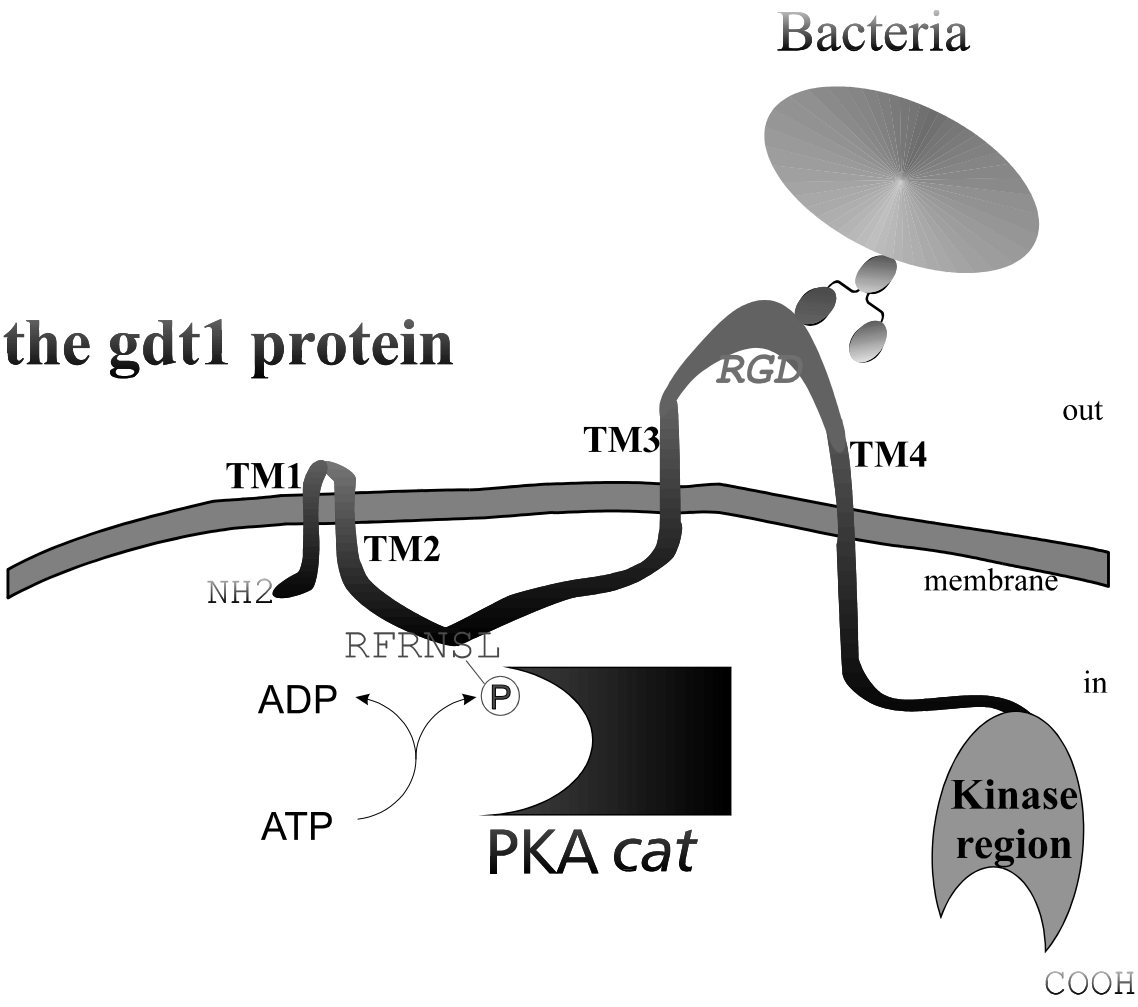
Dissertation

Faculty of Biology/Chemistry at the University of Kassel (GHK)

Molecular Analysis of the Growth-Differentiation-Transition in *Dictyostelium*

by

Changjiang Zeng



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from

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ABBREVIATIONS

AC	adenylyl cyclase
AP	alkaline phosphatase
ATP	adenosine 5'-triphosphate
Ab	antibody
APS	ammoniumperoxidisulfate
A260	absorbance at 260 nm
b	base
bp	base pair
BCIP	5-Bromo-4-Chloro-3-indolylphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CAR	cAMP receptor
CMF	conditioned medium factor
Ci	Curie
cpM	counts per minute
cDNA	complementary DNA
ddNTP	didesoxynucleotide, ddATP, ddCTP, ddGTP, ddTTP
dNTP	desoxyribonucleotide, dATP, dCTP, dGTP, dTTP
DIF	differentiation inducing factor
DTT	1,4-dithiotreitol
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclase
EDTA	ethylendiamintetraacetic acid
G418	geneticin
GC	guanylyl cyclase
GDT	growth-differentiation transition
G-protein	heterotrimeric GTP binding protein
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
HRPO	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pairs
kDa	kilo Dalton

lacZ	β-Galactosidase gene
mAb	monoclonal antibody
MCS	multiple cloning site, polylinker
MES	2-(N-Morpholino)-ethansulfonic acid
β-ME	beta-mercaptoethanol
min	minute
MOPS	γ-(morpholino)-propansulfonic acid
NP40	ethylenphenylpolyethylenglycol
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDI	phosphodiesterase inhibitor
PEG	polyethylenglycol
PKA	cAMP dependent protein kinase
PLC	phospholipase C
PSF	pre-starvation factor
PIPES	1,4-piperazindiethansulfonic acid
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	RNase inhibitor
NTP	ribonucleotide, ATP, CTP, GTP, TTP
RT	room temperature
sarcosyl	N-lauroyl-sarcosine
SDS	sodium dodecyl sulphate
Strep.	streptomycin
TCA	tri-chloroacetic acid
TBE	Tris/borate electrophoresis buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	tris-(hydroxymethyl)-aminomethane
Triton X-100	octylphenylpoly-(ethylenglycolether)
Tween 20	polyoxyethylen-sorbitan-monolaurate
Vol.	volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

I. ZUSAMMENFASSUNG

Unter Nahrungsmangel gehen *Dictyostelium discoideum* Zellen von der einzelligen amöboiden Lebensform in einen vielzelligen Organismus über, der sich zuletzt in zwei Zelltypen differenziert, nämlich Stielzellen und Sporen. Dieser Prozeß wird durch verschiedene Signale gesteuert, die die differentielle Genexpression regulieren. Unter den ersten Genen, die bei diesem Wachstums-Differenzierungs-Übergang (growth-differentiation-transition, GDT) aktiviert werden, ist die discoidin I Genfamilie, die deshalb auch als ein exzellenter Indikator für den Beginn der Entwicklung dient.

Mit Hilfe von Mutanten, die Defekte in bekannten Signaltransduktionskomponenten haben, und durch pharmakologische Analyse, konnten wir bereits ein Model für einen aktivierenden und einen inhibierenden Signalweg für die discoidinexpression aufstellen. Aktivierung erfolgt über einen unbekannten Rezeptor, ein heterotrimeres G-Protein, das die Gα2 Untereinheit enthält, CRAC (cytosolic regulator of adenylyl cyclase) und PKA (cAMP dependent protein kinase). Inaktivierung erfolgt über extrazelluläres cAMP, den cAMP Rezeptor cARI und Ca^{2+} Influx.

Um weitere Signaltransduktionskomponenten zu identifizieren, haben wir mit Hilfe der REMI-Mutagenese (restriction enzyme mediated integration) nach Klonen mit Discodin Fehlexpression (Unter- oder Überexpression) gesucht. Eine Mutation in einem neuen Gen, das *gdt1* benannt wurde, bewirkt discoidin-Überexpression und beschleunigte Entwicklung. In Western-Blots konnte gezeigt werden, daß das gdt1 Genprodukt ein 175 kDa membranassoziiertes Protein ist. Sequenzanalyse zeigte, daß der C-Terminus eine Kinasedomäne enthält, die zur Tyrosin-Rezeptorkinasefamilie gehört. Nach vorläufigen Daten ist gdt1 höchstwahrscheinlich an der Zell-Substrat-Adhäsion beteiligt. Dafür spricht die "RGD" Sequenz (Arg-Gly-Asp) in einer der extrazellulären Domänen. Wie durch Kombination der gdt1 Mutation mit den $G\alpha2^-$ und PKA^- Mutationen gezeigt werden konnte, reprimiert gdt1 die discoidin Genexpression, während die PKA Signalkaskade dem teilweise entgegenwirkt. Aus vorläufigen *in vitro* Experimenten ergibt sich, daß das gdt1 Genprodukt ein PKA Substrat sein könnte. Die gdt1 Rezeptorkinase ist ein negativer Regulator für discoidin und damit auch des GDT. Der gdt1 Signalweg verläuft parallel zu dem bereits zuvor identifizierten Gα2-CRAC-PKA Signalweg, wird jedoch auch durch PKA reguliert, und zwar höchstwahrscheinlich durch Phosphorylierung von gdt1. Eine Phosphorylierung durch PKA könnte das gdt1 Protein inaktivieren, was zu maximaler discoidin Transkription führen würde. Zerstörung des gdt1 Gens resultiert dementsprechend in deregulierter discoidin Überexpression und beschleunigter Entwicklung.

I. SUMMARY

When cells of *Dictyostelium discoideum* are deprived of nutrients, they undergo a transition from single cell amoebae to a multicellular organism which finally differentiates into two cell types: stalk-cells and spores. This process is organised by various signals which regulate the differential expression of genes. The discoidin I gene family is among the first genes activated at the growth-differentiation-transition (GDT) and thus serves as an excellent indicator for the beginning of development.

Using mutants with defects in known signal transduction components and pharmacological analysis, we have previously established a model for an activating and an inactivating pathway for discoidin expression: Activation occurs via an unknown surface receptor, a heterotrimeric G-protein containing the Gα2 subunit, CRAC (cytosolic regulator of adenylyl cyclase) and PKA (cAMP dependent protein kinase). Inactivation occurs via extracellular cAMP, the cAMP receptor cAR1 and Ca^{2+} influx.

By REMI mutagenesis (restriction enzyme mediated integration) we have screened for clones displaying mis-expression of discoidin (null or overexpression) to identify further signalling components. One mutant is disrupted in a new gene denominated *gdt1* resulting in overexpression of discoidin and accelerated development. Western-blots show that the gdt1 gene product is a 175 kDa membrane-associated protein. Sequence analysis indicates that the C-terminus contains a kinase domain which belongs to the Tyrosine Receptor Kinases family. Preliminary data shows that gdt1 is possibly involved in cell-substratum adhesion as suggested from the "RGD" (Arginine-Glycine-Asparagine) site at one of the extracellular domains. As demonstrated by combinations of the gdt1 mutation with $G\alpha2^-$ and PKA^- mutations, the gdt1 generates a repression of discoidin expression and is partially counteracted by the PKA signalling cascade. *In vitro* experiments suggest that the gdt1 gene product is a PKA substrate. The gdt1 receptor kinase is a negative regulator of discoidin and of GDT. The gdt1 signalling pathway is in parallel with the identified Gα2-CRAC-PKA pathway but is regulated by PKA, most likely, by PKA phosphorylation on gdt1. Phosphorylation by PKA may inactivate the gdt1 protein and allow the maximal level of discoidin transcription. Similarly, disruption of gdt1 results in deregulated discoidin overexpression and accelerated development.

II. INTRODUCTION

1. *Dictyostelium discoideum* as a model system to study development

For all eukaryotic systems, cellular differentiation proceeds through a series of stages that have to be carefully timed to result in the proper tissues and structures. In multicellular organisms, where organs are generated from different cell types, the processes must be temporally co-ordinated among diverse cells.

Usually a specific cell type cannot proceed to the next stage in differentiation until a signal is received from adjacent cells. *Dictyostelium discoideum*, which serves as a model system for the study of development, is an unique eukaryotic developmental system in which multicellularity arises from the aggregation of individual cells. The life cycle of *Dictyostelium* consists of distinct growth and developmental phases, and it is a simple matter to switch between the two modes. When nutrients are plentiful, *D. discoideum* cells grow and divide as individual amoebae. When cells are depleted of nutrients, growth ceases and development is initiated. Development is characterised by a stage in which around 10^5 single amoebae aggregate to form a multicellular organism and cells finally differentiate into two types: vacuolated stalk cells and durable spores (see Fig. 1).

In comparison to other eukaryotic organisms, *Dictyostelium* has some special biochemical and genetic characteristics. Such as:

→ Free-living *Dictyostelium* amoebae are easily grown and maintained (Sussman, 1987), thus clonal cells can be simply identified on a bacterial lawn. Some strains of *Dictyostelium discoideum* can also grow in axenic medium. This gives the possibility to carry out gene technical and cell biological work on cells growing in culture.

→ The differentiation of *Dictyostelium* is completely reversible until the late stages of the program. Growth stops when the nutrients in the environment are exhausted and there is no further replication of the chromosome until a fresh food source is found (Shaulsky *et al*., 1996). On re-introduction of nutrients, cells ″erase″ their developmental markers and resume growth (Soll and Finney, 1987). Thus, once developmental mutations are selected, they can be propagated and maintained, and the phenotypes can be expressed again when development is re-initiated.

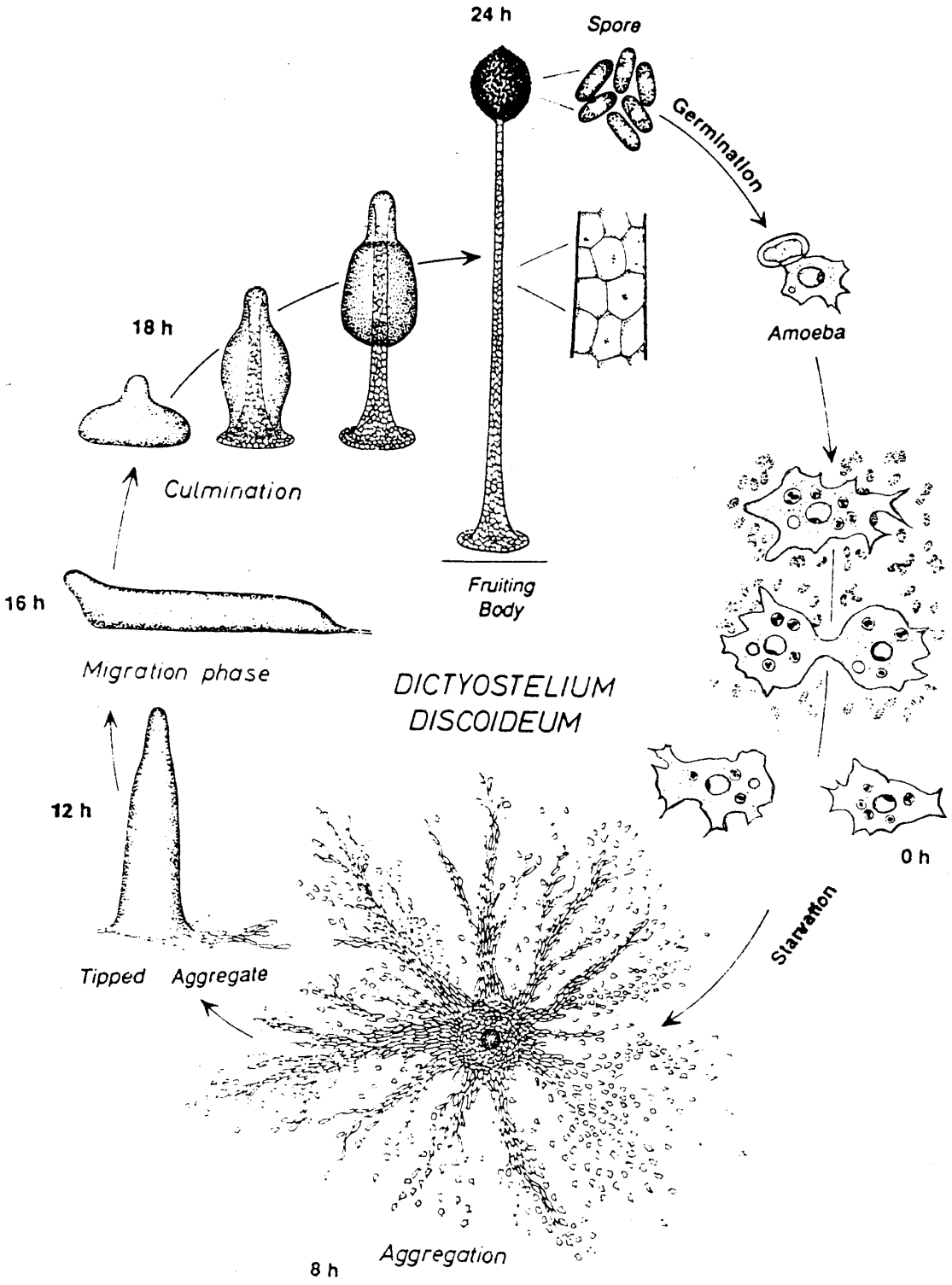


Figure 1. The life cycle of *Dictyostelium discoideum.* *Dictyostelium* is an unique eukaryotic developmental system in which multicellularity arises from the aggregation of individual cells. This process is initiated upon starvation, with vegetative growth and multicellular development being distinct phase of the life cycle. Because *Dictyostelium* generates single clones when fed on its natural food, bacteria, it is possible to screen for clonal *Dictyostelium* mutants with developmental phenotypes on bacterial plate.

→ *Dictyostelium* cells are haploid thus non-lethal recessive mutations can be directly scored. It is relatively easy to mutagenize cells, and selection of mutations is limited only by the screening or selection procedure (Loomis and Gilpin, 1987). However, *Dictyostelium* parasexual diploids can also be obtained by cell fusion. Recessive sensitivity markers were used to select against the haploid cells and allow survival of diploids only (Loomis, 1987). Thus, by use of tester strains marked with recessive resistance markers, mutations can be assigned to complementation and linkage groups (Welker, 1986). More recently, a true sexual mating system for *Dictyostelium* has been established for laboratory work (J. Williams, unpublished data).

Due to the above characteristics, *Dictyostelium discoideum* has long been serving as a useful system in which to study many of the complex processes of development (reviewed in (Loomis, 1975; Loomis, 1982), such as chemotaxis (Gerisch *et al*., 1987; Hall *et al*.,1989; Berlot *et al*.,1987), intercellular communication (Devreotes, 1989), cellular differentiation (Gomer *et al*., 1987; Coffman *et al*., 1993), and signal transduction (reviewed by Firtel, 1991; Devreotes, 1989). The movement and chemotactic response of *Dictyostelium* cells closely resemble those of amoeboid-like cells in higher eukaryotes such as leukocytes and macrophages (Zigmond, 1986), and the signalling properties of individual cells is more clearly understood in *Dictyostelium* than in other organisms.

2. Signal Transduction in *Dictyostelium*

The developmental process of *Dictyostelium* is controlled by cell-cell signalling, by which extracellular stimuli elicit intracellular responses. Various soluble factors are secreted by *Dictyostelium* and serve as signals controlling gene expression, developmental initiation, morphogenesis and cell differentiation (Devreotes, 1982). During exponential growth, most developmental gene products are present at low levels, except a group of V genes which are expressed only in vegetative stage and shut off upon differentiation (Singleton *et al*., 1991). A factor denominated PSF (‘prestarvation factor’) is continuously secreted into the extracellular medium by growing cells to measure the cell density (Clarke *et al*., 1988). On the other side, the bacteria upon which the cells feed inhibit the response to PSF, allowing the cells to monitor their own density in relation to that of their food supply (Clarke *et al*., 1992). Since both PSF and its receptor are not yet cloned, this pathway remains unclear. So far we know that at high PSF/bacteria ratios, which occur during late exponential growth, PSF induces the

expression of several genes whose products are needed for cell aggregation, such as the I-genes, discoidin I, and K5 (Grabel *et al*.,1978; Cardelli *et al*.,1985; Singleton *et al*., 1988). When the food supply has been depleted, PSF production declines, and a second density-sensing pathway is activated. Starving cells secrete conditioned medium factor (CMF), a glycoprotein (Gomer *et al*., 1991) essential for the development of differentiated cell types. CMF activates a so far unknown receptor, and generates an intracellular signalling which leads to cell-cell contacts. Using antisense mutagenesis it has been shown that cells lacking CMF cannot aggregate (Clarke and Gomer, 1995), suggesting that CMF regulates cAMP signal transduction (see below).

