Molecular Cloning of developmentally regulated genes in *Dictyostelium discoideum*

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ABSTRACT

Molecular Cloning of developmentally regulated genes in *Dictyostelium discoideum*

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Dictyostelium is not only a useful model system for the study of development but also for the cloning and characterization of eukaryotic genes.

RNAs from vegetative, and 3 h developing, cells were compared by differential display reverse transcription PCR and EHJ-1 was cloned. Besides EHJ-1 several other cDNAs (Dblp, Dlta4, DdCBS, Drsp24, and Drl7a) were identified as developmentally regulated. The deduced peptide sequences of these clones have about 40 % to 70 % identity to known genes over their entire length in the GenBank DNA data base. The comparison of deduced peptide sequences from DdCBS, Drps24, and Drpl7a to mammalian and yeast homologs showed higher identity between mammalian and *Dictyostelium* sequences. These data support the notion that *Dictyostelium* is more closely related to mammals than is *Saccharomyces cerevisiae.*

The homologs of Dblp and Dlta4 are involved in signal transduction in mammalian cells. A homolog of Dblp in rat, RACK1, has a role in translocation of PKC. In *Dictyostelium*, myosin heavy chain kinase (MHCK), a homolog of PKC, is mobilized from the membrane to myosin heavy chain upon cAMP stimulation during chemotactic movement. A role of Dblp in the translocation of MHCK was proposed.

Leukotrienes act as chemoattractants or second messengers in inflammation or allergic reactions in mammals. However, the presence of leukotrienes or leukotriene synthesis enzymes had not been reported in lower eukaryotes. The significant homology (40 % identity in amino acid sequences) of Dlta4 to mammalian leukotriene A4 hydrolase and conserved residues for this enzyme activity suggested the presence of leukotriene synthesis enzymes and possibly leukotriene related signal transduction in *Dictyostelium*.

To determine the role of cloned genes, antisense (for EHJ-1 Dblp, Dlta4, and DdCBS) or sense (for EHJ-1) RNA producing DNA constructs were introduced. Although the antisense RNA experiments were uninformative, constitutive overexpression of EHJ-1 mRNA caused retardation of development. It may be that EHJ-1 encodes a regulatory protein that controls gene expression in growing cells, overproduction may lead to extended production of vegetative specific genes until late developmental stages causing retardation of development.

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I has been strengthened by the guidance of God whenever I had hard time and I dedicate this mere work to Him.

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Dictyostelium discoideum has been used as a simple model system for developmental studies (Loomis, 1982; Firtel *et al.*, 1989; Devreotes, 1989). Since the growth and developmental processes occur separately, this system is good for analyses of the role of genes in development. Although *Dictyostelium* has a simple program its developmental processes are often found in more complex organisms such as the vertebrate embryo. Unlike vertebrate systems, *Dictyostelium* can be cultured in large amounts and is haploid, thus simplifying genetic and molecular biological analyses (Nellen et al., 1987; Cubitt et al., 1992; Kuspa and Loomis, 1994).

Growth and Development

D. discoideum are soil living amoebae which ingest bacteria by phagocytosis or, in the case of axenic derivates, take up nutrients by pinocytosis. *D. discoideum* can double their number in about 3 h in the presence of bacterial food sources or 8-12 h in axenic broth. Although *D. discoideum* has a true diploid phase, formed from opposite mating types as in yeast, most biological phenomena studied are expressed when the cells are haploid. It has about 40,000 Kb of DNA in the haploid genome on seven chromosomes (Loomis, 1982).

The formation of a multicellular organism is initiated when cells are deprived of a food source or certain amino acids (Marin, 1976). The developmental process of *Dictyostelium* can be divided into four

continuous stages: aggregation, mound formation, slug formation and culmination (Fig. 1 and 2; Cardelli *et al.*, 1985). Starvation enhances the expression of aggregation stage-specific genes and represses vegetative specific genes (Kimmel, 1987; Mann and Firtel, 1987, 1989). Several h after onset of starvation, some cells synthesize and secrete cAMP and nearby cells chemotactically move up the cAMP gradient. The signal relay leads to concentric or spiral cAMP waves that propagate outward at 6 min interval (Gerisch, 1987). When the early development of *Dictyostelium* on agar is observed by dark-field microscopy both concentric and spiral waves are visible due to the cAMP signaling and migration. Bands of moving cells in response to cAMP signal appear bright, whereas intervening bands of rounded unresponsive cells are dark. The first cells receive the cAMP signal and migrate for only 2 min toward positive gradient of cAMP and then stop until another cAMP signal comes after 5-7 min. During that time cells outside of these receive the cAMP signal and migrate to the concentric center (Alcantara and Monk, 1974; Gross *et al.*, 1976). At the end of the aggregation period, the radial rings are transformed to streams of cells in which the elongated cells are attached end to end and migrate more rapidly to form an aggregate.

The cellular differentiation begins during the mound stage of development (12h) and a group of cells arises in the tip of developing aggregate (Kimmel and Firtel, 1991; Williams, 1991). The tip is thought to coordinate the differentiation of the remaining cells in the later developmental stages and considered to have an "embryonic organizer" role (Durston and Vork, 1979). If the tips are excised and grafted to other host mounds it can define new axes and

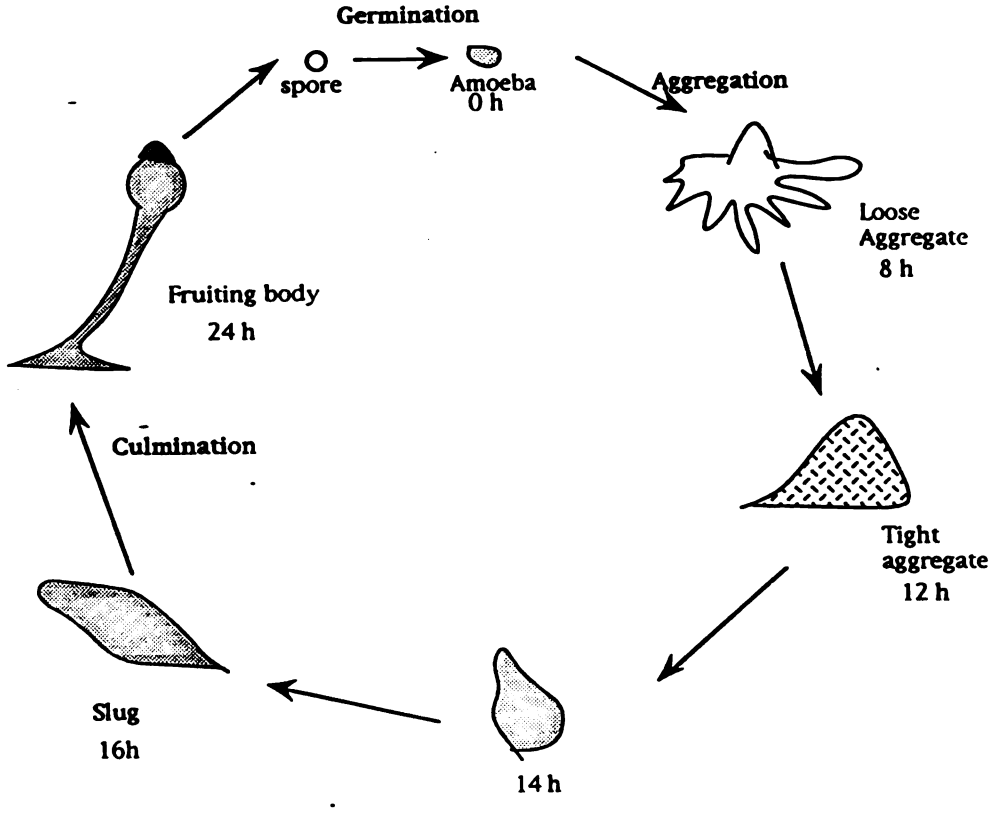
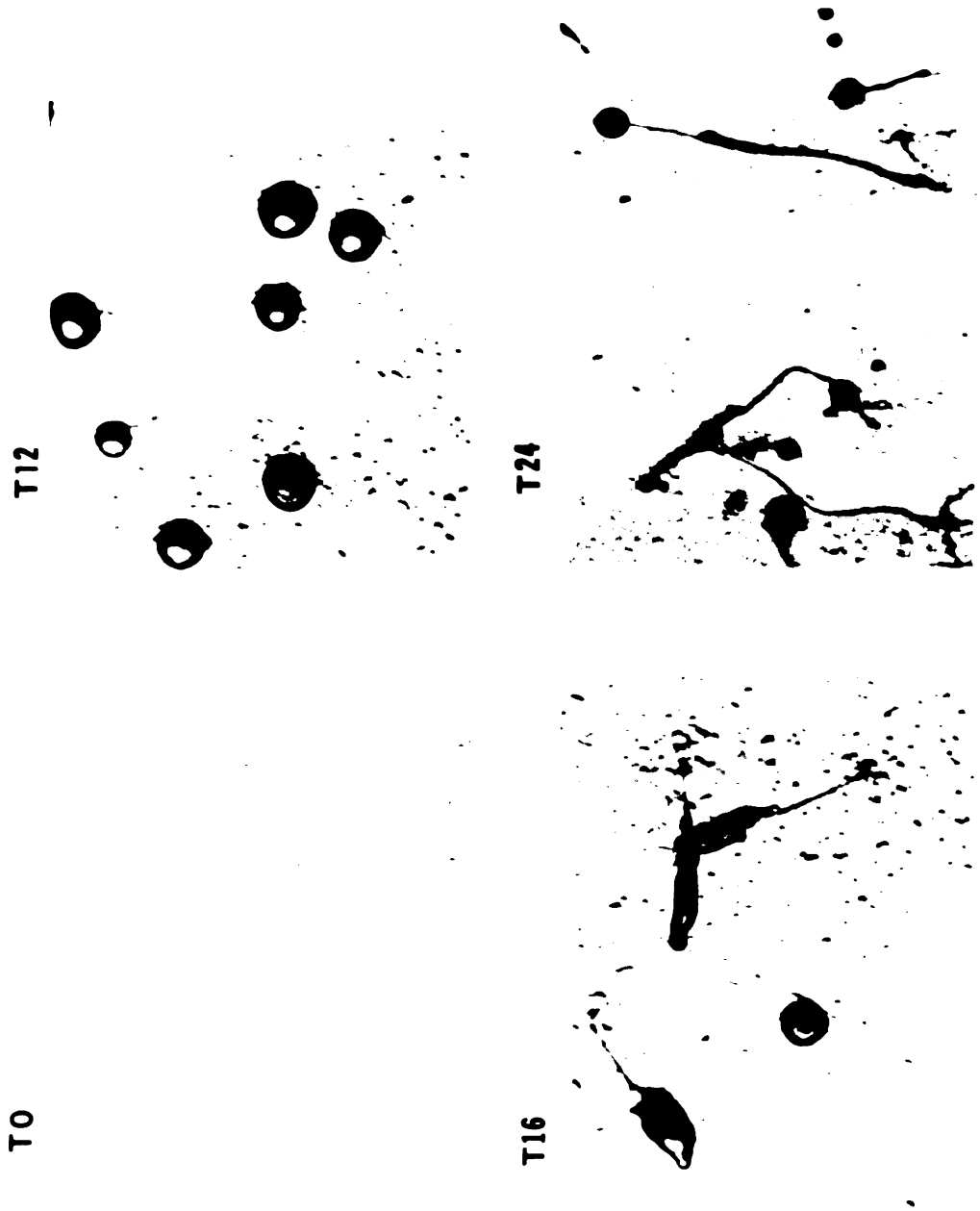


Fig. 1. Life cycle of *Dictyostelium discoideum*

Fig. 2. Development of *D. discoideum* on agar plate. 0 h (T0), 12 h (mound, T12), 16 h (slug, T16) and 24 h (fruiting body, T24) on non-nutrient agar.


T0
T12
T16
T24

thereby cause the formation of several smaller slugs and fruiting bodies (Raper, 1940). The tip secretes cAMP and maintains the gradient of cAMP in the mound and slug. In the slug the tip also coordinates slug migration and appears to be important for cell differentiation (Schaap and Wang., 1984; Williams *et al.*, 1989; Traynor *et al.*, 1992).

In the slug stages (16h) three different types of cells are spatially localized. The spatial patterning of different types of cells has been identified by using antibodies against cell-type specific markers or using cell type specific promoters connected to a reporter gene, such as *lacZ*. The anterior 10 to 15% of slugs are prestalk cells which are precursors of mature fruiting body stalks. The posterior three quarters has prespore cells which will form spores in the fruiting body (Rand and Sussman, 1983; Williams *et al.*, 1989). In the region of prespore some cells are scattered which are indistinguishable from prestalk cells and these types of cells are called " anterior like cells (ALC)" (Sternfeld and David, 1981; Devine and Loomis, 1985). Recently it is clear the prestalk region has at least two subgroups of cells. Prestalk A cells in the anterior of the slug plus ALCs are distinguished by the expression pattern of *ecmA* (a gene which is induced by differentiation inducing factor (DIF)) (Kopachik *et al.*, 1983; Williams *et al.*, 1987; Jermyn *et al.*, 1987). The prestalk B cells are localized as a cone-shaped group within the anterior of the prestalk zone and distingushed by the expression pattern of *ecmB* (Williams *et al.*, 1987, 1989)

The proportion of different cell types are controlled by multiple signaling pathways. High level cAMP maintains prespore specific

gene expression and leads to formation of spores in the fruiting body (Kay, 1989). Adenosine antagonizes the action of extracellular cAMP and prevents the formation of multiple tips in the aggregate (Schaap and Wang, 1986). Adenosine also inhibits the expression of prespore genes. Differentiation inducing factor (DIF) is another morphogen in *Dictyostelium* and it causes the prestalk-specific gene expression and leads the cell fate to stalk (Kay and Jermyn, 1983; Kopachik *et al.*, 1983; Kwong and Weeks, 1989). DIF appears to be involved in regulating extracellular cAMP level by stimulation of phosphodiesterase gene expression (Podgorski et al., 1989; Franke and Kessin, 1992). Ammonia does promote spore cell formation by counteracting the effect of DIF (Williams *et al.*, 1984; Wang and Schaap. 1989).

In the culmination stage (24h), a tube of cellulose is formed in the anterior region of slug. The cells in front of the slug migrate through the cellulose tube, form stalk cells, and die. The prespore cells are pulled toward the upper end of forming stalk and differentiate into spores. Finally a mature fruiting body contains about 100,000 spore cells and the spores are held several millimeters above the substratum by a vacuolated stalk. In the presence of food source the spore cells germinate and repeat their life cycle (Loomis, 1982).

Regulation of Gene Expression during Development

Starvation induces the expression of a class of early genes in development and represses the expression of vegetative-specific

genes (Kopachik *et al.*, 1985; Singleton *et al.*, 1987, 1988). During growth, cells continuously secrete a factor, PSF (Prestarvation factor), that accumulates in proportion to cell density (Rathi *et al.*, 1991; Clarke *et al.*, 1988). PSF or a secreted density sensing factor, conditioned medium factor (CMF), induces the expression of early genes (Mehdy and Firtel, 1985; Gomer and Firtel, 1987). The blocking of CMF expression by antisense RNA inhibits aggregation impling that early gene expression is essential for proper development in *Dictyostelium* (Jain *et al.*, 1992). In many cases, however the induction or repression of gene expression is controlled by cAMP.

About four h after starvation, cells start to secrete cAMP and the gene products for the aggregation process are induced (Mann and Firtel, 1989; Singleton *et al.*, 1988). These genes encode cAMP receptors, guanylyl cyclase, adenylyl cyclase, phospolipase C, cAMP phosphodiesterase, adhesive contact sites A (csA) and G protein $\alpha 2$ subunit (Kessin *et al.*, 1992; Gross, 1994). The activation of adenylyl cyclase increases the intracellular cAMP concentration, mediates actin and myosin mobilization and controls chemotaxis (Newell *et al.*,1987). During this aggregation period some pre-stalk-related genes are positively regulated by both nanomolar cAMP pulses or continuous stimulation with micromolar cAMP concentrations. Cysteine protease and other proteins of unknown function belong to this group of genes (Barklis and Lodish, 1983; Mehdy *et al.* 1983; Mehdy and Firtel, 1985). Several targeted mutants show abnormal development suggesting that aggregation-specific genes are necessary for normal development.

After formation of aggregates, the expression of aggregation-specific genes is reduced. During these stages, spore-specific genes are expressed. Micromolar levels of cAMP is required for spore-specific gene expression. The expression of some prestalk-specific genes, such as *ecmA* and *ecmB* which encode extracellular stalk matrix proteins, are induced by DIF in this slug stage (Jermyn *et al.*, 1987; Williams *et al.*, 1987).

In *Dictyostelium* the expression of many genes are controlled at the transcriptional level. Several cis-acting elements responsible for gene induction by extracellular cAMP, folate, or DIF have been identified (May *et al.*, 1991; Blusch *et al.*, 1992; Early and Williams, 1988; Datta and Firtel, 1987. 1988). In case of discoidin Iγ, transcription is induced by PSF and repressed by cAMP pulses (Clarke et al., 1987; Bozzone and Berger, 1987). Sequence elements for both transcriptional induction and repression have been identified by promoter analysis (Vauti *et al.*, 1990). Most cAMP-inducible promoters have a G/C-rich element in the promoter, termed GBRE (G-box regulatory element) (Datta and Firtel, 1987; Pears and Williams 1987). The removal of GBRE results in a 50 to 100 fold reduction in the level of expression. Firtel's group identified GBRE binding factor (GBF), which is developmentally regulated and inducible by cAMP (Hjorth *et al.* 1990; Schnitzler *et al.*, 1994). They showed GBF is an extracellular cAMP-responsive transcriptional activator which can regulate gene expression.

As in other eukaryotes mRNA stability in *Dictyostelium* is a major control point in the regulation of gene expression (Mullner and Kuhn, 1988; Mangiarotti *et al.*, 1982; Steel and Jacobson, 1988; Shapiro *et*

al., 1988). The level of glycoprotein gp80 mRNA accumulates to a maximum level between 4 to 6 h, remains high until 10 h, and then is reduced rapidly to 10% of the maximum level at 12 h (Kraft *et al.* 1989). By using *in vitro* transcription assays, it was found that the rapid reduction of gp80 mRNA level is due to decreased mRNA stability (Chandrasekhar *et al.*, 1990). Although there are many possible factors determining mRNA stability, no clear cut answer is present. Shapiro *et al.* (1988) showed no correlation between mRNA decay rates and the length of poly A tail, the size of mRNA and the number of ribosome per unit of mRNA. They found that unstable mRNAs were more efficiently translated and suggested a translational role for mRNA modifications that change in a time-dependent manner. An unique example for the usage of endogenous antisense RNA in the stability of mRNA was identified by Hildebrandt and Nellen (1992). The prespore gene, *EB4-PSV* is constitutively transcribed during growth and development but mRNA levels only accumulate when cells form aggregates. They found that the difference between synthesis and accumulation is due to the developmentally regulated endogenous antisense RNA.

During the first hour of development the synthesis of many proteins is rapidly reduced whereas the mRNAs for those proteins persevere in the cell in a translatable form (Alton and Lodish, 1977). It was suggested that a translational control is involved in the reduction of protein synthesis. From the study of distribution of ribosomal mRNAs in polysomes, Steel and Jacobson showed that the blockage of translation initiation is not due to inactivation of these mRNAs by decapping or deadenylation (Steel and Jacobson, 1988).

Both papers suggest that lack of soluble factors such as initiation factors leads to rapid reduction in protein synthesis during early development.

Post-translational modification is another controling step for the proper gene expression in *Dictyostelium*. The ribosomal proteins of *Dictyostelium* are differentially phosphorylated and methylated and those modification are considered an important step for the biosynthesis of the ribosome and (or) its function (Ramagopal, 1990). For proper cell-cell interaction the modification of cell surface glycoprotein is important (Harloff *et al.*, 1986; Stadler *et al.*, 1989). Prespore-specific Antigen (PsA) is a 32 KDa glycoprotein isolated from the surface of prespore cells (Gooley *et al.*, 1992). PsA is post-translationally modified by addition of carbohydrate to the threonine residues of the carboxy-terminal peptide domain, and a glycosyl phosphatidylinositol anchor which attaches glycoprotein to the cell membrane (Gooley *et al.*, 1992).

Signal Transduction in *Dictyostelium*

To coordinate the developmental program, the spontaneous aggregation of thousands of isolated amoebae to a single aggregate, *Dictyostelium* has to have a well controlled signal transduction mechanism. Konijin *et al.* (1968) showed cAMP was an acrasin (a chemoattractant) in *Dictyostelium*. The binding of cAMP to cell-surface receptors is found in many responses associated with chemotaxis (Dinauer *et al.*, 1980; Gerish, 1987; Hall *et al.*, 1989).

Induction of developmentally regulated enzyme activity, mRNAs and proteins by addition of exogenous cAMP confirms that cAMP acts as a hormone by directly binding to cell-surface receptor. Four different types (cAR1 to cAR4) of developmentally regulated cAMP receptors have been cloned. The phenotype of targeted mutants suggests cAMP and cAMP-receptor interaction is essential for development (Saxe *et al.*, 1991a, b; Johnson *et al.*, 1993; Saxe *et al.*, 1993; Louis *et al.*, 1994).

Many recent studies showed heterotrimeric G proteins are coupled to cAMP receptors. Eight different types of $G\alpha$ and one $G\beta$ genes have been cloned (Pupillo *et al.*, 1989; Hadwiger *et al.*, 1991; Wu and Devreotes, 1991; Wu *et al.*, 1994; Lilly *et al.*, 1993). The stimulation of G protein through cAMP bound receptors leads to activation of adenyl cyclase (AC) and phospholiphase C (PLC) (Theibert and Devreotes, 1986; Van Haastert, 1984; Europe-Finner and Newell, 1987). Activation of AC produces cAMP and sends a cAMP signal to outside of cell and causes acitvation of cAMP-dependent protein kinase in the cell. A targeted mutant of adenylyl cyclase A (ACA), which is expressed only in early aggregate, did not aggregate (Pitt *et al.*, 1992).

Dictyostelium has a mechanism similar to mammals for phospholipase-mediated signal transduction (Kimmel and Eisen, 1988; Janssens and Van Haastert, 1987; Newell *et al.*, 1990). Activated PLC synthesizes Ins(1,4,5)P_3 (IP_3) and diacylglycerol (DG) from phosphoinositol biphosphate. DG and IP_3 regulate gene expression.

(A) cAMP receptors.

Four cDNAs for different cAMP receptors (cAR1-cAR4) have been cloned. They have extracellular amino termini, seven transmembrane domains and long cytoplasmic carboxy-termini (Louis *et al.*, 1994). cARs share about 60% amino acid identity in transmembrane domains.

cAR1 mRNA is expressed early in development when the cAMP relay system is being established and its level is decreased in late development (Fig.3; Firtel, 1991). As cAR1 is reduced cAR3 accumulates to a peak at the mound stage and then gradual loss to the fruiting body. The expression of cAR2 is initiated during mound stage and peaks at culmination stage. The cAR2 mRNA is enriched in prestalk cells. cAR4 mRNA is initially expressed during tip elongation and continues to accumulate into culmination. The diverse expression of cARs implies that cARs may mediate specific functions at different developmental stages.

cAR1 appears to couple to a $G\alpha 2$ protein. The addition of guanidine nucleotides to the cell membrane from aggregation competent cells converts the cAMP binding sites from high affinity to low affinity indicating the involvement of G proteins (Janssens *et al.*, 1986; Van Haastert *et al.*, 1986). The expression pattern of $G\alpha 2$ and cAR1 is parallel. A targeted mutant of cAR1 by homologous recombination fails to bind or sense cAMP and arrests in early development (Sun and Devereotes, 1991). The expression of cAR1 utilizes two promoters that are activated at distinct stages of development and respond to different extracellular cAMP conditions

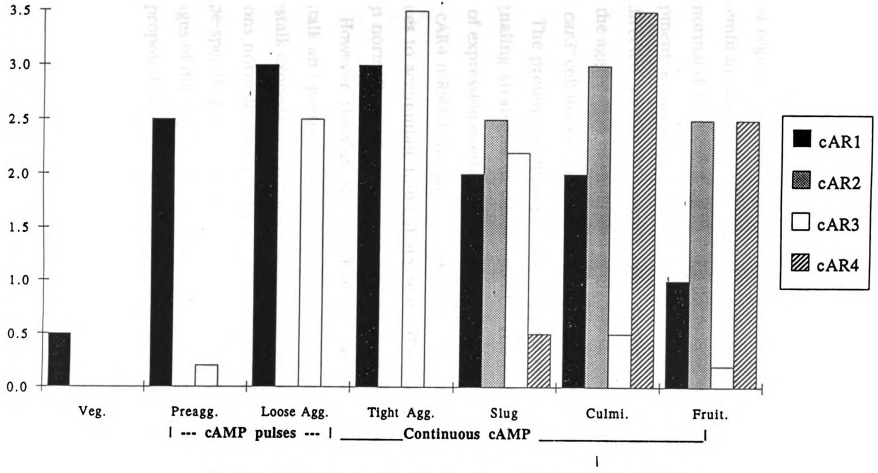


Fig. 3. Developmental Regulation of cAMP Receptors mRNA

(Louis *et al.*, 1993). One promoter is active with low-level oscillation of cAMP; exposure to high cAMP concentrations will repress this promoter and induce a second promoter.

cAR2 is structually similar to cAR1. Outside of the carboxy terminal region cAR1 has about 75% sequence identity to cAR1 in transmembrane domain and loop region. The null mutant of cAR2 shows normal development up to the tight mound stage but arrests development at this stage. This suggests cAR2 may be required for cAMP-directed sorting of prestalk cells during pattern formation within the aggregation mound (Saxe *et al.*, 1993).

The *car3*$^-$ cell lines display no obvious phenotype (Johnson *et al.*, 1993). The presence of multiple cARs suggests redundancy in cell-cell signaling strategies in development. The cAR3 (Fig. 3) time course of expression overlaps with cAR1 and cAR2.

The cAR4 mRNA is initially expressed during tip elongation and continues to accumulate into culmination. The *car4*$^-$ cells initially develop normally until aggregation and tip formation (Louis et al., 1994). However, the slugs showed abnormal phenotype in the level of prestalk and prespore gene expression. Certain prestalk markers for prestalk expression is reduced, and prespore genes are expressed in regions normally restricted to prestalk cells. cAR4 may regulate cell type-specific gene expression and pattern formation during the late stages of development. The following table shows the summary of the proposed roles for cARs in *Dictyostelium* development.

	Phenotype change of null cells	Proposed role in development
cAR1	Fail to sensing cAMP Arrest in early development	cAMP signaling in early development
cAR2	Arrest development at mound stage.	cAMP-directed sorting of prestalk cells
cAR3	No obvious phenotype changes	cAR1 or cAR2 may substitute the role of cAR3 in car3$^-$ cells
cAR4	Improper gene expression in slug and culmination	cell type-specific gene expression in the late stages of development

(B) G proteins

The *Frigid A* mutants show no chemotaxis to extracellar cAMP and do not aggregate (Kesbeke *et al.*, 1988; Mann *et al.*, 1988). *Frigid A* cells lack the activation of guanylyl cyclase and adenylyl cyclase and developmentally induced genes are not induced by exogenously applied pulses of cAMP. In severe *Frigid A* mutants, inhibition of cAMP binding by GTP (a standard indicator of G-protein linked receptors) is not detectable but GTP stimulates wild type level of adenlylyl cyclase activities. These results suggested that *Frigid A* mutants are defective in a G protein required for proper *Dictyostelium* development.

By using redundant oligonucleotides from the highly conserved sequence in putative guanine nucleotide binding protein of mammalian α subunits, two Gα cDNAs, Gα1 and Gα2, were initially

cloned (Pupillo *et al.*, 1989; Kumagai *et al.*, 1989). Six more different Gα cDNAs have been cloned by PCR (Hadwiger *et al.*, 1991; Wu and Devereotes, 1991). Each Gα subunit shares approximately 50% sequence identity. Only one Gβ subunit cDNA has been cloned (Lilly *et al.*, 1993). The comparision of primary sequences indicates they can not be classified of into any of the G_s, G_i, G_q subtypes in mammals.

Gα1 mRNA is present in vegetative cells through aggregate stages (Fig. 4; Firtel, 1991; ; Wu *et al.*, 1994). Loss of Gα1 shows no detectable effects on growth and development (Kumagai *et al.*, 1991). Possibly other Gα subunits substitute for the role of Gα1. However the over-expression of Gα1 results in large and multinucleated cells. The majority of cells do not aggregate, and some aggregating cells form small and abnormal fruiting bodies (Kumagai *et al.*, 1989). Gα1 expression is preferentially expressed in the prestalk AB cells and anterior-like cells. The developmental phenotype of Gα1 overexpression and cell-type-specific expresison of Gα1 suggest that Gα1-mediated signalling pathways play an important role in regulating multicellular development by controlling prestalk morphogenesis (Dharmawardhane *et al.*, 1994).

Among eight Gα subunits, Gα2 is the most studied. Gα2 mRNA is induced by cAMP pulses and preferentially expressed in aggregation (Kumagai *et al.*, 1989). Gα2 null cells do not aggregate and lack cAMP-mediated activation of adenylyl cyclase, guanylyl cyclase and phospholipase C as do *Frigid A* mutants (Kumagai *et al.*, 1991). Gα2 couples to cAR1 during the aggregation phase of development (Kumagai *et al.*, 1991). An aa substitution in Gα2, Gα2[G206T], a

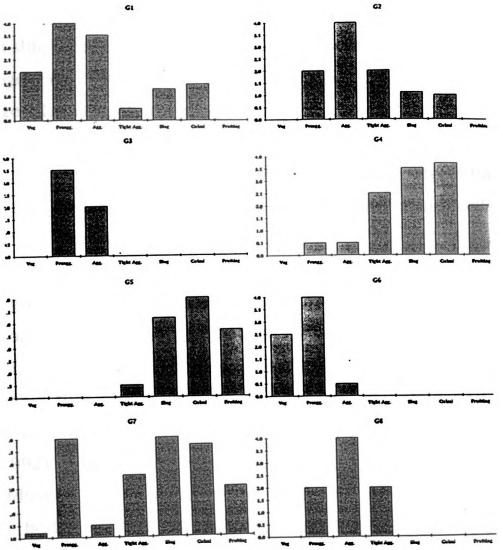


Fig.4. Developmental Changes of Gα mRNAs

putative dominant negative mutation, causes an inhibition of receptor-mediated activation of adenylyl cyclase similar to mammalian system (Osawa and Johnson, 1991). Transformed cells with a preaggregation stage-specific promoter controlling expression Gα2[G206T] do not aggregate. However, cells expressing Gα2[G206T] under the control of ecmA promoter show normal development through slug formation but have culmination with an aberrant stalk morphogenesis. These results suggest that Gα2 plays an essential role in regulating stalk morphogenesis as well as early aggregation (Okaichi *et al.*, 1992; Carrel *et al.*, 1994).

