase and evidence for its upstream regulation by ammonia. EMBO J. 12, 2459-2466.

Hunter, T. (1987). A thousand and one protein kinases. Cell 50, 823-829.

Janssens, P.M.W. and Van Haastert, P.J.M. (1987). Molecular bases of transmembrane signal transduction in *Dictyostelium discoideum*. Microbiol. Rev. 51, 396-418.

Jermyn, K.A., Duffy, K., and Williams, J.G. (1989). A new anatomy of the prestalk zone of *Dictyostelium*. nature 340, 745-755.

Jung, G., and HammerIII, J.A. (1990). Generation and characterization of *Dictyostelium* cells deficient in a Myosin I heavy chain isoform. J. Cell Biol. 110, 1955-1964.

Karin, M. (1991). Signal transduction and gene control. Curr. Opin. Cell Biol. 3, 467-473

Kesbeke, F, Eva Snaar-Jagalska, B. and Van Haastert, P.J.M. (1987) Signal transduction in *Dictyostelium* *fgdA* mutants with a defective interaction between surface cAMP receptors and a GTP binding regulatory protein. J. Cell. Bio. 107, 521-528

Kimmel, A.R. (1987). Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. Dev. Biol. 122, 163-171.

- Kimmel, A.R. and Saxe, C.L. (1986). cAMP- controlled gene expression in *Dictyostelium* mutants exhibiting of developmental cAMP metabolism. *Dev. Biol.* 117, 209-214.
- Klein, P.S., Sun, Saxe CL III, T.J., Kimmel A.R., Johnson, R.L., and Devreotes, P.N., (1988). a chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science* 241, 1467-1472.
- Knecht, D. and Loomis, W.F. (1987). Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. *Science* 236, 1081-1086.
- Knecht, D., Jung J and Matthews L. Quantification of transformation efficiency using a new methods for clonal growth and selection of axenic *Dictyostelium* cells. (1991). *Dev.genet.*,16, 403-409.
- Konijin, T.H. (1975). Chemotaxis in the cellular slime molds. In " Primitive Sensory and Communication Systems" (M.J. Carlile, ed.), pp 102-153. Academic Press, New York.
- Kumagai, A., Pupillo, M., Gunderson, R., Miake-Lye, R., Devreotes, P.N. (1989). Regulation and function of G protein subunits in *Dictyostelium*. *Cell*, 58, 235-239.
- Kumagai, A. Hardwiger, J.A. Pupillo, M. and Firtel, R.A.(1991). Molecular genetic analysis of two G protein subunits in *Dictyostelium*. *J. Biol. Chem.* 266, 1220-1228.
- Kuspa, A. and Loomis, W.F., (1992). Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA*, 89, 8803-8807.
- Luderus, M.E.E., Van der Most, R.G., Otte, A.P. and Van Driel, R. (1989). A protein kinase C related enzyme activity in *Dictyostelium discoideum*. *FEBBS Lett.* 253, 71-75.
- Lilly, P., Wu, L., Welker, D.L., and Devreotes, P.N. (1993). A G-protein β -subunit is essential for *Dictyostelium* development. *Genes & Dev.* 7, 986-995.
- Loomis, W.F. and Smith, W.S. (1990). Molecular phylogeny of *Dictyostelium discoideum* by protein sequence comparison. *Proc. Natl. Acad. Sci. USA*, 87, 9093-9097.
- Loomis, W.F. (1975) *Dictyostelium discoideum*, A developmental system, Academic Press, New York.