Cyclic AMP regulates further cellular differentiation in *Dictyostelium* leading to final morphogenesis. A highly organised network has evolved in *Dictyostelium* to result in intercellular signalling by pulses of cAMP coupled to a chemotactic response (Devreotes, 1982). Approximately 3-4 hours after removal of the food source, a small percentage of cells within a population begins emitting pulses of cAMP. Surrounding cells respond by moving chemotactically towards the signalling cells and by relaying the signal, which is accomplished by activation of adenylyl cyclase and release of cAMP into the surrounding medium. This in turn activates cells further away from the initiating cells and results in the movement of cells in a pulsatile cAMP gradient towards the aggregation center (Firtel *et al*., 1989; Devreotes, 1989). About 50 movement steps result in the formation of a multicellular structure (Gerisch *et al*., 1987; Schaap, 1986). The enzyme responsible for the synthesis of cAMP from ATP, adenylyl cyclase (ACA), rapidly accumulates during the first 8 hours of development and is activated when cAMP binds to a specific cell surface receptor, cARI (Klein *et al*., 1988). cARI is a serpentine seven-transmembrane protein coupled to heterotrimeric G proteins in a manner similar to the ß-adrenergic receptor of vertebrate nerve cells (Klein *et al*., 1988; Saxe and Kimmel, 1988). When cAMP binds to its extracellular domain, the cytoplasmic domain is activated and leads to the exchange of GTP for GDP bound to the Gα2 subunit (Firtel, 1991). The GTP form of Gα2 dissociates from the Gβγ trimeric complex and then activates adenylyl cyclase in conjunction with another protein, CRAC (Insall *et al*., 1994). CRAC carries a PH domain that may mediate its interaction with other proteins. However, the signalling via ACA is not yet fully understood. Within a minute of addition of cAMP to the cells, the activity of ACA can be increased 10 fold while this activity decreases with a half life of 2 minutes. This can result in a burst of cAMP synthesis, and most of the newly made cAMP is secreted for relaying the signal to adjacent cells (Mann *et al*., 1997). Cells lacking ACA or CRAC due to

the disruption of the *acaA* or *crac* genes, fail to aggregate or to show any sign of morphogenesis (Pitt *et al*., 1992). In addition to extracellular cAMP serving as a first messenger, intracellular cAMP functions as a second messenger in *Dictyostelium* like in other eukaryotes. It appears that all the essential responses to cAMP as an internal second messenger are mediated by the cAMP dependent protein kinase. In *Dictyostelium,* PKA plays multiple roles throughout development affecting chemotaxic aggregation, prespore and prestalk differentiation, terminal differentiation, as well as the growth-differentiation-transition (Simon *et al*., 1989; Mann *et al*., 1992; Harwood *et al*., 1992; Parent and Devreotes, 1996; Endl *et al*., 1996).

3. PKA activity appears to be the major effector of cAMP as a second messenger

A wide range of developmental processes in *Drosophila*, vertebrates and *Dicyostelium*, are mediated by PKA. In both *Drosophila* and zebra fish, PKA functions in signal transduction pathways initiated by the intercellular signalling protein *hedgehog* (Li *et al*., 1995; Jiang and Struhl, 1995). In *Drosophila* and mammals (Davis *et al*., 1995) PKA activity also plays a central role in the learning and memory system. The amino acid sequence of both C and R subunits are highly conserved among diverse organisms and the basic enzymatic properties are similar (Meinkoth *et al*., 1993). PKA is a pleiotropic protein kinase and regulates signalling pathways at multiples levels. In mammalian cells, ligand binding to receptors can result in fast activation of cytoplasmic PKA (Hagiwara *et al*., 1993) and in a slower translocation of PKA to the nucleus where it activates gene transcription by phosphorylating CREB (Mayo *et al.*, 1995) and NFκB (Verma, 1995). In *Dictyostelium*, there are multiple signal transduction pathways starting out from surface receptors, and most of them appear to use PKA as the central component. *Dictyostelium* PKA is held in an inactive form by association of a single catalytic subunit (PKA-C) with a single regulatory subunit (PKA-R) thus resulting in an holoenzyme RC. The binding of two molecules of cAMP to the R subunit results in the release of the C subunit. Dissociation of holoenzyme is complete when the intracellular concentration of cAMP raises above 100 nM. Though expression of *acaA*, *carA* (Mann and Firtel, 1991; Mann *et al*., 1997) and discoidin Iγ (Endl *et al*., 1996) have been shown to be regulated by PKA, no transcription factor has yet been determined to be a direct PKA substrate.

Both PKA-C and PKA-R are present at low levels in exponentially growing cells of *Dictyostelium*, but both accumulate about four-fold during the first 12 hours of development and remain at this level until culmination (Leichtling *et al*., 1984; Part *et al*., 1985). The mRNA levels of both *pka*C and *pka*R increase about five-fold during the first four hours of development and then remain constant throughout development (Burki *et al*., 1991; Simon *et al*., 1989). The accumulation of both C and R subunits are co-ordinated, and there is no evidence for differential accumulation of either subunit during aggregation or in the two major cell types at the slug stage (Vaughan and Rutherford, 1987). Overexpression of the C subunit results in a rapid development: cells forming spores in as little as 16 hours, and expression of some early developmental genes, like discoidin, are accelerated (Anjard *et al*., 1992). The R subunit was mutated in both cAMP binding sites, resulting in a stable dominant inhibitor of PKA (Rm). Strains overexpressing Rm during vegetative growth fail completely to develop (Simon *et al*., 1989). The same phenotype is also expressed by the PKA C^- strain, which carries a null mutation of the C subunit (Mann and Firtel, 1991). PKA plays a core role on the whole developmental process of *Dictyostelium*. However, there is as yet no evidence on its function neither in exponential growth nor at the growth-differentiation-transition. Both Rm and PKA C^- strains grow well in axenic medium, but the PKA C^- strain grows slower than wild type cells on bacteria (Kessin and Anjard, personal communication). This suggests that PKA is not essential for growth but is involved in the signalling pathways related to the food source. The regulation of the very early developmental gene discoidin by PKA strongly supports this.

4. The discoidin I gene family is an indicator for the onset of differentiation in *Dictyostelium*

As *Dictyostelium discoideum* amoebae differentiate from the non-cohesive to the cohesive state, they synthesise two galactose-binding lectins, discoidin I and II. These two proteins have common regions (49% identity in the amino acid sequence): a carbohydrate-binding site (Berger and Armant, 1982) and an Arg-Gly-Asp (RGD) sequence which has been found in vitronectins and fibronectins in mammalian cells (Pierschbacher and Ruoslahti, 1984). discoidin I is expressed at the growth-differentiation-transition (GDT), and concentrated in the slime coat and around stalk cells (Devine *et al*., 1982; Cooper and Barondes, 1984). discoidin II is expressed at the onset of aggregation (8 h of development), and prominent in and around prespore cells (Cooper and Barondes, 1984; Fukuzawa and Ochiai, 1996). There are three members in the discoidin I gene family, which begin to coordinately accumulated approximately three generations before the onset of development (Devine *et al*., 1982). Thus

the discoidin I gene family sense as a marker to study the regulation of GDT. Monoclonal antibodies directed against the discoidin I proteins have been generated (Fukuzawa and Ochiai, 1988; Wetterauer *et al*., 1993).

The functions of the discoidin I protein are not clear. For a long time it was believed that discoidin I is implicated in cell to cell cohesion because it can bind to galactose and has a ″RGD″ site. RGD is the minimal structure recognised by mammalian cells in fibronectins and vitronectin, and constitutes the cell attachment sites of these proteins (Pierschbacher and Ruoslahti, 1984; Suzuki *et al*., 1985). Peptides containing this ″RGD″ signal specifically prevent gastrulation in *Drosophila* embryos (Naidet *et al*., 1987), suggesting that the RGD-sequence may also be used by invertebrates to mediate cell-attachment. In *Dictyostelium,* it has been reported that synthetic peptides containing RGD block cell attachment and spreading on a plastic surface and formation of fruiting bodies (Springer *et al*., 1984). However, immunofluorescence analysis demonstrated that discoidin is distributed throughout the cytoplasm excluding vesicles and nuclei, and there is no indication for either extracellular or membrane localization (Alexander *et al*., 1992). The discoidin proteins are essential for the establishment of the elongated cell morphology, cytoskeletal organisation and the side-to-side alignment of cells during aggregation (Alexander *et al*., 1992). However, the elongated morphology is not a required for rapid and persistent single cell translocation.

At the growth-differentiation-transition (GDT), the discoidin I gene family is among the first activated genes and thus considered as an indicator for the onset of differentiation in *Dictyostelium*. discoidin I serves as a facultative marker of GDT: expression is neither required for development nor does discoidin expression lead to obligatory development. This is demonstrated by disc$^-$ mutants which undergo relatively normal development (Crowley *et al*., 1985; Alexander *et al*., 1986) and by the continuous expression of discoidin in axenic growth medium (Blusch *et al*., 1995). The transcription level of discoidin I is very low when cells feed on sufficient bacteria. Several generations before the on-set of starvation, the PSF factor, which is continuously secreted by the *Dictyostelium* cells, accumulates in the extracellular medium. Above a threshold level of PSF, the expression of discoidin is induced (Rahti and Clarke, 1992). Deviation of this induction pattern can be monitored by measuring discoidin expression at different cell densities (Wetterauer *et al*., 1995). When the food source is exhausted and cells stop growing, a strong induction of discoidin occurs. However, the other factor CMF, which is secreted when cells starve and which activates cell differentiation,

is not responsible for the induction of discoidin. As will be presented in this thesis, a CMF knock-out strain shows normal discoidin expression. Thus another signalling pathway regulates accumulation of discoidin. Later, after 6 hours of development, the high level expression of discoidin is turned off by cAMP, which is synthesised and secreted by cells and is involved in multiple signalling pathways throughout development. A model for discoidin I gene regulation (see Fig. 2) has been presented previously and some components required for regulated expression of the discoidin I genes have been identified (Blusch *et al*., 1992; 1995; Endl *et al*., 1996). An intracellular cAMP signal is generated through a pathway including the G protein α2 subunit, CRAC, an unknown adenylyl cyclase and the activation of cAMP dependent protein kinase (PKA). PKA then stimulates transcription of discoidin I by an unknown phosphorylation linkage (Fig. 2). Mutants with aberrant discoidin expression have been generated to obtain insights into the regulation of GDT, among them are the VI88 strain (Wetterauer *et al*., 1993) and the drsA strain (Alexander *et al*; 1983) which show a discoidin overexpression phenotype. However, these mutants were generated by chemical mutagenesis thus the molecular basis is not yet known. So far, components involved in the signalling network towards regulation of discoidin and the interactions between different pathways are still incompletely understood.

By using 5' deletions in the promoter analysis vector PAV-CAT (May *et al*., 1989) and different artificial gene fusions, several sequence elements have been identified to be the ultimate targets for extracellular signals, and to relay signalling to the transcription of the discoidin I gene. The promoter element dIE (discoidin induction element) which displays some characteristics of an enhancer, responds to both the prestarvation factor PSF (Vauti *et al*., 1990) and the folate repression signalling (Blusch *et al*., 1992). Another element, dNCE (discoidin negative cAMP element), located downstream of the dIE is necessary for down regulation by the cAMP signalling (Vauti *et al*., 1990). Further down stream in the discoidin promoter, there is another element dAE (discoidin axenic element) which is required to maintain the basic expression of discoidin during axenic growth (Vauti *et al*., 1990). Identification of these promoter elements provides the basis for tracing back the transduction chain from the level of gene expression to the events at the membrane.

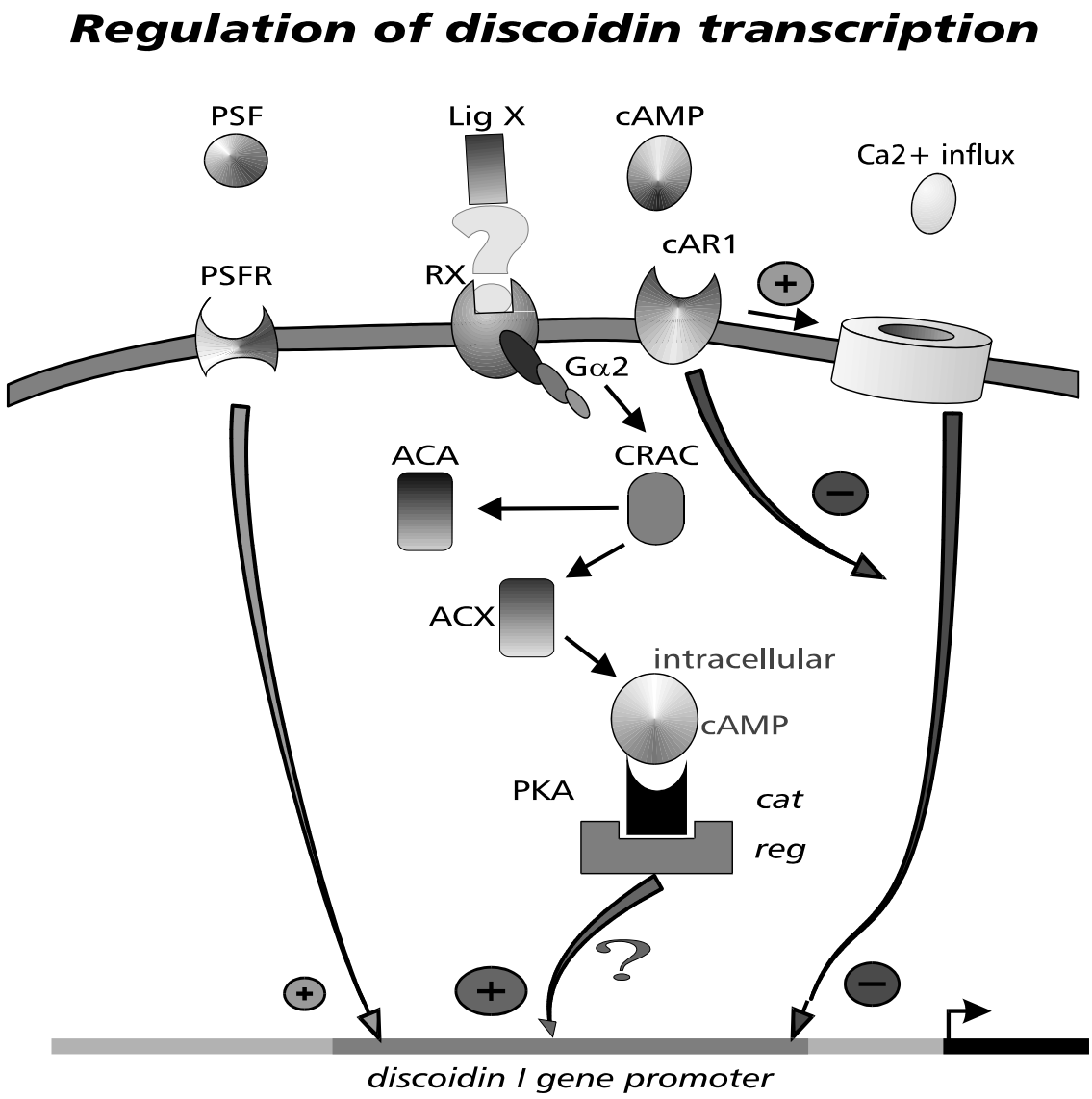


Figure 2. **Initial model for the regulation on discoidin transcription** [from Endl and Nellen, 1996]. Four different regulatory pathways were proposed: PSF and an unknown molecule (ligand X) induce discoidin expression by intracellular signalling through their receptors (PSFR and RX). The pathway by RX involves Gα2, and CRAC which is known to activate ACA. However, ACA was found not to be required for discoidin induction. Therefore another adenylyl cyclase (ACX) was proposed. ACX generates cAMP which activates PKA. The final activation of discoidin transcription via PKA is unknown. Extracellular cAMP and Ca^{2+} influx generate repressing regulations and counteract the induction. Membrane associated proteins like ACA and CRAC are shown inside the cell only for graphical reasons.

5. Using the REMI strategy and discoidin I as a marker to knock out developmental genes at the growth-differentiation-transition of *Dictyostelium*

A new technique for fishing genes by tagged insertion mutagenesis has been established in *Dictyostelium* (Kuspa and Loomis, 1992). Introduction of a restriction enzyme along with a linearized plasmid results in integration of the plasmid at corresponding genomic restriction sites. This method, called REMI (restriction enzyme mediated integration), generates apparently random insertions into genomic DNA. Some integrations cause gene disruptions and thus mutations. After screening for desired phenotypes, the disrupted genes can be isolated together with the inserted plasmid and analyzed. In the next step, to prove that the disruption of the gene by the inserted plasmid is responsible for the observed phenotype, the isolated plasmid is reused to disrupt the gene in wild type cells by homologous recombination. Since development and vegetative growth are distinct phases, it is possible to obtain mutants defective in important signal transduction pathways without impairing the viability of the cells. This REMI system was used to isolate mutants with defects in the expression of discoidin genes.

As described in Fig. 3, an ura$^-$ auxotroph strain of *Dictyostelium* (DHI) was used as a parent for generating the REMI mutants. The DIV-2 vector, which contains the pGem-3 vector and *pyr5-6* gene (coding for UMP synthase) is linearized with *Bam*HI and electroporated into the competent DHI cells together with *Bam*HI. The enzyme cuts occasionally the genomic DNA and the vector is randomly integrated into the genome at the cut site(s). Transformants carrying the *pyr5-6* gene are selected in minimum medium without uracil for about 15 days, then cloned on a bacterial lawn. The REMI mutants with mis-expression of the discoidin I genes were obtained by colony-blot screening with anti-discoidin monoclonal antibodies. The identified mutants were analyzed by Southern-blots probed with the pGEM-3 vector to confirm the altered genomic structure caused by the integration. The disrupted gene was then isolated by a proper restriction digestion which recovers the pGEM-3 vector part (containing the origin of replication and the Amp resistance) of the DIV-2 vector and the flanking genomic DNA at the integration site. This step of recovering the flanking genomic fragments is called REMI rescue. The isolated fragments were then analyzed and used for reconstructing the phenotype.

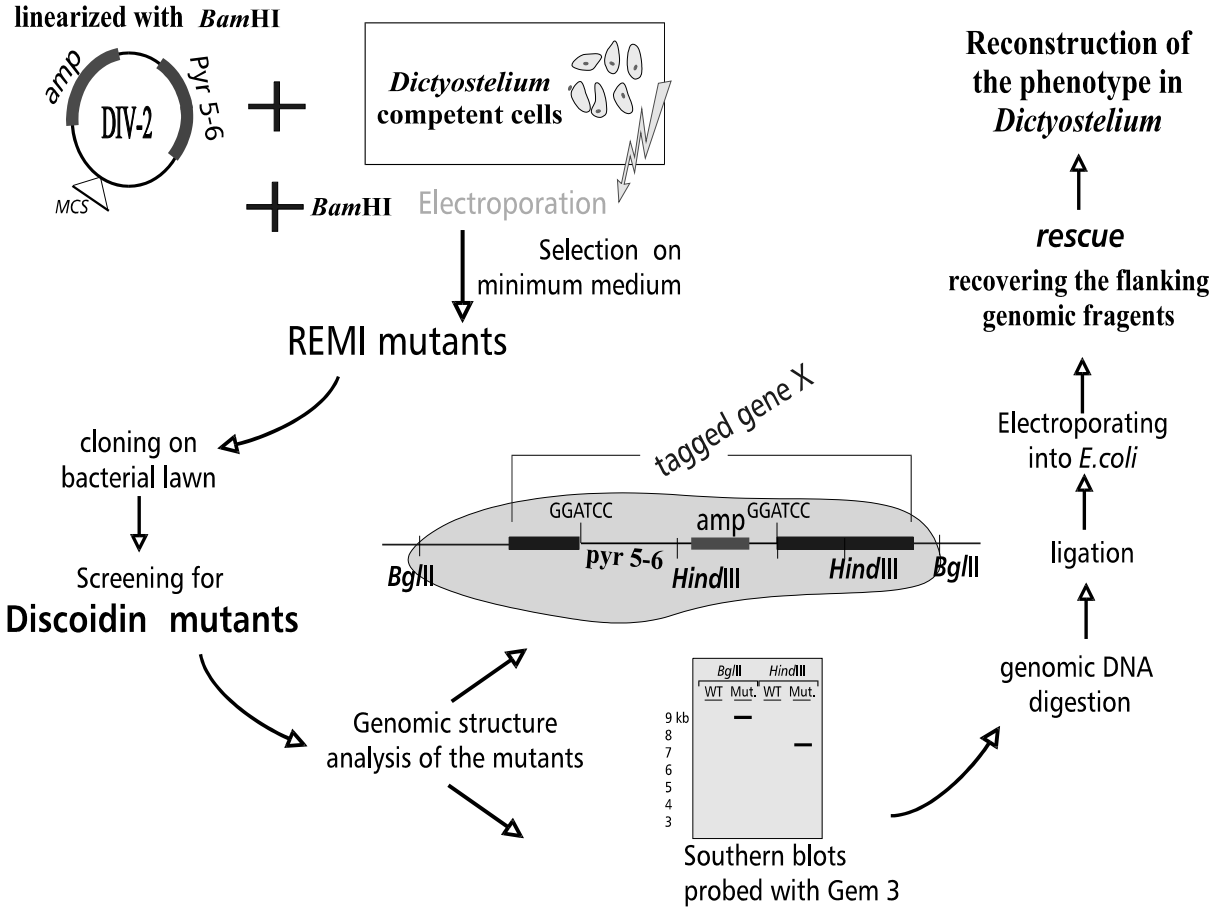


Figure 3. Diagram of REMI strategy for fishing genes involved in the regulation of discoidin. The linearized DIV-2 vector together with *Bam*HI enzyme is electroporated into the competent DHI (ura$^-$ auxotroph strain) cells. The enzyme cuts occasionally inside the genome and the vector is randomly integrated. Transformants are selected in minimum medium then cloned on a bacterial lawn. The mutants with mis-expression of discoidin I are screened by colony-blots with an anti-discoidin monoclonal antibody. The flanking genomic DNA at the integration site of the identified mutant is isolated by a proper restriction digestion together with the pGEM-3 vector. This step of recovering the flanking genomic fragments is called REMI rescue and the isolated fragments are then analyzed and used for reconstructing the phenotype.