Gα3 mRNA is induced by cAMP pulses and preferentially expressed in preaggregation stage (Fig. 4). The data for null cells of Gα3 is not available.

Gα4 is primarily expressed late in development and at a low level during growth and early development. As expected from the expression pattern of Gα4, the Gα4 knock-out cells aggregate and form a tip. However, only the apical portion continues to elongate producing a thin projection that in some cells become "knotted" whereas the basal region remains more rounded (Hadwiger and Firtel, 1992). This mutant forms fewer spores than does the wild type. When gα4$^-$ cells co-aggregate with wild type cells normal fruiting bodies. Possibly Gα4 is essential for multicellular development by producing and secreting intercellular signals.

Gα5 is mainly expressed in late development, whereas Gα6 is expressed primarily during growth and very early development (Fig. 4; Hadwiger *et al.*, 1991; Wu and Devreotes, 1991).

Gα7 mRNA reaches a maximum level in preaggregation and followed by lower levels in aggregation and increasing levels until culmination. The Gα7 null cells show no defects in growth and morphology in development (Wu *et al.*, 1994).

Gα8 mRNA level is very low during growth and reaches a maximum level during aggregation followed by declining levels (Wu *et al.*, 1994). In contrast to mRNA expression the protein is constitutively expressed. Like Gα7 null cells, Gα8 null cells do not have detectable phenotypic change. The transformants overexpressing Gα8 do not show any phenotypic change. These results suggest that Gα7 and Gα8 subunits are functionally redundant with other Gα subunits.

Only one Gβ subunit has been cloned. The mRNA and protein are constitutively expressed. Its sequence has about 60% identity to the homolog of other systems. It is suggested that this β-subunit interacts with other eight Gα subunits which are transiently expressed during development. Targeted mutants in Gβ subunit are viable, but unable to aggregate (Lilly *et al.*, 1993). The $G\beta^-$ cells lack the ability to move towards chemoattractant and their adenylyl cyclase or guanylyl cyclase activity can not be stimulated by CAMP. These results suggest that Gβ links the chemoattractant receptor to effectors and Gβ is essential in many chemoattractant-mediated processes (Wu *et al.*, 1995).

The following table summarizes the phenotype of null cells and possible roles of G protein subunits.

	Phenotype changes of null cells	Possible roles
Gα1	No change	Prestalk morphogenesis
Gα2	No aggregation; resemble *Frigid A* mutants	Link to cAR1 and signal transduction in early development and stalk cell morphogenesis
Gα4	Normal to mound; abnormal fruiting bodies	Produce intercellular signals for development
Gα7	No change	May be fuctionally redundant with other subunits.
Gα8	No change	May be fuctionally redundant with other subunits.
Gβ	No aggregation	Not essential for cell viability. Involved in many chemoattractant-mediated processes

(C) Effectors for signal transduction

Binding of cAMP to surface receptors activates adenylyl cyclase (AC), via a G protein (Kesbeke *et al.*, 1988; Klein *et al.*, 1988). AC catalyzes the production of cAMP from ATP and leads to increased intracellular and extracellular cAMP level. An increase of intracellular cAMP leads to activation of cAMP-dependent protein kinase A (PKA). The extracellular cAMP is used for cAMP relay and then destroyed by extracellular phosphodiesterase (PDE). The cells

are then ready for another pulse wave of cAMP signalling (Wang et al., 1988).

A group of mutants, *Synag7*, fail to aggregate due to the lack of adenylyl cyclase (Theibert and Devreotes, 1986) Two different types of adenylyl cyclase, ACA (aggregation-specific) and ACG (germination -specific) were cloned (Pitt *et al.*, 1992). ACA is expressed during early development whereas ACG is present only during spore germination. cAR1 and unidentified other receptors are linked to $G\alpha 2$ and activates ACA activity (Pupillo *et al.*, 1992).

The targeted mutants of ACA are blocked in development and remain as single amoebae. The mutants show chemotaxis to cAMP but do not have adenylyl cyclase activity. Mutants and wild type cells synergize to restore normal development. These results suggest the *aca*$^-$cells can respond to, but cannot produce, a cAMP signal. Moreover cAMP is not required for chemotaxis, growth and cell division which are unaffected in *aca*$^-$cells.

cAMP relay signal activates a phospholipase C (PLC) coupled pathway (Newell *et al.*, 1988). Stimulation of receptors, through G proteins, activate production of the intracellular messengers diacylglycerol (DG) and 1,4,5 inositol triphosphate (IP_3) by PLC. The evidence suggests gene regulation is mediated by the second messenger IP_3 and DG rather than by intracellular cAMP (Ginsburg and Kimmel, 1989). IP_3 can mobilize intracellular Ca^{+2} ions which interact with calmodulin (Marshak *et al.*, 1977) or activate a protein kinase C (PKC). DG binds to PKC and activated PKC may lead to regulation of gene expression.

Guanylyl cyclase is another enzyme activated by the cAMP receptor. Evidence suggests the accumulation of cGMP is essential for chemotaxis. First, cGMP accumulation is induced by chemoattractant folic acid in vegetative, and cAMP in developing, cells (Mato *et al.*, 1977). Second, the acrasin of *Dictyostelium minatum* and *Dictyostelium lacteum* induces cGMP (De Wit *et al.*, 1983). Third, mutant *stm* (Streamer)*F* remain in the elongated state during chemotaxis for about 5-fold longer than the parental wild type due to lack of cGMP specific phosphodiesterase (Ross and Newell, 1981).

Fig. 5 shows a current model for signal transduction in *Dictyostelium.*

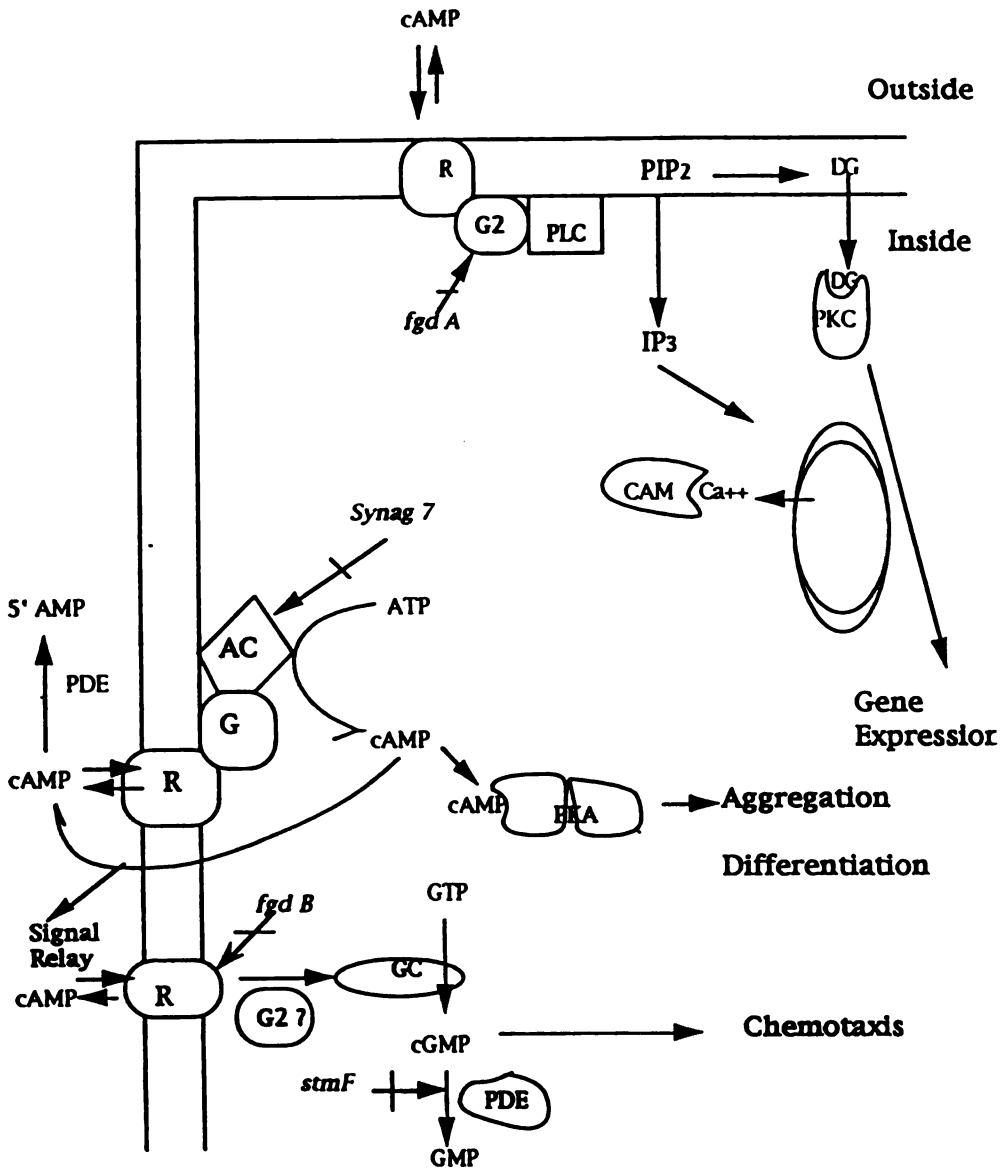
Objectives and Organization of the Thesis

Many people in *Dictyostelium* research have tried to clone genes which are induced by cAMP pulse or spore and stalk cell specific genes. However, I have interested in the cloning of early developmental stage specific genes and the role of these genes during the transition from growth to development.

To isolate genes differentially expressed, a method, termed DDRT-PCR (differential display reverse transcription PCR) was designed by Liang and Pardee (1992). Since this method was invented, the DDRT-PCR method has become popular for cloning of genes (Sager *et al.*, 1993; Aiello *et al.*, 1994; Joseph *et al.*, 1994; Wong and McClelland, 1994; Zimmerman and Schultz, 1994).

The DDRT-PCR approach can be used to find many *Dictyostelium* genes whose mRNAs are induced or reduced by cAMP or otherwise

Fig. 5. A model for signal transduction in *Dictyostelium.*
Look at text for details. CAM, calmodulin; DG, diacylglycerol; PIP_2, phosphoinositol biphosphate; IP_3, 1,4,5 inositol triphosphate; PLC, phospholipase C; R, cAMP receptors; G2, Gα2; G, G proteins; PKC, protein kinase C; PKA, cAMP dependent protein kinase; AC, adenylyl cyclase; GC, guanylyl cyclase; PDE, phosphodiesterase.


cAMP
Outside
R
PIP2
DG
G2
PLC
Inside
fgd A
IP3
DG
PKC
CAM
Ca++
Synag 7
ATP
5' AMP
AC
PDE
G
cAMP
cAMP
R
cAMP
PKA
Aggregation
Differentiation
Gene
Expressior
fgd B
GTP
Signal
Relay
cAMP
R
GC
G2 ?
cGMP
Chemotaxis
stmF
PDE
GMP

regulated. The constitutively expressed genes will be displayed as equal intensity bands. The developmentally regulated genes will be displayed as bands with reduced or induced intensity. I will use DDRT-PCR to clone induced or repressed genes on early development.

After cloning of genes using DDRT-PCR, effort should be made to determine the role of cloned genes. The roles of *Dictyostelium* myosin heavy chain II and other genes on development were analyzed using antisense RNA method (Crowley *et al.*, 1985; Knecht and Loomis, 1987; Fang et al., 1993). Antisense RNA producing transformation vectors can be easily introduced into cells to block the expression of targeted genes. Sometimes the transformation vectors are used for the overexpression of genes (Luo *et al.*, 1994). The following are the main objectives for the thesis.

1. Cloning of developmentally regulated genes by DDRT-PCR. To clone early induced or repressed genes I will compare RNAs from vegetative and 3 h developing cells.
2. Determine the expression of cloned genes by Northern blot analysis. DNA fragments from differentially expressed bands on DDRT-PCR will be used as probes.
3. Screening cDNA library and sequencing for cloned genes. To get full size cDNA, cDNA libraries of vegetative, or developing cells will be screened. Sequencing and sequence analysis will be done.
4. Determine the role of cAMP in regulation of cloned gene expression. Regulation of RNAs during development in shaken

suspension cultures with or without cAMP pulses will be checked using Northern blot analysis.

5. Determine the role of cloned genes in growth and development by using antisense RNA. The portion of cloned cDNAs will be inserted into a transformation vector and the growth and development of transformants checked. If transformants have Phenotypic changes, Northern blot analysis will be done to check the level of endogenous RNAs.
6. Overexpression of cloned genes to determine the role of cloned genes in development. The full, or close to, size cDNA will be inserted into a transformation vector and the growth and development of transformants checked.

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I. CHAPTER ONE : Cloning of a *Dictyostelium discoideum* developmentally regulated gene EHJ-1 and analysis of overexpression

Chapter One was written to submit to J. of Cell Science.

INTRODUCTION

Dictyostelium discoideum is a soil amoeba which feeds on bacteria. In the presence of a food source *Dictyostelium* proliferates as single cells. Upon starvation some cells secrete cAMP and other cells recognize the cAMP signal, through a cAMP receptor which is coupled to G-proteins (Klein *et al.*, 1988), and then migrate toward the cAMP secreting cells. After starvation about 10^5 cells form a tight aggregate at 12 h, slugs at 16 h and finally a fruiting body at 24 h. Fruiting bodies contain spores which germinate in the presence of a food source (Loomis, 1982). Developmental regulation of gene expression at the transcriptional and post-transcriptional level occurs during the transition from vegetative growing to developmental stages (Mangiarotti *et al.*, 1985; Firtel, 1991; Gross, 1994).

Differential screening and subtractive hybridization, have been used to clone developmentally regulated genes (Sargent, 1987). Liang and Pardee (1992) designed a method, differential display reverse transcription PCR (DDRT-PCR), to clone differentially expressed eukaryotic mRNA. DDRT-PCR is straightforward and useful under various circumstances (McClelland *et al.*, 1995). DDRT-PCR was used to clone highly expressed genes in neonatal mammalian brain and cancer specific genes (Joseph *et al.*, 1994; Liang *et al.*, 1992).

D. discoideum is a haploid organism, whose gene expression can be manipulated by transformation (Knecht *et al.*, 1986). Specific gene expression can be blocked by antisense RNA or homologous recombination (Crowley *et al.*, 1985; Knecht *et al.*, 1987; De Lozanne and Spudich, 1987). In addition to blocking experiments, overexpression of truncated cyclin B gene was done to determine the

role of cyclin B in *Dictyostelium* (Luo *et al.*, 1994).

In this chapter I report the cloning of a developmentally regulated gene (clone EHJ-1) by using DDRT-PCR and retardation of development by overexpression of truncated EHJ-1 cDNA.

MATERIALS AND METHODS

Cell growth and development

D. discoideum strain KAX4 (a gift from Dr. Rich Kessin, Columbia University) was used for this work. KAX4 was grown in HL-5 (Watts and Ashworth, 1970) in shaking culture (150 rpm) at 22°C. Harvested amoebae were allowed to develop on 1.5% Bacto-agar (Difco) with developmental buffer (DB, 5mM Na_2HPO_4, 5mM NaH_2PO_4, 2mM $MgSO_4$ and 200μM $CaCl_2$, pH6.5). For development in suspension, vegetative cells were harvested and resuspended in DB and shaken at 230 rpm as described by Hassanain and Kopachik (1989).

Differential display reverse transcription PCR

First strand cDNA was synthesized from 2 μg total RNA of vegetative and 3h developing cells on agar by using SuperScript™ RNase H$^-$ reverse transcriptase (Gibco, BRL). PCR (94°C, 30 seconds; 42°C, 1 minute; 72°C, 30 seconds; 30 cycles) was done with arbitrary 10 mer(primer kit, Operon) and anchored poly T (5'-TTTTTTTTTTTTTTGC-3') primer by using the first strand cDNAs as templates. PCR products were separated on 6% nondenaturing polyacrylamide sequencing gels. A differentially expressed band

was cut from the dried sequencing gel and DNA extracted by boiling the gel piece in 150 μl of H_2O for 15 min. After separation of insoluble material by centrifugation, DNA was precipitated by addition of linearized polyacrylamide and cold ethanol (Gaillard and Strauss, 1990). Redissolved DNA was used for PCR (94°C, 30 seconds; 40°C, 2 minutes; 72°C, 30 seconds; 40 cycles) with the same primer pair used for DDRT-PCR. PCR amplified product was separated on 1% agarose gel and a DNA insert was isolated by adsorption to silica gel particles using the QIAEX DNA gel extraction kit (Qiagen). Purified DNA insert was ligated into pCR™II vector (TA cloning kit, Invitrogen).

Screening of cDNA library and DNA sequencing

The insert from the clone (EHJ-1) in pCR™II vector was sequenced and used for screening a λ ZAP cDNA library made from vegetative cells (gift from Dr. Herbert Ennis, Roche Institute, NJ). The largest clone from the library screening had 1.8 kb insert. The λ ZAP clone containing 1.8 kb insert was isolated as a phagemid by *in vivo* excision of the cloning vector as described by Short (Short *et al.* 1988). The phagemid clone(pEHJ-1) was subcloned and sequenced using the Sequenase DNA sequencing kit (United States Biochemical).

Northern blot analysis

Total cellular RNA was isolated by centrifugation of a guanidinium thiocyanate extract through a cesium chloride cushion or by using RNA isolation kit RNA STAT-60™ (TEL-TEST "B", INC.).

Northern blot analysis was done previously described (Kopachik *et al.*, 1985).

Southern blot analysis

A 10 μg of KAX4 genomic DNA was digested by restriction enzyme EcoR I, BamH I/EcoR I, Hind III, and Hind III/Xho I and separated on 0.7% agarose gel electrophoresis. DNA was electroblotted onto a GeenScreen (NEN) membrane according to the manufacture's procedures. The blot was prehybridized and hybridized as described previously (Hu *et al.*, 1992) and washed twice for 30 minutes in 0.5x SSC, 1% SDS at 65^{o}C and then exposed to X-ray film.

Transformation of *D. discoideum*

The 1.8 kb insert was ligated into pDNeoII transformation vector to overexpress truncated EHJ-1 mRNA constitutively. Twelve μg of cesium chlroride purified pDNeo(EHJ-1) was used to transform exponential phase KAX4 cells by the Ca^{2+} DNA precipitation method (Nellen *et al.*, 1987). Stable transformants were selected and grown clonally in HL-5 medium containing 40 μg/ml of G418.

RESULTS

Cloning of EHJ-1 by DDRT-PCR and screening λ ZAP cDNA library

DDRT-PCR was done with a arbitrary 10 mer(OPA-02; 5'-TGCCGAGCTG) and anchored poly T (NB16; 5'- TTTTTTTTTTTTGC -3')

Fig. 1. Differential Display PCR sequencing gel. DDRT-PCR was done with OPA-02/NB16 and OPA-03/NB16 primer pairs with RNAs from vegetative and 3 h development on agar. PCR products were separated on 6% nondenaturating polyacrylamide sequencing gels. V4 and v5 are vegetative specific bands. Cont1 shows even expression in two different stages.

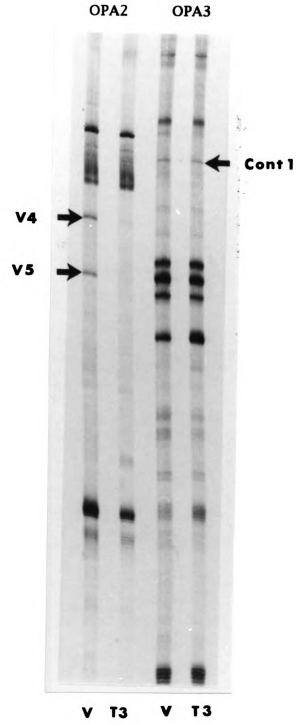
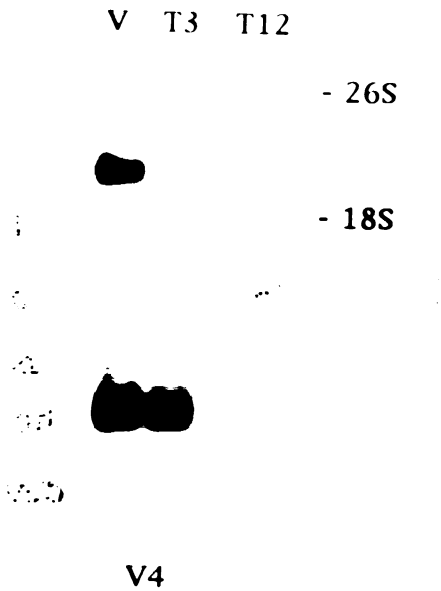
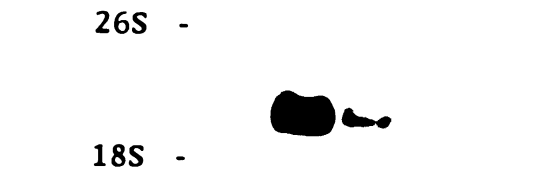

OPA2
OPA3
Cont 1
V4
V5
V
T3
V
T3

Fig. 2. Northern blot analysis.
(A) The blots containing 10 μg of total RNAs from cells of vegetative(V), 3 h and 12 h development on agar was probed with PCR amplified v4 DNA from DDRT-PCR sequencing gel. (B) DNA insert from a clone, v4-7, was used as a probe. The blot contains 10 μg of total RNAs of vegetative, 2 h, 6 h and 15 h development on agar.

A



B



primer by using first strand cDNA made from vegetative and 3 h developmental stage cells (T3) as templates. A sequencing gel showed two vegetative stage specific bands were in OPA-02 and NB16 primer pair (Fig. 1). With OPA-03 (5'-AGTCAGCCAC-3') and NB16 primer pair all detectable DNA was evenly amplified between vegetative and T3. Vegetative specific bands v4 and v5 were isolated from sequencing gel and reamplified by PCR with OPA-02 and NB16 primer pair. On agarose gel electrophoresis about 400 bp DNAs were identified. PCR amplified v4 and v5 DNAs were isolated and radiolabeled by the random primer method and then used to probe Northern blot. The signal for v5 was undetctable and for v4 showed two mRNAs whose level is lower in development (Fig. 2A). The two bands identified could be a result of multiple types of amplified DNA in the probe. In order to clone DNA for both developmentally regulated RNAs, PCR amplified DNA for v4 was cloned into pCR™II cloning vector and inserts were isolated from independent clones. When cloned DNA was used as a probe a single band was detected (Fig. 2B). A insert from pv4-1 plasmid hybridized to lower band and another insert from pv4-7 bound to upper band (Fig. 2B). The sequencing data for pv4-1 showed 53% sequence identity to mouse 60S ribosomal protein L7a in 136 C-terminal amino acids overlap (Giallongo *et al.*, 1989). The deduced potential peptide sequences from pv4-7 did not have significant homology to known genes in GenBank DNA data base search. A λ ZAP cDNA library made from vegetative cells was screened using insert DNA from pv4-7 as a probe. The largest λ ZAP clone hybridizing to the probe whose 3' end was identical to that of the v4-7 sequence had