- Mann, S. and Firtel, R.A. (1987). Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum*: mediation via the cell surface cyclic AMP receptor. *Mol. Cell. Biol.* 7, 458-469.
- Mann, S.K.O. and Firtel, R.A. 1991. A developmentally regulated putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech. Dev.* 35, 89-101.
- Mann, S.K., Pinko, C. and Firtel, R.A. (1988). cAMP regulation of early gene expression in signal transduction mutants of *Dictyostelium*. *Dev. Bio.* 294-303
- Mann, S.K.O., Yonemoto, W.M., Taylor, S.S., and Firtel, R.A. (1992). DdPk3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc. natl. Acad. Sci.* 89, 10701-10705.
- Manstein, D.J. Titus, M.A. De Lozanne, A. and Spudich, J. (1989). Gene replacement in *Dictyostelium*: generation of myosin null mutants. *EMBO.* 8,923-932.
- Newell, P.C. and Sussman, M. (1969). Uridine diphosphate glucose pyrophosphorylase in *Dictyostelium*. *J. Biol. Chem.* 244, 2990-2995.
- Nellen, W., Silan, C. and Firtel, R.A. (1984). DNA-mediated transformation in *Dictyostelium discoideum*: regulated expression of an actin gene fusion. *Mol. Cell. Biol.* 4, 2890-2898.
- Newell, P.C. Europe-Finner, G.N., Small, N.V., and Liu, G. (1988). Uridine Diphosphate glucose pyrophosphorylase in *Dictyostelium discoideum*. *J. Cell Sci.* 89, 123-127
- Nishizuka, Y., (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulations. *Nature*, 334, 661-665.
- Olive, L.S. (1975). *The Mycetozoans*. Academic Press, New York.
- Orr-Weaver, T.L., Szostak, J.W., and Rothstein, R.J. (1981). Yeast transformation: A model system for the study of recombination. *Proc, Natl. Acad. Sci. USA* 78, 6354-6358.
- Oyama M. and Blumberg, D.D. (1986). Interaction of cAMP with the cell-surface receptor induces cell-type specific mRNA accumulation in *Dictyostelium discoideum*. *Proc Natl Acad Sci USA* 83, 4819-4823.

Pavlovic, J., Haribabu, B., and Dottin, R.P. (1989). Identification of a signal transduction response sequence element necessary for induction of a *Dictyostelium discoideum* gene by extracellular cyclic AMP. *Mol. Cell. Biol.* 9, 4660-4669.

Pelech SL, Sanghera JS and Daya-Makin M (1990). Protein kinase cascades in meiotic and mitotic cell cycle control. *Biochem. Cell. Biol.* 68, 1297-1330

Peters, P.J.M., Van Lookeren Campagne, M.M., Van Haastert P.J.M., Speck, W. and Schaap, P. (1989). Lithium ions induce prestalk-associated gene expression and inhibit prespore gene expression in *Dictyostelium discoideum*. *J. Cell Sci.* 93, 205-210.

Pitt G.S., Milona N, Borieis J, Lin KC Reed RR and Devreotes PN. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell* vol. 69, 305-315.

Pitt, G.S., Brant, R., Lin, K.C., Devreotes, P.N., and Schaap, P. (1993). Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in *Dictyostelium* cells lacking the aggregation adenylyl cyclase (ACA). *Genes & Dev.* 7, 2172-2180.

Pupillo, M., Kumagai, A., Pitt, G.S., Firtel, R.A. and Devereotes, P.N. (1989). Multiple alpha subunits of guanine nucleotide-binding proteins in *Dictyostelium*. *Proc Natl. Acad. Sci. USA.* 86, 4892-4896.

Ragheb, J.A. and Dottin R.P. (1987). Structure and sequence of a UDP glucose pyrophosphorylase gene of *Dictyostelium discoideum*. *Nucl. acid. Res.* 15, 3891-3906.

Rubino, S, Mann, S.K.O., Hori, R., Pinico, C. and Firtel, R.A. (1989). Molecular analysis of a developmentally regulated gene required for *Dictyostelium* aggregation, *Dev. Biol.* 131, 27-36

SaxIII, C.L., Ginsberg, G.T., Louis, J.H., Johnson, R., Devreotes, P.N. and Kimmel, A.R. (1993). CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. *Genes & Dev.* 7, 262-272.

Schaap, P., and Van Driel, R. (1985). Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP: evidence of involvement of cell surface cAMP receptor. *Exp Cell Res.* 159, 388-398.