By REMI, both discoidin^{null} and discoidin^{over} mutants have been isolated, among them formerly determined components like CRAC (Riemann, unpublished data). This work will allow to dissect the signal transduction pathways which trigger the transition from cell proliferation to cell differentiation.

6. Protein phosphorylation and protein kinases

Since the *gdt1* gene encodes a putative protein kinase, this chapter will give a brief introduction on the general concept of protein phosphorylation and protein kinase.

Of all the post-translational modifications, protein phosphorylation plays the most dominant role in almost all events in cellular regulation, including proliferation, differentiation, signal transduction, metabolism and cell death. Phosphorylation of a target protein is regulated by the balance of activity of protein kinases and protein phosphatases. The activity of protein kinase and phosphatase is modulated both temporally (different time scales) and spatially (different cellular and subcellular locations). Some protein kinases have multiple substrates and can act at multiple sites. These properties provide an extraordinarily sensitive regulation for physiological functions, while the activity of a protein can be enhanced or diminished in a quick and reversible manner. In a typical mammalian cell, an estimated one-third of all proteins are phosphorylated, and approximately 2-5% of the genes encode protein kinase and phosphotases (Hubbard and Cohen, 1993). To date, more than 400 protein kinases and more than 100 protein phosphatases have been identified (Taylor *et al*., 1995; Hunter, 1997).

The signal transduction cascade involves a large number of protein kinases, by which the primary signal is amplified and the complex cellular regulatory pathways are generated. However, the cellular response of phosphorylation on a signalling component is quite diverse. Some enzymes require phosphorylation for activity (e.g., phosphorylase b), while the others are inactivated by phosphorylation (e.g., glycogen synthases and pyruvate kinase) (De Meyts *et al*., 1995; Denton and Tavare, 1995). Phosphorylation provides regulation in a variety of ways, including allosteric activation or inhibition, the formation of protein-protein interactions, and protein translocation from one compartment of the cell to another (Jans, 1995). Both extracellular ligands, such as polypeptide growth factors, and key intracellular signalling elements such as the concentration of cAMP or calcium regulate the activities of kinase and phosphatase, which themselves are subject to modification by phosphorylation (e.g. PKA) .

The inherent complexity of signal transduction pathways is intensively analyzed in the activation of the mitogen-activated protein kinase (MAPK) cascade (Seger and Krebs, 1995). The MAPK pathway involves a series of serine/threonine protein kinases or serine/tyrosine protein kinases, where phosphorylation of the next kinase in the series results in its activation.

Signalling through this cascade results in approximately 10,000 fold amplification of the initial signal. During the course of this signalling, other signalling pathways participate and either stimulate (e.g., PKC signalling) or inhibit (e.g., PKA signalling and protein phosphatase activity) the MAPK pathway. An activated MAPK enzyme in turn stimulates a variety of cytosolic enzymes (e.g., glucose metabolism) and also transfers to the nucleus, where it further phosphorylates / activates a series of transcription factors (Graves *et al*., 1995).

Many cytokines initiate cellular responses through their interaction with members of the cytokine receptor superfamily which contain no catalytic domains in their cytoplasmic domains. Irrespective, ligand binding induces tyrosine phosphorylation, which requires a membrane proximal region of the cytoplasmic domain. The Janus kinase (JAK) family, which belongs to the protein tyrosine kinases, associates with the membrane proximal region and are rapidly phosphorylated following ligand binding and their kinase activity is activated (Ihle, 1994; Xia *et al*., 1996). Moreover, different cytokines activate the same JAK kinase and the same cytokine ligand can activate more than one member of the JAK superfamily (Foxwell *et al*.,1995; Gaffen *et al*., 1996). The Jak-STAT pathway is a newly discovered intracellular signal transduction pathway that is used by a growing number of extracellular signalling proteins (ESPs) for transcriptional activation of target genes. The activated receptor-JAK kinase complexes recruit members of the STAT family and activate them by phosphorylation. As a consequence, the phosphorylated STAT proteins dimerize, translocate into the nucleus, bind response elements in the promoter of target genes and stimulate the transcription of these genes (reviewed by Heim, 1996).

Protein kinases can be classified according to their functional properties. One categorisation is based on the specific amino acid residue that serves as the phospho-acceptor. This includes the serine-threonine protein kinases, which transfer the phosphate group onto serine and /or threonine residues; the protein tyrosine kinases (PTKs), which transfer the phosphate group onto tyrosine residues; and the dual specificity protein kinase, which transfers the phosphate group onto serine, threonine and tyrosine residues (Taylor *et al*., 1995). A second classification scheme categorises kinases based on their requirement for second messengers (e.g. cAMP), but the boundaries between groups are less definite. Table 1. provides some examples (by Promega) on various classes of protein kinases.

Table 1. Classification of protein kinases based on second messenger requirement.

Messenger-Independent Protein Kinase (Cofactor)	
Enzyme class	**Examples**
Receptor Serine/Threonine PK	transforming growth factor β (TGFβ) receptor and activin receptor, etc.
soluble Serine/Threonine PK	Casein kinase 1 and 2, MAP kinase (ERKs, p38, JNK), MEKs, MEKKs, etc.
Receptor Tyrosine Kinase (RTKs)	Insulin receptor (IR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), nerve growth factor receptor (NGFR or TRK), fibroblast growth factor receptor (FGFR), etc.
Messenger-Dependent Protein Kinase	
Enzyme	**Second Messenger**
PKA	cAMP
JAK	cytokines
cyclic GMP-dependent PK (PKG)	cGMP
Calcium and phospholipid-dependent PK	Ca^{2+}, diacylglycerol (DAG), and phospholipids
Calmodulin-dependent PK (CaMK)	Ca^{2+}, Camodulin (CaM)
DNA-dependent PK (DNA-PK)	dsDNA
dsRNA-dependent PK (RNA-PK)	dsRNA
phosphorylase kinase (Phos K)	Ca^{2+}, Camodulin (CaM)
Myosin light chain PK (MLCK)	Ca^{2+}, Camodulin (CaM)
Cyclin-dependent PKs (CDK)	Cyclins A, B, D, E

III MATERIALS AND METHODS

1. Materials and Sources

1.1. Enzymes and kits

Alkaline Phosphatase from Shrimp (SAP)	USB
DNase RQ, RNase free	Promega
ECL™ detection system	Amersham
Erase-a-Base™ system	Promega
3'-RACE System	Gibco BRL
JETsorb DNA Extraction kit	Genomed
Lambda DNA Extraction kit	Qiagen
Oligotex-dT mRNA kit	Qiagen
Proteinase K	Boehringer
restriction enzymes	Boehringer, Gibco BRL, MBI, Promega, Pharmacia, USB
RNase A	Boehringer
Rnase Inhibitor (RNasin)	Promega
SP6 RNA polymerase	Boehringer
Super-Script Reverse Transcriptase	Gibco BRL
Taq DNA Polymerase	Home made
T4 DNA ligase	MBI
T7 DNA polymerase	Boehringer
T7 sequencing kit	Pharmacia
T-cloning kit	MBI

1.2. Antibiotics

Ampicillin	Sigma
Chloramphenicol	Sigma
Geneticin (G418)	Sigma
Penicillin/Streptomycin	Gibco BRL

1.3. Antibodies

monoclonal anti-discoidin antibody 80-52-13	(Wetterauer *et al*, 1993)
IgG, goat-anti-mouse , Alkaline Phosphatase coupled	Dianova
IgG goat-anti-mouse, Peroxidase coupled	BioRad
IgG, goat-anti-rabbit, Alkaline Phosphatase coupled	Dianova

1.4. Protease Inhibitors

PMSF (Phenylmethylsulfonylfluorid)	Sigma
Aprotinin	Sigma

1.5. Chemicals

Agarose	Sigma
Acetic acid	Serva
Acrylamide Protogel (30%)	National Diagnostics
Amido-black	Merck

Amylose Resine	New England Biolabs
ATP	Boehringer
Bacto-Peptone	Oxioid
Bacto-Tryptone	Oxioid
BCIP	Gerbu
Bisacrylamide	Serva
BSA	BRL
Bromophenol blue	Merck
cAMP	Serva
Chloroform	Riedel-de Haen
Coomassie Brilliant Blue R250	Serva
DMSO	LKB
dNTP	MBI
DTT	Sigma
EDTA	Merck
Ethanol	Roth
Ethidiumbromide	Sigma
Ficoll 400	Pharmacia
Formaldehyde, 37%	Merck
Formamide	Merck
Glycerol	Merck
Glycine	Merck
Guanidine thiocyanate	Fluka
Guanidine chloride	Fluka
HEPES	Serva
IPTG	Boehringer
MOPS	Serva
Ni-NTA Agarose	Qiagen
Nonidet P40	Sigma
ONPG	Serva
Phenol/Chloroform	Roth
PMSF	Serva
Ponceau S	Sigma
Polyvinylpyrollidone	Sigma
Protein Marker	Gibco
Rainbow Protein Marker	Amersham
Repel-Silane	LKB
γ-NTP	MBI
Sephadex G-25, G-50	Pharmacia
Spermidine	Serva
TEMED	Serva
Triton X-100	Serva
Triton X-114	Serva
Tween 20	Sigma
X-Gal	Roth
Urea	Merck
Xylene Cyanol FF	Serva

1.6. Chromatography Columns Media

His Trap ^{TM} Kit (for purification of poly-His tagged protein)	Pharmacia

1.7. Radioactive Materials

α-^{32}P-dATP (12.5 mCi/ml)	Amersham
γ-^{32}P-dATP (12.5 mCi/ml)	Amersham
α-^{32}P-UTP (12.5 mCi/ml)	Amersham
α-^{35}S-dATP (10 mCi/ml)	Amersham

1.8. Biological Materials

Dictyostelium discoideum

Ax2 - 214	(Watts and Ashworth, 1970)
NC4	(Raper, 1935)

Bacteria Strains

E.coli BL21	(Studier and Moffat,1986)
E.coli JM105	(Yanisch-Peron *et al.,* 1985)
E.coli DH5α	(Hanahan,1983)
E.coli Y1090	(Huynh *et al.,* 1983)
E.coli XL1 blue	(Hanahan,1983)
Klebsiella aerogenes	(Williams and Newell, 1976)
Lambda Phage gt11	(Frischauf *et al*.,1983)

1.9. Plasmids

pGEM-3/3Z/4/7Z	Promega
pUC 57 for T-cloning	MBI
DIV-2	(Kuspa and Loomis, 1992)
pET 15b	Novagen

1.10. Oligonucleotide Primers

Name	Sequence	Analysis
Anchor Primer	Gibco BRL	3'-RACE
UAP	Gibco BRL	3'-RACE
SP6	5'-GATTTAGGTGACACTATAG	Sequencing
T7	5'-AATACGACTCACTATAG	Sequencing
5'-FPC	5'-TTCATATGGGAGGATCATTATCATTG	PCR
Primer 166	5'-CCAATCAATGATAATGATCCTCCC	PCR & Seq.
FPC-seq1	5'-GAATCTAGAGCACCACTCA	Sequencing
$Primer_1$(3')	5'-TGGACCTATTACCAATG	PCR
FPC-seq2	5'-TGGGTCATCGATTACTCCA	Sequencing
$Primer_2$5'	5'-ATGGAACCATTCGCTGGCGA	PCR
$Primer_2$3'	5'-TGGCGGCAACATGTCA	PCR
Primer 167	5'-AAAGTGAATCCTCGACAAG	PCR & Seq.

Primer 184	5’-CCAAACTTCCAAGGGGTGGA	PCR & Seq.
His-pDNeo2 (Short)	5’-pCATCATCATCATCACCATATG	Annealing
His-pDNeo2 (long)	5’-pGATCCATATGGTGATGATGATGATGCATG	Annealing
poly-His Linker for pDNeo2	5’-CATCATCATCATCACCATATG GTACGTAGTAGTAGTAGTGGTATACCTAG-5’	linker
FPC_{BGLII}	5’-CCAATGAAAATGCAGAGGTTTG	PCR & Seq.
pGEM 2602-14	5’-GCCACGCGTCCG	Sequencing

1.11. General Buffers

TE buffer	10 mM	Tris-HCl, pH 8.0
	1 mM	EDTA
TBE buffer	90 mM	Tris-borate
	2 mM	EDTA
TAE buffer	40 mM	Tris-acetate
	2 mM	EDTA
100 x Denhardt	2%	Ficoll 400
	2%	Polyvinylpyrollidone
	2%	BSA
20 x SSC	3 M	NaCl
	0.3 M	Sodium Citrate
2 x Protein sample buffer	80 mM	Tris-HCl, pH 6.8
	2%	SDS
	10%	Glycerol
	5%	β-ME
	0.02%	Bromphenol blue
5 x Protein running buffer	25mM	Tris base
	380 mM	Glycine
	0.1%	SDS
Protein transfer buffer	25 mM	Tris base
	190 mM	Glycine
PBS buffer	8 mM	Na_2HPO_4
	2 mM	NaH_2PO_4
	140 mM	NaCl
10 x NCP buffer	12.1 g	Tris base
	87 g	NaCl
	5 ml	Tween
	2.0 g	Sodium azide
	add water to 1 L	
9 x Protein sample buffer	30 ml	Glycerol

	5 ml	β-ME
	2.3 g	SDS
	0.76 g	Tris
	1 mg	Bromophenol blue
	add H_2O to 100 ml and adjust pH to 6.8	
Phosphate buffer (pH 6.0)	20mM	Na_2HPO_4
	20 mM	NaH_2PO_4
6 x DNA loading Dye	40%	Sucrose
	0.5%	SDS
	0.25%	Bromphenolblue
	0.25%	Xylene Cyanol FF

1.12. Media

AX medium (pH 6.7): (Watts & Ashworth, 1970)

14.3 g	Bacto-Peptone
18.0 g	Maltose
0.616 g	Na_2HPO_4 x 2 H_2O
0.486 g	KH_2PO_4
7.15 g	yeast-extract
add H_2O to 1 L	

G0 medium: AX medium plus

50 µg/ml	Ampicillin
1/100 Vol.	Penicillin/Streptomycin
(10000 U/ml in 0.85% Saline)	

SM agar plates (pH 6.5): (Sussman, 1951)

15 g	Bacto-agar
10 g	Peptone
10 g	Glucose
1 g	yeast-extract
1 g	$MgSO_4$ x 7 H_2O
2.2 g	KH_2PO_4
1 g	K_2HPO_4
add H_2O to 1 L, 20 ml of the medium were used for one petridish.	

Phosphate agar plates:

2 mM	$NaHPO_4$
15 mM	KH_2PO4
1.5%	Bacto-agar

LB medium:

10 g	Bacto-Tryptone
5.0 g	yeast-extract
5.0 g	NaCl
add H_2O to 1 L	

1.13. Equipments and other materials

Biodyne A Nylonmembrane	Pall
centrifuge RC 5C	Sorvall
centrifuge Rotanta A,R	Hettich
Corex tube 15 ml, 30 ml	Corex
Electrophoresis Constant Power Supply	Pharmacia
Electroporation cuvettes	BioRad
Easy system	Herolab
Falcon tubes 15 ml, 5 ml	Falcon
Film exposion cassettes	Appligene
Filter HABP 45mm, 22mm	Millipore
Gene Pulser	BioRad
Gradient maker (SG30)	Hoefer Pharmacia Biotech
hybridization tube	Schott
intensifying Screens	DuPont
Microconcentrator, Centricon 10, 30	Amicon
Microscope M 35 III	Zeiss
PCR-“Crocodile″	Appligene
Petridish	Greiner/Schuett
Photometer Gene Quant	Pharmacia
Pump	BioRad
Rotor SW 41	Beckmann
Rotor SS34	Sovall
Speed Vac Concentrator	Savant
Semi-dry blotter	Sartorius
Sonicator (UP 200s)	dr. Hielscher
Tank blotter	Sartorius
UV transilluminator (312nm, 180w)	Bioblock
Whatman 3MM paper	Whatman
X-ray Films X-OMAT AR	Kodak
X-ray Films X-OMAT	Kodak

2. Cell biological methods

2.1.) Cell growth

Dictyotelium discoideum Ax2 and the derived transformants were grown either in AX medium or with Klebsiella aerogenes (KA) as a food source. For growth in suspension, KA were grown on SM plates at room temperature for 2 days. Bacteria were washed off the plates with 30 ml phosphate buffer resulting in an OD_{600} of 0.8. *Dictyostelium* cells were inoculated at 5 x 10^4 cells/ml, shaken at 150 rpm at 22°C and harvested at the cell densities indicated.

2.2.) Cloning *Dictyostelium* on SM plates

To obtain single clones of *Dictyostelium*, around 50-200 cells were suspended in 100 µl phosphate buffer and plated on KA-covered SM plates. Plates were grown at 22°C for 3 days until colony plaques appear on the bacterial lawn. Single clones were picked up with tooth picks, transferred to new KA plates or grown in G0 medium to obtain strains without KA contamination.

2.3.) Differentiation conditions

Vegetative cells were harvested from cultures on bacteria at densities below 1x10^6 cells/ml and washed free of bacteria by differential centrifugation (1200, 1100, and 1000 rpm) with phosphate buffer. Washed cells at 2x10^7 cells/ml were allowed to develop in shaking phosphate buffer suspension for 5 hours. Axenic growing cells were harvested at 2x10^6 cells/ml by a single centrifugation (1200 rpm) at 4°C for 6 minutes. The cell pellet was then washed with 20 mM phosphate buffer, and resuspended in the same buffer at a cell density of 1x10^7 cells/ml. Cells were then shaken for 5-6 hours as described above.

2.4.) Morphogenesis assay on polyacrylamide gel

Cells were harvested at 2x10^6 after axenic growth, washed with phosphate buffer, and resuspended at 5x10^6 cells/ml. The chemical-linked polyacrylamide gels (both glucoside- and hexanol-derivatized gels) were kind gifts from S. Bozzaro (Turin, Italy). These gels (ca. 7mm x 5mm) were stored in isopropornol at 4°C, washed with phosphate buffer 4 times before using, and placed on a plastic surface (petridish). Then ca. 10^5 cells in 20 µl phosphate buffer were pipetted on the gel and developed at 22°C.

2.5.) *Dictyostelium* transformation

Axenic Ax2 cells were harvested at a density of $2x10^6$ and then transformed either by the Ca-phosphate method (Nellen *et al*., 1984) or electropration (Howard *et al*., 1988). All gene-knock out mutants were done by electropration, and the overexpression transformants with G418 resistance were generated by the Ca-Phosphate method.

The Ca-phosphate method

10 ml Ax2 cells were plated on a petri dish and left at room temperature for 10-20 minutes. The medium was then carefully taken out without destroying the cell layer and the MOPS medium was added for 30 minutes. After this step the MOPS medium was pipetted out, and the Ca-phosphate-DNA-precipitate was added and carefully spread over the cell layer. The plate was left at RT for 20 min., 10 ml MOPS medium was added and cells were incubated at 22°C for 3 hours. At the end, all medium was removed, and 2 ml glycerol solution was used for a "glycerol-shock" treatment for 5-9 minutes. Then the glycerol solution was completely removed and G0 medium was added, and incubated overnight at 22°C. On the next day the medium was changed to G_{20} medium (G0 plus 20 µg/ml G418) and maintained for around 20 days until the transformants were obtained. For generating the *pka*C and R transformants, up to 200 µg/ml G418 were used for maintaining high copy number of C and R subunits.

MOPS medium:

5.0 g yeast extrat
10.0 g glucose
10.0 g proteose Peptone
1.3 g MOPS
add H_2O to 1L and filter sterilise

2 x HBS:

4.0 g NaCl
0.18 g KCl
0.05 g NaH_2PO_4
2.5 g HEPES
0.5 g glucose
adjust pH to 7.05, add H_2O to 250 ml and filter sterilise

glycerol solution:

3 ml 60% glycerol
2 ml H_2O
5 ml 2 x HBS

the Ca-phosphate-DNA-precipitation:

12 µg DNA was diluted with 0.6 ml 1 x HBS and strongly vortexed while adding 38 µl 2M $CaCl_2$ (the final concentration is 125 µM). The DNA was then precipitated at RT for 25 min.