Fig. 3. DNA and deduced peptide sequence for EHJ-1. A λ ZAP clone EHJ-1 was sequenced by subcloning or using synthesized primers. Two possible polyadenylation signals were underlined. GenBank accession number is U27540.

```
CTTCATTTGCTCACTACTCATCAAGCCGTACTGCTCTTAAGAGAATTAGAGTTGTAGGTC
  S  F  A  H  Y  S  S  S  R  T  A  L  K  R  I  R  V  V  G  Q
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  A  S  M  P  M  H  V  L  A  K  V  D  L  V  L  G  L  S  D  F
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  I  E  D  N  K  M  S  E  S  M  R  A  T  K  K  Q  V  Q  G  S
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  T  V  S  I  T  P  A  V  I  K  Q  Y  Y  G  I  P  T  G  Q  I
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  G  V  E  S  E  N  F  Q  S  I  A  A  F  S  D  F  Y  S  S  G
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  A  L  Q  F  F  D  Q  K  F  G  I  D  S  S  T  V  R  V  K  N
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  V  G  Q  N  C  I  A  Q  N  C  D  Q  M  E  S  D  L  D  V  Q
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  Y  M  T  A  I  G  N  N  I  T  T  L  F  L  S  S  G  N  G  E
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  W  I  I  D  W  A  T  A  I  Q  Q  Y  N  P  I  P  K  I  A  S
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  S  G  N  C  P  I  D  G  T  K  Q  Y  C  P  L  G  G  C  N  H
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  K  S  S  Q  C  P  M  I  T  I  M  E  S  N  G  T  Q  C  F  F
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  Q  P  D  Y  P  A  S  S  P  F  I  T  S  V  G  A  T  Q  I  T
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  D  V  T  K  P  E  I  V  C  S  V  A  T  G  A  I  I  T  G  G
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  G  G  V  A  I  T  Q  A  Q  P  S  Y  Q  A  D  A  V  A  T  Y
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  I  L  L  L  L  V  M  L  M  N  R  V  P  N  T  L  T  S  N  T
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  C  P  C  A  L  E  S  V  D  G  T  S  C  S  S  P  T  L  A  G
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  M  I  S  L  I  N  D  K  L  I  G  A  G  K  P  T  L  G  F  L
TAAATCCATTATTATACCAAGCTGCCAAAGAACAACCAAACGTTTTCAATGATATTACCA
  N  P  L  L  Y  Q  A  A  K  E  Q  P  N  V  F  N  D  I  T  T
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  G  A  N  N  C  N  R  A  Y  C  C  Q  Y  G  Y  T  A  T  T  G
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  Y  D  A  A  S  G  L  G  S  I  N  F  K  N  F  E  Q  Y  V  L
TAAGTTTAAACTAAATAATATAATATAATAAATATATAAATAAATATATAATCGATTTAA
  S  L  N
CATTGAATTTTATTAATAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```
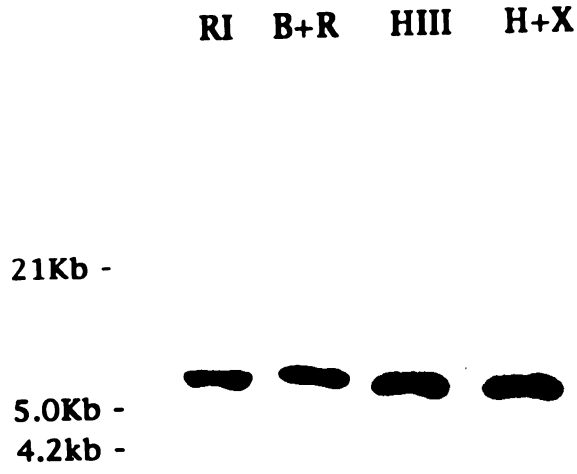

Fig. 4. Southern blot analysis. 10 μg of genomic DNA from KAX4 was digested by EcoR I, BamH I/EcoR I, Hind III and Hind III/Xho I and separated on agarose gel and electroblotted. Blot was probed with radiolabeled 1.8 kb insert DNA from the λ ZAP clone EHJ-1.


RI
B+R
HIII
H+X
21Kb -
5.0Kb -
4.2kb -

1.8 kb insert (EHJ-1). Northern blot analysis determined the EHJ-1 mRNA to be 2.3 Kb. The 1.8 kb DNA (EHJ-1) sequence was deposited in GenBank with an accession number U27540 (Fig. 3). The sequence has a conceptual 536 amino acids open reading frame, but may lack about 500 bp more for full size cDNA. The deduced peptide sequence did not have significant homology to other genes in GenBank data base search.

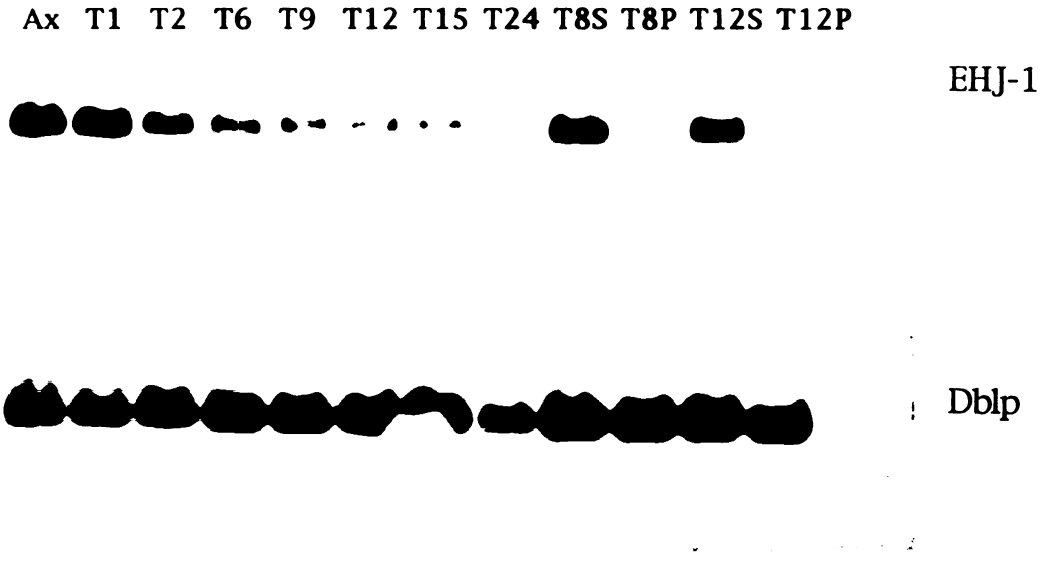
To determine the copy number of EHJ-1 gene Southern blot analysis was done. A single 6 Kb hybridized band was identified in restriction enzyme cut genomic DNA. Southern blot analysis suggests EHJ-1 may be present as a single copy gene in *D. discoideum* haploid genome (Fig. 4).

Developmental regulation of EHJ-1 mRNA and down-regulation of EHJ-1 mRNA by cAMP pulse

Northern blot analysis with the 1.8 kb EHJ-1 insert DNA as a probe showed that a single 2.3 kb RNA is hybridized and EHJ-1 mRNA level was highest in the actively growing vegetative, but reduced in developmental, stages (Fig. 2B and 5).

The expression of some developmentally regulated genes is controlled by cAMP (Mann and Firtel, 1987). To check whether EHJ-1 mRNA level was regulated by cAMP, a suspension culture was given cAMP pulses (Hassanain and Kopachik, 1989). Vegetative cells were harvested and resuspended in development buffer (2×10^6/ ml) and shaken for 4 h at 230 rpm. A pulse of 50nM cAMP was given every 10 min between 4 and 8 h (T8P and T12P in Fig. 5). The cyclic AMP pulse concentration and delivery interval replicate the

Fig. 5. Regulation of EHJ-1 mRNA on development and by cAMP pulse. RNAs were isolated at Vegetative (Ax), 1h (T1), 2h (T2), 6h (T6), 9h (T9), 12h (T12), 15h (T15) and 24h (T24) stages of development on agar. To check the regulation of EHJ-1 mRNA by cAMP pulses RNAs were isolated from cells of shaken suspension culture in DB. Vegetative amoebae were resuspended at 2 x10^7 cells/ml in DB buffer. After 4hr some cultures received additions of 50nM cAMP every 10min for the next 8hr. Total RNA was isolated from cells without(T12S) and with cAMP pulses(T12P) in suspension culture. The blot was probed with a radiolabeled 1.8 kb insert DNA from the λ ZAP clone EHJ-1. The constant expression of Dblp mRNA was used for even loading control.


Ax T1 T2 T6 T9 T12 T15 T24 T8S T8P T12S T12P
EHJ-1
Dblp

physiological rise and fall experienced by aggregating cells as amoebae relay and then degrade a cyclic AMP signal. The EHJ-1 mRNA level in cAMP-pulsed cells was about 10 fold less by 4h than control (without cAMP) and undetectable by 8 h (Fig. 5). Suspension cultured cells without cAMP pulse in development buffer had a nearly identical amount of EHJ-1 mRNA as do vegetative cells. The hybridization pattern for clone Dblp shows that an equal amount of RNA was loaded in all lanes.

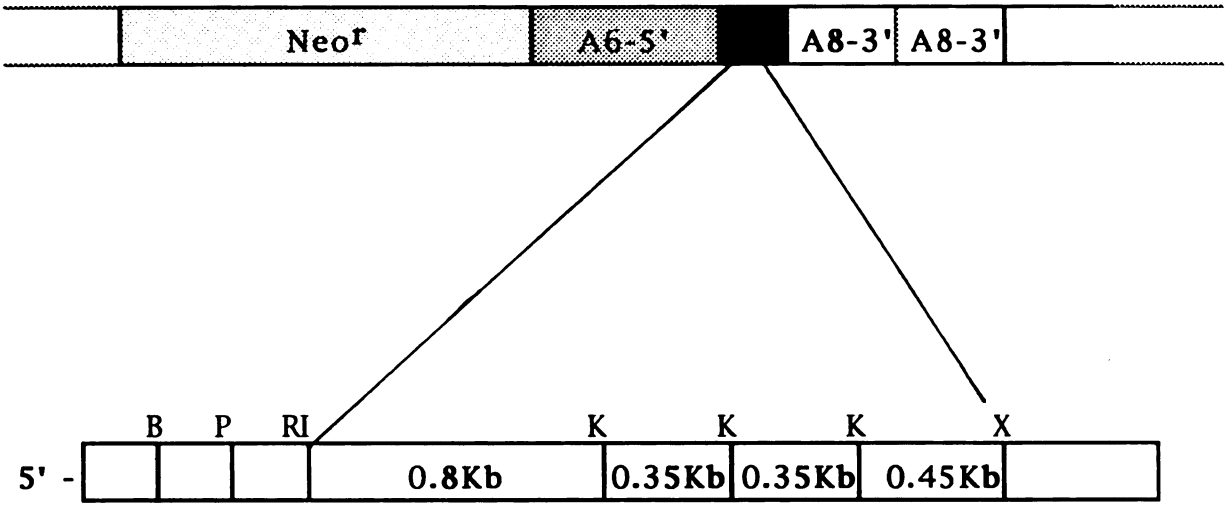
Whether EHJ-1 mRNA level is controlled at the transcriptional level or post-transcriptional level is unknown. We conclude that the cAMP relay during normal development on agar plate is essential for down-regulation of EHJ-1 mRNA.

Retardation of development by overexpression of truncated EHJ-1 mRNA

The largest EHJ-1 clone has 1.8 kb insert (Fig. 3). A translation product from EHJ-1 may lack the N-terminal region of the EHJ-1 protein. A 512 amino acid polypeptide should be synthesized from the 1.8 kb insert in transformation vector pDNeoII. Pst I and Xho I cut 1.8 kb DNA was inserted into pDNeoII and transformed into exponential growth phase KAX4 cells (Fig. 6). Several tiny colonies appeared after 7 days selection with 40 μg/ml of G418. Three transformants were isolated and two were further analyzed. The Southern blot analysis of two transformants showed the expected 1.3 kb Pst I/Bgl II fragment in both transformants, implying no illegitimate recombination in those transformants (Fig. 7). Both transformants grow at the same rate in axenic medium as control

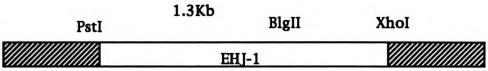
Fig. 6. Construction of transformation vector (pDNeo (EHJ-1)) for overexpression of EHJ-1. The insert of EHJ-1 in pBluescript II SK (Stratagene) was cut with Pst I/Xho I and ligated into Pst I/Xho I cut pDNeoII vector. K stands for restriction enzyme Kpn I sites in EHJ-1.

pDNeoII

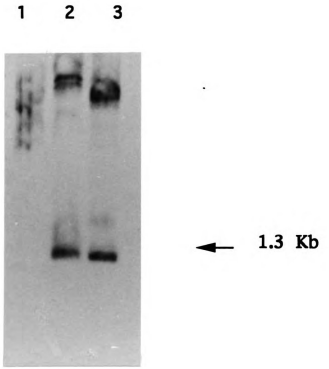


EHJ -1 in BS-SK

Fig. 7. Southern blot analysis for transformants overexpressing EHJ-1 mRNA. Geneomic DNA eas isolated from vegetative cells in axenic medium using mini-prep method (Reymond, 1987). DNA was cut with Pst I and Bgl II. Since EHJ-1 cDNA has a Bgl II site with 1.3 kb distance from a Pst I site in pDNeoII transformation vector, the cells transformed with EHJ-1 cDNA expected to have the 1.3 kb band in southern blot. Lane 1, vector alone transformant; Lane 2 and 3, two independent clone for EHJ-1 transformants.



EHJ-1 in pDNeoII



cells transformed with vector alone. However, the development of transformants on agar plate showed obvious phenotypic changes (Fig. 8). At 12 h development control cells form tight aggregates with few single cells (Fig. 8B). Two independent transformants showed delayed development (Fig. 8C and D). Transformants did not form tight aggregates and many single cells remained. Both control and transformed cells formed fruiting bodies at about 24h but the fruiting bodies in transformants were significantly smaller (Fig. 8E and F). The experiment was repeated several times to confirm the retardation of development. To determine the RNA level total RNA was isolated from vegetative, T3 and T12 cells of control and two transformants. Northern blot analysis showed the 1.8 kb mRNA was overproduced constitutively (Fig. 9). Possibly the retardation of development in transformants was due to over-production of truncated EHJ-1 mRNA in developmental stages.

DISCUSSION

A vegetative specific gene, EHJ-1, was cloned using differential DDRT-PCR. EHJ-1 mRNA is reduced in developing cells and down-regulated by cAMP pulse. Overexpression of EHJ-1 cDNA caused the retardation of development in transformants. The function of EHJ-1 is unknown. The deduced peptide sequence EHJ-1 did not have significant homology to known genes.

Starvation enhances the expression of aggregation stage-specific genes and represses vegetative specific genes (Kimmel, 1987; Mann and Firtel, 1987, 1989). Four hours after starvation, secretion

Fig. 8. The development of pDNeo(EHJ-1) transformants. (A) 0 h development of transformants. (B, F) 12 h and 24 h development of vector alone transformant. (C, D) 12 h and (E) 24 h development of pDNeo(EHJ-1) transformants clone 1 and 2. The phenotypes of transformant clone 1 and 2 were the same. The development of clone 1 is shown (E).

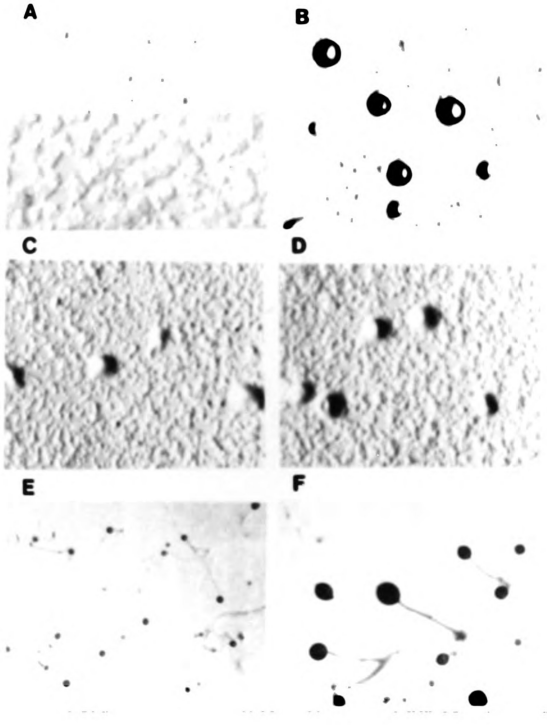

A
B
C
D
E
F

Fig. 9. Northern blot analysis of pDNeo(EHJ-1) transformants. Total RNA of vegetative (V), 3 h (T3) and 15 h (T15) development was isolated from transformants. 10 μg of total RNAs were used for each lanes. The blot was probed with a radiolabelled 1.8 kb insert of pEHJ-1.

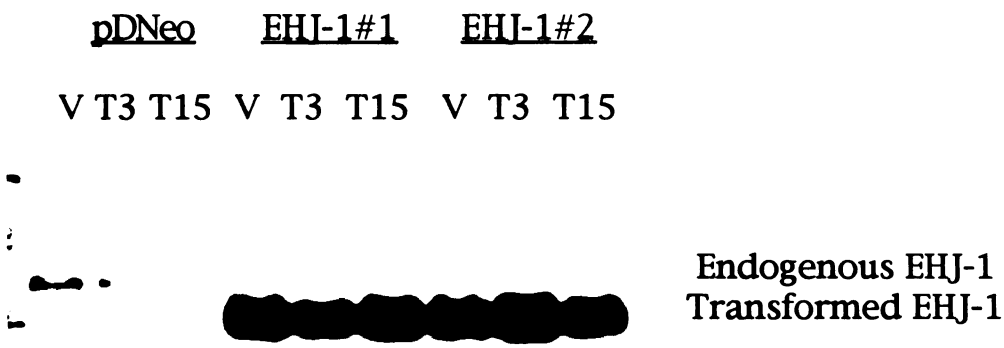
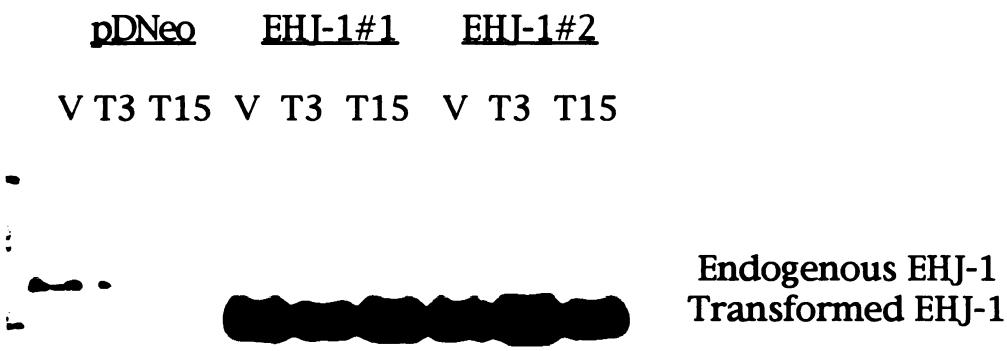

pDNeo
EHJ-1#1
EHJ-1#2
V T3 T15 V T3 T15 V T3 T15
Endogenous EHJ-1
Transformed EHJ-1

Fig. 9. Northern blot analysis of pDNeo(EHJ-1) transformants. Total RNA of vegetative (V), 3 h (T3) and 15 h (T15) development was isolated from transformants. 10 μg of total RNAs were used for each lanes. The blot was probed with a radiolabelled 1.8 kb insert of pEHJ-1.


pDNeo
EHJ-1#1
EHJ-1#2
V T3 T15 V T3 T15 V T3 T15
Endogenous EHJ-1
Transformed EHJ-1

of cAMP regulates the expression of some developmentally expressed genes (Gross, 1994). EHJ-1 mRNA level is controlled in somewhat different ways. The same level of EHJ-1 mRNA in cells in non-nutrient DB and growing cells (Fig. 5) suggests that starvation is not a signal for reduction of EHJ-1 mRNA. The reduction of EHJ-1 mRNA in 2 h development on agar (Fig. 5) implies other mechanisms besides cAMP related regulation. Nuclear run-on experiments (Nellen *et al.* 1989) will show if EHJ-1 mRNA expression is controlled at transcriptional or post-transcriptional level.

The pDNeoII vector uses an actin 6 promoter to control the expression of insert. Actin 6 mRNA is present at a very low level in vegetative cells, increased in pre-aggregated cells and reduced in post-aggregative cells (Knecht *et al.*, 1986). To determine the role of EHJ-1 in growth and development, blocking of EHJ-1 expression by antisense RNA and overexpression of EHJ-1 mRNA expriments were done. The antisense RNA experiment was not informative due to not enough production of antisense RNA to block EHJ-1 expression. Nevertherless, constant overexpression of transformed EHJ-1 mRNA in both transformant clones was observed (Fig. 8). It is possible that 5' end of untranslated EHJ-1 mRNA regulates the stability. To test that possibility transformation of full size EHJ-1 cDNA and determination of transgene mRNA level may be an interesting experiment. The low level of transgenic EHJ-1 will prove that possibility. To confirm that possibility transformation of chimeric DNA and checking the level of transgene mRNA can be done. Chimeric DNA will contain 5' end of EHJ-1 in its 5' end and DNA producing stable mRNA in 3' end.

It is not clear how overexpression of a specific gene, which is abundant in vegetative and early developing cells, causes retardation of development. Since no clue for the function of EHJ-1 is available, it is difficult to guess the mechanism for retardation. Several explanations, however, are possible. First, if EHJ-1 encodes a regulatory enzyme which may control gene expression in growing cells, overproduction may lead to extended production of vegetative specific genes until late developmental stages causing retardation of development. Second, EHJ-1 is maybe essential for transition from vegetative to developmental stages. McPherson and Singleton (1992) showed that blocking of vegetative specific gene, V4, caused retardation of development. A truncated EHJ-1 might not produce functional protein and the competition between non-functional protein with endogenous EHJ-1 results deficiency of functional EHJ-1 in transition from vegetative to developmental stages. The lack of functional EHJ-1 binding to active site may cause retardation of development. Third, too much expression of exogenous RNA (and maybe protein) resulted in poor development. The presence of excess EHJ-1 might not harm to cells in the presence of good nutrient condition but it possibly is a burden to starving developmental cells.

Acknowledgment

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II. CHAPTER TWO : Cloning and characterization of a *Dictyostelium discoideum* cDNA encoding a G protein β like protein

Chapter two was submitted into Molecular General Genetics.

Introduction

Guanine nucleotide binding proteins (G proteins) are involved in signal transduction in eukaryotic systems (Gilman 1987; Birnbaumer 1992). G proteins are generally found as a heterotrimeric complex of α,β and γ subunits (Neer 1995). The activated forms of α subunit regulate many enzyme activities including adenylyl cyclase and potassium channels (Birnbaumer 1992; Codina *et al.* 1987). The $\beta\gamma$ dimer regulates phospolipase A_2, targeting of the β-adrenergic receptor kinase and the yeast mating and pheromone response (Kim *et al.* 1989; Pitcher *et al.* 1992; Whiteway *et al.* 1989, 1990).

cDNAs for different types of Gα and β subunits have been isolated from many eukaryotes (Simon *et al.* 1991). The deduced peptide sequences for the Gβ subunits show about 60 to 70% sequence identity in seven repeat domains. Guillemot *et al.* (1989) isolated a chicken cDNA C12.3 and a human cDNA homolog H12.3 which although had seven homologous repeats had only 26% amino acid sequence identity to Gβ subunits. Other cDNA clones with weak homology to Gβ subunits but strong homology (60 to 70 % amino acid identity) to clone C12.3 and H12.3 have been isolated from algae, tobacco, and rat (Schloss 1990; Ishida *et al.* 1993; Ron *et al.* 1994). The rat homolog, in the Gβ like family, was identified as a receptor activated C kinase (RACK1) (Ron *et al.* 1994). Gβ-like proteins may be trans-membrane signaling molecules (Schloss 1990; Ishida *et al.* 1993).

A diverse set of eukaryotic regulatory proteins have a variable number of the amino acid sequence motif repeat, called the WD-40

(Trp-Asp 40) repeat (Neer *et al.* 1994; Voorn and Ploegh 1992). For example, Gβ subunit, Tup1 and RACK1 proteins have seven WD-40 repeats and PRP4 has four (Neer *et al.* 1994). The function, however, of the WD-40 repeats of those proteins is obscure.

In *Dictyostelium discoideum*, eight different types of Gα, and one type of Gβ, cDNAs were cloned (Wu *et al.* 1994; Lilly *et al.* 1993). Gene targeting revealed an important role in cAMP mediated responses for Gα2 (Kumagai *et al.* 1991) and in multicellular structure formation Gα4 (Hadwiger and Firtel 1992). Disruption of the β-subunit indicates it is required for cell aggregation (Lilly *et al.* 1993).

In response to starvation *D. discoideum* amoebae initiate differentiation. About 10^5 amoebae aggregate in response to cAMP pulses to form a tipped aggregate at 10 to 12h after starvation. Differentiation continues with formation of a migrating slug and ends with construction of a mature fruiting body at 24 h (Gross 1994).

In this chapter I show the primary sequence of a *Dictyostelium* cDNA encoding a Gβ-like protein and expression of the corresponding mRNA in vegetative and developmental stages.

Materials and methods

A λ ZAP clone from a vegetative cell cDNA library was isolated as a phagemid by *in vivo* excision of the cloning vector as described by Short *et al.* (1988). The 1.0 kb insert of the phagemid clone was sequenced using the Sequenase DNA sequencing kit (United States

Biochemical). This clone lacked a start codon at the 5' extremity. The missing 5' fragment was obtained by anchored PCR (Loh *et al.* 1989) using a primer from the λ ZAP clone (5'-ACGGCAATAGAGGTGAC-3') and BS-SK primer with the λ ZAP cDNA library as a template. RNA isolation and Northern blot analysis were done as previously described (Hassanain and Kopachik 1989). For Southern blot analysis genomic DNA was isolated from *D. discoideum* strain KAX4 and digested with several different enzymes. The 1.0 kb insert of λ ZAP clone was used to probe the blot. The 1.0 kb DNA sequence encoding Dblp has been deposited in the GenBank/EMBL nucleotide sequence database with accession number U27537.

Results and discussion

The nucleotide sequence of Dblp contains a single open reading frame (ORF) of 996 bp (Fig. 1). The conceptual translation of the ORF gives a protein of 332 amino acid residues, with a calculated M_r of 36,430. A search of the GenBank database with the FASTA program (Devereux 1991) showed the amino acid sequence had 57% identity over its entire length with the rat RACK 1, and 56% to *Chlamydomonas*, Gβ-like polypeptide (Fig. 2). Its sequence, however, had only 26% identity to the *Dictyostelium* Gβ (Lilly *et al.* 1993). Dblp met three criteria suggested by Neer *et al.* (1994) to identify true functional homologues among WD-repeat proteins: (1) Dblp has seven WD repeats as do Gβ-like proteins. (2) The repeating units are at equivalent positions to other Gβ-like proteins and the sequence of

Fig. 1 Nucleotide and deduced amino acid sequence of Dblp cDNA. A possible initiator methionine is present at nucleotide position 68 and an open reading frame extends to nucleotide position 1063. The underlined sequence was used to make an antisense anchored PCR primer.

```
        10                  30                  50
GGCACGAGGAGAGAGAGAGAGAGAACTAGTTTCAGTTTTTCTTTAGCCAATAGTATTC
        70                  90                  110
ATCAGAAATGGAACAACAAAAAGCACCACAAGTTACTTACTTAGAAGTCGGATCATTAGT
       M  E  Q  Q  K  A  P  Q  V  T  Y  L  E  V  G  S  L  V
       130                 150                 170
TGGTCACAACGGTTTTGTCACCTCTATTGCCGTTTCACCAGAAAACCCAGATACCATCAT
 G  H  N  G  F  V  T  S  I  A  V  S  P  E  N  P  D  T  I  I
       190                 210                 230
TTCATCATCACGTGATAAGACTGTTATGGTATGGCAATTAACCCCAACTGATGCCACCTC
 S  S  S  R  D  K  T  V  M  V  W  Q  L  T  P  T  D  A  T  S
       250                 270                 290
ACCAGGTAAAGCCCACAGATCACTCAAGGGTCACTCACACTTTGTTCAAGATGTTGTCAT
 P  G  K  A  H  R  S  L  K  G  H  S  H  F  V  Q  D  V  V  I
       310                 330                 350
TTCCCACGACGGTCAATTCGCCTTATCAGGTTCATGGGATAATACCTTAAGATTATGGGA
 S  H  D  G  Q  F  A  L  S  G  S  W  D  N  T  L  R  L  W  D
       370                 390                 410
TATCACCAAAGGTGTTTCAACCCGTCTCTTCAAAGGTCACACTCAAGATGTTATGTCTGT
 I  T  K  G  V  S  T  R  L  F  K  G  H  T  Q  D  V  M  S  V
       430                 450                 470
TGCCTTCTCATCAGACAACCGTCAAATCATTTCAGGTTCACGTGATGCCACCATCAAAGT
 A  F  S  S  D  N  R  Q  I  I  S  G  S  R  D  A  T  I  K  V
       490                 510                 530
TTGGAACACCCTCGGTGAATGTAAATTCACTTTAGAAGGTCCAGAAGCTCATCAAGCCGT
 W  N  T  L  G  E  C  K  F  T  L  E  G  P  E  A  H  Q  A  V
       550                 570                 590
ACTGATTGGGTTTCATGTATCAGATTCTCACCAAAACACCCCAACCATCGTTTCAGGTTC
 L  I  G  F  H  V  S  D  S  H  Q  N  T  P  T  I  V  S  G  S
       610                 630                 650
ATGGGATAACAAAGTTAAGATCTGGGATATCAAGAGCTTCAAATGCAACCACACCTTAAC
 W  D  N  K  V  K  I  W  D  I  K  S  F  K  C  N  H  T  L  T
       670                 690                 710
TGACCATACCGGTTACGTCAACACTGTCACCATCTCTCCAGACGGTTCATTATGTGCCTC
 D  H  T  G  Y  V  N  T  V  T  I  S  P  D  G  S  L  C  A  S
       730                 750                 770
TGGTGGTAAAGATACCTTTGCTTGTCTCTGGGAATTATCATCTGGTAAACCATTATACAA
 G  G  K  D  T  F  A  C  L  W  E  L  S  S  G  K  P  L  Y  K
       790                 810                 830
ATTAGAAGCTCGTAACACCATCAATGCTCTTGCTTTCTCACCAAACAAATATTGGTTATC
 L  E  A  R  N  T  I  N  A  L  A  F  S  P  N  K  Y  W  L  S
       850                 870                 890
TGCTGCCACTGATGACAAAATCATCATTTGGGATCTCCTCACCAAACAAGTTCTCGCTGA
 A  A  T  D  D  K  I  I  I  W  D  L  L  T  K  Q  V  L  A  E
       910                 930                 950
AATCGTCCCAGAAGTCAAAGAACAAGCTTTCGACTCAAAGAAAAAGAAAGAATCAAAACC
 I  V  P  E  V  K  E  Q  A  F  D  S  K  K  K  K  E  S  K  P
       970                 990                 1010
AAAAGCACCAGCTTGTCTCTCCCTCGCTTGGTCTGCTGATGGTTCAGTCTTATATGCTGG
 K  A  P  A  C  L  S  L  A  W  S  A  D  G  S  V  L  Y  A  G
      1030                1050                1070
TTACAATGATGGTTTAATCCGTGTTTACAAATCATCATCCCAATAAATTTTTTAATTAAA
 Y  N  D  G  L  I  R  V  Y  K  S  S  S  Q
      1090
ATTTCAAAAAAAAAAAAAAAAA
```

Fig. 2 Alignment of the deduced amino acid sequences of Gβ subunit-like protein cDNAs using Genetics Computer Group Software (Devereux 1991). Dblp, *Dictyostelium* (this work); Cblp, *Chlamydomonas* (Schloss 1990); H12.3, human (Guillemot et al. 1989); RACK 1, rat (Ron et al. 1994); *arcA*, tobacco (Ishida et al. 1993) Gβ subunit-like proteins. Seven WD-40 repeats are shown as described by Fong (Fong et al. 1986). (•) : All five sequences are identical. (o) : Conservative replacement. Conservative groupings are (A,G), (I,L,V,M), (S,T), (R,K), (D,E), (D,N), and (E,Q). V1 and V2 stands for variable regions in number of amino acids among repeats.

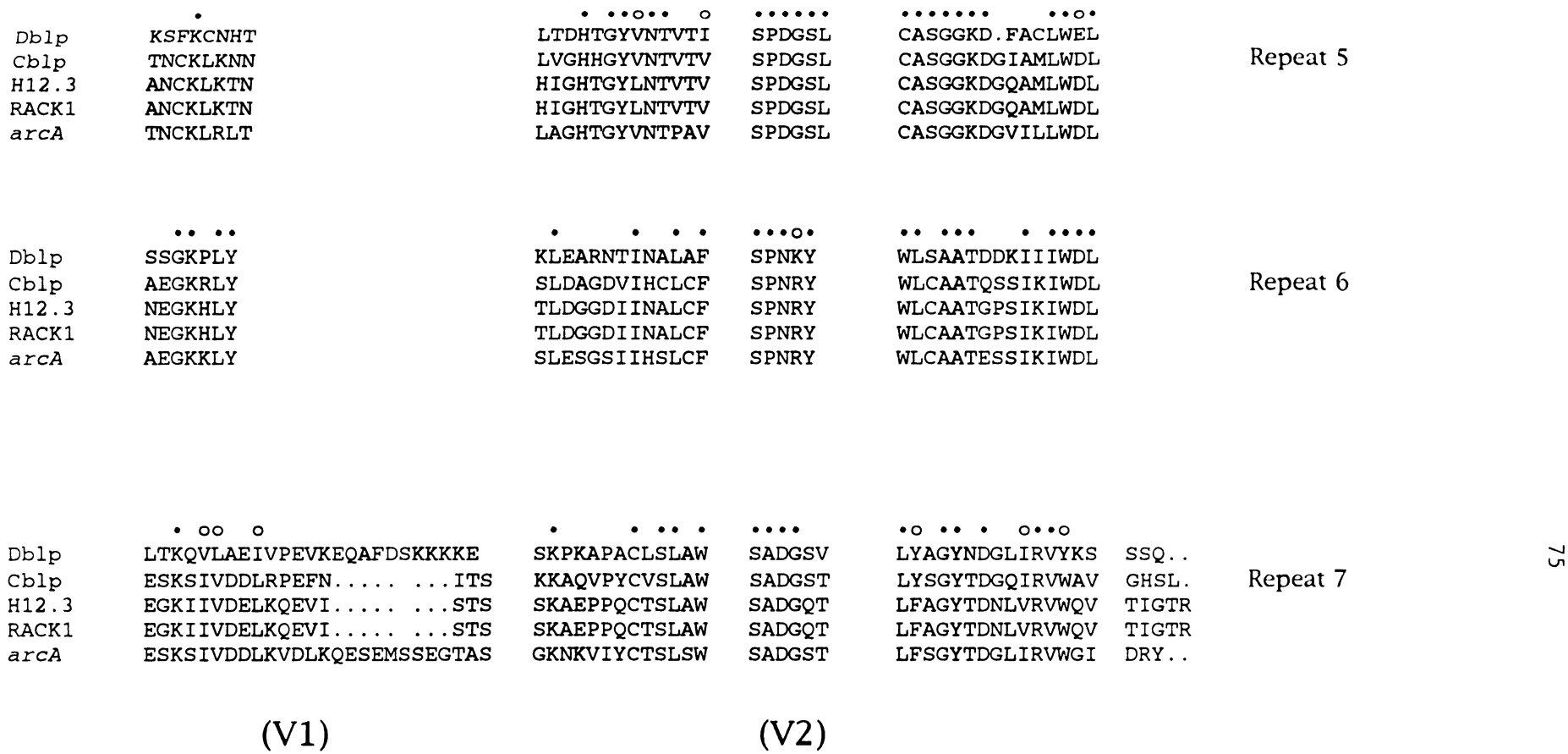
```
                          oo                o o•  o•• ••          o    ooo ••••oooo• o
Dblp       MEQQKAPQVTYLEVGS                 LVGHNGFVTSIAV   SPEN.PDT   IISSSRDKTVMVWQL
Cblp       ......MAETLTLRAT                 LKGHTNWVTAIAT   PLDPSSNT   LLSASRDKSVLVWEL
H12.3      ......MTEQMTLRGT                 LKGHNGWVTQIAT   TPQ.FPDM   ILSASRDKTIIMWKL
RACK1      ......MTEQMTLRGT                 LKGHNGWVTQIAT   TPQ.FPDM   ILSASRDKTIIMWKL
arcA   .....   MSQESLVLRGT                 MRAHTDWVTAIAT   AVDN.SDM   IVTSSRDKSIIVWSI

                    •  oo                  • •• ••• •••o   • •• •     •o•••• ••••••
Dblp       TPTDATSPGKAHRS                   LKGHSHFVQDVVI   SHDGQF     ALSGSWDNTLRLWDI
Cblp       ER.SESNYGYARKA                   LRGHSHFVQDVVI   SSDGQF     CLTGSWDGTLRLWDL
H12.3      TR.DETNYGIPQRA                   LRGHSHFVSDVVI   SSDGQF     ALSGSWDGTLRLWDL
RACK1      TR.DETNYGIPQRA                   LRGHSHFVSDVVI   SSDGQF     ALSGSWDGTLRLWDL
arcA       TK.DGPQYGVPRRR                   LTGHGHFVQDVVL   SSDGMF     ALSGSWDGELRLWDL