Schaap, P., Peters, D.J.M., Haribabu, B., and Dottin, R.P. (1993). Gene regulation by hormone-like signals in *Dictyostelium*. In "Signal Transduction" (ed. Kurjan, J and Taylor, B.L.), pp 353-376.

Shears, S.B., (1989). Metabolism of the inositol phosphates produced upon receptor activation. *Biochem. J.* 260, 313-324.

Shenolikar, S. (1987) Control of cell function by reversible protein phosphorylation. *J. Cycl. Nucl. Prot. Phos. Res.* 11, 531-541.

Sibley, D.R., Strasser, R.H., Benovic, J.L., Daniel, K., Lefkowitz, R.J. (1986). Phosphorylation and dephosphorylation of the beta adrenergic receptor regulates its functional coupling to adenylyl cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. USA* 83, 9408-9412.

Simon, M., Pelegriani, O., Veron, M. and Kay, R.R. (1992). Mutation of Protein kinase A cascades heterochronic development of *Dictyostelium*. *Nature* 356, 171-172.

Sternfeld, J., and David, C. (1981). Cell sorting during pattern formation in *Dictyostelium*. *Differentiation*, 20, 10-20.

Strathmann, M., Wilkie, TM., and Simon, MJ. (1989). Diversity of the G protein family: sequences from five additional α subunits in the mouse. *Proc. Natl. Acad. Sci. USA* , 86, 7407-7409.

Sun TJ, Van Haastert, PJM and Devreotes, PN (1990) Surface cAMP receptors mediate multiple responses during development in *Dictyostelium*: evidence by antisense mutagenesis. *J. Cell Bio.* 110, 1549-1554

Sun T.J. and Devreotes, P.N. (1991). gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. *Gene. & Dev.* 5, 572-582.

Sussmaan, M. and Osborn, M.J. (1964), UDP-galactose polysaccharide transferase in the cellular slime mold, *Dictyostelium discoideum*: appearance and disappearance of activity during cell differentiation. *Proc. Natl. Acad. Sci. USA* 52, 81-87.

Theibert, A. and Devreotes, P.N. (1986), Surface receptor- mediated activation of adenylate cyclase in *Dictyostelium* regulated by guanine nucleotide in wild type cell and aggregation deficient mutants. *J. Biol. Chem.* 261, 15121-15125.

Ullrich A and Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* 61, 203-212.

Van Haastert, P.J.M., Janssens, P.M.W., and Erneux, C. (1991). Sensory transduction in eukaryotes. *Eur. J. Biochem.* 195, 289-303.

Vaughen, R.A. and Devreotes. P.N. (1988). Ligand-induced phosphorylation of the cAMP receptor from *Dictyostelium discoideum*. *J. Biol. Chem.* 263, 14538-14543.

Witke, W., Nellen, W. and Noegel, A. (1987). Homologous recombination in the *Dictyostelium* alpha actinin gene leads to an altered mRNA and lack of the protein. *EMBO.* 6, 4143-4148.

Wright, B.E. and Dahlbers, (1967). Cell wall synthesis in *Dictyostelium discoideum*. II. Synthesis of soluble glycogen by a cytoplasmic enzyme. *Biochem.* 6, 2074-2079.

Zabel V and Baeuerle PA (1990) Purified Human I κ B can rapidly dissociate the complex of the NF κ B transcription factor with its cognate DNA. *Cell*, 61, 255-265.