2.6.) Electroporation

$2x10^7$ cells were collected, washed once with EP buffer, then resuspended in 0.8 ml EP buffer. 20 µg DNA was added to the cells and incubated on ice for 10 min. Electroporation was performed at 1 kV, 3 µF in a 4mm electroporation cuvette (time constant was around 1.0 msec). Cells were plated on a petri dish, mixed with 8 µl 0.1 M $CaCl_2$/ 0.1 M $MgCl_2$ (each) and kept at RT for 15 min to allow the cells attachment. Then 10 ml G0 medium was added for an overnight incubation. On the next day the medium was changed to the selection medium and cells were kept under selection until the transformants were obtained.

electroporation buffer (EP buffer):

Soerensen-buffer +
50 mM Sucrose

2.7.) β-Galactosidase assay

As described by Dingermann *et al*. (1989), a β-Galactosidase assay was used to detect the promotor activity in *Dictyostelium*. The DAG strain was generated from Ax2 cells by transformation with the vector DAG-Gal (a gift from H. MacWilliams). Around $2x10^7$ cells were harvested and washed with 1 x phosphate buffer. The cells were resuspended in 1 ml 1x phosphate buffer, sonicated at maximum power for 10 sec., then mixed with 900 µl Z-buffer and 600 µl ONPG solution. The reaction was incubated 1 hour at 37°C. Then 1 ml from the reaction mix was centrifuged at 14,000 rpm for 5 minutes, and the clear lysate was used for determination of the optical density at 405 nm. Since only relative values were required, no calibration of the enzymatic reaction was done.

Z-buffer:

60 mM Na_2HPO_4
40 mM NaH_2PO_4
10 mM KCl
1 mM $MgSO_4$
2.7 µl/ml β-ME

ONPG solution:

4 mg/ml in
1xPhosphate buffer

3. Molecular biological methods

3.1.) REMI mutagenesis

The ura$^-$ strain DHI (Kuspa and Loomis, 1992) was used as the parent for generating REMI mutants. DHI cells were grown in FM medium (Franke and Kessin, 1977, purchased from Gibco-BRL) supplemented with 20µg/ml uracil. 20µg DIV-2 vector (Kuspa and Loomis, 1992) was linearized with *Bam*HI and electroporated into DHI cells together with 100 units of *Bam*HI at 2.5kV/cm, 3.0µF (Howard *et al*., 1988). After electroporation, cells were distributed on 5 Petri dishes (9 cm diameter) and transformants were selected in FM medium. When clones could be detected on the plates (after 10 to 15 days), cells were washed off, counted and plated in association with *Klebsiella aerogenes* on SM plates for cloning.

3.2.) Isolation of the disrupted gene from REMI mutants

The theory of this experiment is shown in Fig. 3.
To recover the flanking genomic DNA at the vector integration site in the 2-9 REMI mutant, plasmid rescue was done as described by Kuspa and Loomis (1992). Genomic DNA from the 2-9 mutant was digested with *Hind*III, religated in a diluted solution and transformed into *E.coli*. A plasmid (2-9 rescue) containing 3.7 kb of one side flanking genomic sequence was recovered and used for further analysis.

3.3.) Genomic DNA preparation from *Dictyostelium*

As described by Nellen *et al*., 1987, genomic DNA from *Dictyostelium* was prepared from the isolated nuclei.
Up to 10^9 cells from axenic culture were collected, and washed twice in ice-cold phosphate buffer and resuspended in NP-40 lysis buffer. The nuclei fraction was obtained by centrifugation at 6,000 g for 10 min. The nucleus pellet was carefully resuspended in 5 ml SDS-lysis buffer, and incubated with 100 µl Proteinase K stock solution at 50°C for 3 hrs. The genomic DNA was extracted twice with phenol/chloroform (1:1 volume), then precipitated by adding 1/10 vol. 8 M LiCl and 2 vol. 100% ethanol. The DNA precipitate was carefully taken out with a glass needle, washed with 70% ethanol, air dried and then dissolved in a proper volume (200-500 µl) of ddH_2O.

<u>NP-40 Lysis buffer:</u>

30 mM HEPES, pH7.5
10 mM Mg(OAc)2
10 mM NaCl

10% Sucrose
2% NP 40
filter sterilise.

SDS-Lysis solution:

0.7% SDS in TE buffer

Proteinase K solution:

25 mg/ml in ddH_2O

3.4.) Total RNA preparation from *Dictyostelium*

Up to 5x10^7 cells were pelleted and dissolved in 500 µl solution D. After adding 50 µl of 3 M Sodium acetate (pH 4.7) and 500 µl Phenol/Chloroform, the sample was strongly vortexed and incubated on ice for 15 min. After centrifugation 15 min at 12,000 rpm, the upper phase was collected and precipitated by adding 1 vol. isopropanol. The RNA was then pelleted, washed twice with 70% Ethanol, dried in the speed vacuum, and dissolved in 100 µl DEPC water. Up to 3 µg/µl concentration of RNA was obtained.

Solution D:

4 mM Guanidine Thiocyanate
25 mM Sodium citrate
0.1 M β-Mercaptoethanol
0.5% Sarcosyl

DEPC water:

treat Millipore water with 0.1 % DEPC overnight. Then autoclave.

3.5.) Isolation of mRNA from *Dictyostelium*

The Oligotex-dT mRNA kit from Qiagen was used for mRNA preparation.
Total RNA, DEPC water, 6 x binding buffer were mixed to a total volume of 0.5 ml, treated 3 min at 65°C to destroy secondary structures, then loaded on a prewarmed (37°) Oligo-dT column and incubated at RT for 5 min. The column was centrifuged at 12,000 rpm for 30 sec., then washed twice with wash-buffer. At the end mRNA was eluted with 100 µl prewarmed (65°C) elution-buffer. Around 20% of the total RNA was obtained as polyA RNA.

6 x binding buffer:

60 mM Tris-HCl, pH 7.5
3 M NaCl
6 mM EDTA

washing buffer:

10 mM Tris-HCl, pH 7.5
0.15 M NaCl
1 mM EDTA

elution buffer:

5 mM Tris-HCl, pH 7.5

Oligo-dT spin column:

from Qiagen

3.6.) Screening of the λgt11 cDNA library

A λgt11 cDNA Liberia was made from poly(A)$^+$ RNA of vegetative Ax2 cells (growing in bacteria suspension) and was a gift from Hudson Freeze (San Diego, USA).

E.coli Y1090 strain was grown overnight in 50 ml NZYM-plus medium at 37°C. The cells were pelleted by centrifugation 5 min at 3000 rpm, 4°C, then resuspended in 25 ml 10 mM $MgSO_4$ solution. This suspension was used as plating-bacteria for cDNA screening.

5 µl diluted Phage lysate (ca. 2x10^6 pfu/ml) were added to 1.7 ml of plating-bacteria and incubated for 30 min. at RT. 100 µl of this infected bacteria suspension was mixed with 5 ml Top-agarose (prewarmed in 45°C water-bath), briefly vortexed, and immediately poured on a 11cm x 11cm NZYM Agarplate (also prewarmed to 45°C). The plates were then incubated at 42°C for about 5 hrs until the plaques became visible (2mm diameter).

Plaques were transferred on to nitrocellulose membranes, lysed for 5 min with denaturing buffer (0.5M NaOH/1.5 M NaCl), then neutralised with neutralising buffer (0.5 M Tris-HCl, pH 7.0/1.5 M NaCl). The blots were washed in 2xSSC and used for hybridization with a radioactive probe. The positive clones were picked after the first screening, resuspended in 1 ml SM buffer, and stored overnight at 4°C. 1 µl of this storage was then mixed with 200 µl plating-bacteria and used for the second screening. Positive clones were only confirmed after a third screening.

NZYM medium (pH 7.5):

10 g NZamin A
5 g NaCl
5 g Bacto Difco yeast extract
2 g $MgSO_4$ x $7H_2O$
add H_2O to 1 L

NZYM-Agarplate:

NZYM medium
+ 1.5% Agar-Agar

Top-agarose:

0.7% agarose in NZYM medium

SM buffer:

5.8 g NaCl
2 g $MgSO_4.7H_2O$
50 ml 1M Tris-HCl, pH 7.5
5 ml 2% (w/v) gelatin
add H_2O to 1 L

3.7.) isolation of plasmid DNA from *E.coli* (maxi, midi-prep)

Qiagen kits were used for these experiments according to the suggestions of the supplier.

3.7.) Mini-preps for plasmid DNA

For small culture (1ml) of *E.coli* transformants, the alkaline lysis method (Birnboim and Doley, 1979) was used to extract the plasmid DNA. *E.coli* were collected by centrifugation at 4,000 rpm for 5 min., and resuspended in 200 µl solution I by strong vortexing. After adding 200 µl solution II, the cells were lysed at RT for 5 min., then mixed with 200 µl solution III. After 15 min. centrifugation at 12,000 rpm (4°C), the supernatant was collected and precipitated by adding 600 µl isopropanol.

Solution I:

25 mM Tris-HCl, pH 7.4
10 mM EDTA pH 7.4
15% Sucrose

Solution II:

0.2 M NaOH
1% SDS

Solution III:

3M NaAC pH 4.7

3.8.) Isolation of DNA fragment from agarose

JETsorb DNA Extraction kit from Genomed was used for these experiments according to the suggestion of the supplier.

3.9.) Dephosphorylation of DNA fragments

For cloning DNA fragment into vectors, usually the 5'-phosphate of the vectors were dephosphorylated by shrimp alkaline phosphatase (SAP, from USB). 5.5 µl SAP buffer and 5 U SAP (USB) were added to a 50 µl digestion mix and incubated for 30 min. at 37°C. Then SAP was inactivated by heating 10 min at 70°C, and extracted once with Phenol/Chloroform.

optimal conditions:

20 mM Tris-HCl, pH 8.0
10 mM $MgCl_2$
20 µg/ml DNA

3.10.) "Erase-a-base"

By using the Erase-a-Base^{TM} system from Promega, a DNA fragment can be deleted in a single direction. This deletion is carried out by ExoIII, which can only digest the DNA from 5'-overhangs but is blocked by 3'-overhangs.

5-8 µg plasmid DNA was digested with two different restrictions enzymes to generate one 5'-overhang/one 3'-overhang ends. The linearized DNA was extracted once with Phenol/Chloroform, precipitated and dissolved in 60 µl 1 x ExoIII buffer. 250-500 U ExoIII were added and mixed immediately at the proper temperature (37°C). 2.5 µl samples were taken every 30 sec. and added to 7.5 µl prepared SI stop solution (on ice). After the deletion series was finished, all reaction mixes were moved to RT and incubated for further 30 min before 1 µl of stop solution (Promega) was added. 2 µl aliquots from each time point were checked on an agarose gel to see whether the expected deletions were obtained. Then 1 µl Klenow mix was added to the remaining samples, and incubated 3 min at 37°C. 1 µl dNTP mix was quickly mixed to each tube, and incubated for 5 min. At the end, all samples were transferred on ice and ligation was started by adding 40 µl ligation mix. After 1 hr incubation at RT, the religated plasmids were transformed into *E.coli*.

S1 mix (for 25 time points) :

172 µl ddH_2O
27 µl 7.4 x S1 buffer
60 U S1 nuclease

Klenow mix:

30 µl 1 x klenow buffer
3-5 U Klenow DNA polymerase

ligation mix:

790 µl ddH_2O
100 µl 10 x ligase buffer
100 µl 50% PEG
10 µl 100 mM DTT
5 U T4 DNA ligase

10 x ExoIII buffer

7.4 SI buffer

SI stop buffer

1 x Klenow mix

dNTP mix

10 x ligase buffer:

all from Promega Erase-a-Base^{TM} kit

3.11.) Ligation

After restriction digestion, DNA fragments and the appropriate plasmid were mixed at a ratio of 3:1 (for cohesive ends) or 1:1 (for blunt ends), The total DNA amount was less than 1 µg. Ligase buffer (final 1 x) and 2 U T4 DNA ligase were added and incubated at 16°C for overnight.

5 x and 10 x ligation buffer:

from MBI

3.12.) Transformation of *E.coli*

3.12.1 competent cells by improved $CaCl_2$ method:

2 ml overnight culture of *E.coli* was added to 100 ml LB medium and grown at 37°C until an OD_{600} of 0.5. The bacteria were then pelleted for 15 min. at 4,000 rpm (4°C), and incubated in 50 ml ice-cold Tfb1 buffer for 30 min. After that the cells were collected by centrifugation and carefully resuspended in 6 ml Tfb2 buffer. The competent cells were then stored in 200 µl aliquots at -80°C and ready for use.

Tfb1 buffer (pH 5.8):

100 mM RbCl
50 mM $MnCl_2$
30 mM KAc
10 mM $CaCl_2$
15% glycerol

Tfb2 buffer (pH 8.0):

10 mM MOPS
10 mM RbCl
75 mM $CaCl_2$
15% glycerol

3.12.2 Transformation of competent *E.coli*:

About 0.1 µg DNA and 200 µl competent cells were mixed together and incubated on ice for 30 min. The cells were then heat-shocked at 42°C for 90 sec and mixed with 1 ml of LB medium. After growing at 37°C for 45 min, the *E.coli* were collected by centrifugation at 4,000 rpm for 5 min. 1 ml of the supernatant was discarded, the *E.coli* were resuspended in the rest of the medium and spread on LB-Amp plates (containing 50 µg/ml Ampicillin). The transformants were then grown overnight at 37°C.

3.12.3 Competent *E.coli* for electroporation:

2 ml overnight grown *E.coli* was added to 1 L LB medium and grown at 37°C by vigorous shaking (300 rpm). When OD_{600} reached to 0.5, the bacteria were collected by centrifugation 10 min at 4,000 rpm (4°C), and resuspended in ice-cold 15% Glycerol. After centrifugation at 6,000 rpm for 15 min. (4°C), the supernatant was removed carefully and the cells were resuspended successively in 100 ml, 50 ml, 25 ml, and 12 ml Glycerol. About 6 ml of the supernatant was left at the last washing. The cells were then resuspended in the rest solution and stored in 100 µl aliquots at -80°C.

3.12.4 Transformation of *E.coli* by electroporation:

20 ng DNA (in ddH_2O, no salt) were mixed with 100 µl competent cells in a 2 mm BioRad cuvette and incubated on ice for 10 min. The DNA was then electroporated into *E.coli* at 3 kV, 25 µF, 200 Ω. After that, 1 ml prewarmed LB medium (37°C) was immediately added and incubated at 37°C for 1hr without shaking. At the end the bacteria were collected by centrifugation (4,000 rpm) and spread on LB-Amp plates. The transformants were then grown overnight at 37°C.

3.13.) DNA sequencing

After denaturation, plasmid DNA was sequenced with the Sanger method (Sanger *et al*., 1977). The T7 Sequencing kit from Pharmacia was used for all sequencing reactions.

Sequencing gels

25.2 g urea, 6 ml 10 x TBE, 25.2 ml ddH_2O and 6 ml 38% Acrylamide/2% Bis-Acrylamide were mixed, and carefully warmed with a microwave (not over 40°C) until all urea was

dissolved. 60 µl TEMED and 200 µl 10% ammonium peroxidisulfate were added for polymerisation.

3.14.) Polymerase-Chain-Reaction (PCR)

Polymerase-chain-reaction (Saiki *et al*., 1985) was used to amplify DNA in vitro. For amplification of plasmid and genomic DNA templates the following protocol was used:

reaction mix:

1 ng DNA
50 pmol each oligo-nucleotide primers I+II
100 µM dNTPs
5 µl 10 x PCR buffer (Boehringer)
2 µl home-made Taq-polymerase (about 2 U)
add H_2O to 50 µl

a typical reaction protocol:

1. step:	5 min. 94°C
2. step (30 cycle):	30 sec. 94°C
	30 sec. 40°
	30 sec. 72°C (for less than 1 kb fragment)
	or
	1.5 min 40°C
	2 min 72°C (for more than 1 kb fragment)
3. step:	10 min 72°C
4. step:	5 min 25°C

3.15.) inverse PCR

10 µg genomic DNA was thoroughly digested with an appropriate restriction enzyme, and then treated at 65°C for 15 min. to inactivate the enzyme. After 1 time phenol/chloroform extraction, the DNA was dissolved in ddH_2O and set up for self ligation in a volume of 100 µl. The ligation was performed at RT for 1 hr, then 1 µl (1:10 and 1:100 dilutions were also checked) from the reaction mix was used for inverse PCR as a template. The unligated DNA was used as a negative control which should give no PCR products.

3.16.) 3'-RACE PCR

0.1 µg mRNA from *Dictyostelium* (purified with Qiagen mRNA kit) was used as a template to synthesise the first-strand cDNA. The 3'-RACE system kit from Gibco BRL was used for this work.

Protocol for first-strand cDNA synthesis:

0.1 µg	mRNA
10 µl	10 x Super transcriptase buffer
5 µl	10 mM dNTP
10 µl	0.1 M DTT
2 µl	Super transcriptase
10 µl	AP primer

DEPC treated water was added to a final volume of 100 µl, the first-strand cDNA was synthesised at 37°C for 45 min, then treated at 94°C for 2 min.

final conditions for amplification of the target cDNA:

0.1 U/µl	Taq Expander (Stratagene)
0.1 U/µl	Taq polymerase (Stratagene)
1 x	Expander buffer
100 pmol	specific primer
100 pmol	primer UAP
0.5 µM	dNTPs (each)
1 mM	$MgCl_2$

PCR protocol:

1. step:	4 min. 94°C
2. step (40 cycle):	30 sec. 94°C
	1 min 50°C
	2 min 68°C
3. step:	10 min 68°C
4. step:	5 min 25°C

3.17.) Southern-blot analysis

DNA fragments (PCR product, or digested genomic and plasmid DNA) were separated on a 0.8% agarose gel, and photographed under UV light to document DNA migration related to a molecular weight marker. The DNA was denature by incubating in 0.5 M NaOH/ 1.5 M NaCl buffer for 30 min., then neutralized for another 30 min in 0.5 M Tris-HCl pH7.0/ 1.5 M NaCl. The gel was then washed briefly and used for Southern-blot as described by Southern (Southern, 1975). After overnight transfer with 20xSSC buffer, the DNA was cross-linked to a nylon membrane under 314 nm UV light (0.120 J/cm^2), and prepared for hybridization.

3.18.) Northern-blot analysis

10 µg purified total RNA (or 1 µg polyA-mRNA) were dried in the speed vacuum, resuspended in 10 µl RNA sample buffer, and treated at 95°C for 5 min. The denatured

samples were loaded on a denaturing gel, and separated by electrophoresis at 40 mA at 4°C. The gel was photographed under UV light (the 2 ribosome RNA bands were used as molecular weight markers), washed briefly with ddH_2O, then used for Northern-transfer. After overnight transfer with 20 x SSC, the RNA was cross-linked to nylon membrane under 314 nm UV light (0.120 J/cm^2), and prepared for hybridization.

RNA sample buffer:

66% formamide
1 x gel buffer
8% formaldehyde
0.1% Xylene Cyanol FF
0.1% bromophenol blue
0.1% ethidium bromide (10 mg/ml)

RNA gel:

0.84 g agarose
7 ml 10 x gel buffer
52 ml ddH_2O
11.3 ml formaldehyde

10 x Gel buffer:

200 mM MOPS
50 mM sodium acetate
10 mM EDTA
pH 8.0

10 x running buffer:

same as 10 x Gel buffer but pH 7.0

3.19.) Radioactive labelling of DNA

The "oligo-labelling" method was used for labelling DNA fragments in a rapid and effective way. 0.1-0.3 µg purified DNA template was denatured by heating at 95°C for 5 min. Then the reaction was prepared as follows:

10 µl	OLB-mix
32 µl	DNA
5 µl	α-^{32}P-dATP
2 U	Klenow fragment

ddH_2O was used to adjust the volume to 50 µl.

The reaction was done at 37°C for 1 hr, then stopped by adding 100 µl phenol-saturated Tris (pH 7.5). The free nucleotides were separated by centrifugation through a Sephadex G50 spin

column. The purified radioactive probe was then denatured by heating at 90°C for 5 min, cooled on ice, and used for hybridization.