              • o •                        • ••• ••o•••• • ••••     •o•o••• o•oo•••
Dblp       TKGVSTRL                         FKGHTQDVMSVAF   SSDNRQ     IISGSRDATIKVWNT
Cblp       NTGTTTRR                         FVGHTKDVLSVAF   SVDNRQ     IVSGSRDKTIKLWNT
H12.3      TTGTTTRR                         FVGHTKDVLSVAF   SSDNRQ     IVSGSRDKTIKLWNT
RACK1      TTGTTTRR                         FVGHTKDVLSVAF   SSDNRQ     IVSGSRDKTIKLWNT
arcA       QAGTTARR                         FVGHTKDVLSVAF   SVDNRQ     IVSASRDKSIRLWNT

           •• ••o•o                         o •   o         •     •    ••• ••  ••o•oo
Dblp       LGECKFTLEG                       PEAHQAVLIGFHV   SDSHQNTPT  IVSGSWDNKVKIWDI
Cblp       LGECKYTIGE                       PEGHTEWVSCVRF   SPMTTN.PI  IVSGGWDKMVKVWNL
H12.3      LGVCKYTVQ                        DESHSEWVSCVRF   SPNSSN.PI  IVSCGWDKLVKVWNL
RACK1      LGVCKYTVQ                        DESHSEWVSCVRF   SPNSSN.PI  IVSCGWDKLVKVWNL
arcA       LGECKYTIQD                       GDSHSDWVSCVRF   SPNNLQ.PT  IVSGSWDRTVKIWNL
```

Repeat 1

Repeat 2

Repeat 3

Repeat 4


Dblp KSFKCNHT LTDHTGYVNTVTI SPDGSL CASGGKD.FACLWEL
Cblp TNCKLKNN LVGHHGYVNTVTV SPDGSL CASGGKDGIAMLWDL
H12.3 ANCKLKTN HIGHTGYLNTVTV SPDGSL CASGGKDGQAMLWDL
RACK1 ANCKLKTN HIGHTGYLNTVTV SPDGSL CASGGKDGQAMLWDL
arcA TNCKLRLT LAGHTGYVNTPAV SPDGSL CASGGKDGVILLWDL
Repeat 5
Dblp SSGKPLY KLEARNTINALAF SPNKY WLSAATDDKIIIWDL
Cblp AEGKRLY SLDAGDVIHCLCF SPNRY WLCAATQSSIKIWDL
H12.3 NEGKHLY TLDGGDIINALCF SPNRY WLCAATGPSIKIWDL
RACK1 NEGKHLY TLDGGDIINALCF SPNRY WLCAATGPSIKIWDL
arcA AEGKKLY SLESGSIIHSLCF SPNRY WLCAATESSIKIWDL
Repeat 6
Dblp LTKQVLAEIVPEVKEQAFDSKKKKE SKPKAPACLSLAW SADGSV LYAGYNDGLIRVYKS SSQ..
Cblp ESKSIVDDLRPEFN..... ...ITS KKAQVPYCVSLAW SADGST LYSGYTDGQIRVWAV GHSL.
H12.3 EGKIIVDELKQEVI..... ...STS SKAEPPQCTSLAW SADGQT LFAGYTDNLVRVWQV TIGTR
RACK1 EGKIIVDELKQEVI..... ...STS SKAEPPQCTSLAW SADGQT LFAGYTDNLVRVWQV TIGTR
arcA ESKSIVDDLKVDLKQESEMSSEGTAS GKNKVIYCTSLSW SADGST LFSGYTDGLIRVWGI DRY..
Repeat 7
(V1)
(V2)

the repeats are more similar to each other than to any other repeating units. For example, the third WD-repeat in Dblp has 94% amino acid sequence similarity and 85% identity to the third WD-repeat of human homolog. Only one exception was identified. The fourth WD-repeat in Dblp has the highest homology to the third WD-repeat in rat RACK1. However, the fourth WD-repeat in Dblp shows highest homology to the fourth repeat of *Chlamydomonas*, and tobacco, Gβ-like protein. (3) Dblp has very short amino- and carboxy-terminal extentions as do other Gβ-like proteins.

To determine the copy number of the Dblp gene, Southern blot hybridization analysis was done. The autoradiograph from each lane of the blot containing genomic DNA digested with a restriction enzyme had a single band that hybridized to the ^{32}P-labeled 1.0 kb probe DNA (Fig. 3). This evidence suggests there is a single copy of the Dblp gene present in the genome.

The regulation of Dblp mRNA level at different developmental stages was assessed by Northern blot analysis. A 1.1 kb of Dblp mRNA was constitutively expressed from the vegetative and developing cells (Fig. 4). The closeness of the mRNA to the cDNA size suggests the cDNA sequence is full length.

Ron *et al.* (1994) have a model for the control of the transition from inactive to active PKC. In the inactive conformation a pseudo-RACK domain of six amino acid sequence in the C2 region on PKC (homologous to a RACK1 six amino acid domain) occupies the RACK1 binding site and prevents access of substrate to the substrate-binding site. In the active PKC conformation RACK1 displaces the pseudo-RACK sequence from the RACK1 binding site and allows

Fig. 3 Southern blot hybridization analysis of *Dblp* gene per haploid genome of *D. discoideum*. 10μg of genomic DNA for *D. discoideum* was digested by BamH I (lane 1), EcoR I (lane 2), BamH I and EcoR I (lane 3), and Hind III (lane 4). The blots were probed with ^{32}P-radiolabeled 1.0 kb insert of the λ ZAP clone.

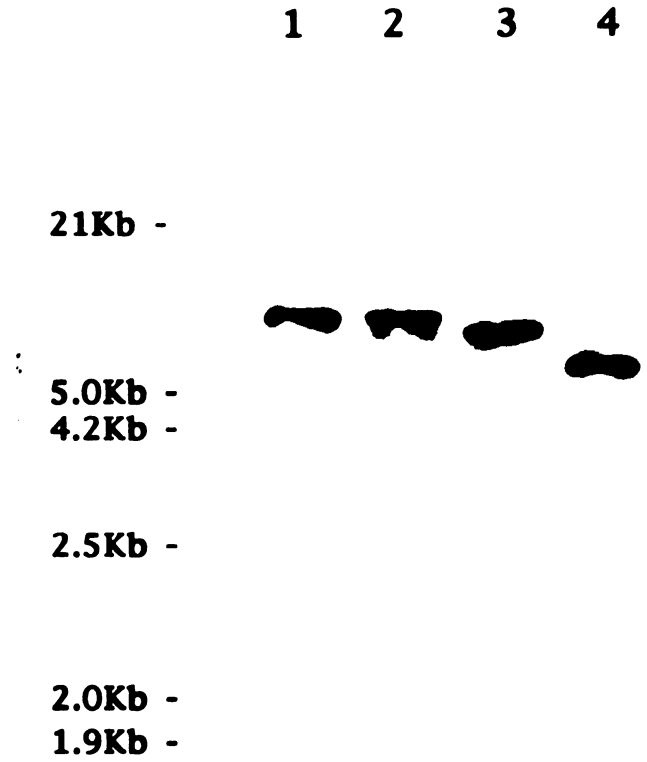
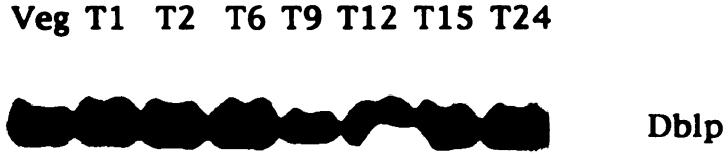

1
2
3
4
21Kb -
5.0Kb -
4.2Kb -
2.5Kb -
2.0Kb -
1.9Kb -

Fig. 4 Northern blot hybridization analysis. Expression of Dblp mRNA during vegetative and development stages on agar plate (5mM Na_2HPO_4, 5mM NaH_2PO_4, 2mM $MgSO_4$ and 200μM $CaCl_2$, pH6.5). RNAs were isolated at vegetative (veg), 1h (T1), 2h (T2), 6h (T6), 9h (T9), 12h (T12), 15h (T15) and 24h (T24) stages. 10μg of total RNAs were used for each lane and the blot was probed with ^{32}P-radiolabeled 1.0 kb insert of the λ ZAP clone.


Veg T1 T2 T6 T9 T12 T15 T24
Dblp

access to the substrate binding site. In *Dictyostelium* the myosin heavy chain kinase (MHCK), a member of the protein kinase C family, is translocated to myosin in response to cAMP (Ravid and Spudich, 1992). Translocated MHCK phosphorylates the myosin heavy chain, and leads to inhibition of myosin thick filament formation during chemotaxis (Ravid and Spudich, 1989; Pasternak *et al.* 1989). It is possible that Dblp plays a role in the translocation of MHCK. A comparison of Dblp and MHCK amino acid sequence by the Bestfit program (Devereux, 1991) found the amino acid sequence VMVWQL (46-51) in Dblp showed strong homology to MHCK sequence VMIWHL (311-316). The homologous sequence falls in the C2 region of MHCK as does the pseudo-RACK sequence of rat PKC (Fig. 5).

The single Gβ subunit may participate in the formation of heterotrimers with all of the α subunits in *Dictyostelium* (Wu *et al.* 1995). The normal growth of Gβ-null cell suggested that heterotrimeric G proteins are not necessary for cell growth (Wu *et al.* 1995). It is not known if the Gβ-like proteins form heterotrimers. Therefore it will be important to determine if Dblp can interact with other Gα subunits to assess the role of G protein related signal transduction in cell growth. Disruption of Dblp gene by homologous recombination should enable us to determine the role of the Gβ like protein in growth and development of *Dictyostelium*.

Acknowledgments We thank Drs. Neal R. Band and Ronald J. Patterson for helpful comments on the manuscript and Dr. Herbert L.

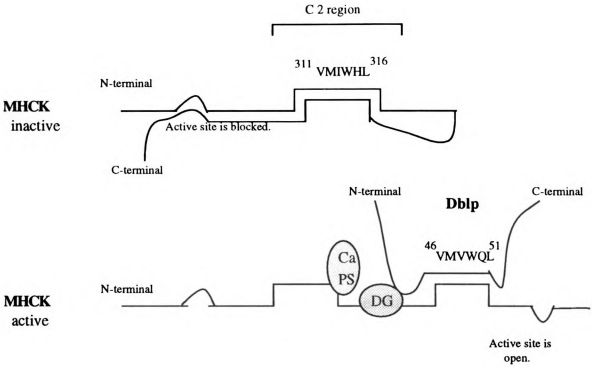


Fig. 5. Model of inactive and active forms of MHCK.

DG, Diacylglycerol; Ca, Calcium; PS, phosphatidylserine.

Ennis (Roche Institute of Molecular Biology) for providing the vegetative λ ZAP cDNA library.

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III. CHAPTER THREE : Evidence for leukotriene-related signal transduction in *Dictyostelium discoideum* .

Introduction

A G protein-coupled receptor stimulates phospholipase A2 or the combined action of phospholipase C and a diacylglycerol-lipase, and leads to synthesis of arachidonic acid in mammals (Van den Bosch, 1980; Irvine, 1982). Arachidonic acid can be metabolized in mammals through lipoxygenase and cycloxygenase pathways (Fig. 1; Parker, 1987). The cyclooxygenase pathway leads to formation of prostaglandins. Arachidonic acid is metabolized to 5-hydroperoxytetraenoic acid (5-HPETE) and HPETE is subsequently converted to unstable epoxide intermediate, leukotriene A_4 (LTA_4) by 5-lipoxygenase. Leukotrienes are a class of bioactive compounds which have diverse roles in inflammation and hypersensitivity reactions (Samuelsson *et al.*, 1987; Samuelsson and Funk, 1989).

Arachidonic metabolites including HPETE and leukotrienes have been shown as signal transduction molecules in several different systems. 1) In human blood polymorphonuclear leukocytes, occupancy of leukotriene B_4 (LTB_4) receptors induces the release of lysosomal enzymes, superoxide generation and the chemotactic migration and adhesion of leukocytes to endothelial cells (Ford-Hutchison *et al.*, 1980; Samuelsson *et al.*, 1987). 2) In mammalian systems, G protein coupled activation of LTD_4 receptors sequentially

increases intracellular Ca^{++}, inositol (1,4,5) triphosphate (IP_3), diacylglycerol and then activates protein kinase C (PKC). Activation of PKC increases expression of genes for signal transduction for another cycle of leukotriene synthesis. The newly synthesized leukotrienes are secreted and used for signal transduction molecules (Crooke *et al.*, 1990). 3) The opening of cardiac muscarinic K^+-channel can be regulated by arachidonic acid metabolites (Kurachi *et al.* 1989; Kim *et al.*, 1989). 4) In the nervous system of the marine mollusc *Aplysia*, arachidonic acid metabolites are used for second messengers in the direct modulation of K^+ channels (Piomelli *et al.* 1987; Buttner *et al.*, 1989).

LTA_4 is converted into leukotriene B_4 (LTB_4) by leukotriene A_4 hydrolase. LTA_4 hydrolase (EC 3.3.2.6) is a cytosolic enzyme and its cDNA has been cloned from human, mouse and rat (Funk et al., 1987; Minami et al., 1987; Medina et al., 1991; Makita et al., 1992). The primary sequence of LTA_4 hydrolase has weak homology (about 20%) to aminopeptidases (Malfroy *et al.*, 1989) and a zinc binding motif characteristic of aminopeptidase is present. LTA_4 hydrolase also possesses peptidase activity (Haeggstrom *et al.*, 1990)

Here the partial cDNA sequence of *Dictyostelium discoideum* LTA_4 hydrolase homolog (Ddlta4) and the developmental regulation of this mRNA is examined. Previously the cDNA for LTA4 hydrolase was only cloned from mammalian system. Therefore this is the first cloning of LTA_4 hydrolase from a non-mammalian cell.

Prc

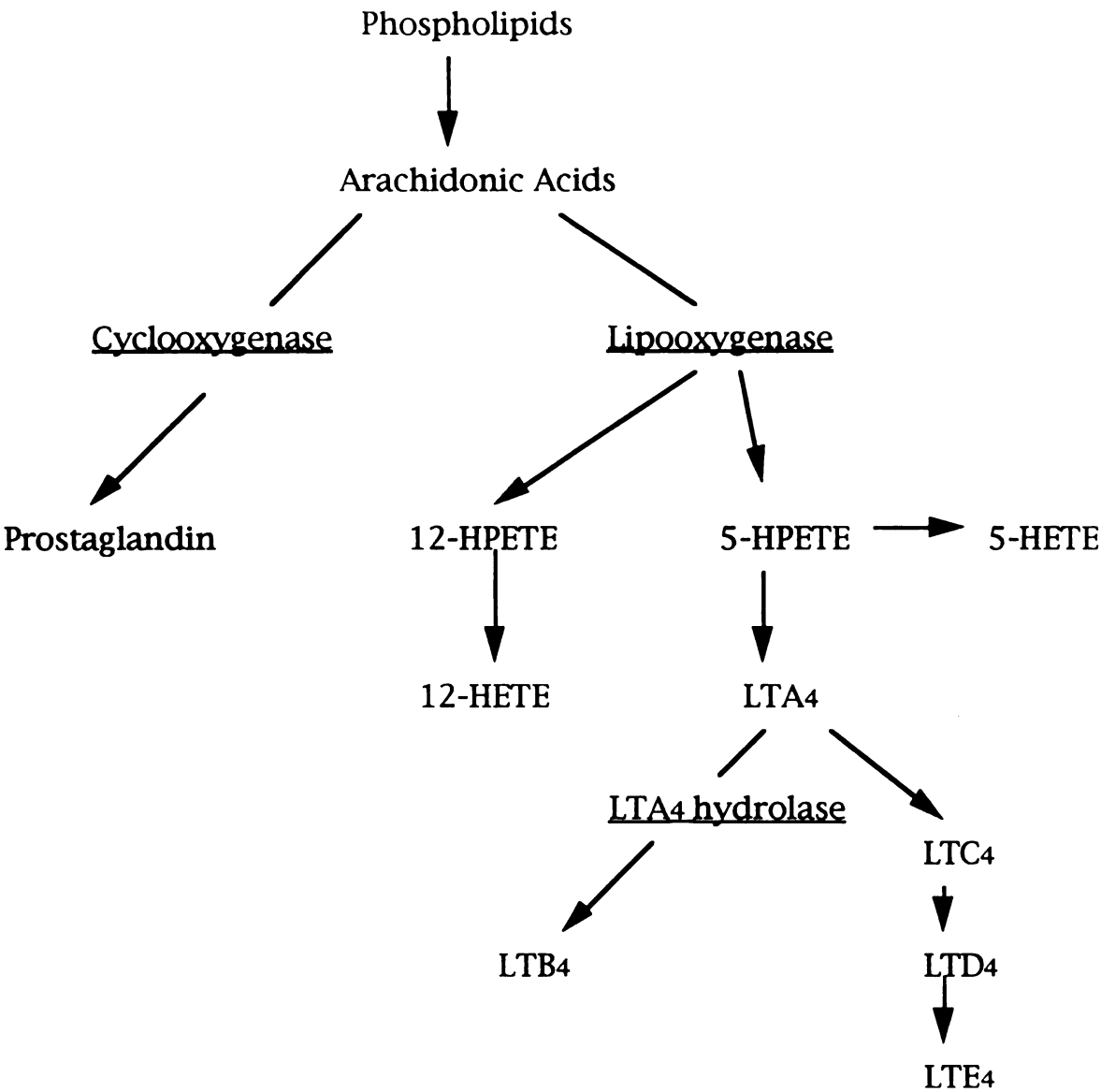


Fig.1. Pathways of arachidonic acid metabolism

Material and Methods

A λ ZAP cDNA clone (Ddlta4) with a 1.0 kb insert was isolated from a λ ZAP cDNA library made from *D. discoideum* vegetative cells. Its primary sequence was determined by using sequenase DNA sequencing kit (United States Biochemical). RNA isolation and Northern blot analysis were done as previously described (Hassanain and Kopachik 1989). For Southern blot analysis genomic DNA was isolated from *D. discoideum* strain KAX4 and digested with several different enzymes. The 1.0 kb insert of λ ZAP clone was used to probe the blot. The 1.0 kb DNA sequence encoding Ddlta4 has been deposited in the GenBank/EMBL nucleotide sequence database with accession number U27538.

Results and discussion

The nucleotide sequence of Ddlta4 contains a single open reading frame (ORF) of 948 bp (Fig. 2). The deduced peptide sequence of clone Ddlta4 has 59% sequence similarity (39.2% and 36.2% identity) to the carboxy terminal region of the human and mouse leukotriene A_4 hydrolase (Fig. 3). Ddlta4 amino acid sequence showed weak homology to rabbit aminopeptidase (27% identity in 167 aa overlap) same as other mammalian LTA_4 hydrolases do. Northern blot analysis shows that the full size of mRNA should be 2.2 Kb which is similar to the size of mammalian LTA_4 hydrolase mRNA (Fig. 4; Funk *et al.*, 1987). Besides significant homology in size and amino acids

Fig. 2. Nucleotide and deduced amino acid sequence of Ddlta4 cDNA. Two putative polyadenylation sequences are underlined. EcoR I and Hind III restriction enzyme sites in this clone was marked.

```
        10                  30                  50  EcoR I
GGCACGAGGCTCATAGTTGGTGTGGTAATTTAGTAACAAATAAATATTGGTCAGAATTCT
  H  E  A  H  S  W  C  G  N  L  V  T  N  K  Y  W  S  E  F  F
        70                  90                 110
TTTTAAATGAAGGTTTTACAGTATTTGTTGAAAGAAAGATTCTTGGTCGTCTTTATGGTG
  L  N  E  G  F  T  V  F  V  E  R  K  I  L  G  R  L  Y  G  E
      130                 150                 170
AAGAAATGTTTGATTTTGAAGCAATGAATGGTTTGAAACATCTTCATGATGATGTTGATT
  E  M  F  D  F  E  A  M  N  G  L  K  H  L  H  D  D  V  D  L
      190                 210                 230
TATTCACACATAAACATCAAGAAGAATTGACAGCATTAATTCCAAATCTTAATGGTATTG
  F  T  H  K  H  Q  E  E  L  T  A  L  I  P  N  L  N  G  I  D
      250                 270                 290
ACCCAGATGATGCATTCTCATCTGTACCATATGAAAAAGGTTTCAATCTCTTATGTTATC
  P  D  D  A  F  S  S  V  P  Y  E  K  G  F  N  L  L  C  Y  L
      310              HindIII                350
TTCAATCATTGGTTGGTGTTGCCGATTTTGAAGCTTGGTTAAAATCATACATTTCCAAAT
  Q  S  L  V  G  V  A  D  F  E  A  W  L  K  S  Y  I  S  K  F
      370                 390                 410
TCTCTTATCAAAGTATTGTCGCCACCCAAATGAAAGATTATTTCATTGAATATTTCACAG
  S  Y  Q  S  I  V  A  T  Q  M  K  D  Y  F  I  E  Y  F  T  E
      430                 450                 470
AGAAGGGTAAATCCGAGCAAATCAGTGTTGTAAATTGGAATGATTGGTTCAATAAACCAG
  K  G  K  S  E  Q  I  S  V  V  N  W  N  D  W  F  N  K  P  G
      490                 510                 530
GTATGCCAATTGAACAAGTTGTCTTTGTTTCCCCAGCTGCTAAAGTTGCCAAGGATTTAG
  M  P  I  E  Q  V  V  F  V  S  P  A  A  K  V  A  K  D  L  A
      550                 570                 590
CTGAAATCACTTGGATCAAAGATCAAGGTGTCAATGCAACCAAAGATGATATTAAATCAT
  E  I  T  W  I  K  D  Q  G  V  N  A  T  K  D  D  I  K  S  F
      610                 630                 650
TCAAAACTCAACAAATCATTCTCTTTTTGGATACTCTCATTCATTCAACCTCTGAAAAAC
  K  T  Q  Q  I  I  L  F  L  D  T  L  I  H  S  T  S  E  K  P
      670                 690                 710
CATTATCAGTCGATGTTTTAGAGAAAATGGATTCTCTCTATGGTTTCACCGATGTCGTTA
  L  S  V  D  V  L  E  K  M  D  S  L  Y  G  F  T  D  V  V  N
      730                 750                 770
ATAGTGAATACAAATTCAGATGGCAAACATTATGTCTTCACTCTGGTTTAAAGAGAATTG
  S  E  Y  K  F  R  W  Q  T  L  C  L  H  S  G  L  K  R  I  E
      790                 810                 830
AACCAAAAGTTGTTGAATTTTTAATCTCTCAAGGTCGTATGAAATTCGTTAGACCACTCT
  P  K  V  V  E  F  L  I  S  Q  G  R  M  K  F  V  R  P  L  Y
      850                 870                 890
ATCGTGAATTAAATAAGGTTAACCCTGAATTGGCTAAATCCACTTTTAATAAATACAAAT
  R  E  L  N  K  V  N  P  E  L  A  K  S  T  F  N  K  Y  K  S
      910                 930                 950
CTCAATATCATATTATCGCTTCAAAGATGGTTGCAAAAGATTTAGGTTTATAAATGTAAA
  Q  Y  H  I  I  A  S  K  M  V  A  K  D  L  G  L
      970                 990                1010
TTAATAAACTCTTTTTTTTTAAAAAAAAAAAAAAATAGTAAATATTTCAATAAAAAAAAAA
     1030
AAAAAAAAAA
```

Fig. 3. Alignment of the deduced amino acid sequences of *Dictyostelium* and mammalian LTA_4 hydrolase cDNAs using pileup program (Devereux, 1991).

• : conserved sequences in all three clones.

Bold letters : putative zinc-binding site.

: putative active site.

o : putative proton donor residue.

```
        1                                                   50
Human   PEIVDTCSLA SPASVCRTKH LHLRCSVDFT RRTLTGTAAL TVQSQEDNLR
Mouse   PEVADTCSLA SPASVCRTQH LHLRCSVDFA RRTLTGTAAL TVQSQEENLR

        51                                                  100
Human   SLVLDTKDLT IEKVVINGQE VKYALGERQS YKGSPMEISL PIALSKNQEI
Mouse   SLTLDTKDLT IEKVVINGQE VKYTLGESQG YKGSPMEISL PIALSKNQEI

        101                                                 150
Human   VIEISFETSP KSSALQWLTP EQTSGKEHPY LFSQCQAIHC RAILPCQDTP
Mouse   VIEISFETSP KSSALQWLTP EQTSGKQHPY LFSQCQAIHC RAILPCQDTP

        151                                                 200
Human   SVKLTYTAEV SVPKELVALM SAIRDGETPD PEDPSRKIYK FIQKVPIPCY
Mouse   SVKLTYTAEV SVPKELVALM SAIRDGEAPD PEDPSRKIYR FNQRVPIPCY

        201                                                 250
Human   LIALVVGALE SRQIGPRTLV WSEKEQVEKS AYEFSETESM LKIAEDLGGP
Mouse   LIALVVGALE SRQIGPRTLV WSEKEQVEKS ANEFSETESM LKIAEDLGGP


        251                                                 300
                                                          #
                                                        ••  ••
   Dd                                                  HE AHS
Human   YVWGQYDLLV LPPSFPYGGM ENPCLTFVTP TLLAGDKSLS NVIAHEISHS
Mouse   YVWGQYDLLV LPPSFPYGGM ENPCLTFVTP TLLAGDKSLS NVIAHEISHS

        301                                                 350
        • •••••••  •  • ••••  ••  •• • • •• ••    •  •  •   •
   Dd   WCGNLVTNKY WSEFFLNEGF TVFVERKILG RLYGEEMFDF EAMNGLKHLH
Human   WTGNLVTNKT WDHFWLNEGH TVYLERHICG RLFGEKFRHF NALGGWGELQ
Mouse   WTGNLVTNKT WDHFWLNEGH TVYLERHICG RLFGEKFRHF HALGGWGELQ


        351                                                 400
                                                  o
             •   •     • •    •   ••• •  •• ••••••  •• ••  • •
   Dd   DDVDLFTHKH QEELTALIPN LNGIDPDDAF SSVPYEKGFN LLCYLQSLVG
Human   NSVKTFGETH P..FTKLVVD LTDIDPDVAY SSVPYEKGFA LLFYLEQLLG
Mouse   NTIKTFGESH P..FTKLVVD LKDVDPDVAY SSIPYEKGFA LLFYLEQLLG


        401                                                 450
            •   ••  •  •••• •       •       •  • •       • ••
   Dd   VAD.FEAWLK SYISKFSYQS IVATQMKDYF IEYFTEKGKS EQISVVNWND
Human   GPEIFLGFLK AYVEKFSYKS ITTDDWKDFL YSYF..KDKV DVLNQVDWNA
Mouse   GPEVFLGFLK AYVKKFSYQS VTTDDWKSFL YSHF..KDKV DLLNQVDWNA


        451                                                 500
        •   •• •                   •       •             • •
   Dd   WFNKPGMPIE QVVFVSPAAK VAKDLAE..I TWIKDQGVNA TKDDIKSFKT
Human   WLYSPGLPPI KPNYDMTLTN ACIALSQRWI TAKEDDLNSF NATDLKDLSS
Mouse   WLYAPGLPPV KPNYDVTLTN ACIALSQRWV TAKEEDLSSF SIADLKDLSS
```

```
        501                                                   550
          •   ••           ••         •    • •     ••   •••  ••
   Dd   QQIILFLDTL IHSTSEKPLS VDVLEKMDSL YGFTDVVNSE YKFRWQTLCL
Human   HQLNEFLAQT LQRA...PLP LGHIKRMQEV YNFNAINNSE IRFRWLRLCI
Mouse   HQLNEFLAQV LQKA...PLP LGHIKRMQEV YNFNAINNSE IRFRWLRLCI

        551                                                   600
          •      •         ••• ••• •••    •          •  •
   Dd   HSGLKRIEPK VVEFLISQGR MKFVRPLYRE LNKVN..PEL AKSTFNKYKS
Human   QSKWEDAIPL ALKMATEQGR MKFTRPLFKD LAAFDKSHDQ AVRTYQEHKA
Mouse   QSKWEEAIPL ALKMATEQGR MKFTRPLFKD LAAFDKSHDQ AVHTYQEHRA