REFERENCES

Part I

- Alton, T.H, and Lodish, H.F. (1977). Developmental changes in messenger RNA and protein synthesis in *Dictyostelium discoideum*. *Dev. Biol.* 60:180-206.
- Ashworth, J.M. and Sussman, M. (1967). The appearance and disappearance of UDPG Pyrophosphorylase during development of the cellular slime mold *Dictyostelium*. *J. Biol. Chem.* 242:1696-1700.
- Brede, G., Fjervik, E., and Valla, S. (1991). Nucleotide sequence and expression analysis of the *Acetobacter xylinum* uridine diphosphoglucose pyrophosphorylase gene. *J of Bact.* 173:7042-7045.
- Buck, L. and Axel, R. (1991). A Novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* 65:175-187.
- Clegg, J.S. and Filosa, M.F. (1961). Trehalose in the cellular slime mold *Dictyostelium mucoroids*. *Nature (London)* 192:1077-1078.
- De Lozanne, A. and Spudich, J.A. (1987). Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. *Sci.* 236:1086-1091.
- Devereux, J., Haeblerli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* 12:387-395.
- Dimond, R.L., Farnsworth, P.A. and Loomis, W.F. (1976). Isolation and characterization of mutations affecting UDPG pyrophosphorylase activity in *Dictyostelium*. *Dev. Biol.* 50:169-181.
- Dynes, J.L. and Firtel, R.A. (1989). Molecular complementation of a genetic marker in *Dictyostelium* using a genomic DNA library. *Proc. Natl. Acad. Sci.* 86:7966-7970.
- Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Firtel, R.A., (1991). Signal transduction pathways controlling multicellular development in *Dictyostelium*. *TIG* 7:381-388.

Fishel, B.R., Ragheb, J.A., Rajkovic, A., Haribabu, B., Schweinfest, C.W. and Dottin, R.P. (1985). Molecular cloning of a cDNA complementary to a UDP glucose pyrophosphorylase mRNA of *Dictyostelium discoideum*. Dev. Biol. 110:369-381.

Gustafson, G.C., Kong, W.Y. and Wright, B.E. (1973). Analysis of Uridine diphosphate-glucose pyrophosphorylase synthesis during differentiation in *Dictyostelium discoideum*. J. Biol. Chem. 248:5188-5186.

Haribabu, B. and Dottin R.P. (1986). Pharmacological characterization of cAMP receptors mediating gene regulation in *Dictyostelium*. Mol. Cell. Biol. 6:2402-2408.

Haribabu, B. and Dottin, R.P. (1991). Identification of a protein kinase multigene family of *Dictyostelium discoideum*: Molecular cloning and expression of a cDNA encoding a developmentally regulated protein kinase. Proc. Natl. Acad. Sci., USA 88:1115-1119.

Kamb, A., Weir, M., Rudy, B., Varmus, H. and Kenyon, C. (1989). Identification of genes from pattern formation, tyrosine kinase, and potassium channel families by DNA amplification. Proc. Natl. Acad. Sci., USA 86:4372-4376.

Katsube, T., Kazuta, Y., Mori, H., Nakano, K., Tanizawa, K., and Fukui, T. (1990). UDP-glucose Pyrophosphorylase from Potato Tuber: cDNA Cloning and Sequencing. J. Biochem. 108:321-326.

Kazuta, Y., Omura, Y., Tagaya, M., Nakano, K., and Fukui, T. (1991 a), Identification of lysyl residues located at the substrate-binding site in UDP-glucose pyrophosphorylase from potato tuber: Affinity labeling with uridine di- and triphosphopyridoxal. Biochemistry 30, 8541-8545.

Kazuta, Y., Tanizawa, K., and Fukui, T. (1991 b), Comparative affinity labeling with reactive UDP-glucose analogues: Possible locations of five lysyl residues around the substrate bound to potato tuber UDP- glucose pyrophosphorylase. J. Biochem. 110, 708-713.

Katsube, T., Kazuta, Y., Tanizawa, K., and Fukui, T. (1991), Expression in *Escherichia coli* of UDP-Glucose Pyrophosphorylase cDNA from Potato Tuber and Functional Assessment of the Five lysyl Residues Located at the Substrate-Binding Site, Biochemistry 30, 8546-8551.

Keepling, P.L., Wood, J.R., Tyson, R.H. and Bridges, I.G.

(1988). Starch Biosynthesis in Developing Wheat Grain. *Plant Physiol.* 87:311-319.

Knecht, D., Jung, J. and Matthews, L. (1991). Quantification of transformation efficiency using a new methods for clonal growth and selection of axenic *Dictyostelium* cells. *Dev. Genet.* 11:403-409.