OLB mix:

200 mM Tris-HCl, pH 7.5
25 mM $MgCl_2$
10 mM β-ME
1 M HEPES pH 6.6
13.5 U A_{260} oligos (MBI)
0.25 mM dCTP, dGTP, dTTP

3.20.) Radioactive labelling of RNA

RNA radioactive probes were made by in vitro transcription. pGEM vector containing the appropriate DNA fragment was linearized with an unique restriction enzyme, then extracted with phenol/chloroform. The labelling reaction was set up at 37°C and incubated for 1 hr. The reaction was stopped by adding 100 µl phenol saturated ddH_2O. After purification on a sephadex G50 spin column, the probe was denatured by heating (90°C) and prepared for hybridization.

transcription reaction:

8µl ddH_2O
2 µl T7-buffer
1µl linearized plasmid (1µg/µl)
2 µl NTP (5 mM each)
1 µl RNasin
1 µl RNA polymerase (10 U/µl)
5 µl ^{32}P-UTP

3.21.) Hybridization

Southern- or Northern-blots were washed briefly with ddH_2O, and placed into a hybridization tube. The prehybridization was performed in hybridization solution for 1 hr at 37°C with 2 mg/ml Herring sperm DNA (Boehringer). Then the denatured probe was and incubated overnight at 37°C (Southern-blots) or 42°C (Northern-blots). When a discoidin RNA probe was used for Northern-blots, the temperature was raised to 50°C.
After hybridization the blots were washed twice with 2xSSC/0.2% SDS, then once with 0.1xSSC/0.2% SDS. The blots were then wrapped in Saran and exposed on X-ray film.

Hybridization solution:

50% formamide

3 x SSC
0.12 M Phosphate buffer, pH 6.8
0.2% SDS
3 x Denhardt solution
2 mM EDTA
0.2% sarcosyl

100 x Denhardt solution:

2% Ficoll 400
2% polyvinylpyrolidone
2% BSA

3.22.) Construction of the expression vectors

D1-pET15b and D2-pET15b

Two parts of the gdt1 gene, denominated Domain1 (D1) and Domain2 (D2, see Fig.15a in results) were amplified by PCR and cloned into the pET15b vector. For D1, the 920 bp PCR product (amplified with Primer 5'-FPC / $Primer_1$3') was digested with *Nde*I/*Bam*HI, then cloned into the *Nde*I/*Bam*HI site of pET15b. For D2, the 940 bp fragment (amplified with $Primer_2$5'/$Primer_2$3') was first cloned in vector pAS2-cYH1, then re-obtained with *Nde*I/*Bam*HI digestion, and cloned in frame with the 6-His epitope of pET15b vector by insertion at the *Nde*I/*Bam*HI site. Both D1 and D2 clonings were confirmed by sequencing.

4. Methods of protein analysis

4.1.) Protein colony-blot for REMI mutants

Dictyostelium clones (approx. 0.5 cm diameter) on KA plates were transferred onto nitrocellulose filters and treated as described by Wallraff and Gerisch, 1991. discoidin expression was detected with a monoclonal antibody 80-52-13 (Wetterauer *et al*., 1993) and a phosphatase coupled secondary goat-anti-mouse antibody. Filters were then stained with Ponceau S to detect all *Dictyostelium* cellular protein. Colonies displaying no discoidin expression and colonies showing stronger expression than wild type cells were picked, recloned and blotted again to confirm the phenotype and its stability.

4.2.) Preparation of total protein from *Dictyostelium*

1 to $5x10^7$ *Dictyostelium* cells were washed in 1 x phosphate buffer, and pelleted by centrifugation. Total protein was prepared by lysing the cells in 100-500 µl Laemmli buffer (Laemmli, 1970).

For detection of the protein expression in different cell lines, equal amounts (20 µg) of protein were loaded on a discontinuous polyacrylamide gel and blotted. Gels were blotted and incubated with antibodies in parallel to allow for a quantitative comparison.

Laemmli buffer (6 x):

7 ml 4 x Tris-HCl/SDS, pH 6.8
3 ml glycerol
1 g SDS
0.93 g DTT
1.2 mg bromphenol blue

4.3.) Preparation of membrane fraction proteins

For detecting the expression of the gdt1 protein, membrane fraction proteins were prepared as described by Bordier (1989). Around $5x10^7$ *Dictyostelium* cells were collected and kept on ice. Cells were then resuspended in 1 ml ice-cold TBS buffer to a final protein concentration of ≤4 mg/ml. 1/5 Vol. precondensed Triton X-114 stock solution was added to a final concentration of 2%, and incubated 15 min on ice for extraction. After centrifugation for 10 min. at 10,000 x g, 4°C, the supernatant was transferred to a fresh tube. The membrane fraction was separated from the cytosolic part by warming in a 37°C water bath. The solution was then centrifuged 10 min at 1000 x g, RT, and separated into upper and lower phases. Both phase were collect in separate tubes. 1/6 Vol. of 9 x sample buffer was added to the soluble phase, and an equal Vol. of 3 x Laemmli buffer to the membrane fraction. After denaturation for 5 min at 95°C, the samples were briefly centrifuged and 20 µl were loaded on a gradient gel.

TBS buffer:

10 mM Tris-HCl, pH 7.5
150 mM NaCl
20 µg/ml aprotinin
1 mM PMSF

precondensed Triton X-114:

1.5 g Triton X-114 was dissolved in 50 ml TBS (without proteinase inhibitors), warmed to 37°C in a water bath, then centrifuged 10 min. at 1000 x g, RT. The upper phase was removed and discarded, and the lower phase was re-dissolved in an equal volume of ice-cold TBS. This partitioning was repeated 3 times for eliminating the hydrophilic contaminants.

4.4.) Preparation of nuclear proteins

To detect the localization of the gdt1 protein, the nuclear protein fraction was also checked by immunoblotting with the anti-D1 polyclonal antiserum.

5 x 10^7 growing cells were harvested from bacteria suspension and lysed in 20 ml NP 40-lysis buffer. The nuclear fraction was obtained by centrifugation 10 min. at 6,000 rpm, 4°C. The pellet was washed twice with washing buffer, then resuspended in 100 µl Laemmli buffer. 20 µl of the sample was used for analysis.

Lysis buffer:

10 mM MES, pH 6.2
10 mM NaCl
1.5 mM $MgCl_2$
10% Glycerol
1 mM EDTA
5 mM DTT
1% NP-40

washing buffer:

Lysis buffer without NP-40

4.5.) Expression of recombinant proteins in *E.coli*

Expression of Domain1 and Domain2 of the gdt1 protein as 6xHis-tagged peptides

Vector D1-pET15b and D2-pET15b were transformed into BL 21 cells (Novagen) for expressing two domains of the gdt1 protein. Both domains, D1 and D2, were expressed as N-6xHis-tagged proteins around the same size of 36 kDa. After purification under denaturing conditions, both D1 and D2 were used for PKA assays and for generation of polyclonal antibodies. The purification was performed by using FPLC (Biologic system from BioRad) and the His Trap ^{TM} Kit from Pharmacia. A brief protocol is given bellow:

1. grow 5 ml preculture overnight at 30°C.
2. set up large scale culture (1 L) until OD_{600} reaches 0.5.
3. induction for 3 hrs with 0.3 mM IPTG.
4. pellet cells and resuspend in 20 ml Buffer A.
5. sonicate 12 x 1 min. at max. efficiency until all cells are lysed.
6. centrifuge 30 min at 14,000 rpm, 4°C.
7. load the supernatant to a Ni^{2+} coupled His Trap column.
8. wash the column with 15 ml Buffer B + 20 mM imidazole.

9. elute the His-tag protein with 200 mM imidazole.

Buffer A (pH 8.0):

6 M Guanidine-HCl
0.1 M NaH_2PO_4
0.01 M Tris-HCl

Buffer B (pH 8.0):

8 M Urea
0.1 M NaH_2PO_4
0.01 M Tris-HCl

4.6.) Immunoprecipitation

$5x10^7$ cells were lysed in lysis buffer, sonicated for 10 sec., then centrifuged for 15 min. at 10,000 rpm, 4°C. 5 µl polyclonal serum was added to the supernatant and incubated on ice for 1 hr. Then 40 µl Protein A pre-equilibrated in lysis buffer was added and incubated for 1 hr at 4°C with thorough rocking. At the end the immunprecipitate was collected by centrifugation for 5 min. at 4°C, washed twice in TG buffer, and resuspended in 100 µl Laemmli sample buffer. 20 µl of each sample was used for immunoblotting.

Lysis buffer:

20 mM HEPES, pH 7.5
10% glycerol
1% Triton X-100
1.5 mM $MgCl_2$
150 mM NaCl
20 µg/ml aprotinin
1 mM PMSF
2 mM NaF
5 mM EDTA

TG (Triton-glycerol) buffer:

5 x PBS
1% Triton X-100
10% glycerol
20 µg/ml aprotinin
1 mM PMSF
2 mM NaF

4.7.) Discontinuous SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

12% polyacrylamide gels were prepared according to Laemmli (1970). The samples were denatured by heating at 95°C for 5 min., then loaded on the stacking gel. Electrophoresis was carried out in 1 x running buffer at 40 mA for about 3 hours.

12% resolving gel	stacking gel

30% Acrylamide/Bis-Acrylamide 37.5:1 [ml]	17.6		1.8
Gel buffer [ml]	13.6		10.1
10% APS [ml]	0.96	0.48	
TEMED [µl]	10.7		8.0

SDS-stacking gel buffer 10 x :
(or 4 x Tris-HCl/SDS, pH 6.8)

0.5 M Tris-HCl, pH 6.8
14 mM SDS

SDS-resolving gel buffer 10 x :

(or 4 x Tris-HCl/SDS, pH 8.8)

1.5 M Tris-HCl, pH 8.8
14 mM SDS

protein running buffer 10 x :

1.9 M glycin
0.25 M Tris-HCl, pH 8.8
1.0% SDS

4.8.) Gradient SDS-PAGE

5% - 12% gradient SDS-PAGE was carried out to detect the gdt1 gene expression. As described by Matsudaira and Burgess (1978), the gradient gel was prepared by mixing light and heavy Acrylamide gel solutions with the help of a gradient maker (SG30, Hoefer Pharmacia Biotech). Protein samples were mixed with Laemmli buffer, boiled for 5 min, and loaded on the stacking gel. Electrophoresis was performed in 1x running buffer at 6 mA overnight.

light Acrylamide gel solution (5%):

2.5 ml	30% Acrylamide/0.8% Bis-Acrylamide
3.75 ml	4 x Tris-HCl/SDS pH 8.8
8.75 ml	H_2O
0.05 ml	10% ammonium persulfate

add 2.3 µl TEMED to every 7 ml solution before use.

heavy Acrylamide gel solution (12%):

5.0 ml	30% Acrylamide/0.8% Bis-Acrylamide
3.75 ml	4 x Tris-HCl/SDS pH 8.8
5.0 ml	H_2O
2.25 g	sucrose
0.05 ml	10% ammonium persulfate

add 2.3 µl TEMED to every 7 ml solution before use.

4.9.) Staining of proteins with Coomassie Blue

The resolving protein gel was immersed in Coomassie Blue R250 staining solution with gentle shaking. Destaining was performed by shaking in several changes of the destaining solution.

Coomassie solution:

0.1% Coomassie-blue R 250
10% acetic acid

destaining solution:

5% methanol
7% acetic acid

4.10.) Protein quantification

Protein concentration was determined by using the Amido-Black assay. 5 µl protein sample was added to 0.5 ml Amido-black solution, mixed briefly and centrifuged at 10,000 rpm for 4 min. The supernatant was discarded and the pellet was carefully washed once in 0.5 ml washing solution without destroying the pellet. At the end the pellet was resuspended in 0.1 N NaOH, and optical density was measured at 615 nm. By using BSA (1-50 µg) as standards, the concentration of the protein sample was derived from the standard curve.

Amido-black solution:

0.26 g amido-black
90% methanol
10% acetic acid
in 1 L ddH_2O

washing solution:

90% methanol
10% acetic acid

4.11.) Electroblotting of proteins "Semi-dry Blotting"

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by using a semi-dry blotting system (as described by Bjerrum, 1986). This method was used to detect proteins of less than 70 kDa molecular weight.
The transfer was performed with the "Semi-Dry"-Blot apparatus at 2mA/cm^2 membrane (maximal 40 V) for 1 hr.

transfer buffer:

6 g Tris base
29 g glycine
add ddH_2O to 2 L. The pH should be 8.2-8.4.

4.12.) "Tank Transfer"

For proteins bigger than 70 kDa, another system the "Tank Transfer" (Sartorius) was used to improve the transfer efficiency. This transfer was carried out at 50 V (around 600 mA) for 4 hrs or at15 V (around 150 mA) overnight. Transfer buffer was the same as above.

4.13.) Immunodetection with enzyme conjugated secondary antibodies

The Western-blots were immersed in blocking buffer (1xNCP/2% BSA), then incubated with the primary antibody at a proper dilution. The blots were washed and exposed to an enzyme conjugated secondary antibody directed against the primary antibody (e.g. goat anti-mouse and goat anti-rabbit IgG). Antigens are finally identified by chromogenic or luminescent visualization. The secondary antibodies were conjugated with either alkaline phosphates (AP) or horseradish peroxidase (HRPO), and were detected with BCIP substrate solution (for AP-conjugate) or ECL detection system (for HRPO-conjugate).

1 x NCP:

150 mM NaCl
10 mM Tris-HCl, pH 8.0
0.05% Tween 20
0.25% sodium azide

BCIP substrate buffer:

0.2 mg/ml in 0.1 M Na_2CO_3

ECL detection system:

from Amersham.

4.14.) *in vitro* phosphorylation assay of PKA

Since D1 and D2 were purified under denaturing conditions, they had to be dialysed before use for the PKA assay. The samples were dialysed first against 2M urea buffer for 2 hrs, then the dialysis was changed to buffer without urea for overnight dialysis. A substantial precipitate was observed with D1 but not with D2. The soluble D1 concentration was around 10 ng/µl while the concentration of D2 was 250 ng/µl. The PKA assay was carried out as

described by Etchebehere *et al.* (1997). Recombinant PKA from *Dictyostelium* (generous gifts from M. Véron) was first saturated with cold ATP, then used for the phosphorylation assay on substrates with γ-^{32}P-ATP. A specific inhibitor of PKA (PKI) was used to prove the specificity of phosphorylation by PKA (also a gift from M. Véron).

Step 1. Pre-incubation of PKA

4 µl	10 x kinase buffer
10 µl	PKA
1 µl	ATP (10 mM)
24 µl	H_2O

incubate 1 hr at RT, then go to step 2.

Step 2. phosphorylation on substrates with γ-^{32}P-ATP

add to the reaction from step 1:

1 µl	γ-^{32}P-ATP
10 µl	protein substrate
± 1 µl	PKI

incubate at 30°C for 1 hr then stops with adding 1 x Vol. Laemmli buffer.

10 x kinase buffer:

500 mM MOPS
50 mM $MgCl_2$
20 mM NaF

4.15.) autophosphorylation of the gdt1 protein

10 µl Triton X-114 extracted membrane fraction (from ca. $2x10^6$ cells) were used for autophosphorylation assays. The following buffers were mixed on ice and incubated overnight at 4°C. For samples after immunoprecipitation, 10 µl of the immunoprecipitates were incubated with the same reaction mix, but the reaction was performed at 30°C for 1 hour.

10 µl	membrane fraction (or immunoprecipitates)
10µl	10 x kinase buffer
1 µl	100 mM PMSF
5 µl	Aprotinin (20,000U/ml)
2 µl	γ-^{32}P-ATP

10 x kinase buffer:

200 mM	HEPES (pH 7.5)
50 mM	$MgCl_2$
20 mM	NaF

4.16.) Generation of polyclonal antisera against the recombinant D1 and D2 protein

4.16.1.) Preparation of antigens

About 200 µg recombinant D1 or D2 protein were separated on a 12% preparative SDS-polyacrylamide gel and stained with Coomassie-Blue. After destaining, the gel was washed in ddH_2O for 15 min with several changes of water, then the band of the recombinant protein was cut out and broken by passing several times through metal-nets. An approximate weight of the gel was measured and a corresponding volume of PBS buffer was added. After sonication at maximum power for 10 sec, the sample was ready for immunization.

4.16.2) Production of polyclonal antiserum in rabbits

Rabbit FU 42 was used for D1, and rabbit FU 43 was used for D2. A standard protocol for subcutaneous immunization was used for both. Before the first injection, pre-immunserum was taken from both rabbits and used for detecting the background. About 5 ml blood was taken from the ear vein, incubated 1 hr at 37°C for clotting, then centrifuged at 4°C, 4000 rpm for 30 min. The supernatant was aliquoted and frozen at -80°C. 28 days after the first injection, a boost injection was performed. The further boosts were performed at a time distance of 10-15 days. From the 2nd immunization, up to 15 ml blood was taken at 10 days after each injection and used for preparing polyclonal antiserum. The method for preparation was the same as described for pre-immunserum. Specific antiserum was obtained for D1, but failed for D2.

4.17.) Affinity-purification of antibody

About 500 µg D1 protein were loaded on a preparative gel, separated and then transferred on a nitrocellulose membrane by “semi-dry” blotting. The membrane was washed in 1 x NCP, then stained with Poncea S solution to determine the exact position of the D1 band. The band was cut out, washed in 1xNCP until the colour less. Then the membrane was washed 7 times in 5 ml Tween buffer (10 mM Tris-HCl pH 9.0, 0.9% NaCl, 0.05% Tween 20) for 10 min. After that the membrane was incubated with 5ml diluted antiserum (1:1 in Tween-buffer), at 4°C overnight for adsorption. The filter was further washed successively with Tween-buffer, PBS pH 7.2, PBS pH 7.2/1 M NaCl and PBS pH 7.2, every washing was repeated 3 times for 5 min. At the end the membrane was eluted for 3 min with 0.5 M acetate acid (pH 2.5). The eluate was immediately neutralized with 0.5 M Tris/BSA buffer (1M Tris-HCl pH 9.0,

2 mg/ml BSA), and dialysed against PBS pH 7.2 buffer overnight. The antibody was aliquoted, and frozen at -80°C.

However, after this purification, the D1 antibody lost most ist activity and could not be used for further analysis.

4.18.) Purification of IgG antibodies with protein A-Sepharose

As described by Ey *et al.* (1978), protein A-sepharose was used to fractionate IgG antibodies using a stepwise pH elution. The buffer systems was described by Oi and Herzenberg (1980), to allow efficient purification of most IgGs and occasional IgM antibodies at RT.

Around 0.5 g Protein A-Sepharose was fully swelled in H_2O and poured into a chromatography column, then equilibrated with Tris-buffer at RT. The collected sera was diluted in 3 vol. Tris-buffer and applied to the column at a flow rate of 2 ml/min. The column was washed with Tris-buffer until all unbound proteins were eluted. The bound proteins were eluted with 2 vol. of glycine buffer directly into test tubes containing neutralising buffer (1/4 of the collected volume). At the end all eluted fractions were assayed for antigen-specificity.

Tris-buffer (pH 8.6):

50 mM Tris-HCl
150 mM NaCl
0.02% NaN_3

glycine buffer (pH 2.3):

50 mM glycine
150 mM NaCl

Neutralising buffer (pH 7.7):

0.5 M Na_2HPO_4/NaH_2PO_4

IV. EXPERIMENTS AND RESULTS

1. Identification of the *REMI* mutant 2-9

The expression of discoidin protein is distinct from growth to development, which is easily observed in colony-blots. During vegetative growth on bacteria, expression in wild type cells is below the detection level. This results in an outer ring which is stained by Ponceau S but not by the specific anti-discoidin antibody. With the onset of development, discoidin expression is induced, resulting in antibody staining of an inner ring of preaggregation cells. Even though transcription is down-regulated in later development, the protein is still stable and can be detected in late stages. discoidin null mutants can be detected by the lack of antibody staining. Since colony-blots are semi-quantitative, overexpression mutants which display stronger antibody staining than the wild type can also be identified.

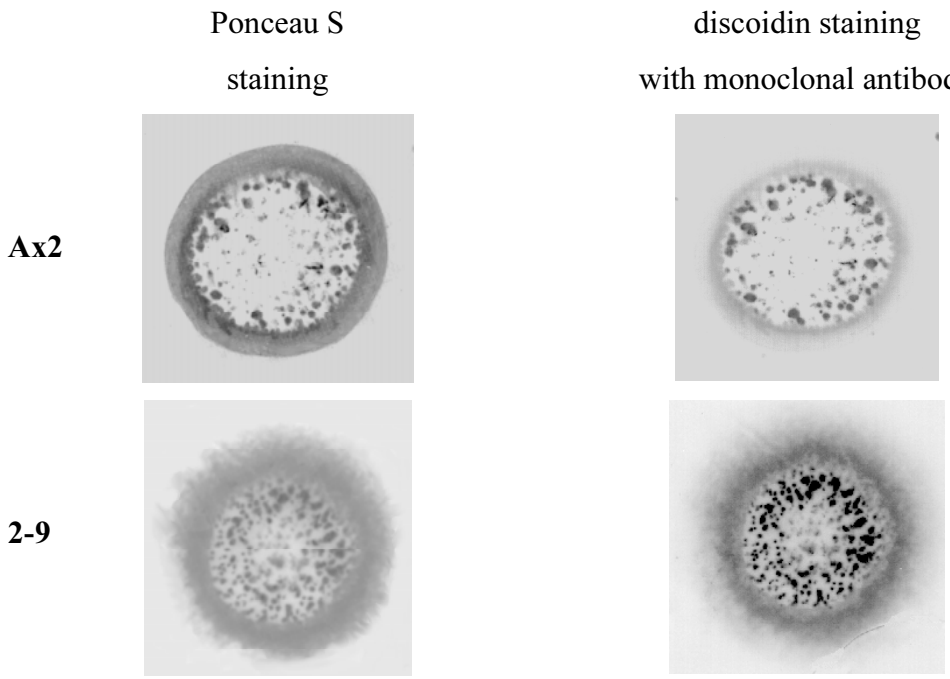


Figure 4a. Colonies of 1-2 cm diameter growing on a lawn of *Klebsiella aerogenes* were blotted, lysed and processed for detection of discoidin I with the monoclonal antibody 80-52-13 as described (Wetterauer *et al.*, 1993). In order to visualise total protein, the blots were counter-stained with Ponceau S. The 2-9 mutant shows antibody staining in the feeding edge and a stronger signal than Ax2.