        601            617
          •      •   ••
   Dd   QYHIIASKMV AKDLGL.
Human   SMHPVTAMLV GKDLKVD
Mouse   SMHPVTAMLV GRDLKVD
```

sequence with other homologs, Ddlta4 hydrolase has putative zinc binding sites, an active site and proton donor residue which are conserved in other clones (Fig. 3; Medina, *et al.*, 1991; Wetterholm *et al.*, 1992).

Northern blot analysis indicates that *Ddlta4* mRNA levels are developmentally regulated (Fig. 4). Both axenically grown amoebae and vegetative cells grown with bacteria have similar levels of mRNA. Upon starvation the mRNA level is reduced and increased again around 6 h development. The mRNA level shows a peak at 9 h and a steady decrease in later developmental stages.

To determine the number of genes for Ddlta4 Southern blot analysis was done with the 1.0 Kb insert probe DNA from the λ ZAP cDNA clone (Fig. 5). Because the λ ZAP cDNA clone has EcoR I and Hind III restriction enzyme sites, two bands were expected in EcoR I or Hind III cut lanes if the gene encoding Ddlta4 is single copy in the *D. discoideum* haploid genome (Fig. 2). Southern blot data suggests that a single copy is present for the gene encoding Ddlta4 (Fig. 5).

The presence of an enzyme for arachidonic acid metabolism suggests several possibilities. First, arachidonic acid metabolites involving signal transduction mechanism may be present in *D. discoideum.* It is possible that either leukotrienes are new types of acrasin for chemotaxis in *D. discoideum* or act as second messenger molecules or act in both ways. Second, it is known that LTA_4 hydrolase has weak homology to aminopeptidase and has peptidase activity. LTA_4 hydrolase may act only as a peptidase in *D. discoideum.* Third, LTA_4 hydrolase has a role in arachidonic acid metabolism and the metabolites have nothing to do with signal

Fig. 4. Northern blot hybridization analysis.

A) Expression of Ddlta4 mRNA during vegetative and development stages on agar plate (5mM Na_2HPO_4, 5mM NaH_2PO_4, 2mM $MgSO_4$ and 200μM $CaCl_2$, pH6.5). RNAs were isolated at vegetative (veg), 1h (T1), 2h (T2), 6h (T6), 9h (T9), 12h (T12), 15h (T15) and 24h (T24) stages. 10μg of total RNAs were used for each lane and the blot was probed with ^{32}P-radiolabeled 1.0 kb insert of the Dlta4 clone.

B) Same blot was probed with ^{32}P-radiolabeled 1.0 kb insert of the Dblp for even loading control.

A)

veg T1 T2 T6 T9 T12 T15 T24

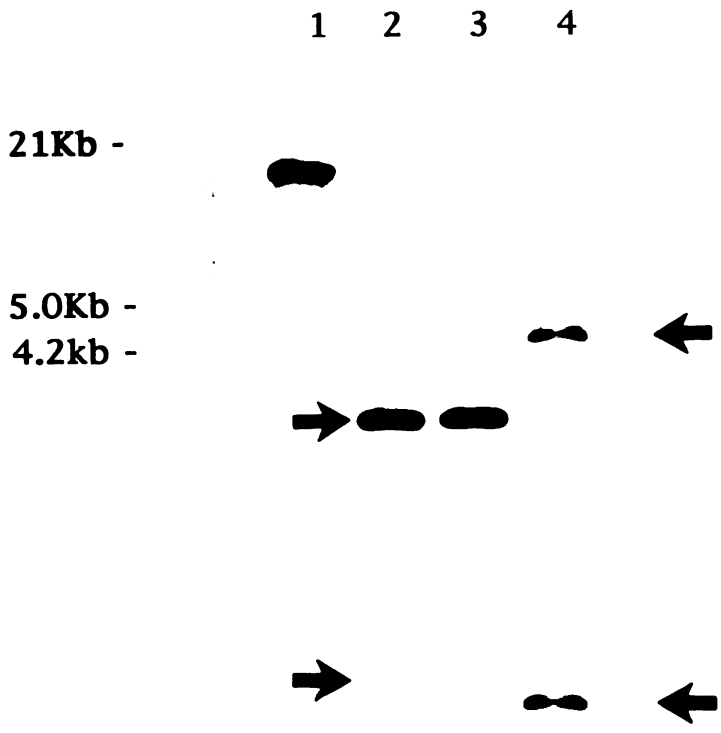
Ddlta4

B)

Veg T1 T2 T6 T9 T12 T15 T24

Dblp

Fig. 5. Southern blot hybridization analysis of *Ddlta4* gene per haploid genome of *D. discoideum*. 10μg of genomic DNA for *D. discoideum* was digested by BamH I (lane 1), EcoR I (lane 2), BamH I and EcoR I (lane 3), and Hind III (lane 4). The blots were probed with ^{32}P-radiolabeled 1.0 kb insert of the λ ZAP clone. Expected two bands were marked with arrows in EcoR I or Hind III cut lanes.


1 2 3 4
21Kb -
5.0Kb -
4.2kb -

transduction and are only present as intermediates. It is too early to say which possibility is real in *D. discoideum.* The cloning of a full size cDNA and a sequence analysis remains to be done. To check the LTA_4 hydrolase activity, expression of *Ddlta4* in *E. coli* can be done (Minami *et al.* 1988; Medina *et al.*, 1991). LTB_4 is a potent chemoattractant for human blood polymorphonuclear leukocytes (Samuelsson *et al.*, 1987). It is not known whether LTB_4 is a chemoattractant for *D. discoideum.*

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Discussion

Efforts are underway to sequence the genomic DNA of humans and other prokaryotic and eukaryotic organisms (Watson, 1990; Stevens, 1992; Fleischmann *et al.*, 1995). The genome projects are changing the way research is done in biological sciences. Although much progress in gene identification has occurred, in the future a bigger goal of understanding the function of known genes awaits. Access to the whole genomic sequence will have tremendous influence on applied and academic research. More disease-related genes may be discovered and used for gene therapy. It is expected that more productive and pathogenic resistant crops may result. Fundamental knowledge on how gene expression is regulated and the developmental process is controlled will also be gained.

Dictyostelium is not only a model system for the study of development, it is useful in the search for, and analysis of eukaryotic genes. The genome size is about 40 Mb which is less than 2% that of humans. It is easier to clone genes because fewer cDNA or genomic clones need to be screened. The short size of introns (about 100 nucleotides) permits easier isolation of full size genomic DNA. For example, rat cystathionine β synthase (CBS) genomic DNA is about 25 Kb however, the cDNA is only 1.7 Kb due to lengthy introns (Swaroop et al., 1992). In contrast, Southern blot analysis of *Dictyostelium* CBS suggested that the size is less than 4 Kb (Fig. 3 in Ch. 4) whereas the cDNA was 1.6 Kb. The 80-95% AT rich 5' untranslated sequences on mRNA help to identify coding regions. Because AT rich sequences are likely to have a stop codon, TAA (the only one used in

Dictyostelium), an ATG following TAA in the 5' end is a likely candidate for the initiation codon. The haploid life cycle is an advantage in analyzing the function of cloned genes by gene targeting through homologous recombination. The formation of diploids between normal haploid cells allows this system to be used for parasexual genetic study. When cultures of two strains of the same mating type are mixed they occasionally fuse to form heterozygous diploids. Since the diploids are stable for a number of generations, mutations can be determined to be dominant or recessive. However, the 1 X 10^{-5} rate of forming diploids is low (Loomis, 1980) and infrequent mitotic recombination results in low-resolution mapping.

A *Dictyostelium* physical map with about 30 different markers was constructed by use of YAC vectors (Kuspa *et al.* 1992). From several cDNA-mRNA and single-copy DNA-mRNA hybridization studies (Firtel, 1972; Blumberg and Lodish, 1980, 1981; Jacquet *et al.*, 1981) it is known that about 4000 to 5000 different mRNAs are present in growing cells and about 2000 to 3000 developmental stage genes are expressed in post-aggregation cells. Loomis (1978) claimed that only 150 to 300 genes among 2000 to 3000 developmental stage genes are needed for all of development from biochemical and genetic evidence. He suggested that most developmental genes may play minor and supportive roles during development without affecting morphogenesis under standard laboratory conditions.

Diverse ways are used for cloning genes. Screening *Dictyostelium* genes by homology using other eukaryotic DNAs or

guessmers as probes and differential screening for the vegetative or developmental stage genes are typical methods. Since *Dictyostelium* has biased codon usage by favoring A or T in the wobble position in synonomous codons (Spudich, 1989) the low number of redundancy, which increase possibility of picking target gene specifically, is possible for designing guessmers. The recently invented restriction enzyme mediated integration (REMI) method is valuable in especially the cloning of genes with roles in development. For yeast, Schiestl and Petes (1991) found the efficiency of transformation was about 7-fold higher in a presence of the restriction enzyme than in its absence . Kuspa and Loomis (1992) showed that introducing a restriction enzyme used to linearize a transformation plasmid into *Dictyostelium* along with the plasmid DNA increases more than 20-fold the frequency of integration into genomic restriction sites recognized by the specific enzyme. This integration of vector DNA causes abnormal development when DNA is integrated into genes which have significant roles in development. The vector DNA is used for cloning targeted genes and cloned genes are used for complementation of mutant phenotypes. By using this method the *lagC* gene which is required for muticellular development (Dynes *et al.*, 1994) was cloned. Isolation of a cytokinesis mutant showed the possibility of cloning of some genes expressed in vegetative cells by REMI method (Adachi *et al.*, 1994). This method, however, does not allow the identification of two classes of genes: those essential for cell viability or these functionally or physically redundant. Although these genes may have significant roles in development, they will not be recovered. Recently Chang *et al.* (in press) used modified REMI

method, so called promoter trap, to clone genes whose disruption produces no obvious phenotype. They transformed lacZ coding gene using REMI and lacZ expressing transformants on development to select developmentally induced promoter. This method may allow us to clone developmentally induced genes no better than differential screening does. It, however, does not allow cloning of specific genes which have significant roles in development. Anyway, if Loomis (1978) is true there are many developmentally regulated genes to be cloned by methods other than classic REMI.

My approach was different than described above and led to isolation of genes expressed both in growth and development. Two novel genes (EHJ-1 and IFK) and five genes (Dblp, Dlta4, DdCBS, Drps24, and Drpl7a) which have known homologs in other organisms were found. Anchored PCR and DDRT-PCR methods were used to clone these genes. Guessmers from known conserved amino acid sequences and λ ZAP DNA specific oligomers were used for anchored PCR with λ ZAP cDNA library as template. The use of DDRT-PCR for cloning of induced or repressed genes in early development was also successful. When RNA from vegetative and 3 h developmental cells was compared, two or three differentially expressed bands were found with only one primer pair. More differentially expressed bands were identified between RNA from vegetative and slug cells. In one trial, 2 or 3 repressed and induced bands were identified amongst 50 detectable bands (data not shown). Kopachik *et al.* (1985) found that about 3% of genes were induced in differential screening using solution hybridization which is similar to what I can get from DDRT-PCR with RNA from vegetative and slug. Bands

repressed on development were isolated and Northern blot analysis was done to check if the expression of cloned genes are reduced. EHJ-1 and Drpl7a cDNA sequences were cloned using DDRT-PCR.

The successful use of the DDRT-PCR method opens the possibility for quickly and efficiently cloning many developmentally regulated genes. Some genome projects involve sequencing cDNAs because the sequence encoding proteins are obtained with less cost. *Dictyostelium* has about 70-75 % single copy sequence of which about 30 % is expressed in cytoplasmic mRNA (Firtel and Bonner, 1972; Blumberg and Lodish, 1981). Therefore about 21 % (0.7 X 0.3) of total genome is expressed in cytoplasmic mRNA and the cost for whole cDNAs sequencing will be five times less than genomic DNAs. Although methods for equalization of cDNAs in a library (Ko, 1990; Takahashi and Ko, 1994) are possible, some rare and important cDNAs can be missed while many housekeeping gene sequences will still be repetitively sequenced. The use of DDRT-PCR methods, however, may avoid these problems.

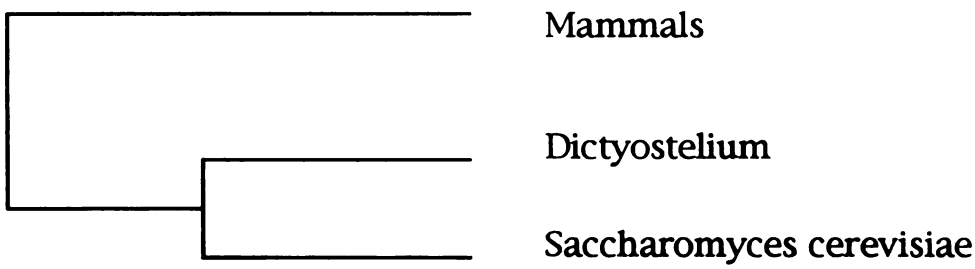
The DDRT-PCR approach could be used to find many genes whose mRNAs are induced or reduced by cAMP or otherwise regulated. The constitutively expressed genes will be displayed as equal intensity bands. The developmentally regulated genes will be displayed as bands with reduced or induced intensity. About 50 distinct bands per primer pair were displayed, therefore differential displays with only 160 primer pairs (50 X 160) are required for 8000 different cDNAs. In practice, however, isolation of developmental stage genes requires more primer pairs because constitutive bands overlap differentially expressed bands.. Usually

the 300-500 bp inserts can be obtained by DDRT-PCR and used for hybridization to a YAC library.

Use of DDRT-PCR has several advantages than other methods for cloning of useful markers. 1) This method will normalize cDNAs in a cDNA library. Although there will be a difference in bands intensity, abundant mRNAs will be shown as a single band like rare mRNAs. Theoretically, different types of cDNAs are displayed as individual bands if primer sequences are not repeated in the same cDNA. Therefore use of this method can avoid the problem of repetitive cloning of house keeping genes. 2) More developmentally regulated genes including those essential for cell viability and functionally or physically redundant ones missed by the REMI-based method can be cloned. For example, the Gα7 or Gα8 null mutants which did not show abnormal phenotype could be cloned by the DDRT-PCR method. 3) This method will be fast and economical. The construction of several cDNA libraries for different developmental stages is not necessary. To include all different types of cDNAs for genes induced by cAMP, DIF or ammonia, three normalized cDNA libraries are needed. For the purpose of constructing better physical maps using DDRT-PCR, all that is needed is first strand cDNA with mRNAs from different stages of cells.

Loomis and Smith (1990) suggested that comparison of amino acid sequences is more reliable than untranslated nucleic acid sequences for evolutionary comparisons (Fig. 1). They claim that *Dictyostelium* is more closely related to mammals than is yeast in contrast to the analysis based on 18S rRNA (McCarroll et al., 1983).

McCarroll *et al.* (1983) : Based on comparison of 18S rRNA.



Loomis and Smith (1990) : Based on eight different protein sequences

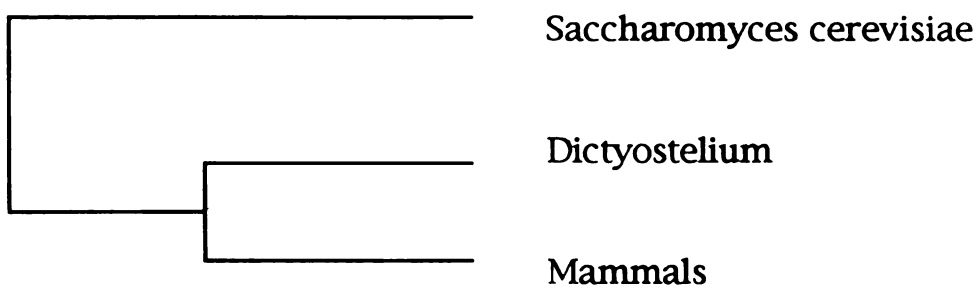


Fig. 1. Parsimony of three different organisms.

The homologous sequences for DdCBS, Drpl7a, and Drps24 are known in yeast and mammalian systems. Without exceptions the *Dictyostelium* sequences have greater identical amino acids throughout their entire length to homologs of mammals than they do to homologs in yeast (54 % vs. 49 % for DdCBS; 55 % vs. 48 % for Drpl7a; 73 % vs. 67 % for Drps24). Therefore the comparison of deduced amino acid sequences cloned in this thesis supports Loomis and Smith's claim. Since it is thought that *Dictyostelium* and mammals diverged 1000-1200 million years ago, the sequence identity is remarkabe. The essential role of these proteins might act as a intense selection pressure to keep these sequences conserved.

As I mentioned in the introduction cAMP receptor linked G-proteins have main roles in signal transduction in *Dictyostelium.* Eight clones for different types of Gαs and one for Gβ are identified. A constitutive Gβ is thought to interact with eight developmentally regulated Gαs for the formation of heterotrimeric G protein complexes. Surprisingly, however, the Gβ knock-out mutant is viable although the developmental processes are completely blocked. One possible explanation is that G-protein related signal transduction has no role in growth. The other explanation is that other protein(s) may substitute for the role of Gβ in growth. A possible candidate for that role is the Gβ-like protein.

In this thesis a *Dictyostelium* homolog of the Gβ-like protein (Dblp) was cloned. Dblp mRNA is constitutively expressed (Fig. 4 in Ch. 2). Dblp has a weak homology (26 % over middle 200 amino acids) to the the *Dictyostelium* Gβ (Lilly *et al.* 1993). However, Dblp

has seven WD-40 repeats, which are considered as domains for protein-protein interaction, as do other Gβ proteins.

The rat Gβ-like protein is a known receptor for activated C-kinase (RACK1) whose proposed role is mobilization of activated protein kinase C (PKC) to target region. The proposed role of this protein in plants is different. In tobacco, the homolog of this protein is induced by treatment of auxin and may participate in regulation of cell division. However, whether the Gβ-like protein can substitute for the role of Gβ in both systems is unknown.

Ron *et al.* (1994) proposed a model, in which a similar sequence between RACK1 and a pseudo-RACK1 sequence in PKC is a possible contact site in mobilization of activated PKC. I identified a similar sequence shared between Dblp and *Dictyostelium* myosin heavy chain kinase (MHCK; Ravid and Spudich, 1992), the only cloned protein kinase C in *Dictyostelium*. MHCK is translocated to myosin in response to cAMP. Phosphorylation of the myosin heavy chain inhibits myosin thick filament formation and leads to mobilization of myosin to the posterior cortex of the polarized cell during chemotaxis (Yumura and Fukui, 1985; Ravid and Spudich, 1989).

Dblp may have a role in the translocation of MHCK. MHCK has the amino acid sequence VMIWHL (311-316) which is similar to VMVWQL (46-51) in Dblp. The homologous sequence falls in the C2 region of MHCK as does the pseudo-RACK sequence of rat PKC. In inactive form of MHCK, VMIWHL may be covered by a specific region of MHCK. In active form of MHCK upon cAMP stimulation, the specific region of MHCK may be bound by VMVWQL in Dblp (Fig. 5 in Ch. 2). To test protein protein interaction the yeast two-hybrid

system (Fields and Song, 1989; Vojtek *et al.*, 1993) can be used with MHCK and Dblp cDNA clones. Another way is to check if *in vitro* translated proteins from the two cDNAs bind. When an antibody for Dblp is available, immunofluorescent detection (Yumura and Fukul, 1985) can be used to see colocalization of Dblp and MHCK in the posterior cortex of polarized cell during chemotaxis occurs.

The proposed role of Dblp in development does not explain why Dblp mRNA is present during the vegetative stage. Although the Dblp protein level was not measured, for the purpose of discussion I assume that mRNA presence means active protein presence. Vegetative and early developmental cells are chemotactic to folic acid (Tillinghast and Newell, 1987). Although MHCK is not present in vegetative cells (Ravid and Spudich, 1992), but several related MHCKs have been purified from *Dictyostelium* (Cote and Bukiejko, 1987). Therefore Dblp may have a role in folic acid mediated chemotaxis in translocation of other MHCKs.

Dblp may interact with Gαs and form functional heterotrimeric G proteins as Gβ does. This functional redundancy could allow Gβ null cells to grow normally. In the past research on mammalian cells, the G$\beta\gamma$ subunit was assumed to have no role in signal transduction.. Some evidence, however, now exists that G$\beta\gamma$ is directly involved in regulation of effector molecules in signal transduction. It is possible G$\beta\gamma$ and Dblp have different effectors in *Dictyostelium* development. Dblp may control the localization of MHCK and G$\beta\gamma$ may have other unknown effector molecules, therefore Gβ null cells showed abnormal development in the presence of Dblp.

Although leukotriene receptors and signal transduction processes have been extensively characterized in mammalian cells, they were unknown in lower organisms such as *Dictyostelium* before a cDNA was cloned which encodes an enzyme, leukotriene A_4 hydrolase (Dlta4). Dlta4 mRNA level is about four fold reduced in 2h developmental cells and increased to vegetative cell levels at the time cAMP relays start. The level was gradually reduced up to 20 folds after aggregation. This expression pattern is not common. In most cases, mRNAs are gradually reduced or induced during development. The down and up regulation pattern suggests that Dlta4 may have a significant role in leukotriene-related signal transduction during growth and early development. Mutant cells made null for cAR1 which is highly expressed in cells on early developmental stage, fail to bind or sense cAMP and arrest in early development. The cAR1 null cell phenotype implied that leukotriene may not substitute for the role of cAMP in signal transduction. Another possible explanation is that the leukotriene system is controlled by cAR1 related signal transduction. Therefore lack of cAR1 causes improper regulation of Dlta4 and blocking of leukotriene related signal transduction. However, since leukotriene A_4 hydrolase has not only hydrolase but also peptidase activity in the mammalian system, it is unclear leukotriene signal transduction exists in *Dictyostelium.*

To test if Ddlta4 has roles in LTA_4 hydrolase and peptidase activity, expression of the full size cDNA for Ddlta4 in *E. coli* can be attempted (Minami *et al.*, 1988). LTA_4 hydrolase converts LTA_4 into LTB_4 and LTB_4 is known as a potent chemoattractant for the human

blood polynuclear leukocytes (Samuelsson *et al.*, 1987). Perhaps *Dictyostelium* secretes and responds to LTB_4. Because Dlta4 is a single copy gene gene targeting is possible. The targeted mutant should be affected at the aggregation stage if leukotriene-related signal transduction is necessary.

Although five clones (Dblp, Ddlta4, DdCBS, Drps24, and Drpl7a) reported here had 40-70 % sequence identity to known sequence in entire deduced peptide sequences, whether they have similar roles needs to be tested. Complementation of yeast mutants, when they exist, with *Dictyostelium* cDNAs can be done. For example, complementation of a yeast cystathionine β synthase mutant (Cherest *et al.*, 1993) by DdCBS cDNA can be tried.

A 1.3 Kb cDNA (IFK) had a conceptual 355 amino acids open reading frame which was homologous at 50 % identity over 61-186 amino acids region (data not shown) to eukaryotic initiation factor 2α (eIF 2α) kinase clone by Roussou *et al.* (1988) and Meurs *et al.*, (1990). Northern blot analysis suggested that full size IFK cDNA should be 4 Kb, whereas the human eIF 2α kinase mRNA size is 2.5 Kb and the yeast is 5 Kb. IFK and human eIF 2α kinase mRNA levels increase upon starvation (Meurs *et al.*, 1990). eIF2α kinases have 11 conserved domains in their catalytic region (Wek et al., 1989; Meurs *et al.*, 1990). The first five conserved domains were identified in IFK. Another features are homopolymer repeats of Gln, Asn, and Thr in the N-terminal region shared those within several other *Dictyostelium* protein kinases (Mann and Firtel, 1991). However, the sequences, except conserved domains, did not have significant

homology to that of eukaryotic initiation factor 2α (eIF2α) kinase. It will be important to check whether other conserved domains are present in the full size cDNA and if IFK has eIF2α kinase activity using *in vitro*-translated product as Chen *et al.*(1991) did for the rabbit homolog. A yeast eIF2α kinase (GCN2) mutant may be complemented by the full size IFK cDNA.

To determine the role of cloned genes (IFK, EHJ-1, Dblp, Dlta4, and DdCBS) in growth and development I tried to block gene expression using antisense RNA. However, the antisense experiments were not informative possibly because not enough antisense RNA was produced. For the production of antisense RNA pDNeoII vector was used. The pDNeoII vector uses an actin 6 promoter to control the expression of insert. Actin 6 mRNA is present at a very low level in vegetative cells, increased in pre-aggregated cells and reduced in post-aggregative cells (Knecht *et al.*, 1986). The antisense RNA production pattern was the same as that of actin 6 mRNA. Northern blot analysis, however showed that less antisense RNA was present than endogenous RNA in the transformant cells except for the IFK antisense producing cells. IFK antisense transformants had two times more antisense RNA in vegetative and 10 times more in 3 h developing, cells. Although more antisense, than endogenous RNA, was produced the reduction of IFK RNA was not detected. Lilly *et al.* (1993) reported similar failure of antisense experiments. In attempts to block expression of *Dictyostelium* Gβ using antisense RNA, endogenous Gβ mRNA was not reduced although more antisense RNA was produced than endogenous RNA. The reason for

failure is unknown, but inappropriate localization of endogenous RNA is a possibility.

Scherczinger *et al.* (1992) analyzed variables affecting antisense RNA inhibition of gene expression. They showed that the critical factor for the inhibition of myosin heavy chain II (MHC II) gene expression by antisense RNA was the particular fragment of the gene used to produce the antisense. The fragments that produce the greatest inhibition were from the 3' end region of the gene. About 400 bp chosen at random amongst 8000 bp did not inhibit the expression of MHC II gene expression. If Scherczinger *et al.*'s claim (1992) is true, the failure of antisense experiment in my case can be explainable. I used 5' end of cDNAs when I construct antisense RNA producing vector and the size of DNA was about 500 bp in every case. Although many successful antisense RNAs are from other than 3' end or short oligomers in other systems (Crowley *et al.* 1985; Watkins *et al.*, 1992), 3' end or full size cDNAs or genomic DNA can be used again for antisense experiments in my case.

An advantage of working with *Dictyostelium* is having a relatively simple and easy method for isolation of gene targeted cells. Genes for cAMP receptors, Gα, Gβ and protein kinase were targeted and their roles studied. Gene targeting of my cloned genes will give significant information for the function of these genes. The knock-out cells of Dblp or Dlta4 may arrest at early developmental stages. The function of EHJ-1 is obscure. I will get some idea for the role of EHJ-1 in growth and development if null cells have detectable phenotypic changes. Although genes expressed in growth are not all

essential for cell viability, IFK, Dblp, Dlta4, DdCBS and EHJ-1 knock-out cells will not be found if they are. To circumvent this problem, the pVEII vector, which uses the discoidin I gamma promoter for expression of genes (Blusch *et al.*, 1992), or the use of the Cre/*loxP* recombination system, allowing inducible deletion in mice (Gu *et al.*, 1994; Kuhn *et al.*, 1995), are alternatives.

Inducible expression will be useful to examine the effects of protein overexpression or antisense-mediated down-regulation since it allows for a direct comparison of the 'on' and 'off' state of the promoter. The discoidin I gamma promoter is inducible by folate and the expression of antisense RNA can be controlled by folate. The growth rate and development would be checked in antisense producing cells. To avoid the problem of not enough expression of antisense RNA, a discoidin overproducer mutant can be used (Wetterauer *et al.* 1993). The discoidin promoter is overexpressed by a factor of 10 to 100 under control by folate as in wild type cells. Thus the pVEII vector allows overproduction of inducible antisense RNA or proteins.

A recombinase called Cre from bacteriophage P1 deletes any phage genome integrated into chromosomes. Cre lines up short sequences of phage DNA called *loxP* sites and removes the DNA between them, leaving one *loxP* site behind. The Cre/*loxP* recombination system works in transgenic mice. Gu *et al.* (1994) and Kuhn *et al.* (1995) demonstrated that a target gene flanked with *loxP* sequences could be eliminated by conditionally induced Cre. If the Cre/*loxP* recombination system works in *Dictyostelium*, inducible gene targeting may be possible. The expression of *Cre* gene could be

controlled by the discoidin promoter and target genes flanked by *loxP* sequences in the *Cre*$^{+}$ cells. Removal of folic acid from growth media or initiation of development will cause deletion of the target gene and the role of that gene in growth or development can be studied.

The overexpression of genes causes phenotypic changes in several cases (Simon *et al.*, 1989; Faix *et al.*, 1990). For example, the overexpression of regulatory (R) subunit of cAMP dependent protein kinase in vegetative cells when endogenous R subunit is not normally present causes an inability of cells to aggregate (Simon *et al.*, 1989). In my research, constitutive overexpression of EHJ-1 leads to delay of development and formation of smaller fruiting bodies. It is not clear how overexpression of EHJ-1 causes that phenotypic changes. Several possible explanations for this phenotype change were described in chapter one.

In summary, I cloned and sequenced several cDNAs with new approaches and characterized the regulation of mRNA expression for those clones. Conserved sequences in signal transduction-related genes of other systems were identified in Dblp and Dlta4 and the role of those genes were described. The comparison of deduced peptide sequences implied that *Dictyostelium* is more closely related to mammals than is yeast. The next step should be determination of the role of these cloned genes. This research opens several possibilities which were not recognized so far in *Dictyostelium* research.

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APPENDIX I : Primary sequence and developmental regulation of *Dictyostelium discoideum* ribosomal protein S24 and L7a mRNA

The work for Drps24 in appendix I was submitted to gene.

Introduction

In eukaryotes about 70 different types of ribosomal proteins are part of the functional ribosome (Warner, 1989). The expression of ribosomal proteins and formation of ribosomes are controlled according to the rate of cell growth and developmental stages (Angerer *et al.*, 1992; Ken and Singleton, 1994). *Dictyostelium discoideum*, a cellular slime mold, initiates development upon starvation. During development expression of several mRNAs for *D. discoideum* ribosomal protein is reduced (Steel and Jacobson, 1988; Singleton *et al.*, 1989; Proffitt *et al.*, 1991; for review, Ramagopal, 1992). In *D. discoideum* development cAMP acts as a signaling molecule and regulates many developmentally expressed genes (Kimmel, 1987; Mann and Firtel, 1987).

Two *D. discoideum* cDNAs which appear to encode a protein homologous to the yeast 40S ribosome protein S24 and the human 60S ribosomal protein L7a were cloned and sequenced.

Results and Discussion

1) Homolog to the yeast 40S ribosomal protein S24

The cDNA would encode a protein of 130 amino acids with a molecular weight of 14,895 daltons (Fig. 1). The protein has a net positive charge of +10.3 as do other S24 ribosomal proteins (Bonham-Smith *et al.*, 1992; Angerer *et al.*, 1992). The protein may correspond to S17 or S18 of the nomenclature for *Dictyostelium* ribosomal protein when the size and pI are considered (Ramagopal and Ennis,

Fig. 1. Nucleotide sequence of *D. discoideum* ribosomal protein Drps24. The sequence was determined by the dideoxy techniques using Sequenase 2.0 (United States Biochemical). The putative polyadenylation site was underlined.