Loomis, W.F. (1975). *Dictyostelium discoideum*, A developmental system Academic Press, New York.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning. A laboratory mannual. Cold Spring Harbor laboratory, Cold Spring Harbor, New York.

Mangiarotti, G., Lefebvre, P. and Lodish, H.F. (1982). Differences in the stabilization controls the expression of a class of developmentally regulated genes in *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* 82:5786-5790.

Manrow, R.E. and Jacobson, A. (1988). mRNA decay rates in late developing *Dictyostelium discoideum* cells are heterogenous, and cyclic AMP does not act directly to stabilize cell-type-specific mRNAs. *Mol. Cell. Biol.* 8:4088-4097.

Manstein, D.J., Titus, M.A., De Lozanne, A. and Spudich, J. (1989). Gene replacement in *Dictyostelium*: generation of myosin null mutants. *EMBO.* 8:923-932.

May T., Blusch J., Sachse A., and Nellen W. (1991). A cis-acting element responsible for early gene induction by extracellular cAMP in *Dictyostelium discoideum*. *Mech. of Devel*, 33, 147-156.

Newell, P.C. and Sussman, M. (1969). Uridine diphosphate glucose pyrophosphorylase in *Dictyostelium*. *J. Biol. Chem.* 244:2990-2995.

Pavlovic, J., Haribabu, B. and Dottin, R.P. (1989). Identification of a signal transduction response sequence element necessary for induction of a *Dictyostelium discoideum* gene by extracellular cyclic AMP. *Mol. Cell. Biol.* 9:4660-4669.

Pears, C.J., and Williams, J.G. (1987). Identification of a DNA sequence element required for efficient expression of a developmentally regulated and cAMP-induced gene. *EMBO J.*, 6, 195-200.

- Ragheb, J.A. and Dottin R.P. (1987). Structure and sequence of a UDP glucose pyrophosphorylase gene of *Dictyostelium discoideum*. *Nucleic Acids Res.* 15:3891-3906.
- Sussman, M. (1966). Biochemical and genetic methods in the study of cellular slime mould development. *Meth. Cell. Physiol.* 2:397-410.
- Sussman, M. and Osborn, M.J. (1964), UDP-galactose polysaccharide transferase in the cellular slime mold, *Dictyostelium discoideum*: appearance and disappearance of activity during cell differentiation. *Proc. Natl. Acad. Sci. USA* 52:81-87.
- Thomas, K.R. and Capecchi, M.R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503-512.
- Thomas, K.R., Den, C. and Capecchi, M.R. (1992). High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Mol. Cell. Biol.* 12:2919-2923.
- Updegraff, D.M. (1969). Semimicro determination of cellulose in biological materials. *Anal. Biochem.* 32:420-424.
- Van Haastert, P.J.M., Janssens, P.M.W. and Erneux, C. (1991). Sensory transduction in eukaryotes. *Eur. J. Biochem.* 195:289-303.
- Warrik, H.M. and Spudich, J.A. (1988). Codon preference in *Dictyostelium discoidium*. *Nucleic Acids Res.*, 6617-6635.
- Watts, D.J. (1984). Protein synthesis during development and differentiation in the cellular slime mould *Dictyostelium discoideum*. *Biochem. J.* 220:1-14
- Witke, W., Nellen, W. and Noegel, A. (1987). Homologous recombination in the *Dictyostelium* d-actinin gene leads to an altered mRNA and lack of the protein. *EMBO* 6:4143-4148.
- Wright, B.E. and Dahlberg, D. (1967). Cell wall synthesis in *Dictyostelium discoideum*. II. synthesis of soluble glycogen by a cytoplasmic enzyme. *Biochem.* 6:2074-2079.
- Wright, B.E. (1991). Construction of kinetic models to understand metabolism in vivo. *J. Chromatography*

566:309-326.