The REMI 2-9 mutant was detected in a REMI screen as a discoidin overexpressor (Fig. 4a). In contrast to wild type colonies, discoidin protein was found in cells beyond the visible border of the colony, i.e., in growing cells which still have sufficient supply of nutrients. In addition, from the microscopical observation, the 2-9 mutant cells aggregated close to the growing edge and tended to aggregate where they were in the middle of bacteria. This resulted in a ragged colony shape compared to the smooth edge observed in wild type clones (Fig. 4b).

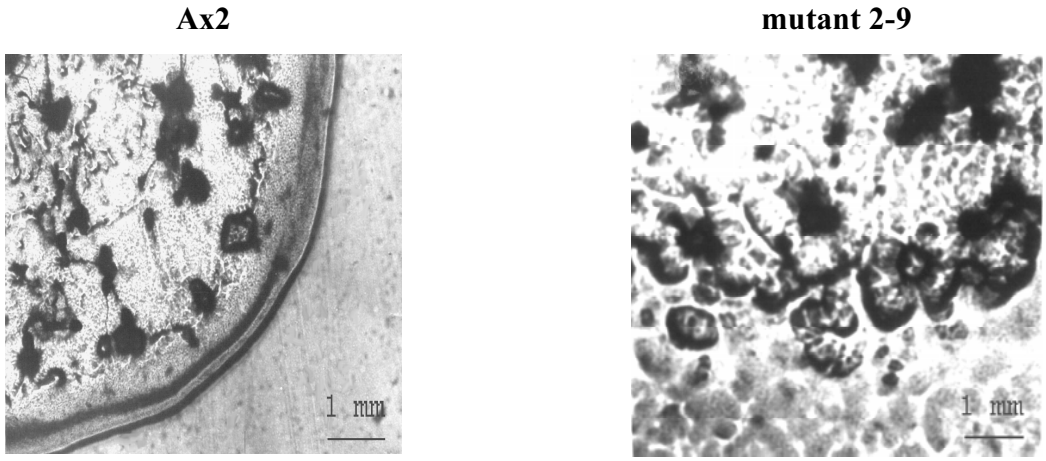


Figure 4b. In the microscopical observation, the 2-9 mutant formed aggregates close to the feeding edge and the preaggregation zone as visible in Ax2 was missing. In 2-9 there were clearly visible cell masses outside of the plaque. Upon further growth these cells formed satellite plaques which ultimately led to an irregular boundary of the colony.

The discoidin overexpression of mutant 2-9 was confirmed by Northern-blots (Fig. 7) and Western-blots (Fig. 26): discoidin mRNA accumulated at lower cell density, and discoidin protein was significantly increased compared to Ax2 cells.

Unless otherwise stated, all the following experiments were done with the reconstructed gene disruption mutant L8 because this strain was derived from the common laboratory strain Ax2 (see section 5.2).

For all the experiments with growing cells, the generation time during exponential growth has been measured. Interestingly, in comparison to the wild type, the L8 mutant cells grow more slowly in bacterial suspension, but in axenic medium they grow normally (Table 2). During axenic growth *Dictyostelium* cells absorb medium by pinocytosis, while in bacterial growth

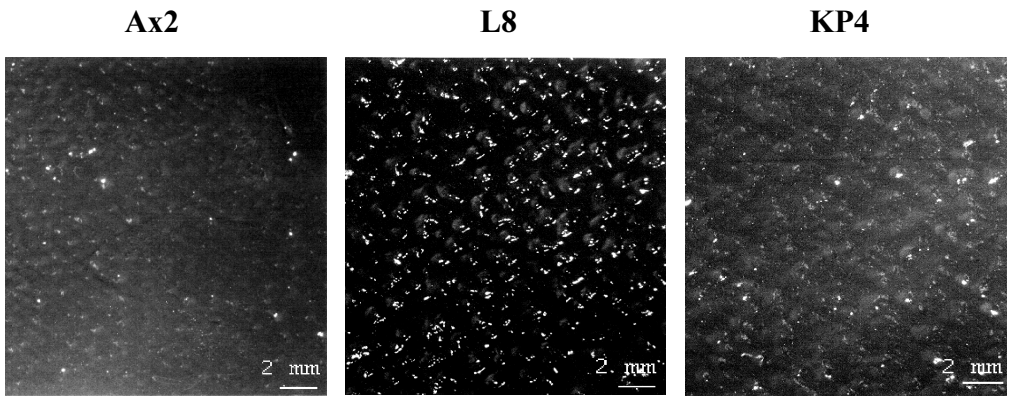
they need to chemotacticly move toward bacteria and engulf food by endocytosis. Therefore the L8 mutant is probably affected in sensing the food source, or in endocytosis.

Table 2. Generation time for L8 and Ax2 cells

	growth in axenic medium	growth in bacteria suspension
Ax2	8 hours	3 hours
L8	8 hours	4-5 hours

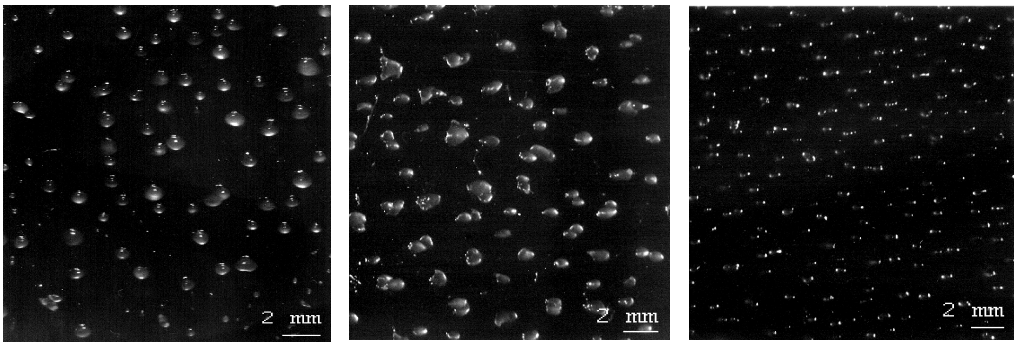
2. The developmental process is accelerated in the 2-9 mutant

To further investigate the defect in the developmental process in the mutant, a developmental timing experiment was performed for the reconstructed L8 mutant, wild type Ax2 and a rapid-development strain, KP4, which overexpressed the PKA C subunit (Anjard *et al.*, 1992). All cell lines were harvested at a density of $1x10^6$ from axenic culture, washed with phosphate buffer and resuspended to $1x10^8$ cells/ml in phosphate buffer. $5x10^6$ cells were then spotted on a phosphate agar plate (see METHODS 1.12) and developed at 22°C as described (Newell *et al.*, 1969). Development was monitored under a binocular microscope (Olympus SZ-PT) and photos were taken at the times indicated. As shown in Fig. 5, the developmental process was accelerated in both L8 and KP4 cells in comparison to wild type Ax2. However, there were obvious differences between the two strains: in L8, cells aggregated earlier thus the whole developmental process was accelerated; whereas in the KP4 strain, cells started to make small aggregates at the normal time but then rapidly achieved development as described previously (Anjard *et al.*, 1992). These data suggested that the mutation in L8 resulted in accelerated aggregation in contrast to accelerated slug formation/culmination in KP4.



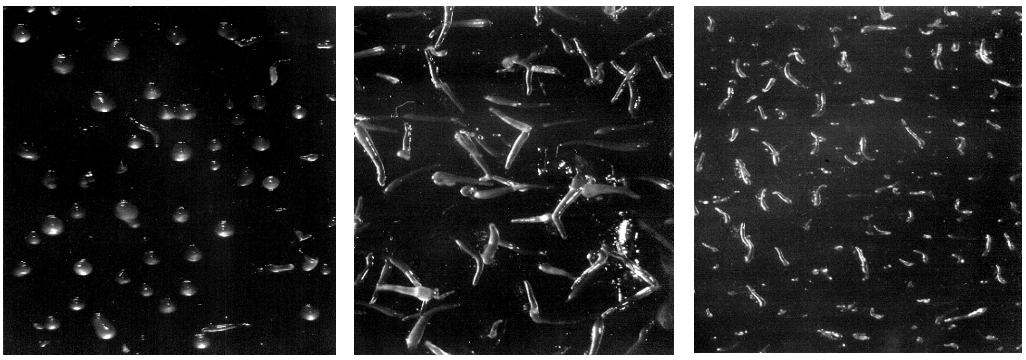
7-9 hours:

Loose-aggregates for L8, ripples (i.e. first signs of aggregation) for Ax2 and KP4.



13 hours:

Tipped-aggregates for L8, aggregates for Ax2 and KP4.



15 hours:

Slugs for L8 and KP4, tipped-aggregates plus few slugs for Ax2.

19 hours:

Fruiting bodies for KP4, late-culmination for L8, early-culmination for Ax2.

Figure 5. The developmental process in Ax2, KP4 and L8 cells when starved on phosphate agar-plates. Note that KP4 cells make smaller aggregates, culminations at 19 hrs are therefore not clearly seen.

3. The 2-9 mutant is cell autonomous

In order to investigate the cell autonomous function of the 2-9 mutant, a β-galactosidase assay was used for reporting the regulation of discoidin. A transgenic strain, denominated DAG (Wetterauer *et al.*, 1993), was constructed to express β-galactosidase under the control of discoidin Iγ promoter in Ax2 cells. The L8 mutant and DAG cells grown in bacterial suspension to a density of $3x10^5$ were mixed at ratios of 1:9, 1:1 and 9:1 and then grown for further 6 hours in bacterial suspension. Cells were harvested at a density of approx. $1x10^6$, and investigated for the activity of the discoidin promoter measured by the β-galactosidase assay (described in METHOD 2.7). 100% L8 cells and 100% DAG cells were used as controls. As shown in Fig. 6a, the β-galactosidase activity increased in parallel with the proportion of the DAG cells. This indicated that the L8 cells did not secret any extracellular factor able to stimulate the discoidin expression in wild type cells.

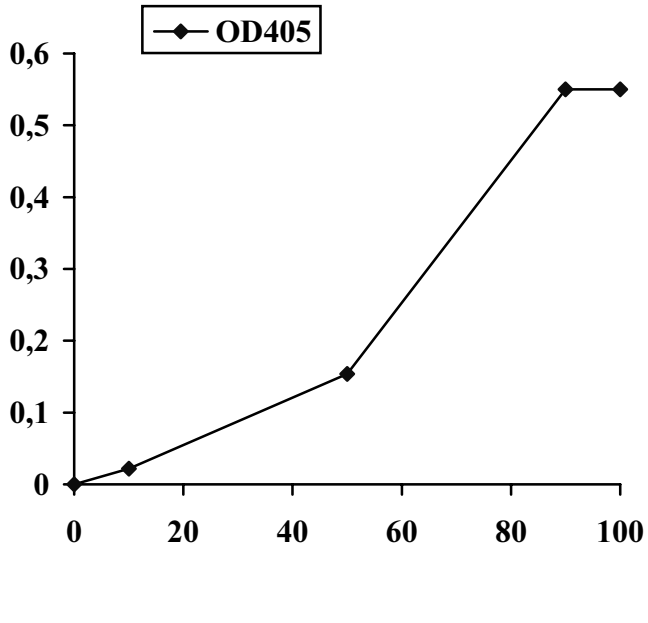


Figure 6a. The β-galactosidase assay showed that L8 cells did not induce disproportional activity of the discoidin promoter in DAG cells.

The reverse experiment was done for Ax2 and the L8 mutant to determine whether Ax2 cells influenced the disc^{over} phenotype in L8. As described in Fig. 6b, Ax2 and L8 cells were mixed at different ratios and discoidin I expression was detected by Western-blot. The expression of discoidin I increased proportionally with the percentage of the L8 cells, and no inhibition by DAG cells was observed. These data suggest that, the L8 mutant is cell autonomous.

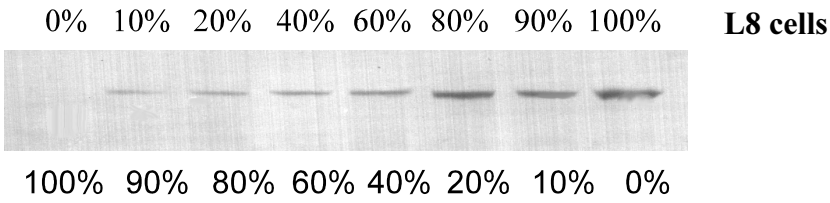


Figure 6b. Mixes of L8 and Ax2 cells were prepared as described for the β-galactosidase assay. Whole cell lysates were prepared and an equal amount of proteins for each point was separated by 12% SDS-PAGE, blotted and detected with the monoclonal discoidin antibody 80-52-13. Note that under these conditions no discoidin protein expression was observed in Ax2, therefore all discoidin protein originated from L8 cells. There was no disproportional reduction of discoidin in the mixes, thus Ax2 cells did not inhibit the mutant phenotype of L8.

4. The 2-9 phenotype: mis-regulation of early-developmental genes

In wild type Ax2 cells, discoidin expression is induced approximately 3 generations before the onset of starvation when cells are cultured in a standard bacterial suspension. Deviation of this expression pattern can be monitored by harvesting cells at different densities during growth and measuring the amount of discoidin (Wetterauer *et al.*, 1995). Thus to confirm the premature expression of discoidin in the mutant, L8 cells were cultured and harvested at different cell densities ($1x10^6$, $2x10^6$ and $5x10^6$ cells/ml) during bacterial growth. As described in the introduction, there are two autocrine factors, the prestarvation factor (PSF) and the conditioned medium factor (CMF), secreted from *Dictyostelium* cells at the GDT and regulating gene expression during growth and early development. However, so far only CMF has been cloned and PSF remains unknown. A *cmf* gene knock-out mutant (ΔCMF strain) was recently generated (Gomer, unpublished). Preliminary work suggested that CMF regulated cAMP signal transduction (Clarke and Gomer, 1995) and could also be involved in the regulation of discoidin. Thus the ΔCMF strain was included as a comparison. Expression of discoidin I was detected for Ax2, ΔCMF and L8 by Northern-blots hybridized with an *in vitro* transcript of *discoidin Iγ* . As shown in Fig. 7, a normal pattern was observed for ΔCMF and a premature expression of the discoidin mRNA was observed in L8: discoidin was already expressed at high level in growing cells at low density ($1x10^6$ cells/ml), and the accumulation was accelerated.

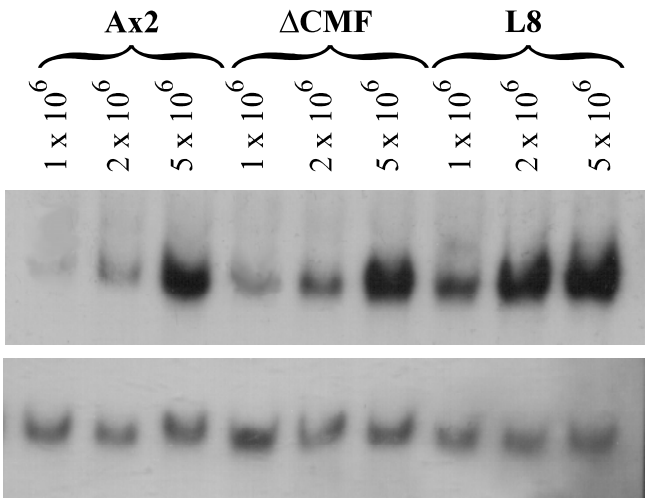


Figure 7. Total RNA was isolated from Ax2, ΔCMF and L8 at the indicated cell densities. 10 µg of each sample were used for the Northern-blot and hybridized with an *in vitro* transcript of the discoidin Iγ gene. After washing, the filter was exposed to Kodak Biomax film overnight. To ensure that equal amounts of RNA had been used for each sample, the same filter was washed and rehybridized with an actin 6 probe (generated by ″oligo-labelling″, see METHODS 3.19). Equal signals were obtained for all samples.

The premature expression of discoidin suggested that other genes in the developmental program might also be prematurely expressed in L8. Therefore other developmental genes (e.g., *carA*, *pde* and *csA*) were analyzed by comparing wild type Ax2 and L8 mutant in parallel (Fig. 8). In Ax2 cells, *csA* (Noegel *et al*., 1986) was induced in the stationary phase when cells had depleted the food source for several hours. The same pattern was observed for L8, but the expression was higher than in Ax2. *Pde* (Faure *et al.*, 1990) was transcribed in Ax2 as small message of 1.9 kb in a cell density dependent fashion, the larger transcript (2.4 kb) becomes prominently expressed in stationary cells. In L8, both the small 1.9 kb transcript and the larger size 2.4 kb transcript were prematurely expressed, but they were not induced by development. In both Ax2 and L8 the expression of *carA* (Klein *et al.*, 1988) was induced in stationary phase, but the expression level was significantly lower in L8 than in Ax2.

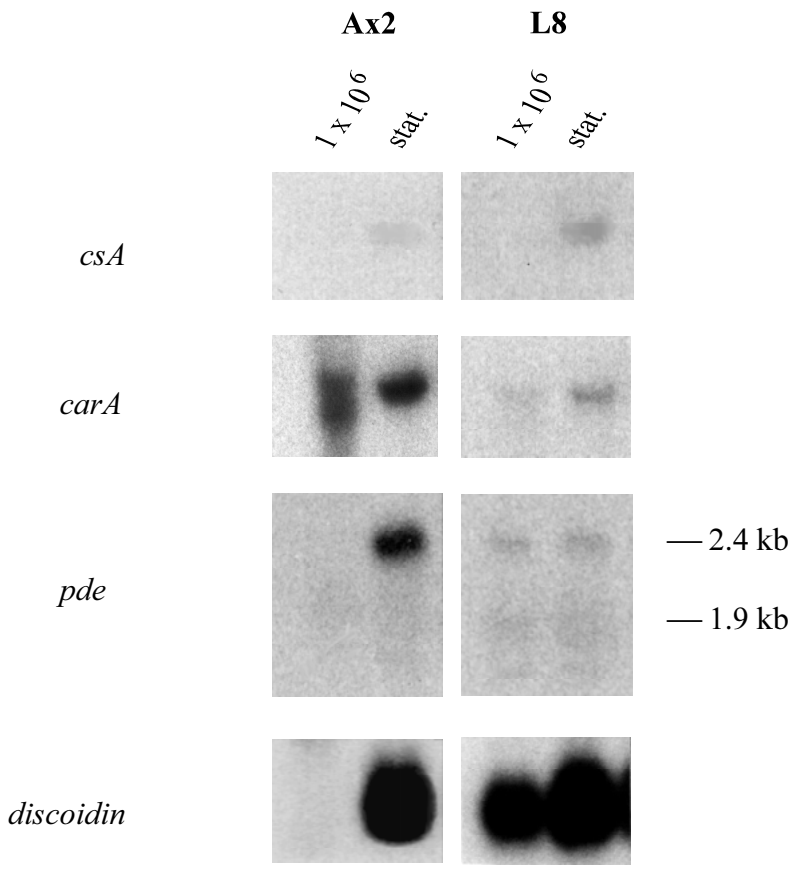


Figure 8. RNA was prepared from Ax2 and L8 at cell densities of $1x10^6$ and the stationary phases (several hours after the cells had depleted the food source, roughly equivalent to the first stages of development in suspension culture). 10 µg RNA were used for each sample and all samples were processed on the same gel. An *in vitro* transcript was used as *discoidin* probe, other probes were made by ″oligo-labelling″ for *csA*, *carA* and *pde*. The discoidin hybridization was exposed to Kodak Biomax film overnight, the *pde* exposure for 2 days, and 7 days for *csA* and *carA*. For *pde* the 1.9 kb mRNA is barely visible in this blot but can be seen in the original.