```
       10                  30                  50
GCACGAGGTTATACTTGAAACAGATACAAAATGGTCAGAATCAGTGTTTTAAACGATTGC
                              MetValArgIleSerValLeuAsnAspCys
       70                  90                  110
TTATACTCCATTGTCAATGCCGAAAGACAAGGTAAAAGACAAGTCTTAGTCAGACCATCA
LeuTyrSerIleValAsnAlaGluArgGlnGlyLysArgGlnValLeuValArgProSer
      130                 150                 170
TCAAAAGTCATCGTTAAATTCTTAGAAGTTATGATGAAAAAGAGATACATTGGTGAATTC
SerLysValIleValLysPheLeuGluValMetMetLysLysArgTyrIleGlyGluPhe
      190                 210                 230
GAAATCGTTGATGACCATCGTTCCGGTAAAATTGTCATTGATTTAATCGGTCGTATCAAC
GluIleValAspAspHisArgSerGlyLysIleValIleAspLeuIleGlyArgIleAsn
      250                 270                 290
AAATGTGGTGTCATCTCCCCAAGATTTGACGTTACTTTAGACGAAATCGAAAAATGGGCC
LysCysGlyValIleSerProArgPheAspValThrLeuAspGluIleGluLysTrpAla
      310                 330                 350
TCTTACTTACTCCCATCCCGTCAATTCGGTCATATCGTCCTCACCACCTCCCTCGGTATC
SerTyrLeuLeuProSerArgGlnPheGlyHisIleValLeuThrThrSerLeuGlyIle
      370                 390                 410
ATGGACCACAACGAAGCCAAAACCAGACACACTGGTGGTAAATTATTAGGTTTCTTCTAT
MetAspHisAsnGluAlaLysThrArgHisThrGlyGlyLysLeuLeuGlyPhePheTyr
      430                 450                 470
TAAATTGCTAGTCTTTTTTAAATAAAATTAAAATATTATTTAAATCCTTTAAAAAAAAAA

AAAAAAAA
```

1980). From the homology to the yeast ribosomal protein subunit S24, the cDNA was named *Dictyostelium* ribosomal protein S24 (Drps24). The consensus polyadenylation signal AATAAA was found at 442-447 nucleotides (Steel *et al.*, 1987). The deduced peptide sequence (Fig. 2) showed 72% sequence identity (85% similarity) to sea urchin (Angerer *et al.*, 1992), and 67% identity (82% similarity) to yeast, ribosomal protein (Leer *et al.*, 1985). An autoradiograph of a northern blot shows a 0.5 kb band which is similar to the size of Drps24 cDNA. Drps24 mRNA level was highest in actively growing cells and reduced up to 10 fold in cells developing on agar plate (Fig. 3A). To check if Drps24 mRNA level was regulated by extracellular cAMP during development, we isolated total RNA from cells shaking in DB buffer with or without a cAMP pulse as described (Hassanain and Kopachik, 1989). The Drps24 mRNA level was reduced in cells given cAMP (Fig. 3B).

The GenBank/EMBL accession number for *D. discoideum* Drps24 is U27539.

2) Homolog to the mouse 60S ribosomal protein L7a

The λ ZAP clone which has about 1.0 Kb DNA insert was cloned and sequenced (Fig.4). Its conceptual translation product contains an open reading frame with 278 amino acids. The deduced peptide sequence (Fig.5) has 55% identity (68% similarity) to the human (Ziemiecki *et al.*, 1988), and 48% identity (64% similarity) to yeast, ribosomal protein L7a (Arevalo and Warner, 1990). Due to the significant homology to L7a this cDNA was named *Dictyostelium* ribosomal protein L7a (Drpl7a). Northern blot data suggest that the

Fig. 2. Alignment of RPS24 sequences. Drps24, *D. discoideum* ribosomal protein S24 (this report); YS24, yeast ribosomal protein YS24 (Leer *et al.*, 1985); rps15a, *Brassica napus* ribosomal protein rps15a (Bonham-Smith *et al.*, 1992); SpS24, Sea urchin ribosomal protein SpS24. The alignment was optimized by the Pileup program (The University of Wisconsin Genetics Computer Group software, Devereux, 1991). •, residues strictly conserved in the four sequences.

```
          1                                                  50
          . .  .. .  .    ...    .... ..  .... ... . . .........
Drps24    MVRISVLNDC LYSIVNAERQ GKRQVLVRPS SKVIVKFLEV MMKKRYIGEF
  YS24    MTRSSVLADA LNAINNAEKT GKRQVLIRPS SKVIIKFLQV MQKHGYIGEF
rps15a    MVRISVLNDA LKSMFNAEKR GKRQVMIRPS SKVIIKFLIV MQKHGYIGEF
 SpS24    MVRMNVLADA LRSICNAEKR CKRQVLIRPC SKVTVKFLMV MMKHGYIGEF

          51                                                 100
          .  .... .. .   . .. . .........  .     . .    ... ....
Drps24    EIVDDHRSGK IVIDLIGRIN KCGVISPRFD VTLDEIEKWA SYLLPSRQFG
  YS24    EYIDDHRSGK IVVQLNGRLN KCGVISPRFN VKIGDIEKWT ANLLPARQFG
rps15a    EYVDDHRSGK IVVELNGRLN KCGVISPRFD VGVKEIEGWT ARLLPSRQFG
 SpS24    EIVDDHRGGK IIVNLNGRLN KCGVISPRFD VPINEMEKWT SNLLPSRQFG

          101                          130
             .... .. ... ..       .. ...
Drps24    HIVLTTSLGI MDHNEAKTRH TGGKLLGFFY
  YS24    YVILTTSAGI MDHEEARRKH VSGKILGFVY
rps15a    YIVLTTSAGI MDHEEARRKN VGGKVLGFFY
 SpS24    YVVLTTSGGI MDHEEARRKH VGGKILGFFF
```

Fig. 3. Expression of *Dictyostelium* Drps24 transcripts. A) RNA was isolated from vegetative cells and during development on agar for 3, 6, 9, 12, 15 and 24h. pLK326 mRNA was used to show equivalent amount of intact mRNA were loaded in each lane. B) Vegetative amoebae were resuspended at $2x10^7$ cells /ml in DB buffer and shaken at 250rpm for 10 h without cAMP addition (T10S) or for four h at which time 50 nM cAMP was given every 10 min for 6 h (T10P). The blots were probed with ^{32}P radiolabeled insert of the λ ZAP clone.

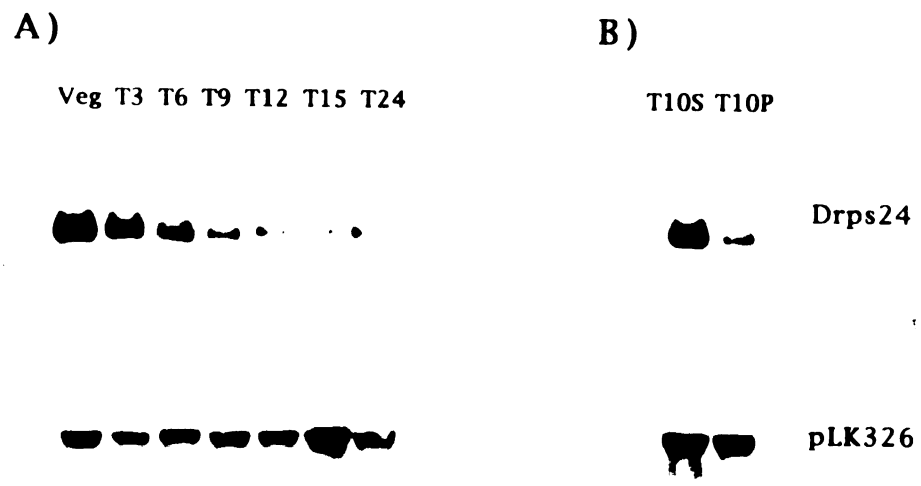
A)
Veg T3 T6 T9 T12 T15 T24
B)
T10S T10P
Drps24
pLK326

Fig. 4. Nucleotide and deduced peptide sequence of *D. discoideum* ribosomal protein Drpl7a. The putative polyadenylation site was underlined.

```
        10                  30                  50
CACGAGGCTGCCACCAAAGCTGCTCCAGCCAAAACCGCTGTTGCCACCACCAAATCAAAG
HisGluAlaAlaThrLysAlaAlaProAlaLysThrAlaValAlaThrThrLysSerLys
        70                  90                  110
AAAGTCGTCAAGAAGGGTGAGAAGAAAATCAAAACTAGAACCTTATTCAACGCCTTATAC
LysValValLysLysGlyGluLysLysIleLysThrArgThrLeuPheAsnAlaLeuTyr
       130                 150                 170
ACCAAAAACGTCAAAAACTTTGGTACTGGTTTCGGTGTTCAACCAAAGAGAGATTTAACT
ThrLysAsnValLysAsnPheGlyThrGlyPheGlyValGlnProLysArgAspLeuThr
       190                 210                 230
CATTTCACTCACTGGCCAAGATACATCAAATTACAAAGACAAAGACGTGTTTTATTAAAG
HisPheThrHisTrpProArgTyrIleLysLeuGlnArgGlnArgArgValLeuLeuLys
       250                 270                 290
AGATTAAAGGTTCCACCAACAATCAACCAATTCACCCGTGTCTTTGACAAAAACACCGCT
ArgLeuLysValProProThrIleAsnGlnPheThrArgValPheAspLysAsnThrAla
       310                 330                 350
GTCCATTTATTCAAATTATTAGATAAATACAGACCAGAAGAAGCCTCAGTCAAGAAAGCT
ValHisLeuPheLysLeuLeuAspLysTyrArgProGluGluAlaSerValLysLysAla
       370                 390                 410
AGATTATTGAAAATCGCTGAAGCCCGTGCTGCCACTCCAAAAGGTCAAGCTGCTCCAAAA
ArgLeuLeuLysIleAlaGluAlaArgAlaAlaThrProLysGlyGlnAlaAlaProLys
       430                 450                 470
GCTGAAAAACCAGTCCGACACTTACGTTTCGGTATTAACTCTGTCACCAAATTAATCGAA
AlaGluLysProValArgHisLeuArgPheGlyIleAsnSerValThrLysLeuIleGlu
       490                 510                 530
AAGAAGAAAGCTAAATTAGTCGTCATTGCCCACGATGTTGACCCAGTTGAACTCGTCTTA
LysLysLysAlaLysLeuValValIleAlaHisAspValAspProValGluLeuValLeu
       550                 570                 590
TACATACCAACCCTCTGCAGACGTATGGATGTCCCATACTGTATCGTCAAATCTAAATCC
TyrIleProThrLeuCysArgArgMetAspValProTyrCysIleValLysSerLysSer
       610                 630                 650
AGATTAGGTGAATTAGTTCACATGAGAAACGCTTCATGTGTTGCCCTCACTGGTGTCAAC
ArgLeuGlyGluLeuValHisMetArgAsnAlaSerCysValAlaLeuThrGlyValAsn
       670                 690                 710
TCTGCTGACTCAAACGAACTCGCTTTATTAGTTGAATCCGCCAAACAAATGTTCGACAAT
SerAlaAspSerAsnGluLeuAlaLeuLeuValGluSerAlaLysGlnMetPheAspAsn
       730                 750                 770
AACAGTGAACACAGAAAGACCTGGGGTGGTAACACTTTATCTGGTCCAGCTCGTGCTATC
AsnSerGluHisArgLysThrTrpGlyGlyAsnThrLeuSerGlyProAlaArgAlaIle
       790                 810                 830
TTAGCCAAACGTCAAAAAGCTGAAGCCAAAGAAAGTTTAGCCAAATCAAAGATCTAAGCT
LeuAlaLysArgGlnLysAlaGluAlaLysGluSerLeuAlaLysSerLysIle
       850                 870                 890
CTTTAATAGTTTAGCGAGACTCTCACTCTTTTTTTAAATAATCAAATAAAATAAAGGTTC
       910                 930                 950
TTTAAAAAAAAAAAAATACTAAAAAAAAAAATTTTATTCTTTTAAAAAAAAAAATAAAAAAA
       970
AAAAAAAAAAAAAA
```

Fig. 5. Alignment of RPL7a sequences. L7aDd, *D. discoideum* ribosomal protein L7a (this report); L7aChicken, chicken ribosomal protein L7a (Maeda *et al.*, 1993); L7aHuman, the human ribosomal protein L7a (Ziemiecki *et al.*, 1988); L7aRice, rice ribosomal protein L7a (Nishi *et al.*, 1993); L7aYeast, yeast ribosomal protein RPL4A (Arevalo and Warner, 1990). The alignment was optimized by the Pileup program (The University of Wisconsin Genetics Computer Group software, Devereux, 1991). •, conserved residues in all sequences or in four sequences including L7aDd.

```
            1                                                  50
                     .  ...          ..                .  .  .  ..
     L7aDd  ....HEAATK AAPAKTAVAT TKSKKVVKKG EKKIKTRTLF NALYTKNVKN
L7aChicken  PKGKKAKGKK VAPAPAVVKK QEAKKVV... .......... NPLFEKRPKN
  L7aHuman  PKGKKAKGKK VAPAPAVVKK QEAKKVV... .......... NPLFEKRPKN
   L7aRice  ......MAPK RGGRAPVPAK KKTEKVT... .......... NPLFEKRPKQ
  L7aYeast  .....APGKK VAPAPFGAKS TKSNKAK... .......... NPLTHSTPKN

            51                                                 100
            ..  .   ...  ...  .  ..  .  .....   .  ......  ....   .
     L7aDd  FGTGFGVQPK RDLTHFTHWP RYIKLQRQRR VLLKRLKVPP TINQFTRVFD
L7aChicken  FGIGQDIQPK RDLTRFVKWP RYIRLQRQRS ILYKRLKVPP AINQFSQALD
  L7aHuman  FGIGQDIQPK RDLTRFVKWP RYIRLQRQRA ILYKRLKVPP AINQFTQALD
   L7aRice  FGIGGALPPK KDLHRFVKWP KVVRIQRQRR ILKQRLKVPP ALNQFTRTLD
  L7aYeast  FGIGQAVQPK RNLSRYVKWP EYVRLQRQKK ILSIRLKVPP TIAQFQYTLD

            101                                                150
              ..  . ..   .....     .. ...  .    .   .      .    .
     L7aDd  KNTAVHLFKL LDKYRPEEAS VKKARLLKIA EARAATPKGQ AAPKAEKPVR
L7aChicken  RQTATQLLKL AHKYRPETKQ EKKQRLLARA EQKAAG.KGD TPTK.RPPV.
  L7aHuman  RQTATQLLKL AHKYRPETKQ EKKQRLLARA EKKAAG.KGD VPTK.RPPV.
   L7aRice  KNLATNLFKM LLKYRPEDKA AKKERLLKRA QAEAEG.KT. VEAK.KPIV.
  L7aYeast  RNTAAETFKL FNKYRPETAA EKKERLTKEA AAIAEG.KSK QDASPKPYA.

            151                                                200
                . .  ..  . . ... . .........  ...   . ..  . .  .....
     L7aDd  HLRFGINSVT KLIEKKKAKL VVIAHDVDPV ELVLYIPTLC RRMDVPYCIV
L7aChicken  .LRAGVNTVT TLVENKKAQL VVIAHDVDPI ELVVFLPALC RKMGVPYCII
  L7aHuman  .LRAGVNTVT TLVENKKAQL VVIAHDVDPI ELVVFLPALC RKMGVPYCII
   L7aRice  .VKYGLNHVT YLIEQSKAQL VVIAHDVDPI ELVVWLPALC RKMEVPYCIV
  L7aYeast  .VKYGLNHVV SLIENKKAKL VLIANDVDPI ELVVFLPALC KKMGVPYAII

            201                                                250
            .  .  ...  ..  .       .  .  .   .    ..  ...        .   . .
     L7aDd  KSKSRLGELV HMRNASCVAL TGVNSADSNE LALLVESAKQ MF.DNNSEHR
L7aChicken  KSKARLGRLV HRKTCTCVAF TQVNPEDKGA LAKLVEAVKT NYNDRYDEIR
  L7aHuman  KGKARLGRLV HRKTCTTVAF TQVNSEDKGA LAKLVEAIRT NYNDRYDEIR
   L7aRice  KGKARLGSIV HKKTASVLCL TTVKNEDKLE FSKILEAIKA NFNDKFDEVR
  L7aYeast  KGKARLGTLV NQKTSAVAAL TEVRAEDEAA LAKLVSTIDA NFADKYDEVK

            251                              283
              ...  .      .   .   .  ...
     L7aDd  KTWGGNTLSG PARAILAKRQ KAEAKESLAK SKI
L7aChicken  RHWGGNVLGP KSVARIAKLE KAKAKELATK LG.
  L7aHuman  RHWGGNVLGP KSVARIAKLE KAKAKELATK LG.
   L7aRice  KKWGGGVMGS KSQAKTKARE KLLAKEAAQR MT.
  L7aYeast  KHWGGGILGN KAQAKMDKRA KTSDSA.... ...
```

full size cDNA should be about 1.2 Kb. The 1.0 Kb insert DNA appears to lack of an initiation codon. The consensus polyadenylation signal AATAAA was found at 885-890 nucleotides (Steel *et al.*, 1987). Northern blot analysis showed that the same pattern of mRNA expression as Drps24 (data not shown).

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Appendix II : Cloning and characterization of *Dictyostelium discoideum* cDNA encoding cystathionine β-synthase

Chapter four was submitted to Gene.

INTRODUCTION

Cystathionine β-synthase (CBS, EC 4.2.1.22) is a key enzyme for cysteine and methionine biosynthesis in eukaryotic systems (Griffith, 1987). In a pyridoxal 5'-phosphate (PLP) dependent manner CBS catalyzes the condensation of homocysteine with serine to form cystathionine. Cystathionine is converted to cysteine by another PLP dependent enzyme cystathionine γ-lyase (EC 4.4.1.1). CBS deficiency in humans is an autosomal recessive disease, homocystinuria (Mudd et al.,1989).

Dictyostelium discoideum is a soil amoeba which feeds on bacteria. When the food source is depleted, the cells initiate a program of multicellular development (Loomis, 1982). Four h after removal of the food source, a small percentage of starving cells begin emitting pulses of cyclic adenosine monophosphate (cAMP). Neighboring cells sense cAMP via cAMP receptors expressed on the cell surface, move chemotactically towards the source and relay the cAMP signal. By nine h, a tight aggregate of cells is established. Differentiation continues with formation of a tipped aggregate at about 12 h, a migrating slug at about 16 h, and a mature fruiting body at 24h (Firtel, 1991).

Here we report the *D. discoideum* sequence of a cDNA encoding cystathionine β-synthase and the regulation of the mRNA during development.

EXPERIMENTAL AND DISCUSSION

(a) Cloning and sequence analysis

Among a group of developmentally regulated genes we isolated a partial cDNA whose deduced amino acid sequence showed strong homology to human and rat cystathionine β-synthase. By using the cDNA insert as a probe of a cDNA library made to vegetative cell mRNA we isolated a plasmid with a cDNA 1.6kb insert.

A contiguous 1,611 bp stretch of DNA was sequenced (Fig.1). The nucleotide sequence contains a single open reading frame (ORF) of 1,491 bp. Conceptual translation of the ORF gives a protein of 497 amino acid residues, with a calculated Mr of 54,400. Potential AATAAA polyadenylation signals were found (Steel et al., 1987) A GenBank database search with the FastA program showed that its deduced amino acid sequence had 54% identity and 71% similarity over its entire length with the human CBS (Fig.2). Yeast CBS showed 49% identity and 65% similarity to DdCBS(Fig.2). DdCBS also had significant homology (37 and 39% identity) with bacterial cysteine synthase A and B encoded by *cysK* and *cysM* (Byrne *et al.*, 1988; Sirko *et al.*,1990). DdCBS has a putative pyridoxal phosphate attachment site (Lysine 73) which is used as a cofactor for CBS and cysteine synthase.

(b) Southern blot analysis

To check the copy number of the DdCBS gene, Southern hybridization analysis was performed. Genomic DNA was isolated from *D. discoideum* strain KAX4 and digested with several different enzymes. Labeled probes from the 1.6 kb insert or EcoRI/SalI fragment (1-1088bp) were found to hybridize to a single restriction

Fig. 1. Nucleotide sequence of the *D. discoideum* DdCBS gene and deduced amino acid sequence. Putative PLP binding sites are marked by bold letters at amino acids 62 and 73. Potential overlapped polyadenylation sites were underlined from base 1527 to 1542. The sequence of the DdCBS cDNA was determined by the dideoxy-termination method using synthetic oligonucleotide primers and the Sequenase DNA sequencing kit (United States Biochemical).

```
1     ACGAGTTTTTTTTTTTTTTTTAAACCTCTCTAAAACAAACAAATAACTAAAAATGTCAGCA
                                                        M  S  A        3
61    CCAGAAGGACCATCAAAATGCACTTGGACTCCAAATACCACTGAAAACACTCCACATACC
      P  E  G  P  S  K  C  T  W  T  P  N  T  T  E  N  T  P  H  T     23
121   ACCAGAAGAACTCCAAAGAAATTAATTATGGATAATATTCTTGATAATATTGGTGGAACA
      T  R  R  T  P  K  K  L  I  M  D  N  I  L  D  N  I  G  G  T     43
181   CCATTAGTTAGAGTTAATAAAGTTTCATCAGATTTAGAATGTGAATTAGTTGCAAAATGT
      P  L  V  R  V  N  K  V  S  S  D  L  E  C  E  L  V  A  K  C     63
241   GAATTTTTCAATGCAGGTGGTTCAGTTAAGGATCGTATTGGTCATCGTATGATTGTTGAT
      E  F  F  N  A  G  G  S  V  K  D  R  I  G  H  R  M  I  V  D     83
301   GCAGAAGAGAGTGGTAGAATTAAGAAAGGAGATACATTAATTGAACCAACCTCTGGTAAC
      A  E  E  S  G  R  I  K  K  G  D  T  L  I  E  P  T  S  G  N    103
361   ACTGGTATTGGTTTAGCATTGACAGCAGCCATCAAAGGTTACAAAATGATCATTACACTC
      T  G  I  G  L  A  L  T  A  A  I  K  G  Y  K  M  I  I  T  L    123
421   CCAGAGAAAATGTCACAAGAGAAAGTTGATGTCTTGAAAGCATTGGGAGGAGAGATCATT
      P  E  K  M  S  Q  E  K  V  D  V  L  K  A  L  G  G  E  I  I    143
481   CGTACACCAACTGAAGCAGCATTTGATGCACCAGAGTCACATATTGGTGTTGCAAAGAAA
      R  T  P  T  E  A  A  F  D  A  P  E  S  H  I  G  V  A  K  K    163
541   TTAAATTCAGAGATTCCAAATTCCCACATTTTAGATCAATACGGTAACCCATCCAATCCA
      L  N  S  E  I  P  N  S  H  I  L  D  Q  Y  G  N  P  S  N  P    183
601   TTGGCCCATTACGATGGTACCGCCGAAGAACTCCTCGAACAATGTGAGGGTAAGATTGAT
      L  A  H  Y  D  G  T  A  E  E  L  L  E  Q  C  E  G  K  I  D    203
661   ATGATCGTTTGCACAGCCGGTACCGGTGGTACAATCACTGGTATTGCCAGAAAGATCAAA
      M  I  V  C  T  A  G  T  G  G  T  I  T  G  I  A  R  K  I  K    223
721   GAAAGACTTCCAAACTGTATCGTCGTTGGTGTCGATCCACATGGTTCAATTCTCGCTCAA
      E  R  L  P  N  C  I  V  V  G  V  D  P  H  G  S  I  L  A  Q    243
781   CCAGAATCACTCAACAATACCAACAAGAGTTACAAAATCGAAGGTATCGGTTACGATTTC
      P  E  S  L  N  N  T  N  K  S  Y  K  I  E  G  I  G  Y  D  F    263
841   ATTCCAAACGTTCTCGAACGTAAATTAGTCGATCAATGGATCAAAACCGACGATAAGGAA
      I  P  N  V  L  E  R  K  L  V  D  Q  W  I  K  T  D  D  K  E    283
901   TCTTTCATCATGGCTCGTCGTCTCATTAAAGAAGAAGGTCTCCTTTGCGCTGGTAGTTCA
      S  F  I  M  A  R  R  L  I  K  E  E  G  L  L  C  A  G  S  S    303
961   GGTTCCGCTATGGTTGGTGCACTCCTAGCCGCCAAACAATTGAAAAAAGGTCAACGTTGT
      G  S  A  M  V  G  A  L  L  A  A  K  Q  L  K  K  G  Q  R  C    323
1021  GTTGTCTTATTAGCCGATTCCATTAGAAACTATATGACCAAACATTTAAATGATGATTGG
      V  V  L  L  A  D  S  I  R  N  Y  M  T  K  H  L  N  D  D  W    343
1081  TTAGTCGACAATGGTTTCGTTGATCCAGAATACAAAACTAAAGATCAACAAGAAGAAGAG
      L  V  D  N  G  F  V  D  P  E  Y  K  T  K  D  Q  Q  E  E  E    363
1141  AAATATCATGGTGCCACCGTCAAAGATTTAACACTCCCAAAACCAATCACCATCTCTGCC
      K  Y  H  G  A  T  V  K  D  L  T  L  P  K  P  I  T  I  S  A    383
1201  ACCACCACTTGTGCTGCCGCAGTTCAACTCCTCCAACAATATGGTTTCGATCAATTACCA
      T  T  T  C  A  A  A  V  Q  L  L  Q  Q  Y  G  F  D  Q  L  P    403
1261  GTCGTTAGTGAATCAAAAAAAGTTTTGGTCAACTCACTCTTGGTAACTTCTCTCACATAT
      V  V  S  E  S  K  K  V  L  V  N  S  L  L  V  T  S  L  T  Y    423
1321  GCCTCTAAAAAAGCTGTCCCAACTGATGCTGTCAGTAAAGTTATGTTCCGTTTCACTAAA
      A  S  K  K  A  V  P  T  D  A  V  S  K  V  M  F  R  F  T  K    443
1381  AATGAAAAATATATTCCAATCACTCAATCAACTTCTTTAGCTACTCTTAGCAAATTTTTC
      N  E  K  Y  I  P  I  T  Q  S  T  S  L  A  T  L  S  K  F  F    463
1421  GAAAATCATAGCAGTGCTATCGTAACTGAAAATGATGAAATCATTTCAATTGTAACTAAA
      E  N  H  S  S  A  I  V  T  E  N  D  E  I  I  S  I  V  T  K    483
1481  ATTGATTTATTAACTTATTTAATGAAATCTCAACAAAAAAATTAAAAATAAAATAAAATA
      I  D  L  L  T  Y  L  M  K  S  Q  Q  K  N                      497
1541  AAAAAAAAAACACGTAATATTAATAATAATTATAAAAAAAAAAAAAAAAAA
```

Fig. 2. Alignment of cystathionine β-synthase sequences.
CBS-DIC, *D. discoideum* cystathione β-synthase (this report); CBS-HUMAN, human cystathionine β-synthase (Kruger and Cox, 1994); CBS-RAT, rat cystathionine β-synthase (Swaroop et al., 1992; Ishihara et al., 1990); CBS-YEAST, cystathionine encoded by the *S.cerevisiae STR4* gene (Cherest et al., 1993). The alignment was optimized by the Pileup program (The University of Wisconsin Genetics Computer Group software, Devereux et al., 1984):
•) residues strictly conserved in the four sequences
o) residues conserved in three sequences only
#) residues similar in four sequences.

```
                                                                    o# oo
  CBS-DIC  ..........  ..........  ..........  ..........  ..MSAPEGPS
CBS-HUMAN  MPSETPQAEV  GPTGCPHRSG  PHSAKGSLEK  GSPEDKEAKE  PLWIRPDAPS
  CBS-RAT  .PSGTSQCED  GSAGCPQDLE  VQPEKGQLEK  GASGD...KE  RVWISPDTPS
CBS-YEAST  ..........  ..........  ..........  ..........  ..........