REFERENCES

Part II

- Alton, T.H., and Lodish, H.F. (1977). Developmental changes in messenger RNA and protein synthesis in *Dictyostelium discoideum*. Dev. Biol. 60, 180-206.
- Barklis, E., and Lodish, H.F. (1983). Regulation of *Dictyostelium discoideum* mRNAs specific for prespore or prestalk cells. Cell. 32, 1139-1148.
- Bellacosa, A., Testa, J., Staal, S.P., and Tsichlis, P.N. (1991). A retro viral oncogene akt encoding a serine/threonine kinase containing an SH2-like region. Science. 254, 274-277.
- Berger, E.A. and Clark, J.M. (1983). Specific cell-cell contact serves as the developmental signal to deactivate discoidin 1 gene expression in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA. 80, 127.
- Birge, R.B. and Hanafusa, H. (1993). Closing in on SH2 specificity. Science. 262, 1522-1524.
- Bowtel, D., Fu, P., Simon, M., and Senior, P. (1992). Identification of murine homologues of the *Drosophila* Son of sevenless gene: potential activator of ras. Proc. Natl. Acad. Sci. USA. 89, 6511-6515.
- Chelskey, D., Ralph, R. and Jonak G. 1989, Sequence requirements for synthetic peptide nucleotide translocation to the nucleus. MCB, 9, 2487-2492.
- Cicchetti, P., Mayer, B.J., Thiel, G. and Baltimore, D. (1992). Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and Gap-rho. Science. 257. 803-806.
- Devreotes, P. (1989). *Dictyostelium discoideum*: A model system for cell-cell interactions in development. Science 245, 1054-1058.
- Dynes, J.L. and Firtel, R.A. (1989). Molecular complementation of a genetic marker in *Dictyostelium* using a genomic DNA library. Proc. Natl. Acad. Sci. 86, 7966-7970.
- Egan, S.E., Giddings, B.W., Brooks, M.W., Budday, L.,

Sizeland, A.M., and Weinberg, R.A. (1993). Association of Sos Ras exchange protein with Grb2 implicated in tyrosine kinase signal transduction and transformation. *Nature*. 363, 45-51.

Edelman, A.M., Blumenthale, D.K. and Krebs, E.G. (1987). Protein serine/threonine kinases. *Annu. Rev. Biochem.* 56, 567-613.

Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6-13.

Firtel, R.A. (1991). Signal transduction pathways controlling multicellular development in *Dictyostelium*.

Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J., and Bar-Sagi, D. (1993). Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature*. 363, 88-92.

Haribabu, B. and Dottin R.P. (1986). Pharmacological characterization of cAMP receptors mediating gene regulation in *Dictyostelium*. *Mol. Cell. Biol.* 6, 2402-2408.

Haribabu, B., Ragheb, J.A., and Dottin, R.P. (1987). A transmembrane signal transduction model for cAMP mediated gene regulation in *Dictyostelium discoideum*. In Davidson, E.H. and Firtel, R.A. (eds), "Molecular Approaches to developmental Biology." New York, Alan R. Liss, Inc., pp385-399.

Haribabu, B., Pavlovic, J., Bodduluri, S.R., Doody, J.F., Ortiz, B.D., Mullings, S., Moon, B.C. and Dottin, R.P. (1991a). Signal transduction pathways involved in the expression of the uridine diphosphoglucose pyrophosphorylase gene of *Dictyostelium discoideum*. *Dev. genet.* 12, 35-44.

Haribabu, B. and Dottin, R.P. (1991b) Identification of a protein kinase multigene family of *Dictyostelium discoideum*: Molecular cloning and expression of a cDNA encoding a developmentally regulated protein kinase. *Proc. Natl. Acad. Sci.* 88, 1115-1119.

Harwood, A.J., Hopper, N.A., Simon, M.N., Driscoll, D.M., Veron, M. and Williams, J.G. (1992). Culmination in *Dictyostelium* is regulated by cAMP-dependent protein kinase. *Cell*. 69, 615-624.

Haslam, R.J., Kolde, H.B., and Hemmings, B.A. (1993). Pleckstrin domain homology. *Nature*. 363, 309-310.

Henikoff, S and Henikoff, J.G. (1992), Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. USA*. 89, 10915-10919.