5. The *gdt1* gene and reconstruction of the mutant

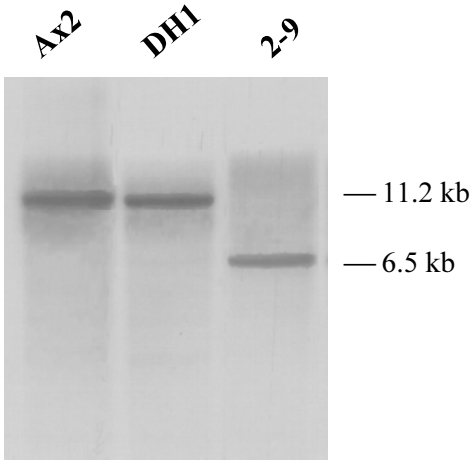
5.1. Isolation of a 3.7 kb genomic fragment of the *gdt1* gene

Part of the affected gene in mutant 2-9 was isolated by "plasmid rescue" (see METHODS 3.2) using *Hind*III digested genomic DNA. Since *Hind*III cuts within the DIV-2 vector, only the part containing the pGem3 vector was recovered, together with a 3.7 kb flanking genomic region (see Fig. 10). This isolated plasmid, denominated 2-9 rescue, was used for sequencing and the constructions of further gene disruption mutants. The isolated 3.7 kb genomic fragment was labelled and applied to probe a genomic Southern-blot for Ax2, the parent strain

DH1 and mutant 2-9 (see Fig. 10 for fragment size). As shown in Fig. 9, a single fragment of 6.5 kb was found in the 2-9 mutant, which contained the whole 3.7 kb fragment and the 2.8 kb pGem3 vector part of the DIV-2 vector. In Ax2, a single fragment of 11.2 kb consisting of the 3.7 kb fragment and 7.5 kb upstream sequence was observed. This proved that the genomic structure in the region of the isolated fragment was altered in the 2-9 mutant.

Sequence analysis revealed a continuous open reading frame (ORF) over the entire 3.7 kb fragment, but no initiation nor a termination codon was found. In addition, the whole ORF showed no homology to any known gene in the databases. This suggested that the phenotype of the 2-9 mutant was generated by the disruption of a new gene involved in the regulation of the growth-differentiation-transition. Thus the putative gene was denominated as *gdt1*, and the isolated 3.7 kb fragment was used for the further analysis.

Figure 9. Genomic DNA from Ax2, DH1 and the REMI 2-9 mutant were digested with *Hind*III, separated on a 0.8% agarose gel, blotted to nylon membrane and hybridized with a ^{32}P labelled probe (by oligo-labelling) of the isolated 3.7 kb fragment.



5.2. Reconstruction of the 2-9 mutant

To confirm that the phenotype of the 2-9 mutant was a direct consequence of the disruption of the *gdt1* gene, two vectors were constructed for targeted gene disruption (see Fig. 10): 2-9-Bs^{R}-XbaI, which created a gene disruption at the *Xba*I site (the L8 mutant); and 2-9-Bs^{R}-*Hind*III, which generated a disruption at the end of the 3.7 kb fragment (the K series).

Genomic maps of the *gdt1* gene in wild type and gene disruption mutants

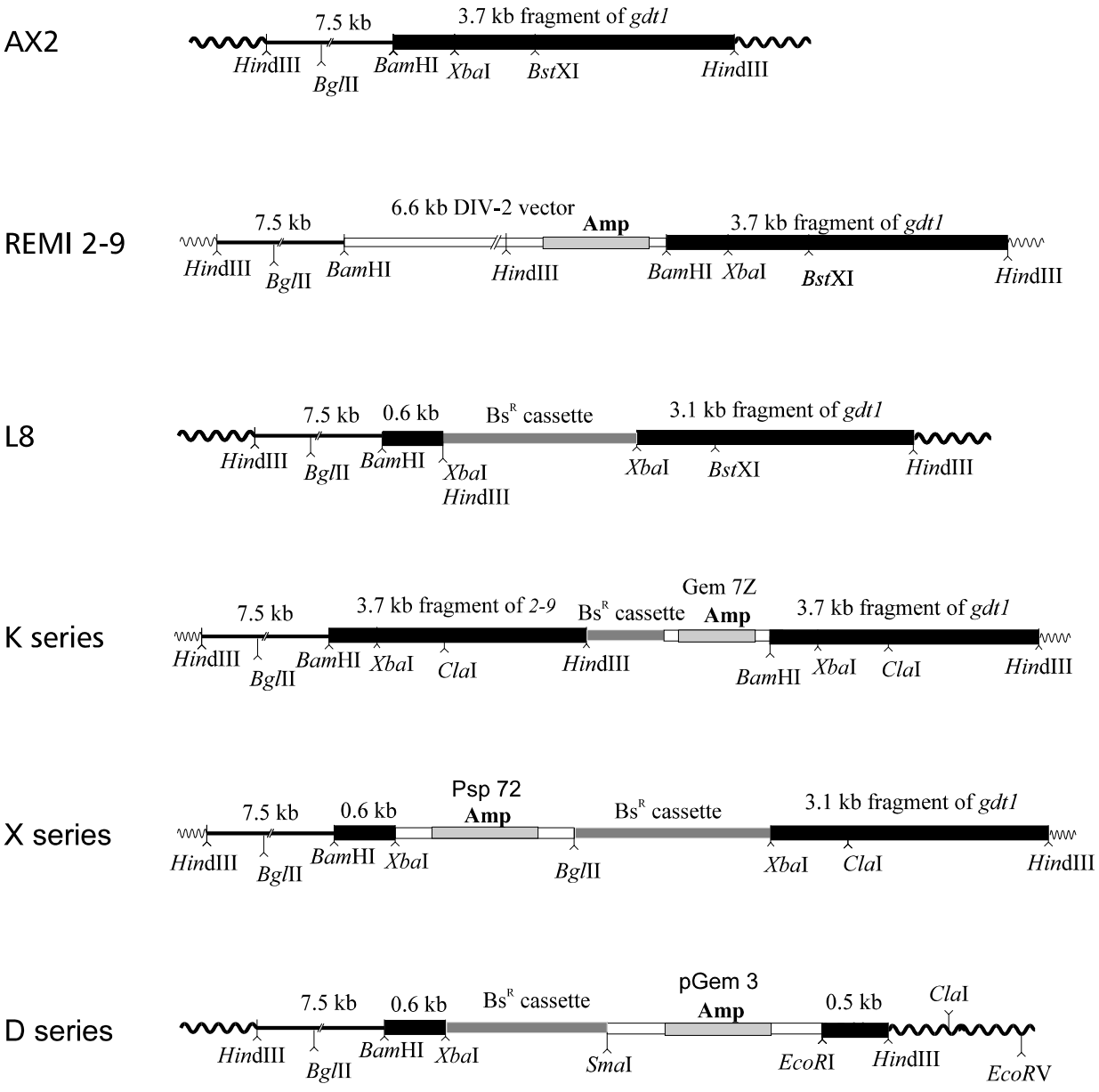


Figure 10. REMI 2-9 was generated by insertion of the DIV-2 vector (see INTRODUCTION 5) into the *Bam*HI site of the *gdt1* gene (191 bp after the start codon ″ATG″). The 3' flanking part of the *gdt1* gene (3.7 kb) was isolated from the REMI 2-9 mutant by plasmid rescue. Other *gdt1* gene disruptants (the L8 mutant, the K series, the X series and the D series) were constructed by homologous recombination (see section 5.2, 5.3 and 5.4.3). The Bs^R cassette (Sutoh, 1993) contained a Blasticidin resistance marker and was used for the selection of mutants. All the gdt1 mutants display the same phenotype: discoidin overexpression and the irregular colony shape.

The vector 2-9-Bs^R-XbaI was constructed by inserting the whole Bs^R cassette (carrying a Blasticidin resistance marker under the control of the actin15 promoter and actin8 terminator; Sutoh, 1993) into the *Xba*I site of the 3.7 kb *gdt1* fragment in the 2-9 rescue plasmid. This vector was cut with *Bam*HI and *Bst*XI (0.9 kb downstream of the *Xba*I site, see Fig. 10) thus resulted in two fragments: one with two arms (0.6 kb and 0.9 kb) of the *gdt1* gene and the Bs^R cassette in the middle; another one containing the rest of the 2-9 rescue vector without Bs^R cassette. Ax2 cells were electroporated with 10 μg of the digestion mix to generate homologous recombination. Over 90% of the transformants (denominated L series) displayed the same colony morphology as the REMI 2-9 mutant: a ragged growing edge, and the tendency to aggregate outside of the plaque. As controls, supercoiled 2-9-Bs^R-XbaI vector, the Bs^R cassette alone and the linearized vector 2-9 rescue (containing only the 3.7 kb fragment in the pGem3 vector) were introduced into cells under the same conditions but no mutants were detected (data not shown). 8 clones from the L series (L1-L8) were randomly picked and all displayed overexpression of discoidin protein during vegetative growth on bacteria (Fig. 11a). On Southern-blots all disruptants showed the restriction fragment pattern as expected for the homologous recombination.

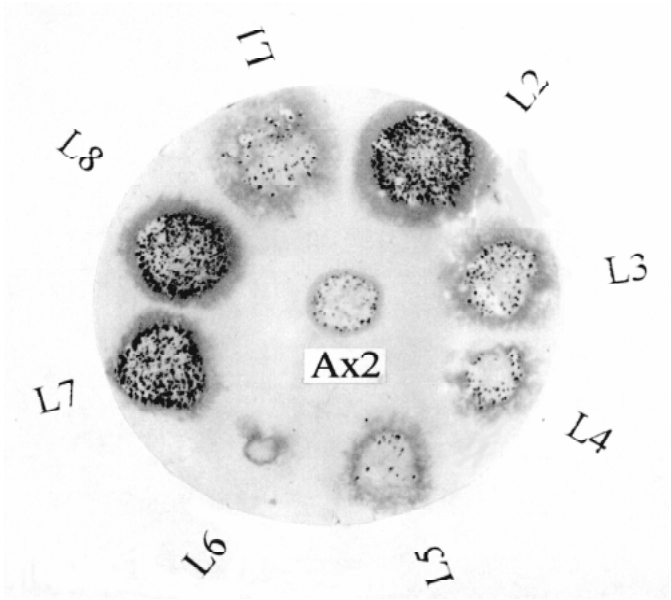


Figure 11a. Targeted gene disruption of the *gdt1* gene in wild type Ax2 resulted in L series. 8 clones (L1-L8) from the L series were randomly picked and analyzed on colony-blots with the monoclonal anti-discoidin antibody. All mutants reconstructed the phenotype of the original REMI 2-9 mutant: discoidin overexpression during vegetative growth on bacteria, and the irregular colony morphology.

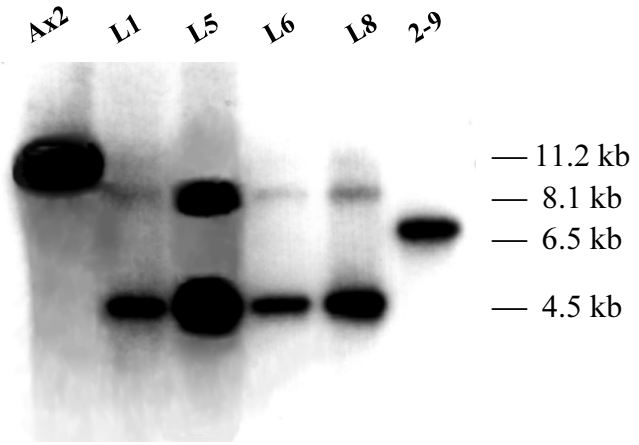


Figure 11b. Genomic DNA was prepared from Ax2, the 2-9 mutant and some clones from the L series. After digestion with *Hind*III, samples were used for Southern-blot analysis and hybridized with a ^{32}P labelled 3.7 kb probe of the *gdt1* gene. All L mutants showed the same insertion pattern as expected: two fragments of 8.1 kb and 4.5 kb resulted by a single insertion of the Bs^R cassette into the *gdt1* gene (see Fig. 10). The 8.1 kb fragment contained 7.5 kb of upstream genomic DNA and 0.6 kb from the 3.7 kb fragment; while the 4.5 kb fragment contained the Bs^R cassette of 1.4 kb and 3.1 kb of the 3.7 kb fragment.

The L series differed from the original 2-9 mutant in several aspects: the parent strain was Ax2 in contrast to DH1, the selection marker was Blasticidine resistance in contrast to uracil auxotrophy and the disruption was done in the *Xba*I site instead of the *Bam*HI site. Since all disruptants were identical, it could be concluded that the reconstructed phenotype was generated by homologous recombination. For further analysis, the reconstructed strain L8 was used because it was in the genetic background of the common laboratory strain Ax2.

A third strain (K series) was generated by disruption of the *gdt1* gene at the *Hind*III site using the vector 2-9-Bs^R-*Hind*III (see Fig. 10). The Bs^R cassette was cut out from vector pUC Bs^R ΔBamHI (Sutoh 1993) and cloned into the *Xba*I/*Hind*III site of the Gem7Z vector. Then the 3.7 kb fragment of the *gdt1* gene was cloned directly after the actin8 terminator inside the Bs^R cassette and resulted in the construction of the vector 2-9-Bs^R-*Hind*III. This vector was linearized with *Cla*I (cutting in the middle of the 3.7 kb fragment), and was then electroporated into Ax2 cells. Over 60% of the transformants displayed the same phenotype as L8. Three clones with the L8 phenotype (denominated K1-3) were randomly picked and analyzed by Southern-blots (data not shown). A same restriction pattern was obtained for all the K series which demonstrated a single integration of the entire vector 2-9-Bs^R-*Hind*III and duplication of the 3.7 kb fragment (see Fig. 10). Thus the K series mutants were generated by

gene disruption at the *Hind*III site, and re-expressed the phenotype as in the strains disrupted at the *Bam*HI (original 2-9 mutant) and the *Xba*I sites (L8). Since the insertion sites *Bam*HI and *Hind*III were on the opposite ends of the 3.7 kb fragment, the results supported the sequence analysis which predicted a single open reading frame over the entire fragment.

5.3. Isolation of the 5' end of the *gdt1* gene

The 5' part of the *gdt1* gene could not be isolated by plasmid rescue from L8 cells, since they contained no ampicillin resistance and *E.coli* replication origin. Therefore a further vector, 2.9-5'Bs^R was constructed for a 4th disruption of the *gdt1* gene, and the 5' end of the *gdt1* gene was recovered by a new gene ″rescue″ from the resulted X series disruptants (see Fig.10). The vector 2.9-5'Bs^R was constructed by two steps of cloning. First, a fragment of 0.6 kb was cut out by *Bam*HI/*Xba*I from the isolated 3.7 kb fragment, and was cloned into the *Bam*HI/*Xba*I site of vector Psp72 (Promega). Then a 2.2 kb fragment containing the Bs^R cassette (1.4 kb) and 0.8 kb of the 3.7 kb fragment (until the indicated *Cla*I site) was cut out of the vector 2-9-Bs^R-XbaI with *Cla*I. This was cloned into the *Cla*I site of the Psp72 plasmid already containing the 0.6 kb *gdt1* fragment. The final vector was linearized with *Bam*HI and *Cla*I, then transformed into Ax2 cells. The resultant gene-disruptants (X series) were first screened for the discoidin ^{over} phenotype by colony-blot, then confirmed by PCR analysis (Fig. 12). Genomic DNA was prepared from X series cells, digested with *Bgl*II, self-ligated and transformed into *E.coli*. A 3.4 kb genomic fragment with 0.6 kb (*Bam*HI/*Xba*I) of known sequence and 2.8 kb of additional 5' upstream sequence was obtained together with the Psp72 vector. Sequencing analysis revealed a 191 bp open reading frame (ORF) within this 2.8 kb which started with an ″ATG″ and went into frame with the former large ORF (see APPENDIX 3). Around 300 bp sequence before the ″ATG″ was analyzed but no ORF nor a possible intron sequence was found (data not shown). The sequence before the ″ATG″ site was quite A-T rich and contained two large polyA blocks of around 50 bp (see APPENDIX 3). Thus the ″ATG″ was most likely the translation initiation site of the *gdt1* gene.

The isolated 5'-end sequence was confirmed by PCR with genomic DNA from Ax2 and X series disruptants (Fig. 12). 3 primers were used: primer 166 (indicated in the APPENDIX 3) inside the known 3.7 kb and close to the *Bam*HI site; primer 183 (indicated in the APPENDIX 3) from the new 5' sequence and the Sp6 primer which was contained in the X series transformants from the Psp72 vector but did not exist in Ax2. Two PCR reactions were

performed to amplify the genomic fragments between primers 183 and 166 (Fig. 12, lane 1-4); and between primers 183 and Sp6 (Fig.12, lane 5-8). In the first case, Ax2 and the X series disruptants gave the same band of 350 bp, whereas in the second case only the X series disruptants had a product of 940 bp and nothing was amplified from Ax2. The results confirmed that the isolated upstream sequence was continuous with the previously isolated 3.7 kb fragment and that the X series was constructed by tagged disruption in the *gdt1* gene by homologous recombination.

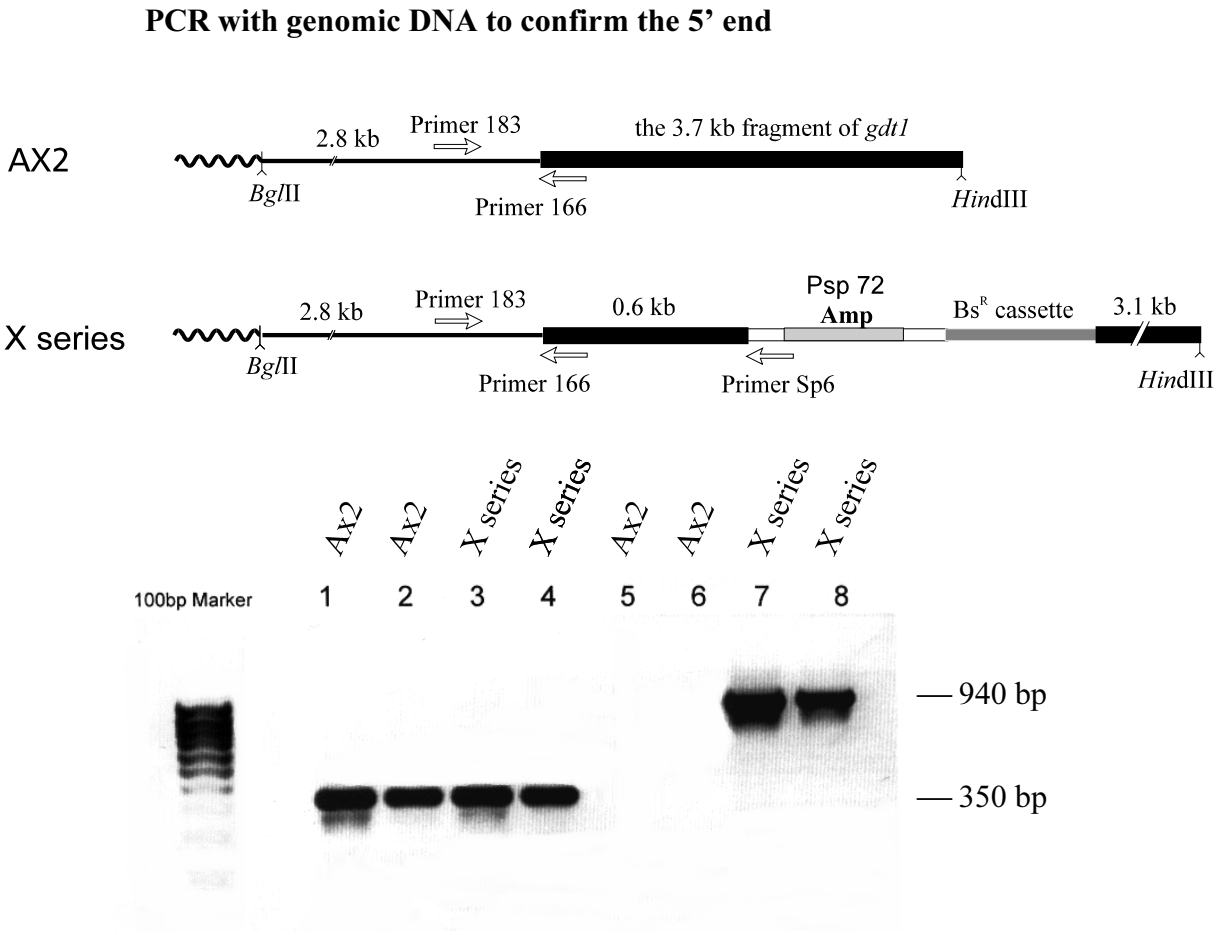


Figure 12. About 20 ng genomic DNA was used as template in the PCR reactions for both Ax2 and X series transformants. The primers are indicated in the *gdt1* sequence in APPENDIX 3. PCR reactions were performed as described (see METHODS 3.14) with primer 183 and 166 (lane 1-4), and primer 183 and Sp6 (lane 5-8). In the former case Ax2 and X series disruptants gave the same band of 350 bp, whereas in the second one only X series disruptants had a product of 940 bp but no amplification was obtained with Ax2.