           #ooo               •         o  #o o  o• ••#o o#o#  o  #
  CBS-DIC  KCTW..TPNT  TENTPHTTRR  TPKKLIMDNI  LDNIGGTPLV  RVNKVSS..D
CBS-HUMAN  RCTWQLGRPA  SESPHHHTAP  AKSPKILPDI  LKKIGDTPMV  RINKIGKKFG
  CBS-RAT  RCTWQLGRPM  ADSPHYHTVP  TKSPKILPDI  LRKIGNTPMV  RINRISKNAG
CBS-YEAST  ..........  ......MTKS  EQQADSRHNV  IDLVGNTPLI  ALKKLPKALG

           ooooo ••o•  oo•o•••o••  ••  o•o o•  •  • #o o  o•#•••••••
  CBS-DIC  LECELVAKCE  FFNAGGSVKD  RIGHRMIVDA  EESGRIKKG.  DTLIEPTSGN
CBS-HUMAN  LKCELLAKCE  FFNAGGSVKD  RISLRMIEDA  ERDGTLKPG.  DTIIEPTSGN
  CBS-RAT  LKCELLAKCE  FFNAGGSVKD  RISLRMIEDA  ERAGTLKPG.  DTIIEPTSGN
CBS-YEAST  IKPQIYAKLE  LYNPGGSIKD  RIAKSMVEEA  EASGRIHPSR  STLIEPTSGN

           ••••••• o•  ##••#  •• #  •••••  •••o  ••#•••#••#  •••• • o•
  CBS-DIC  TGIGLALTAA  IKGYKMIITL  PEKMSQEKVD  VLKALGGEII  RTPTEAAFDA
CBS-HUMAN  TGIGLALAAA  VRGYRCIIVM  PEKMSSEKVD  VLRALGAEIV  RTPTNARFDS
  CBS-RAT  TGIGLALAAA  VKGYRCIIVM  PEKMSMEKVD  VLRALGAEIV  RTPTNARFDS
CBS-YEAST  TGIGLALIGA  IKGYRTIITL  PEKMSNEKVS  VLKALGAEII  RTPTAAAWDS

           ••••#••• # •  •••ooo•  •••• • o••  o•••o •o •  oo#•o#
  CBS-DIC  PESHIGVAKK  LNSEIPNSHI  LDQYGNPSNP  LAHYDGTAEE  LLEQCE....
CBS-HUMAN  PESHVGVAWR  LKNEIPNSHI  LDQYRNASNP  LAHYDTTADE  ILQQCD....
  CBS-RAT  PESHVGVAWR  LKNEIPNSHI  LDQYRNASNP  LAHYDDTAEE  ILQQCD....
CBS-YEAST  PESHIGVAKK  LEKEIPGAVI  LDQYNNMMNP  EAHYFGTGRE  IQRQLEDLNL

            oo#oo#•    ••••••o•• ooo#••# o  o ##•o•• • •••• •• ••
  CBS-DIC  .GKIDMIVCT  AGTGGTITGI  ARKIKERLPN  CIVVGVDPHG  SILAQPESLN
CBS-HUMAN  .GKLDMLVAS  VGTGGTITGI  ARKLKEKCPG  CRIIGVDPEG  SILAEPEELN
  CBS-RAT  .GKVDMLVAS  AGTGGTITGI  ARKLKEKCPG  CKIIGVDPEG  SILAEPEELN
CBS-YEAST  FDNLRAVVAG  AGTGGTISGI  SKYLKEQNDK  IQIVGADPFG  SILAQPENLN

           #•    • #• ••••••o• • •#• #o• •  •# •   •   •• ••  ••#
  CBS-DIC  NTNK.SYKIE  GIGYDFIPNV  LERKLVDQWI  KTDDKESFIM  ARRLIKEEGL
CBS-HUMAN  QTEQTTYEVE  GIGYDFIPTV  LDRTVVDKWF  KSNDEEAFTF  ARMLIAQEGL
  CBS-RAT  QTEQTAYEVE  GIGYDFIPTV  LDRAVVDRWF  KSNDDDSFAF  ARMLISQEGL
CBS-YEAST  KTDITDYKVE  GIGYDFVPQV  LDRKLIDVWY  KTDDKPSFKY  ARQLISNEGV

           •o#•• ••     o# oo      •  oooo• o#o ••#•o• o • o • •#
  CBS-DIC  LCAGSSGSAM  VGALLAAK..  .QLKKGQRCV  VLLADSIRNY  MTKHLNDDWL
CBS-HUMAN  LCGGSAGSTV  AVAVKAAQ..  .ELQEGQRCV  VILPDSVRNY  MTKFLSDRWM
  CBS-RAT  LCGGSSGSAM  AVAVKAAQ..  .ELKEGQRCV  VILPDSVRNY  MSKFLSDKWM
CBS-YEAST  LVGGSSGSAF  TAVVKYCEDH  PELTEDDVIV  AIFPDSIRSY  LTKFVDDEWL

              oo   o                              •##•     o#o#  •
  CBS-DIC  VDNGFVDPEY  KTK.......  ..DQQEEEKY  HGATVKDLTL  PKPITISATT
CBS-HUMAN  LQKGFLKEED  LTE.......  ..KKP...WW  WHLRVQELGL  SAPLTVLPTI
  CBS-RAT  LQKGFMKEE.  LSV.......  ..KRP...WW  WHLRVQELSP  SAPLTVLPTV
CBS-YEAST  KKNNLWDDDV  LARFDSSKLE  ASTTKYADVF  GNATVKDLHL  KPVVSVKETA
```

```
           oo   # #•#   •••• ••o  •   #o     o   •
  CBS-DIC  TCAAAVQLLQ QYGFDQLPVV SESKKVLVNS LLVTSL.TYA S.........
CBS-HUMAN  TCGHTIEILR EKGFDQAPVV DEAGVILGMV TLGNMLSSLL A.........
  CBS-RAT  TCEHTIAILR EKGFDQAPVV NESGAILGMV TLGNMLSSLL A.........
CBS-YEAST  KVTDVIKILK DNGFDQLPVL TEDGKLSGLV TLSELLRKLS INNSNNDNTI

                  •   o o • oo               o     •   •o   •
  CBS-DIC  .......KKA VPTDAVSKVM FRFTKNEKYI PITQSTSLAT LSKFFENHSS
CBS-HUMAN  .......GKV QPSDQVGKVI YK.....QFK QIRLTDTLGR LSHILEMDHF
  CBS-RAT  .......GKV RPSDEVCKVL YK.....QFK PIHLTDTLGM LSHILEMDHF
CBS-YEAST  KGKYLDFKKL NNFNDVSSYN ENKSGKKKFI KFDENSKLSD LNRFFEKNSS