Hopper, N.A., Harwood, A.J., Bouzid, S., Veron., M. and Williams, J. G. (1993). Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP dependent protein kinase and evidence for its upstream regulation by ammonia. *EMBO J*. 12, 2459-2466.

Hunter, T. (1987). A thousand and one kinases. *Cell* 50, 823-829.

Hunter, T and Karin, M. (1992). The regulation of transcription by phosphorylation. *Cell*. 70, 375-387.

Inglese, J., Koch, W., Caron, M.G., and Lefkowitz, R.J. (1992). Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature*. 359, 147-150.

Jones, P.F., Jakubowicz, T., Pitossi, F.J., Maurer, F., and Hemmings, B.A. (1991). Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc. Natl. Acad. Sci. USA*. 88, 4171-4175.

Knecht, D., Jung, J. and Matthews, L. (1991). Quantification of transformation efficiency using a new methods for clonal growth and selection of axenic *Dictyostelium* cells. *Dev. Genet*. 11, 403-409.

Knecht, D., Kern, H., and Scherzinger, C. (1993). Bidirectional transcription from actin promoters in *Dictyostelium*. *Biochimica et Biophysica Acta*, 1216, 105-109.

Koch, W.J., Inglese, J., Stone, W.C., and Lefkowitz, R.J. (1993). The binding site for the $\beta\gamma$ subunits of heterotrimeric G proteins on the β -adrenergic receptor kinase. *J. Biol. Chem*. 268, 8256-8260.

Konijin, T.M., Van de Meene, J.G.C., Bonner, J.T., and Barkely, D.S. (1967). The acrasin activity of adenosine-3',5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. USA*. 58, 1152-1154.

Li, N., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). Guanine-

nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature*.363, 85-88.

Lipman, D.J., and Pearson, W.R. (1985). Rapid and sensitivity protein similarity searches. *Science*, 227, 1435-1441.

Loomis, W.F.(1975). *Dictyostelium discoideum*, A developmental system. Academic Press, New York.

Loomis,W.F. (1982). Essential genes for development of *Dictyostelium*. In Kuchino, J.Y., Muller, W.E.G., and Paine, P.L.(eds), "Progress in Molecular and Subcellular Biology", Springer-Verlag, New York. pp159-182.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning. A laboratory manual. Cold Spring Harbor laboratory, Cold Spring Harbor, New York.

Mann, S.K.O., and Firtel, R.A., (1987). Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum*: Mediation via the cell surface cyclic AMP receptor. *MCB*, 7, 458-469.

Mann, S.K.O and Firtel, R.A. (1989). Two phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*. *Proc. Natl. Acad. Sci. USA*. 86, 1924-1928.

Mayer, B.J., Ren, R., Clark, K.L., and Baltimore, D.(1993). A putative modular domain present in diverse signaling proteins. *Cell*. 73, 629-630.

Margolis, B., Silvenninen, o., Comiglio, F., Roenprapunt, C., Skolnick, E., Ullrich, A. and Schlessinger, J. (1992). High efficiency expression/cloning of epidermal growth factor-receptor binding proteins with Src homology 2 domains. *Proc. Natl. Sci. USA* 89, 8894-8898.

Musacchio, A., Gibson, T., Rice, P., Thompson, J., and Saraste, M. (1993), The PH domain: a common piece in the structural patchwork of signalling proteins. *TIBS*. 18, 343-348.

Obar, R.A., Collins, C.A., Hummarback, J.A., Schpetner, H.S., and Vallee, R.B. (1990). Molecular cloning of the microtubule-associated mechanochemical enzyme dynamin reveals homology with a new family of GTP binding

protein. *Nature*, 347, 256-261.

Otsuka, A.J., Jeyaparakash, A., Garcia-Anoveros, J., Tang, L.Z., Fisk, G., Hartshorne, T., Franco, R., and Born, T. (1991). *Neuron*, 6, 113-122.

Pawson, T. and Schlessinger, J. (1993). SH2 and SH3 domains. *Current Biol.* 3, 434-442.

Pearson, W.R. 1990, Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods in Enzymology*, 183, 63-95.

Rawlings, D.J., Saffran, D.C., Tsukada, S., Largaespada, D.A., Grimaldi, J.C., Cohen, L., Mohr, R.N., Bazan, J.F., Howard, M., Copeland, N.G., Jenkins, N.A. and Witte, O.N. (1993). Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science*. 261, 358-361.

Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. *Science*. 259, 1157-1161.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature*. 363, 83-85.

Saxe, III, C.L., Johnson, R.L., Devreotes, P.N., and Kimmel, A.R. (1991). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. *Gene. Dev.* 5, 1-8.

Schaap, P., and Van Driel, R. (1985). Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP: evidence of involvement of cell surface cAMP receptor. *Exp Cell Res.* 159, 388-398.

Schaap, P., Peters, D.J.M., Haribabu, B., and Dottin, R.P. (1993). Gene regulation by hormone-like signals in *Dictyostelium*. In "Signal Transduction" (ed. Kurjan, J and Taylor, B.L.), pp 353-376.

Shenolikar, S. (1987). Control of cell function by reversible protein phosphorylation. *J. Cycl. Nucl. Prot. Phos. Res.* 11, 531-541.

Shou, C., Farnsworth, C.L., Neel, B.G., and Feig, L.A. (1992). Molecular cloning of cDNA encoding a guanine-nucleotide-releasing factor for Ras p21. *Nature*. 358, 351-354.

Simon, M.A., Bow.A., Bowtell, D.D.L., Dodson, G.S., Lavery, T.R., and Rubin, G.M. (1991). Ras 1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell*. 67, 701-716.

Simon M, Pelegrini O, Veron M and Kay RR (1992). Mutation of protein kinase A cascades heterochronic development of *Dictyostelium*. *Nature* 356, 171-172.

Skehel, P. (1993). PhD thesis. The University of London.

Sussman, M. (1966). Biochemical and genetic methods in the study of cellular slime mould development. *Meth. Cell. Physiol.* 2, 397-410.

Taylor, S.S. (1989). cAMP-dependent protein kinase. Model for an enzyme family. *J. Biol. Chem.* 264, 8443-8446.

Touhara, K., Inglese, J, Pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994). Binding of G protein $\beta\gamma$ -subunits to pleckstrin homology domains.

Trahey, M. Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G.A., Lander, M., Long, C.M., Crosier, W.J., Watt, K., Kohts, K., and McCormick, F. (1988). Molecular cloning of two types of GAP complementary DNA from human placenta. *Science* 242, 1697-1700.

Tyers, M., Rachubinski, R.A., Stewart, M.I., Varrichio, A.M., Shorr, R.G., Haslam, R.J. and Harley, C.B (1988). Molecular cloning and expression of the major protein kinase C substrate of platelets. *Nature*. 333, 470-473.

Twigg, A.J. and Sherratt, D. 1980. Transcompletable copy-number mutants of plasmid ColE1. *Nature*, 71, 2260.

Vaughan, R.A. and Devreotes, P.N. (1989). Ligand-induced phosphorylation of the cAMP receptor from *Dictyostelium discoideum*. *J. Biol. Chem.* 263, 14538-14543.

Vetre, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C. Levinsky, R., Bobrow, M., Smith, C.I.E, and Bentley, D.R. (1993). The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature*, 361, 226-233.

Waksman, G, Kominos, D., Robertson, S., Pant, N.,

Baltimore, D., Birge, R.B., Cowburn, S.C., Hanafusa, H., Mayer, B.J., Overduin, M., Resh, M.D., Rios, C.B., Silverman, L., Kuriyan, J. (1992). Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature*. 358, 646-653.

Williams, J.G., Tsang, A.S., and Mahbubani, H. (1978). A change in the rate of transcription of a eukaryotic gene in response to cyclic AMP. *Proc. Natl. Acad. Sci. USA*. 77, 7171.

Witke, W., Nellen, W. and Noegel, A. (1987). Homologous recombination in the *Dictyostelium* α -actinin gene leads to an altered mRNA and lack of the protein. *EMBO* 6:4143-4148.