           •#o                      #•• o• •• ##
  CBS-DIC  AIVTEND... .......... EIISIVTKID LLTYLMKSQQ KN.
CBS-HUMAN  ALVVHEQIQY HSTGKSSQRQ MVFGVVTAID LLNFVAAQER DQK
  CBS-RAT  ALVVHEQIQY RNNGVSSKQL MVFGVVTAID LLNFVAAREQ TRK
CBS-YEAST  AVITDG.... .........L KPIHIVTKMD LLSYLA.... ...
```

enzyme fragment suggesting that the gene for DdCBS was present as a single copy(Fig.3).

(C) Northern blot analysis

Northern blot analysis was done as previously described (Hassanain and Kopachik, 1989). DdCBS mRNA level was highest in the actively growing vegetative stage and gradually reduced in developmental stages (Fig.4A). Some of the expression of developmentally regulated genes in this organism is controlled by cAMP (Firtel, 1991). To check whether DdCBS mRNA level was regulated by cAMP, 50nM cAMP was given every 10 min for 8 h to cells shaking as a suspension in DB buffer. The DdCBS mRNA level between cAMP-pulsed and control cells (without cAMP) showed about a 5 fold reduction by cAMP pulse (Fig.4B). While other mRNAs show different types of regulation by cAMP; clone Dblp mRNA level had no change and clone EHJ-1 showed 20 fold reduction. The mRNA level in vegetative (Veg) and cells taken after 12 h (T12S) in the absence of cAMP pulses in clone Dblp and clone EHJ-1 was unchanged, whereas the DdCBS mRNA level was clearly reduced (Fig.4B). This suggests that starvation alone as well as cAMP can reduce DdCBS mRNA level in *D. discoideum* development.

(d) Conclusion

(1) An entire coding sequence for *Dictyostelium* cystathionine β-synthase has been reported. The deduced amino acid sequence showed 51% identity with human and 48% with yeast homologues.

Fig. 4. A. Expression of DdCBS mRNA during development on agar DB plate. RNAs were isolated at Vegetative (Veg), 1h (T1), 2h (T2), 6h (T6), 9h (T9), 12h (T12), 15h (T15) and 24h (T24) stages. Northern blot analyses were done as previously described (Hassanain and Kopachik, 1989). The constitutive level of clone Dblp mRNA shows that an equal amount of mRNA was present in each lane. B. Regulation of different types of RNA during development in shaken suspension cultures with or without cAMP pulses. Vegetative amoebae were resuspended at $2x10^7$ cells/ml in DB buffer. After 4hr some cultures received additions of 50nM cAMP every 10min for the next 8hr. Total RNA was isolated from cells without (T12S) and with cAMP pulses (T12P) in suspension culture. The same set of RNA blots were probed with DdCBS (a), clone EHJ-1 (b), and clone Dblp (c).

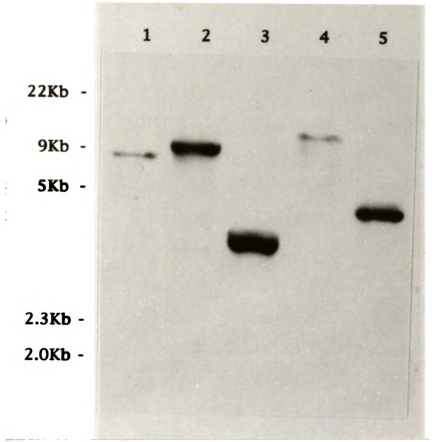
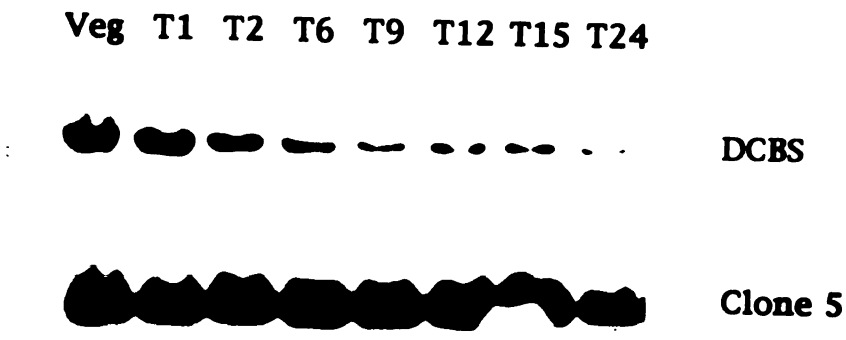
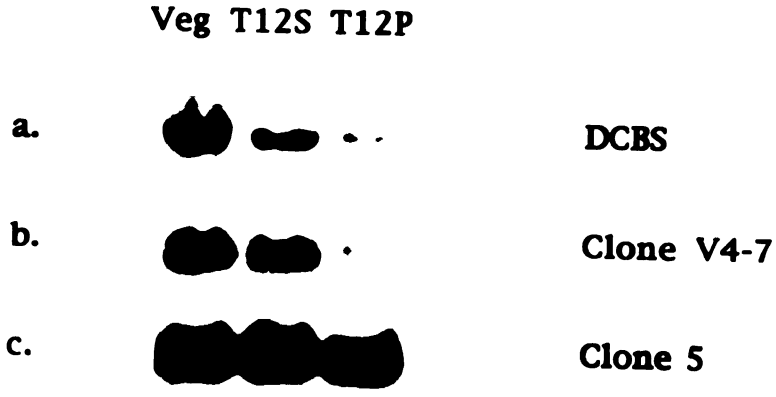

1
2
3
4
5
22Kb -
9Kb -
5Kb -
2.3Kb -
2.0Kb -

Fig. 3. Southern blot analysis of *D. discoideum* genomic DNA.
10 μg of DNA was digested by restriction enzymes and separated on 0.7% agarose gel. DNA was transferred to Genescreen (NEN) membrane. Blot was probed with a ^{32}P-labelled clone 6-1 whole insert or EcoRI/SalI fragment of clone 6-1. Lane 1: Bgl II, lane 2: EcoR I, lane 3: Bgl II/EcoR I, lane 4: Hind III, lane 5: Hind III/Pst I were used.

A)


Veg T1 T2 T6 T9 T12 T15 T24
DCBS
Clone 5

B)


Veg T12S T12P
a.
DCBS
b.
Clone V4-7
c.
Clone 5

(2) Southern hybridization analysis implied that DdCBS is a single copy gene.

(3) DdCBS mRNA level was reduced in developing cells.

(4) DdCBS mRNA level was reduced by starvation as well as by cAMP signaling during *Dictyostelium* development.

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APPENDIX III : MATERIALS AND METHODS

Basic molecular cloning techniques were followed according to "Molecular Cloning (Sambrook *et al.*, 1989).

Growth and Differentiation of *Dictyostelium*

In this work a strain KAX4 from Dr. Kessin was used.

1. To start a culture of axenic cells spread a suspension of *Klebsiella pneumoniae* in SM/2 broth onto 0.1 LP plate. Spread some of the silica gel crystals which contain spores onto the plate and incubate at 19-22°C. Place the plates right side up (lid on top) in a plastic box and cover.

2. When a plate of fruiting bodies is available, collect spores from the fruiting bodies with inoculation loop. Transfer spores into HL-5 broth containing 200 μg/ml streptomycin sulfate and 10 μg/ml penicillin G in 15 ml tube. The final concentration of spores should be more than 3×10^7/ml.

3. Place the tube in the incubator and shake at 150 rpm for about 3-4 days then count the amoebae. When the concentration reaches more than 8×10^6/ml dilute to 2 ml and then allow to grow to that concentration again in about a day. Next day dilute cells to 1×10^5ml in 50 ml flask containing 10 ml of HL-5 media. Shake the cells at 150 rpm in the incubator and subculture every day or every the other day.

4. To harvest cells for suspension development experiments spin the cells down at 1,500 rpm in the table top centrifuge for 2 min.

Wash the pellet with equal amount of Sorensen's buffer or KK2 buffer and spin down again. Resuspend cells in a buffer at 5 x 10^6 to 2 x 10^7/ml and then shake cells at 150 rpm in the incubator.

5. Cells are placed on non-nutrient agar (NNA) for checking normal development. To do this spread 1-2 x 10^7 washed cells out evenly on NNA buffered with KK2 or DB or SB. Adjust the wettness of the plates by adding buffer if too dry or drying with the lid off to evaporate some buffer if too wet. Then place in the incubator.

MEDIA AND BUFFERS

a. SB : Sorensen's phosphate buffer 17 mM pH 6.0. Prepare a 1 M stock solution:

KH_2PO_4	120.33 g
Na_2HPO_4	17.92 g

Nano pure water to 1 L (check pH and adjust to 6.0)

Autoclave 25 min.

b. DB : Development buffer, Sodium phosphate 10 mM pH 6.5.

Prepare a 1 M stock solution of phosphate buffer:

Na_2HPO_4	45.44 g
NaH_2PO_4	93.84 g
NaOH pellets	9.0 g

Nano pure water to 1 L (check pH and adjust to 6.5)

Autoclave 25 min. Add 10 ml of stock and 0.2 ml of 1 M sterile $CaCl_2$ and 2 ml of 1 M $MgSO_4$ to nano pure water to make 1 L. Store at RT.

c. KK2 : Potassium phosphate buffer, 40 mM pH 6.4. Prepare a 10 X stock: To KH_2PO_4 (54.4g) add about 800 ml of nano pure water and about 155 ml of 1 M KOH and pH to 6.4. Adjust to 1 L. Autoclave 25 min. Dilute 1 : 10 with nano pure water to make a 1X solution.

d. SM/2 : Rich nutrient media in plates or as a broth.

Bacto peptone	5 g
Yeast extract	0.5 g
Glucose	5 g
$MgSO_4$ X 7 H_2O	1 g
KH_2PO_4	2.2 g
Na_2HPO_4	1.0 g
d water to	1000 ml

Add BBL or Bacto agar 20 g per liter for plates. Autoclave 40 min.

e. 0.1 LP : Weak nutrient media for plates

Agar	15 g
Lactose	1 g
Bacto peptone	1 g
KH_2PO_4	2.05 g
Na_2HPO_4	0.03 g
d water to	1000 ml

Autoclave 40 min.

f. HL-5 : Axenic broth for KAX4.

Glucose	14 g	Add 100 ml water in flask.
Yeast extract	7 g	
BBL Thiotone	14 g	Add 600 ml water in bottle.
KH_2PO_4	0.5 g	

NaH_2PO_4 0.5 g Add 100 ml water in flask.

Autoclave all three solution 40 min. After autoclaving combine the solutions and make to 1000 ml. Add 1 ml of 1 M HCl to adjust pH to 6.5.

g. Streptomycin sulfate. Prepare a stock solution of 20 mg/ml. Weigh out and dissolve then filter through a 0.45 μm Millipore filter and store at -20°C.

h. Penicillin G. Prepare a stock solution of 100 mg/ml as for 'g' above.

Differential Display PCR

1. First strand cDNA synthesis

5 X Reaction buffer :	4 μl
RNasin (Promega)	0.5μl
dNTP mix (100 μM each)	1 μl
DTT (100 mM)	1 μl
14 mer (25 μM)	2 μl
Total RNA (G/SCN prep)	1.5 μl (2-3 μg)
MMLV reverse transcriptase	1.5 μl (300 U)
Diethylpyrocarbonate treated (Dep'd) water	7.5 μl

Incubate at 37°C for 1 h. After incubation heat at 95°C for 2 min to inactivate enzymes.

2. PCR

1 µl of arbitrary 10 mer + 2 µl of the first strand cDNA + 17 µl of PCR labeling mix + 3 units of Taq polymerase.

Step 1	94°C	30 s
Step 2	42°C	1 min
Step 3	72°C	30 s

Repeat 30 cycles.

3. Load 6.5 µl of PCR product on 6 % sequencing gel. Follow the procedure written in "Sequencing gel preparation and electrophoresis". However, to isolate differentially expressed band do not fix the gel.

PCR labeling mix :

Dep'd water	25.3 µl
10 X Reaction buffer	5 µl
dNTPs (250 µM each)	4 µl
14 mer (50 µM)	2.5 µl
10 mer (10 µM)	2.5 µl
$MgCl_2$ (25 mM)	2.8 µl
^{35}S-dATP	2.8 µl

Recovery and Reamplification of DNA from sequencing gel

DNA should be amplified to subclone into a plasmid.

1. Cut differentially expressed DNA band from sequencing gel and then hydrate in 140 µl of water and boil 15 min.

2. Spin down and transfer supernatant then add NaAc to 0.3 M with 0.4 µl of linear polyacrylamide (5 mg/ml). Add 2 volume of EtOH. Keep in -70°C at least 30 min. Spin down and wash the pellet with 70 % EtOH. Dry pellet and dissolve in 10 µl water.

3. PCR

H_2O	17.9 µl
10 X buffer	4.0 µl
dNTPs (250 µM each)	3.2 µl
14 mer (50 µM)	2.0 µl
10 mer (10 µM)	2.0 µl
EtOH ppt. DNA	8.0 µl
$MgCl_2$ (25 mM)	2.4 µl
Taq Pol (Promega)	0.5 µl

After 40 cycles (94°C, 30 s; 40°C, 2 min; 72°C, 30 s) add 0.5 µl of Taq Polymerase and do PCR 15 more cycles. Link 5 min final extension at 72°C. Run agarose gel and isolate insert by using QIAEX Gel Extraction Kit (QIAGEN) and dissolve in 20 µl of TE.

4. Ligate the amplified insert DNA into pCRII vector (TA cloning Kit, Invitrogen).

Radioactive Probe Preparation (Feinberg and Vogelstein, *et al.*, 1983)

- Random primer method (Oligolabelling)

Dilute 100-200 ng of purified DNA fragment to 12 µl in dH_2O.

Boil for 10 min to denature and then quick-cool in ice-water for 2 min.

Add following:

LS	18 µl	
BSA	1 µl	(16 mg/ml)
dNTPs	3 µl	(dGTP, dATP & dTTP)
{^{32}p}dCTP	5 µl	(50 µCi)
Klenow frag.	8 unit	(DNA polymerase I)
total	40 µl	

Incubate at RT for 5 h to overnight

Note: TM: 250 mM Tris.cl pH 8.0 2.5 ml
25 mM $MgCl_2$ 0.25 ml
50 mM β-Mercaptoethanol 36 µl
Water to total 10 ml

OL: 90 O.D. Units/ml Hexamers
1 mM Tris. Cl pH 8.0
1 mM EDTA pH 8.0

LS: 1 M Hepes (pH 6.6) : TM : OL,
25 : 25 : 7

Screening of λ cDNA library

1. Preparation of blot.

a. Grow LE 392 cells in LB containing maltose and $MgCl_2$ overnight.

100 ml LB + 1 ml of 20 % maltose + 1 ml of 1 M $MgCl_2$

b. Add 0.1 ml of culture to fresh media and grow several h.

c. Spin down culture in the table top centrifuge at maximum speed for 5 min.

d. Pour off supernatant and resuspend cells in half volume of SM buffer.

e. Add 0.1 ml LE392 cells into 5 ml glass tube.

f. Add 2 µl of 1000 fold diluted λ ZAP cDNA library to LE392 cells.

g. Incubate at 37°C for 30 min.

h. Add 3 ml of top agar.

i. Plate out on LB agar and incubate at 37°C for 4-6 h.

j. After plaques are formed leave the plate in the refrigerator for 1 h.

k. Use NEN Colony/PlaqueScreen membrane and follow the manufacture's protocol. To avoid false positives make two blots from a plate.

2. Screening the blot

Use same buffer as used for Southern blot and follow same procedure which was described in Southern blot.

3. Identification of positive and re-screen blot.

a. Find the location of positive plaques by overlapping two X-ray films and the plate.

b. Isolate agar plug from the plate by wide side of pasteur pipet.

c. Transfer agar plug in 1 ml of SM and leave at RT for 1 h.

d. While incubating prepare infectable LE392 cells as described in "Preparation of blot".

e. Add 2 µl or 20 µl of 'c' solution into LE392 cells.

f. Follow same procedure from step 1.g to 2.

g. Isolate single positive plaque by overlapping the plate and X-ray film.

h. To excise plasmid DNA from λ ZAP clone follow the procedure written in "Excision of Plasmids from the λ ZAP cDNA Clone".

SM : NaCl 2.9 g

$MgSO_4$ 1.0 g

1 M Tris 25 ml (pH 7.5)

2 % Gelatin solution 2.5 ml

H_2O to 500 ml

λ Top agarose

Tryptone 2.0 g

NaCl 1.0 g

Agarose 1.6 g

H_2O to 200 ml

Autoclave then add 2 ml of 1 M $MgSO_4$ + 2 ml of 2 % maltose after cool.

Excision of Plasmids from the λ ZAP cDNA Clone

For sequencing and other manipulation of DNA it is required to isolate plasmid DNA from λ ZAP cDNA Clone.

1. Pick out positive plaque and dissolve in 50 μl of SM buffer. Leave it in the refrigerator overnight.

2. Mix 200 μl of OD≈1.0 SURE cells, 10 μl of λ ZAP phage stock, 190 μl of SM buffer, and 20 μl of R408 helper phage in 15 ml test tube. Incubate this mixture at 37°C for 15 min.

3. Add 5 ml of 2X YT medium (10 g NaCl, 10 g yeast extract, 16 g tryptone per liter of H_2O) and incubate at 37°C for 4 to 6 h with shaking.

4. After incubation, heat the mixture to 70°C for 20 min to inactivate the present λ phage and to kill the bacteria.

5. Spin down cells for 5 min at 2,000 rpm in table top centrifuge.

6. Save supernatant which contains the Bluescript phagemid packaged in the f1 phage particle, as well as the f1 helper phages.

7. To recover the excised phagemid from this stock, mix 200 μl of OD≈1.0 DH5 αF' host cells with 200 μl of phagemid stock and then incubate at 37°C for 15 min.

8. Spread 200-300 μl to LB-amp plate with 50 μl of 2% X-gal and 10 μl of IPTG.

9. Select white colonies and check insert.

λ ZAP cDNA library DNA isolation

λ ZAP cDNA library DNA was used for anchored PCR.

1. Prepare infectable LE392 cells as described in "Screening of λ cDNA library". Add 1-2 ul of λ ZAP cDNA library (titer 10^{10}/ml) to 1 ml of fresh LE392 cells. Incubate the tubes at 37°C glass bead bath

for 30 min and transfer into 100-200 ml of LB-Mg^{++} (LB + maltose and magnesium).

2. Shake in the 37°C incubator for 5-7 h. It should be cloudy in 1h and clear upon lysis in 5-7 h.

3. Spin down with 8000 g at 4°C for 10 min. λ is in the supernatant.

4. Add RNase A to 1 μg/ml and 50 μl of crude DNase to the supernatant and incubate at 37°C for 30 min.

5. Add 5.8 g of NaCl. Swirl to dissolve. Keep on ice for 1 h.

6. Spin down with 11,000 g at 4°C for 10 min. Keep the supernatant.

7. Add PEG (8000) to 10 %. Stir slowly at RT then keep on ice for at least 1 h or overnight.

8. Spin down with 11,000 g at 4°C for 10 min. Drain throughly and leave the tube inverted for 5 min or more.

9. Resuspend phage in 3-4 ml SM.

10. Add equal volume of $CHCl_3$ and vortex 30 s. Spin down at maxmum speed in the table top centrifuge for 15 min.Place aqueous phase on the CsCl step gradient after dissolving 0.5 g CsCl per ml. Step gradient in 17 ml polyallomer tube.

3 ml 1.7 g/ml

2 ml 1.5 g/ml

2 ml 1.45 g/ml

Spin AH-629 with 22 K rpm at 4°C for 2 h.

11. Hold the tube in stand. Place light above and use dark paper or board for background if blue band is not easy to see.

12. Use tape over tube and puncture with 21 gauge needle in 1 ml syringe. Pull out 0.5-1 ml.

13. Dialyze against 1 L of 10 mM NaCl/ 50 mM Tris pH 8/ 10 mM $MgCl_2$ for 1 h then replace with fresh buffer and dialyze one more h in the cold room.

14. Collect dialysate and add RNase A to 50 μg/ml. Incubate at 37°C for 30 min.

15. Add EDTA pH8 to 20 mM and proteinase K to 50 μg/ml and SDS (Serva SDS) to 0.5%. Gently mix and keep on 55°C water bath for 1 h.

16. Cool. Then extract with phenol, phenol: $CHCl_3$ (50:50) and $CHCl_3$.

17. Add 3 M NaAc (pH 7.0 not 5.0) to 0.3 M and mix. Add 2 volumes of EtOH. Invert tube to mix and watch for thin thread of DNA. Remove with pipette tip. Redissolve without vigorous mixing in 100 μl of TE.

18. Check yield on agarose gel. If there is too much RNA present, add RNase A to 100 μg/ml. Incubate at 37°C for 30 min to 1 h.

19. Extract RNaseA with Phenol/chloroform and chloroform.

20. Spin through mini G-50 column in STE. See "Plasmid isolation" for directions. EtOH precipitation. Dissolve DNA in 100 μl TE.

Anchored PCR

To get 5'end cDNA of λ ZAP clone sequence anchored PCR was done by using λ ZAP cDNA library DNA as a template.

λ ZAP cDNA (10 ng)	1 µl
10 X buffer	10 µl
$MgCl_2$ (25 mM)	6 µl
dNTPs (250µM each)	8 µl
BS-SK primer (50 µM)	2 µl
Gene specific primer (50 µM)	2 µl
H_2O	72 µl

PCR 35 cycles (94°C, 1 min; 55°C, 1.5 min; 72°C, 3 min). Link 15 min final extension at 72°C. Run agarose gel and isolate insert by using QIAEX Gel Extraction Kit (QIAGEN) and dissolve in 20 µl of TE. Ligate the amplified insert DNA into pCRII vector (TA cloning Kit, Invitrogen).

Plasmid Isolation

I. Mini-preparation methods

A. Alkaline lysis method

1. Grow 5 ml of isolated clone in LB or SOB containing 100 µg/ ml ampicillin. Shake rapidly in the 37°C incubator at least 9 h until stationary phase.

2. Spin down cells in microfuge 1 min.

3. Resuspend pellet with freshly prepared 100 µl of 50 mM glucose/ 25 mM Tris (pH 8) / 10 mM EDTA/ 2 mg/ml lysozyme. Ice 10 min.

4. Add 200 µl 0.2N NaOH/ 1% SDS and mix gently to lysis cells. Do not vortex. Prepare fresh NaOH/ SDS solution. Ice 10 min.

5. Add 150 µl of 3 M sodium acetate (pH 5) and mix by inversion. Leave in -20°C freezer for 10 min.

6. Spin in SH-MT rotor at 13,500 rpm for 10 min at 20°C or maximum speed in microfuge. Recover the supernatant and add 2 volumes of cold EtOH. Precipitate at -20°C or -70°C freezer at least 10 min.

7. Spin in SH-MT rotor for 10 min. Aspirate off supernatant. Wash the pellet with 70% cold EtOH. Dry the pellet in the Speed Vac.

8. Resuspend pellet with TE which contains 100 µg/ ml RNAse A. Incubate for 30 min at 37°C.

9. Add equal volume of 50 : 50 phenol/ chloroform (pH8). Vortex 1 min and spin down 5 min in microfuge. Repeat extraction with one quarter volume of chloroform alone. Collect supernatant (aqueous phase).

10. Prepare mini-G50 column. Add G50 Sephadex equilibrated with 0.1M NaCl in TE pH 8.0 to spin column tubes only up to the neck. Place tube inside of larger collection tube. Spin in microfuge for 1 min. Check G50 level and spin down again for 5 min. Place the G50 column in a new tube then add aqueous phase to G50 and spin for 5 min.

11. Add 2 volumes of EtOH. Precipitate DNA for at least 10 min in -70°C freezer. Spin down and dry as before. Resuspend DNA in 40 µl of TE or water.

B. Speedprep (Goode and Feinstein, 1992)

This method is good for checking insert.

1. Spin down 1.5 ml of overnight cultured bacteria.

2. Aspirate supernatant and resuspend pellet in 100 µl of solution A (50 mM Tris (pH8.0), 4 % Triton X-100, 2.5 M LiCl, 62.5 mM EDTA).

3. Add 100 µl of a Tris-buffered phenol/ chloroform mixture (1:1). Vortex the tube for 10 s and microfuge at top speed for 2 min.

4. Remove the plasmid-containing 200 µl of cold 100% EtOH. Vortex the sample briefly, and microfuge it at top speed for 5 min.

5. Discard the supernatant. Wash the pellet with 1 ml of 70% EtOH and dry in Speed Vac.

6. Resuspend the pellet in 10 µl of TE which contains 100 µg/ ml RNAse A. Incubate at RT for 5 min. This DNA can be used for restriction enzyme digestion.

C. Wizard miniprep (Promega)

After the presence of insert is confirmed by Speedprep method Wizard miniprep kit was used to isolate plasmid for sequencing. The manufacture's protocol was followed.

II. Large Scale Plasmid Isolation

A. Cesium Chloride Method

1. Grow to saturation 100 ml of cells in LB or SOB with 100 μg/ ml ampicilin.

2. Spin down cells in HB-4 or GSA rotor at 7 K for 5 min. Resuspend the pellet of cells in 2 ml of sucrose buffer (25 % sucrose/ 50 mM Tris pH 8/ 10 mM EDTA). Pipet up and down and vortex to resuspend all cells. Transfer to a clear T865 tube.

3. Add 0.6 ml of 5 mg/ ml lysozyme in 50 mM Tris/ 10 mM EDTA. Keep on ice for 5 min.

4. Add 1.2 ml of Tris/ EDTA the 50 μl of 10 mg/ ml boiled RNAse A. Keep on ice for 5 min.

5. Mix to resuspend cells then slowly add with swirling 5 ml of 2 % triton/ 50 mM Tris/ 10 mM EDTA. Leave 10 min at RT.

6. Spin in the ultracentrifuge T865 rotoer for 20 min at 25K at 20°C. The pellet should be slightly fluffy at the top but doesn't have to be.

7. Pour off supernatant into a 15 ml tube and make to 10 ml with 0.4 ml of 5 mg/ml EtBr. Dissolve 9.6 g of CsCl in the solution by warming at 55°C and shaking for 15 min. Spin the tube at maximum speed in the table top centrifuge for 10 min. Decant the solution under the red protein floating on top. Load the sample into two TV865 tubes and spin at 50 K for at least 8 h at 20°C with the reograde mode.

8. Gently pick up rotor and take tubes into the darkroom. Identify the red band with the 366 nm uv lamp and collect DNA with 20 gauge needle and one ml syringe.

9. Extract the EtBr by adding an equal volume of isopropyl alcohol saturated with 5 M NaCl. Repeat several times until the pink color is gone.

10. Dialyze in 10 mM Tris/1 mM EDTA buffer at least 6 h.

11. Extract dialysate with equal volume of 50:50 phenol/ chloroform buffered with 0.1 M Tris pH 8.

12. EtOH precipitation. Add 0.1 volume of 3 M sodium acetate pH 5 and two volumes of cold EtOH. Spin down at 13 K for 10 min and wash the pellet with 70% EtOH. Dry the pellet and resuspend with 200 µl of TE.

B. Qiagen Maxi Plasmid Kit.
Follow the manufacture's protocol.

Transformation of *Dictyostelium*

W. Nellen *et al.*, in Methods in Cell Biology voulme 28; 67-100, Spudich ed. (1987) was a general reference for an early version of transformation.

1. The Kessin strain of AX3 (KAX4) was used for transformation experiment. Cells within a week or two were used. During growth keep the cell concentration in early log phase (i. e. below 2 x 10^6 cells/ml).

2. Dispense 10 ml containing 1 x 10^7 cells onto a 100 mm tissue culture plate. Allow cells to settle out of solution at least 1 h.

3. Remove the HL-5 media and replace with 10 ml of MES/ HL-5 pH 6.3. Tilt plate and pipette off from one marked edge of plate.

Make all subsequent additions from this one area to prevent too much cell loss from pipetting steps. Leave at least 30 min for cells to resettle.

4. Prepare DNA (12 μg) for addition. To 0.6 ml of HBS add 38 μl of 1 M $CaCl_2$ and the DNA while vortexing. Allow to precipitate for 30 min.

5. Pipet off media and then vortex DNA tube to resuspend DNA and dump by drops in the middle of the plate. Rock gently to spread the DNA and leave for 30 min.

6. Add 10 ml of MES/HL-5 pH 6.3 and let stand for 3-5 h.

7. Pipet off media and add 2 ml of 18% glycerol in HBS. Let stand for 2 min and then pipet off residual glycerol. Quickly add 10 ml of HL-5. Let stand overnight (12-18 h).

8. Pipet off media and add 10 ml of HL-5 plus 20-40 μg/ml G418. Let stand for 2 days. Change media with drug every two or three more days.

9. Colonies become visible as faint patches when the plate is held up to the light and tilted. Colonies with the endogenous plasmid (pnDeI or pnDeΔI) becomes visible in about 5 days but colonies with an integrating plasmid (pDneoII, B10S) become visible in 12 to 18 days.

10. When faint patches of colonies visible change media with drug to remove floating cells. Pipet off cells from the middle of patch area by using P-200 pipetman. Transfer those cells to new 100 mm tissue culture plate which contains media with G418. It will take about 3 days to form patches of colonies from isolated clones.

11. When the colonies have become almost confluent or at least covering one half of the plate wash the cells off the plate and into a 10 ml flask for suspension growth with drug selection.

Notes.

- Sterilize DNA by heating in the heating block at 70°C for several hours.
- Sterilize G418 is prepared as 4 mg/ml water by filtration. Make 1 ml aliquots and store at -20°C.
- Sterilize MES buffer (1 M stock) by filtration. MES is not stable to autoclaving.
- 18 % glycerol : To 3 ml of 60 % ultrapure sterile glycerol add 5 ml of 2 x HBS and 2 ml of water.
-HBS is prepared as a 2 x concentrate and stored frozen.

4 g	NaCl
0.18 g	KCl
0.05 g	Na_2HPO_4 or 0.062 g Na_2HPO_4/ H_2O
2.5 g	HEPES
0.5 g	Dextrose

pH to 7.05 with NaOH (proper pH is important)

nanopure water to 250 ml. Filter sterilize and store as 50 ml aliquots in -20°C.

- Sterilize 2 M $CaCl_2$ and stored at -20°C.

Genomic DNA isolation

1. Grow 4 L of KAX4 cells in HL-5 until cell concentration reaches to 0.9-1.1 x 10^7.

2. Harvest cells.

a. Centrifuge cells at 1500 rpm, 0°C for 5 min.

b. Resuspend the pellet with 500 ml of ice-cold 17 mM SB buffer. Spin down cells as above.

c. Repeat step 'b'.

3. Preparation of nuclei.

a. Add 100 ml of nuclei buffer to the pellet.

b. Add 0.65 ml of 100% NP-40 and shake vigorously about 5 min. Check lysis with microscope.

c. If cells are completely lysed centrifuge lysate 8000 rpm at 10°C for 10 min to pellet nuclei.

d. Resuspend nuclei pellet with 100 ml nuclei buffer.

e. Add 0.55 ml of 100% NP-40 and shake vigorously to remove mitochondria.

f. Spin down lysate with 8000 rpm at 10°C for 10 min

4. Isolation of nuclear DNA

a. Resuspend nuclei pellet with 30 ml of 0.1 M EDTA.

b. Add 3.5 ml of 20 % N-lauroyl sarcosine by dripping.

c. Incubate at 65°C for 15 min.

d. Transfer that into 50 ml of yellow cap tube.

e. Add 2.8 ml of 5 mg/ml EtBr and $CsCl_2$ to final density of 1.55 g/ml.

f. After dissolving centrifuge the tube at maximum speed in the tabe top centrifuge for 10 min to remove proteins.

g. Transfer the clear solution to T865 tube and centrifuge with 35K rpm in 20°C for at least 36 h.

h. Isolate DNA band by using 21 gauge needle and 1ml syringe.

i. Extract EtBr by adding an equal volume of isopropyl alcohol saturated with 5 M NaCl. Repeat several times until the pink color is gone.

j. Dialyze in 10 mM Tris/1 mM EDTA buffer at least 6 h.

k. Collect dialysate and add 2 volume of cold EtOH and 1/40 volume of 4 M NaCl. Spool out DNA by using yellow tip.

l. Dissolve in 1 ml of TE. Use shaker for 1 day to dissolve genomic DNA.

Nuclei buffer :

0.025 M Tris-HCl, pH 9.2

5 mM MgAc

5 mM EDTA

5 % sucrose

Bacterial Cell Transformation

1. Grow DH5 α. Pick out about 5 colonies from an LB plate and grow in 5 ml of SOB at 37°C overnight or for at least few h until cloudy.

2. Transfer 100 µl of overnight culture or 0.5 ml of younger culture to 100 ml of SOB in a 500 ml flask. Grow to OD 550 of about 0.5 (in about 2 h).

3. Cool on ice 10 min. Spin down cells with 7000 rpm at 4°C for 5 min. Resuspend cells in 10 ml of Tfb I. Keep on ice 10 min.

4. Spin as above. Resuspend cells in 2 ml of Tfb II. Add 70 µl of DMSO. Keep on ice 10 min.

5. Add 70 µl of DMSO and make 200 µl of aliquots in tube and freeze at -70°C. Keep in -70°C until it is needed for transformation.

6. Thaw frozen competent cells on ice and transfer to Falcon 2059 tube.

7. Add about 10 ng of DNA to the tube and keep on ice for 30 min.

8. Heat shock for 50 s at 42°C and quickly transfer to ice again. Keep on ice for 2 min.

9. Add 0.9 ml of SOC and shake the cells in the 37°C incubator for 2h.

10. For blue-white selection spread out 50 µl of 2% X-gal and 10 µl of 0.1 M IPTG on LB-amp plate. When pate is dry, spread out about 50 to 200 µl of culture from 9. Incubate the plates in the 37°C incubator.

SOC : To SOB add 18.6 ml of filter sterile 20 % glucose per liter.

Tfb I (Transformation buffer I)

10 mM MES pH 6.2	1 ml of 1 M
100 ml $RbCl_2$	1.9 g
45 mM $MnCl_2$ x 4 H2O	0.89 g

10 mM $CaCl_2$ x 2 H2O	1 ml of 1M
3 mM Hexamine cobalt	0.08 g

nanopure water to 100 ml then filter sterile and store at 4°C.

Tfb II (Transformation buffer II)

10 mM KAc	1 ml of 1 M pH 7.0
100 mM KCl	10 ml of 1 M
45 mM $MnCl_2$	0.89 g
10 mM $CaCl_2$	1 ml of 1 M
3 mM $HACoCl_3$	0.08 g
10 % ultrapure glycerol	10 ml

nanopure water to 100 ml the filter and store at 4°C.

Southern Blot

1. Completely digest 10 µg of genomic DNA in a total reaction volume of 100 µl with 5µl restriction enzyme at 37°C for 3 h or overnight. Use only a cut off pipette tip with a wider bore so the DNA is not sheared excessively. Mix the reaction well by pipetting and flicking the tube because the high MW DNA is very viscous.

2. Prepare 0.7% agarose gel with 1 X TBE buffer. Need 400 ml of gel in the 20 X 30 cm gel box and 1.6 L running buffer. Use the large comb slots (0.9 cm X 1.5 cm) or else the DNA may smear during the run. Cover the gel with a thin (2 mm) layer of buffer.

3. Add 10 µl loading buffer to the sample, mix well. Then load the sample in the slots and use lambda DNA (1µg) cut with EcoR I or

Hind III or both as a size marker at the ends of the gel. Start electrophoresis with EC 500 at 80 V for 10 min to make all of the DNA stack in slot. Then lower voltage to 40 V and electrophoresis overnight for about 15 h until the dye reaches about 10 cm. Perform electrophoresis the same way so that blots done on different days could be easily compared later.

4. Shut off power and cut away the excess gel. Transfer the DNA in gel using a X-ray flm as a supporter because the gel breaks easily. Cut off a small bit of one edge of gel as an orientation mark. Place in a large plastic boxs and add about 1 L of buffer then about 2 to 3 drops of EtBr until the solution is just pink. Shake for about 15 min or until the DNA is stained. View on the transilluminator and take a picture with a ruler set along the side of the gel. At the same time, turn on the Haake Cooler set at -10°C.

5. Replace the buffer with 1 L of 0.2 N NaOH /0.6 M NaCl and shake for 30 min to denature the DNA. Replace with 1 L of 25 mM phosphate buffer (pH 6.5). Change buffer twice within about 30 min.

6. Pour 5 L of phospate buffer into the electoblot apparatus. Cut 4 sheets of 3 MM filter paper and 1 sheet of Gene Screen membrane to the size of the gel to be blotted. Soak the Gene Screen for about 10 min. Assemble the blot sandwhich: on the plastic grid in a box with 3 L of phosphate buffer place a layer of foam, 2 layers of prewetted filter paper, the gel, the prewetted Gene Screen membrane, 2 layers of prewettd filter paper, foam and then the other side of the plastic grid. Do all of assembling process under the buffer to prevent air bubbles from forming in the sandwhich. DNA will not be transfered to the membrane in regions containing air

bubbles. Clamp the sandwhich with hands and do not release grip until the sandwhich is within the electroblotter slots and submerged into the buffer.

7. Electroblot with ≈0.4 Amp for 1 h. Check after 10 min. to make sure the current is stable. Increase current to ≈1 Amp and continue for 6 h or longer. At the end remove the filter and dry on a filter paper. View the blot with the short wave UV light and mark lightly with pencil any lambda size bands or rDNA bands.

8. Fix the DNA on the blot by baking 80°C for 2-4 h.

9. Seal the blot in a seal-a-meal bag with 1 X 10 ml hybridization buffer. Prehybridize the blot at 65°C for at least 1 hr using the agitator set for slow movement of the buffer over the blot.

10. Cut the bag at a corner and add a very hot inset probe at ≤ 10 ng/m. Re-seal and incubate at the 65°C shaker for at least 2 day.

11. For the post-hybridization, wash the blot as follows:

a. 2 X 2X SSC RT for 5 min each

b. 2 X 0.5X SSC/1% SDS at 65°C for 30 min.

Check background count (cpm) over region which should not have any counts; if less than 50 cpm, stop here.

If the counts still high:

c. 1 X 0.2X SSC/1% SDS at 65°C for 30 min.

Check counts

d. 1 X 0.1X SSC/1% SDS at 65°C for 30 min.

(There should be almost no detectable cpm even over the DNA which should contain radiolabelled probe DNA.)

Wash away excess SDS with a couple of rinses in 0.1X SSC

12. Wrap in plastic wrap and mount over 3 MM filter paper using tape. Mark all size markers, rDNA and any other bands you saw on the blot with radioactive (^{35}S) ink. Put date, oreintation marks as X on the filter. Do not get outside wet or else the film in contact will be ruined. Set up blot against XAR-5 Kodak film and lay this over an intensifying screen in a cassette. Expose at -70°C for overnight or longer.

13. Keep blot for reuse as many as three times. For reuse, follow procedure for stripping old probe (0.1X SSc/1%SDS, boil 30 min). Store the dry blot at RT in plastic bag.

Solutions for Southern Blot:

1. Phosphate buffer for 1 M stock.

Mix 93.84 g $NaHPO_4$ and 45.44 g Na_2PO_4 in 700 ml water, heat and stir then cool to RT; adjust pH to 6.5 and volume to 1 L.

2. Pre-hybridization buffer:

NaCl	2.92g
1 M tris pH 7.5	2.5 ml
50% dextran sulfate	10 ml
50X Denhardt's solution	5 ml
10% SDS (ultra-pure)	5 ml
10 mg/ml Salmon DNA	0.5 ml
water to total	50 ml

RNA Isolation (Chirgwin, *et al.*, 1979)

A. Isolation of total cellular RNA from D. discoideum by using guanidinium thiocyanate

DAY 1

1. For each 10^8 cells (about 1-2 ml cell pellet from 100 ml shaking culture) dissolve in 5 to 10 ml of 4M guanidinium thiocyanate / 0.1 M β-mercaptoethanol/pH 5. Freeze sample in dry ice or place in -70°C freezer to make sure that all cells lyse. Samples can be stored indefinitely when frozen.

2. Thaw sample then spin at 3000 rpm in a table top centrifuge to pellet insoluble material if any. Centrifugation for 15 to 20 minutes is usually enough.

3. Take an RNAse free AH-629 polyallomer tube and add 2.5 ml of 5.7M CsCl/0.1 M EDTA/pH 5 to tube to make a CsCl cushion. Gently layer the sample over the cushion. Fill the tube up to within a couple of millimeters of the top. The sample can be up to 13 ml. Balance the tubes to within 0.1g and load all of the buckets even if you only 2 have samples. Centrifuge for 24 h at 25K at 15°C.

For the Beckman SW 41 use 2.2 ml of CsCl and spin at 33K and for the SW 28 add 5.5 ml of CsCl and spin at 27 K. Shorter spin times (18 h) could be used without signifcant loss of RNA yield.

DAY 2

4. Remove all of the sample by aspiration down to the CsCl layer and then add 3 ml of dep'd water to the tube to wash the walls. Aspirate the wash water and most of the CsCl so that about 1 ml is left. Invert the tube and keep inverted.

5. Carefully add about 0.5 ml of dep'd water to wash the area around the pellet and drain this out. Do not disturb the small

button-like translucent pellet of RNA which will be in the exact bottom of the tube. The small aggregates around the pellet are not RNA and should be avoided. They can be wiped out with a Kim-Wipe if necessary.

6. Take up 0.5 ml of dep'd water in a blue tip and jab the pellet to break it up. Add the water and take up the pieces to transfer to a microfuge tube.

Add anaother 0.5 ml of water to the cup to rinse and make sure that no RNA is left in the tube or in the blue tip. Now vortex the RNA in the microfuge tube continuously for about 2 min to dissolve the RNA then spin in the microfuge for 5 min to pellet the insoluble material, if any. Take up the supernatant and transfer to a baked Corex 13 ml tube and add 2 ml of dep'd water, 75 µl of 4M NaCl, and 7.5 ml of cold 100% EtOH. Cap with parafilm, invert to mix and store at -20°C for a few hours or overnight or indefinitely.

7. Spin the tube at 13,000 rpm in the HB-4 rotor for 20 min at 4°C to pellet the RNA. Drain or aspirate the supernatant and then gently wash the film of a pellet with cold 80% EtOH using about 0.5 ml to remove the residual NaCl. Remove this wash EtOH by aspirating. Cap the tube with parafilm and poke holes in it and then place in a vacuum for 5 min or until the EtOH and water have evaporated. The pellet is similar to the appearance of a dry cracked lake bed. Take up the pellet in 200 µl of dep'd water and vortex to dissolve the RNA. Transfer to a microfuge tube and then add another 200 µl of water to rinse the tube and transfer this to the microfuge

tube also. RNA dissolves easily within 2 min of vortexing. If there is pellet material that does not dissolve in this time it is not RNA.

This RNA is good enough for use in Northern blots.

8. To further purify the RNA (for in vitro translation and construction of cDNA library) add 0.4 ml of buffered phenol/chloroform and vortex to make a cloudy emulsion. Spin for 2 min in the Speedy Vac centrifuge without vacuum to separate the phases. Remove the upper water phase and leave any interface behind. Reextract the water phase in another tube with choroform/isoamyl alcohol 0.4 ml and spin as before. Remove the lower chloroform phase and reextract with chloroform again. The small amount of residual RNA left in the phenol/chloroform phase could be recovered by adding 0.2 ml of dep'd water to it and vortexing and spinning as before. If done add this to the ca. 0.3 ml first recovered from chloroform. Add 4M NaCl to 0.1 M final concentration (12.5 µl to 0.5 ml) and 2 volumes of cold 100% EtOH (1.0 ml to the 0.5 ml). Vortex to mix and allow to precipitate at -20°C for at least 2 hours or overnight or indefinitely.

9. Spin in the microfuge for 10 min and wash the pellet with 50 µl of 80% EtOH. Dry in the Speedy Vac.

10. Add 1 ml of cold 2 M LiCl to the dry pellet and break up the pellet. Cap and rotate or rock for about 1 hour at 4°C. Spin again in the microfuge to recover the pellet and then wash the pellet with 50 ul of 2M LiCl. Remove the wash and add 0.5 ml of dep'd water to dissolve the pellet and then precipitate with NaCl and EtOH as before

(step 8). Finally dissolve the RNA in about 100 to 200 µl of dep'd water and determine the OD at 260 to 280 nm. Add 5 µl of the RNA to 1 ml of water and read the ODs. Get at least 0.1 OD units at 260nm, if necessary add more sample. Determine the OD and multiply by the dilution factor of 200 and multiply by 40 µg/ml to get the RNA concentration in µg/ml. The usual 260/280 ratio is about 2 to 2.3 but values as low as 1.6 are still good preparations.

NOTES

1. This procedure gives total RNA which can be translated in the reticulocyte assay because inhibitory material has been removed with the extra phenol/chloroform and LiCl washes. Do not use more than about 2 x 10^8 cell in the 10 ml at step 1 because a large amount of gelatinous material will spin if the gradient is overloaded.

2. At step 10 the RNA will not dissolve easily occasionally if the RNA concentration is too high and expecially after phenol/chloroform extraction. These RNA aggregates will need extra help in dissolving such as breaking up the aggregates with a blue tip and vortexing for a long time (over 10 min).

Solutions for RNA preparation:

1. All glassware should be baked to be RNAse free (350 °F for at least 3 hours).

2. All plasticware is OK if not handled with bare hands which have RNAse. Wear gloves throughout the procedure. Have pipette

tips and microfuge tubes and glassware that you will use only for RNA preparation.

3. Water. Use nanopure water that has been treated with diethypyrocarbonate (dep) at a final concentration of 0.2% as follows. Make a 10% solution of dep in 100% EtOH and dilute this 500 fold in the water. Leave overnight or at least 3 hours and then autoclave for 20 min to inactivate the residual dep. Store the water in small baked bottles. Use gloves when handling the dep because it is a strong denaturing agent.

4. 4M NaCl. Treat with dep as above.

5. Polyallomer tubes. Treat with dep as above. Place the tubes in a large beaker with water and add the dep to 0.2% and mix up. Autoclave as before and store the tubes in a closed box.

6. Guanidinium thiocyanate/0.1 M β-mercaptoethanol. Add 47.2 g of Fluka G/SCN or ICN or BRL to about 70 ml of nanopure water and then in a hood add 0.7 ml of β-mercaptoethanol. Cover and stir and gently warm to dissolve and make up to a final volume of 100 ml. Filter through a 0.45 μm Nalgene filter and store at -70°C in 50 ml plastic tubes. Check pH, it should be 5 and will be if Fluka G/SCN is used. Adjust the pH if necessary with acid.

7. 5.7 M CsCl/0.1 M EDTA. Add 48 g of baked CsCl technical grade to about 30 ml of nanopure water and then 5 ml of 0.5 M EDTA and stir and warm to dissolve at a final volume of 50 ml. Adjust the pH to 5 with acid. Filter through a Nalgene filter and store at -20 C or add dep to 0.2 % and treat as for dep'd water.

8. Phenol/Chloroform. Shake phenol with 0.1M Tris (pH8) (usually 25ml of phenol with 25ml of Tris) to equilibrate. Spin in 50

ml plastic tube at 3000 rpm for 5 min to separate the phases. Repeat until the water phase is pH about 7-8. This may be several times. Add a pinch of 8-hydroxyquinoline to give the phenol some brown color for visibility and then add β-mercaptoethanol to 0.1%. This makes the equilibrated phenol which can be stored at 4°C tightly capped. Add equal parts of this phenol to chloroform to make the 50:50 mixture.

9. Chloroform/isoamyl alcohol. Add isoamyl to make a 50 to one solution of chloroform to isoamyl alcohol and store this at room temperature.

B. Isolation of Total RNA by using kit

RNA STAT-60 (TEL-TEST "B") was used for quick isolation of total RNAs. The manufacture's protocol was followed.

Northern Blot

1. Add 5.2 g of agarose in a 500ml flask add 80 ml of 5X MOPS buffer (pH 7) and 250 ml of nanopure water. Put in the microwave and allow to boil throughly to melt all of the agarose. Meanwhile tape the edges of the 20 x 30 cm gel box and put it in the hood and fix the 0.7 cm x 2 mm gel comb in place. When the agarose is melted cool to about 60°C and add 69 ml of 37% formaldehyde while swirling to evenly mix the formaldehyde into the agarose. Pour the mixture in the gel box and smooth out any bubbles. It hardens in about 30 minutes. The concentration of agarose is 1.3% and the gel is

about 0.7 cm thick. Up to about 60 µl of sample can be loaded in the slots.

2. While the gel is cooling denature the RNA samples. Normally about 10 µg of total RNA is run but 50 µg will be successfully separate by this method.

Prepare the following in a microfuge tube:

5X MOPS buffer	4 µl
formaldehyde (37%)	3.5 µl
formamide	10 µl
RNA	2.5 µl to 4 µl

Mix and place at 55°C for 15 min. Add 2 µl of sterile loading buffer (50% glycerol/0.05% bromophenol blue). Make sure that the RNA is assayed correctly for concentration or else the lanes may have different amounts. It is best to determine the OD of all then make a dilution to 1 or 2 mg/ml for all samples. Reassay the OD of the dilution then calculate the amount load.

3. Gently remove the comb from agarose gel. Fill up the chambers in the gel box which require about 800 ml per side and add a covering layer of buffer to about 1 mm to just cover the gel and fill up the slots. Do not overfill because the formaldehyde concentration must remain high. Add the samples and run the gel at about 40 volts overnigh (ca. 11.5 hrs with the EC 500; use 50 V for the EC 600) so that the blue dye has run about 10 cm. Run all of the gels the same way so that in the future you can compare gels run on separate days.

Optional faster runs: 80 V 5.5 h 10 cm EC 500

100 V 5.5 h hrs 10 cm EC 600

Save the running buffer for reuse: can be used at least 3 times.

4. Cut out the gel area containing the lanes and put it in another box and rinse with 1 liter of the Tris or phosphate solution..

5. Meanwhile turn on the Haake circulator cooling bath set at -10°C.

6. Replace with 1 L of the Tris or phosphate buffer solution and shake for 15 min. and then pour that out and replace with one more liter of Tris or phosphate buffer. Shake again for 15 min plus add 5 drops of 5mg/ml Ethidium bromide to stain the RNA. The rRNA on the gel is usually not visible in the stained gel but it will be stained on the blot.

7. Remove the gel and look at the RNA on the ultraviolet light box. The RNA will only be visible well if the Tris buffer had been used and pH is adjusted to 8. The ribosomal RNAs of 26S and 17S should be visible about 5 to 7 cm from the slot. Trim away any extra gel outside of the lanes and cut off the gel below the bromphenol blue area at the bottom of the gel. Good visualization at this atage is not really necessary. The bands will appear well on the membrane after blotting.

8. Cut out 4 pieces of Whatman 3MM paper and one piece of Gene Screen or Gene Screen Plus the same size as the gel and soak these in the Tris (Gene Screen Plus) or phosphate (Gene Screen) buffer. Assemble the electoblot sandwhich in a large plastic box. First add about 1.5 liter of the appropriate buffer and soak one of the sponges. Then add 2 sheets of the paper making sure that no air bubbles are trapped between or under them. Add the gel on top of

them while everything is submerged in the buffer to prevent trapping of air bubbles and then put the Gene Screen or Gene Screen Plus on top of gel. Smooth out the layers and then add the final two sheets of paper under the buffer as before. Add the other side of the sandwich and place in the electroblot aparatus which is filled with 5 L of the buffer.

9. The Gene Screen side of the sandwich is on the positive side of the electoblotter. Start the electrobloting at 0.4 amp for one hour and check the amperage in about 15 min to make sure that it is stable and still on. After one hour increase the amperage to ca. 0.9 amp for 3 hours. Remove the blot and air dry or briefly dry on the filter paper and then bake at 80°C for 2 hours. The air drying step is a good overnight stopping point. Look at the blot under the short wave length uv light and mark the slots, the rRNA bands, the date and the samples in pencil on the blot.

10. Prepare the prehybridization buffer in a 50 ml tube.

50% formamide	25 ml	
50X Denhardt's sol	5 ml	Do not heat over 37° to thaw.
1 M Tris, pH 6.8		2.5 ml
NaCl	2.92g	Dissolve NaCl first in the aqueous ingredients not the formamide or SDS.
10% SDS		5 ml
50% dextran sulfate		10 ml
10 mg/ml salmon DNA		0.5 ml

Check pH with paper. Should be 7 and pale green. If not and the pH is very green the formamide may need to be deionized and the buffer should not be used for hybridizition.

50% dextran sulfate = 100 g plus 155 ml of nd water; heat and stir to dissolve slowly.

11. Seal the blot in the seal a meal bags and add 10 ml of the prehybridization buffer. Place in a 42°C water bath and allow the bubbles to float up to the top. Prehybridize at least one half hour but can be o/n or longer.

12. Add the probe to 10 ml of the prehybridization buffer so that the probe concentration is ≤ 10 ng/ml with a specific activity of at least 1 x 10^7 per μg. Usually add one half of the oligolabelling reaction of 100 ng DNA. Heat the hybridization buffer for about 10 min in boiling water to denature the DNA then quickly cool on ice briefly. Pour off the old prehybridization buffer and add the cooled hybridization buffer to the blot and seal again. Leave in the 42°C bath for two days before the posthybridization washes. Turn on the 65°C shaker bath before the posthybridization so that it heats up in time.

13. Remove the hybridization buffer and save in a plastic tube at room temperature (can be reused at least 3 times within 2 weeks). Wash the blot in 1 L of 2 X SSC twice for 5 min with shaking at RT. Follow with a wash in 1 l of 0.5 X SSC/ 1 % SDS (Sigma 95% SDS) at 65°C for 30 min with shaking. Check counts of blot with Geiger counter held over the top of blot for background which should be low and over the lower part below the rRNA band which should be

noticeabley higher. Usually necessary to wash again at 0.5 X SSC/1 % SDS at 65°C for another 30 min. Remove excess SDS by several quick washes over 30 min with 0.1 X SSC at RT. During the washes do not allow the blot to dry.

14. Wrap the blot in plastic wrap and then mount on 3MM paper. Do not get the outside wet or it will stick to the film during exposure. Mark the rRNA bands location, the date and the orentation of the lanes and someother X marks on the paper with ink spiked with some ^{35}S. Place into light proof cassette against XAR5 film with the film against an intensifying screen. Expose at -70° for at least 1 day.

Sequencing (Sanger, *et al.*, 1977)

Double stranded DNA sequencing is time and labor saving comparing to ss-DNA sequencing. By using the following protocol, we can easily read up to 300 bp.

I. PREPARATION OF DOUBLE-STRANDED DNA FOR SEQUENCING

See Plasmid mini-prep and large preparation DNAs which were isolated by mini and large prep except quick mini prep could be used for sequencing.

II. DENATURATION OF PLASMID FOR SEQUENCE ANALYSIS

1. Take 10 μg. ds plasmid in TE buffer in 1.5 ml microfuge-tube or use all of one mini-prep plasmid DNA.

2. Add up to 0.4 M NaOH, then incubate at 37°C for 15 min.
3. Add 0.1 vol of 3 M NaAC (pH 5.0) to neutralize.
4. Add 2 to 4 vol 100% EtOH (cold), then precipitate. at -70°C for 30 to 60 min or overnight.
5. centrifuge for 15 min at 4°C at 13,000 rpm.
6. Wash with 300 µl of 70% EtOH for 3 min, then spin 3-5 min at micro-centrifuge. Pour out EtOH and remove residual EtOH with micro-pipette.
7. Dry the pellet at room temp. for 30 min.
8. Keep the sample dry at 4°C until ready for sequencing.
9. Before the sequencing, add 7 ul ddH_20 to dissolve the DNA.
10. Add 2 ul 5X sequence kit buffer (United State Biochemical), 1 µl Primer (about 40 ng) and mix with micro-pipette.
11. Put the tube in 65°C water bath for 2 min., then cool at room temp to < 30°C. (about 30 min)
12. Follow the USB brief protocol.
13. We follow the Sequenase Version II protocol supplies by USB modofied as follows:
    1) use 1:2 dilution of labelling mix (instead 2:8)
    2) the termination reaction is done between 40-45°C (instead of 37°C) for 5 min.

III. Gel PREPARATION and ELECTOPHORESIS

1. Thoroughly mix the following components:
   6% : 50.4 g. Urea
   6.84 g. acrylamide (Ultrapure)
   0.36 g. bisacrylamide (Ultrapure)

12 ml 10X TBE
0.12g ammonium persulfate.
68 ml dH_2O

120 ml

2. Filter (Whatman No.1) the solution into a flask on ice.

3. Place gel solution on room temp. until the temperature of the solution reaches 15°C.

4. Add 60 µl of TEMED and swirl gently to ensure thorough mixing, and pour the gel immediately.

5. Insert the comb at the top of the gel in a horizontal position and clamp the plates at the top. Leave the gel flat on the bench horizontally for about 30 min.

6. Buffer preparation: 1 X TBE 1.6 L.

7. After the gel is polymerized, tear off the tape of the bottom and affix plates to the sequencing appratus. Pour running buffer (1X TBE) into upper and lower reservoirs.

8. Remove one comb and flush wells with 1 X buffer to remove any unpolymerized acrylamide; insert one shark-tooth comb in the same position; then, do the same for the other comb.

9. Pre-run the gel for 1 to 2 hours at 60 Watt (constant power). (The running power is 50-55 Watt.) The optimal plate temerature (measured with a thermometer outside the plate) for a good running is about 40°C.

10 Check the leakage of the wells by loading 1 ul stop buffer. (prepare samples while pre-run. See brief protocol supplies by manufacture)

11. After electrophonesis is finished, fixing 15 min. (15% methanol/ 5% acetic acid) with gentle shaking every 5 min. and drying at 80°C for 40-50 min.
12. Place against XAR5 film and expose overnight.

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