5.4. Isolation of the 3' end

For isolating the 3' end of the *gdt1* gene, several methods have been tried: cDNA screening, inverse PCR and a new rescue from the disruption close to the *Hind*III end (see Fig. 10, D series). Each method succeeded in obtaining part of the sequence (Table 4) as indicated in Fig. 15a.

Table 4. Sequence obtained for the 3' end of the *gdt1* gene

	Whole sequence isolated	Overlap to the 3.7 kb fragment	Additional sequence of ORF / genomic DNA
cDNA screen	1.8 kb	1.6 kb	180 bp/180 bp
inverse PCR	530 bp	46 bp	340 bp/340 bp
rescue from D series	3.3 kb	0.5 kb	1.2 kb/2.8 kb

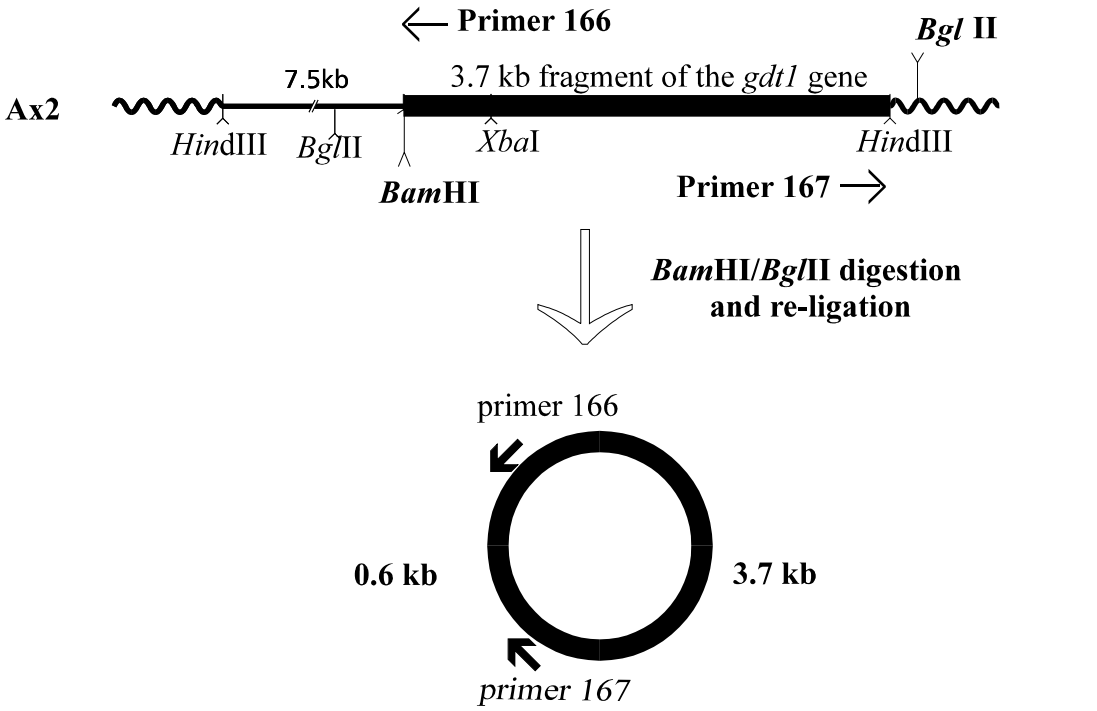
1) cDNA screening

As described in METHODS 3.6, a cDNA library (a gift from H.Freeze's lab) was screened by probing with the isolated 3.7 kb fragment. A 1.8 kb cDNA fragment was obtained after the third round of screening. Sequence analysis showed that this 1.8 kb fragment encodes a continuous ORF which has 1.6 kb overlap with the identified 3.7 kb fragment, and runs 180 bp further downstream. The additional sequence confirmed the ORF but did not contain a stop codon.

2) Inverse PCR

To investigate the genomic structure of the *gdt1* gene, genomic DNA from Ax2 was digested with several restriction enzymes and analyzed in Southern-blots with the 3.7 kb fragment (data not shown). A unique band of 6.7 kb was found for *Bgl*II. The known 3.7 kb fragment contained no *Bgl*II site, this indicated that the 6.7 kb fragment contained 3 kb of genomic sequence in addition to the known 3.7 kb sequence (Fig. 13). Since the 5' end was already completed and contained a *Bgl*II site, a inverse PCR was designed for amplifying up to the second *Bgl*II at the 3' side. Genomic DNA from Ax2 was digested with *Bam*HI/*Bgl*II, and ligated to form a circular structure. With the specific primers 166 and 167 (indicated in APPENDIX 3), a 0.6 kb fragment was amplified by PCR (described in 3.15 METHODS). The

PCR fragment was inserted by T-cloning into the pUC57 vector (see 1.9. in MATERIALS). Sequence analysis showed that this 0.6 kb fragment encoded an ORF which covered sequences from the cDNA, and 200 bp further downstream to the 3' end. A stop codon was not found.



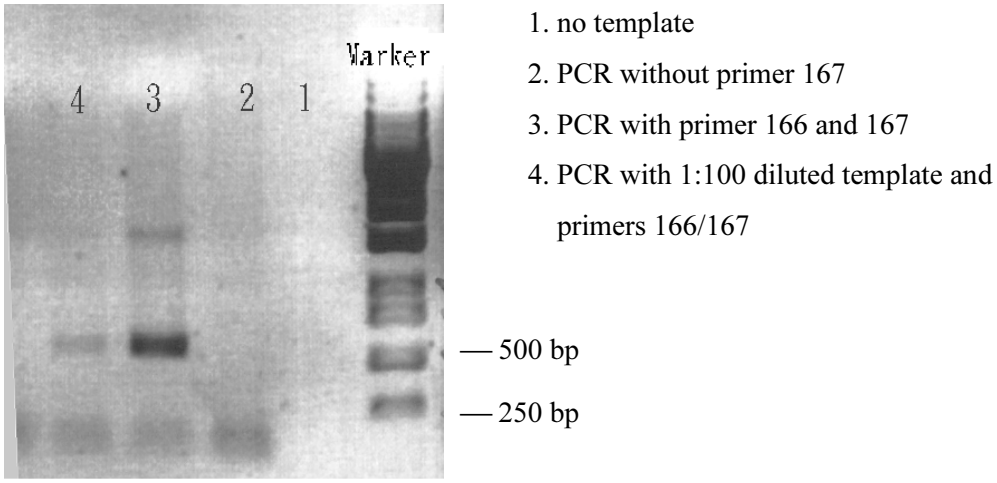


Figure 13. Genomic DNA was digested with *Bam*HI and *Bgl*II and circularized. Primers 166 and 167 were used to specifically amplify a fragment containing 60 bp of known upstream sequence and 470 bp of new 3' sequence.

3) Rescue from the D series gene-disruptant

As shown in Fig. 10, the fifth gene-disruption was generated in the D series mutants. The D series mutants were first identified by their discoidin^{over} phenotype and then confirmed by the isolated rescue sequence. Genomic DNA from the D series mutants was prepared, digested with *Sma*I/*EcoR*V, re-ligated, and transformed into *E.coli*. A plasmid containing an approx. 3.3 kb genomic fragment was obtained and analyzed. Sequencing confirmed that this was from the *gdt1* gene since previously isolated fragments (e.g. from cDNA and inverse PCR) were contained in the 3.3 kb. However, the continuous ORF of the *gdt1* gene stopped at 700 bp after the *Hind*III site, and the size of the ORF was supported by the molecular weight of the gdt1 protein (see section 7).

4) 3'-RACE PCR for amplifying the cDNA ends

A 3'-RACE System kit from Gibco BRL was used for the 3' end amplification (see METHODS 3.16). The first-strand cDNA was synthesized from polyA-mRNA by using primer AP (anchor primer for polyA ends, with 13 mer dT/ Gibco) and the target cDNA was amplified by using primer 167 (see APPENDIX 3) and primer UAP (an universal anchor for the AP primer, with 13 mer dA/ Gibco). A approx. 0.7 kb fragment was amplified with the specific primer 167 and primer UAP, but it was not obtained with only the UAP primer (Fig. 14a). Since the primer 167 is close to the *Hind*III site at 3929 bp, this would suggest that the coding sequence of the *gdt1* gene stops at approx. 0.7 kb after the *Hind*III site. However, the data did not fit with the mRNA size of the *gdt1* gene (approx. 7 kb, see Fig. 17). Thus the 0.7 kb band was most likely amplified from the endogenous polyA sequence just after the stop codon (AAAAAATAAAA, see APPENDIX 3).

lane 1-3 with primers 167/UAP

1. 1 µl first-strand cDNA
2. 2 µl first-strand cDNA
3. 5 µl first-strand cDNA

lane 4-6 with primer UAP only

4. 1 µl first-strand cDNA
5. 2 µl first-strand cDNA
6. 5 µl first-strand cDNA

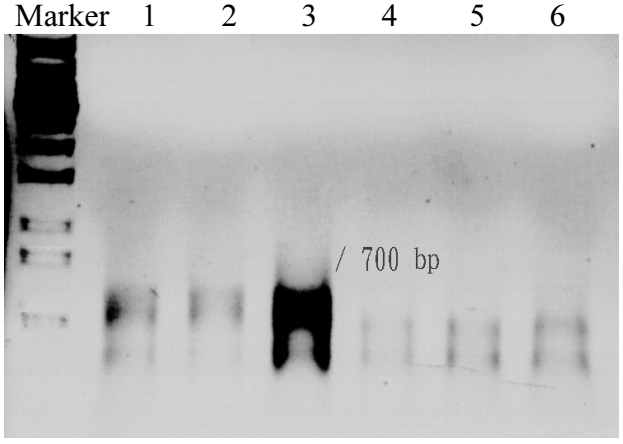


Figure 14a. **3'-RACE PCR for amplification of the cDNA ends**

To improve the specificity to the real 3' polyA ends, an amplification was performed by using a 26 mer polyA primer instead of the primer UAP. Since the 26 mer polyA was much longer, amplification using this and the specific primer 167 should provide a better chance to obtain the real 3' polyA end. PCR amplification was performed with an annealing temperature of 40°C and 1 µl first-strand cDNA (same as in Fig. 14a). As shown in Fig. 14b, a specific band of 2.5 kb was amplified for the *gdt1* gene (lane 3), no amplification was obtained by using the 26 mer polyA primer alone (lane 1), and a specific amplification at the expected size of 700 bp was obtained for the positive control *Erkb* gene (lane 2). This suggested that the polyA end of *gdt1* mRNA was located approx. 2.5 kb after the *Hind*III site, and that the whole mRNA size is around 6.6 kb (containing 3929 bp coding region before *Hind*III, 2.5 kb downstream sequence until the 3' polyA ends, and plus approx. 100 bp polyA signal and approx. 100 bp of the 5' non-coding sequence). The data suggests that the polyA signal at 6431 bp (indicated in APPENDIX 3) within the 3'-untranslated region of the *gdt1* gene was the real polyA signal for the *gdt1* mRNA.

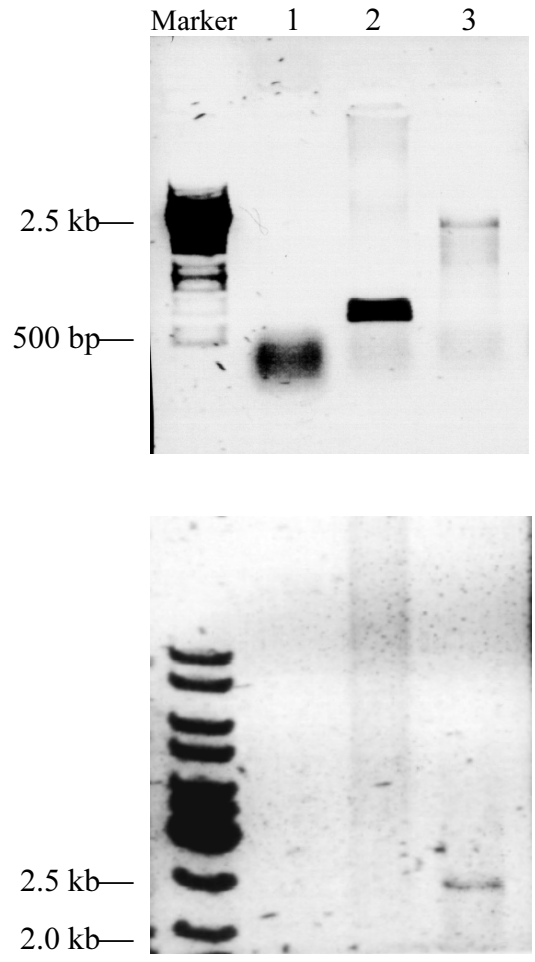


Figure 14b. PCR amplification was performed to obtain the 3' polyA end for *gdt1* (lane 3), negative control (with only the polyA primer, lane 1) and a positive control to amplify a 700 bp fragment of the *Erkb* gene with two internal primers (lane 2). 10 µl of the PCR products were separated on a 0.8% agarose gel by electrophoresis. The result was observed after short run (upper gel) and long run (lower gel). The 1 kb DNA ladder (from MBI) was used to identify the size of the amplified bands. The band in lane 1 represented small heterogeneous non-specific products.

6. The *gdt1* gene and its product

6.1. Sequence

Over all, almost 12 kb of the *gdt1* gene locus have been isolated (see APPENDIX 1). These include 4683 bp of the *gdt1* coding region, 1895 bp of 3' flanking and 113 bp of 5' flanking region (EMBL database, accession number AJ000992). 5' of the *gdt1* gene, in a distance of approximately 2.8 kb sequence, an ORF encoding a putative cationic amino acid transporter was detected in the opposite direction (EMBL database, submitted). Still further upstream there was a 1114 bp ORF encoding the 3' end of a putative glycoprotein with similarity to *csA* (EMBL database, submitted). However, the sequence was not completed, about 2.5 kb of the upstream sequence before the initial ″ATG″ site of the *gdt1* gene is still undetermined.

The sequence of the *gdt1* gene was compiled by various approaches. Figure 15a. provides a map of the genomic locus and indicates the origin of the different subclones. There are no introns in the coding region of the *gdt1* gene, and the 4683 bp contains a single open reading frame. This was confirmed by the gdt1 disruptants: REMI 2-9 mutant, L8, K series, X series and D series (see Fig. 10). All mutants were constructed by disruption at different sites inside the *gdt1* coding region but displayed the same phenotype. This strongly suggested that all mutants were functionally complete knock-outs even though some may express a partial mRNA (see Fig. 17b, a truncated 1.2 kb transcript was observed in L8).

The *gdt1* gene product has a predicted molecule weight of 175 kDa. The amino acid sequence of gdt1 was given in Fig. 15b, with important domains (i.e., the kinase domain, the two putative phosphorylation sites for PKA, one putative nuclear localization signal and the four putative transmembrane domains) indicated.

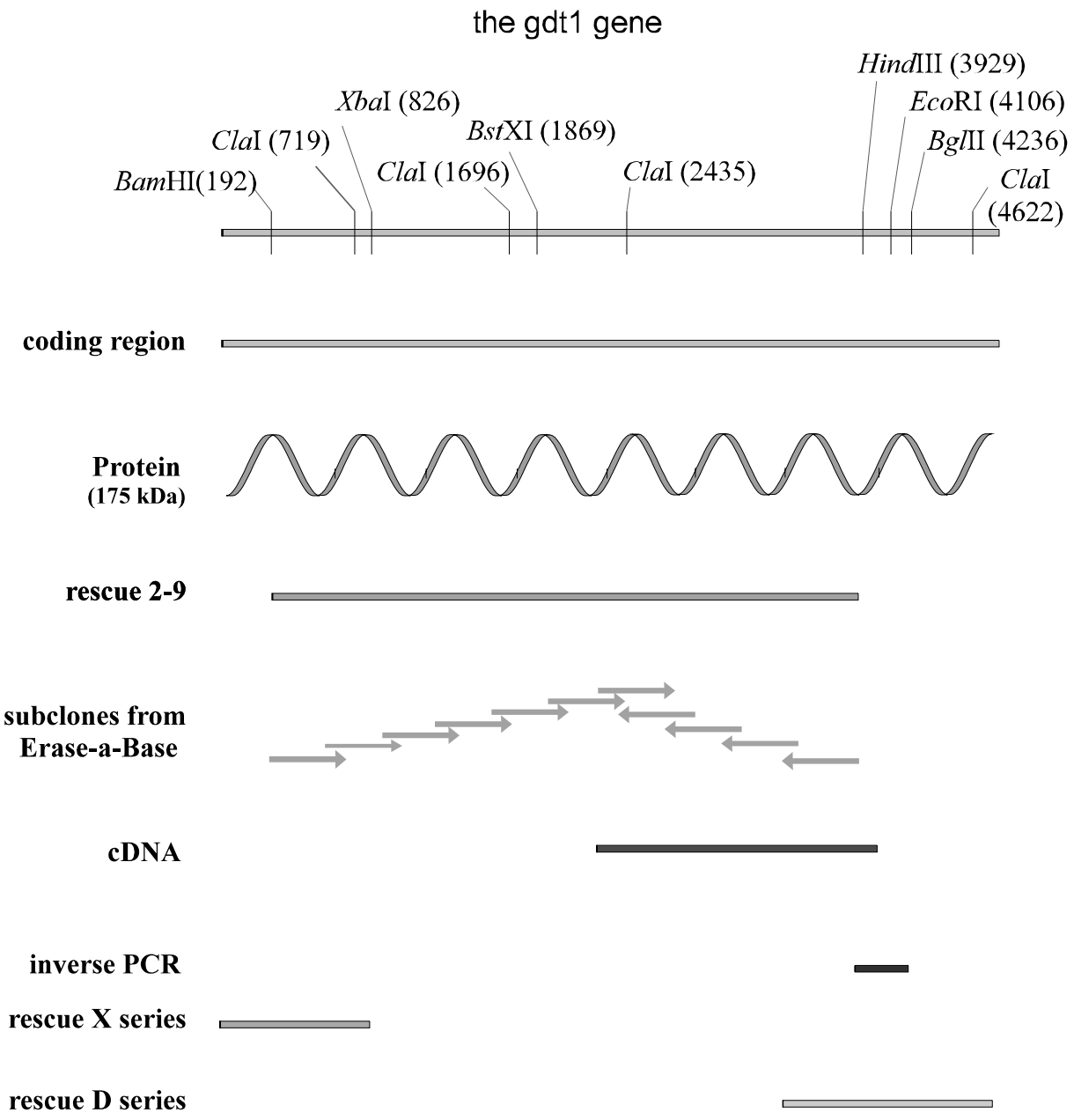


Fig. 15a. Map of the *gdt1* gene indicating the origin of the different subclones. The *gdt1* gene contains a continuous open reading frame of 4683 bp (see APPENDIX 3, from the start codon ATG at 114 bp to the stop codon TAA at 4797 bp) and encodes a protein consisting of 1561 amino acids. The sequence of the *gdt1* gene was completed by different approaches. First by REMI rescue (see section 5.1), 3.7 kb sequence were obtained and subcloned by Erase-a-Base (METHODS 3.10). Then the whole sequence was completed and overlapped by cDNA (1.8 kb), inverse PCR (0.6 kb), rescue from the X series (0.8 kb, see section 5.3) and the D series (1.2 kb, section 5.4) mutants.

1 MKYRVGNGGIPIYKINVLCVEGTLEIPE**GISFFNVGALFILPGGVL**NSKSSIRFTDLDPY
61 NSKMD**PFNFFPGMMVLGGSLSLIG**EKKRIFQATRIDDYQLQIEDFKKIGSLTNNIYLGSK
121 VTIYSQQISEGQTCSFSFGASNDKINLTSSSSSSLRGTNCLPISKNDKNIIVHFNCKYLL
181 GGSSESFIDSTSTVGSSIYITGDSQVQIENFTLDSIGKTTNKLYNDTKLIFSNDKPNQVI
241 DIIKGENQRFRNSLYIEFSNSVVIKGCAIIDRVKESRAPLIFVSSNVSLSESLIVSKSGS
301 NLIAQYGTEFIKSKLNHYFLIPPLPQPFGLNSPINFPSPFSMDYGFEGNGIYSLSPN**VQS**
361 **INDTFISQLIALNFNFIG**NRSIITGFDNDCYSPCTNTSILFSNLIQYPVDFKINNSTYFY
421 KLNNNNNDNNNNTNNFNLLNINNENNNNDDNNNNNNSQNHYLLNINNNGNSPGRFFTIKD
481 LVASDTVSAYLPNSALVFNNLNATENFSFNGTVSRLDFINSSFNTNLTSTQKFFDETTTT
541 VTNFQNTYIYGSPETTEPPEKVFGSSITPIYYFSKSVLDSLKVETVYPNEPHKMFFNSSV
601 SLSVKLNNMGLKTPIICISSSNGMESKEVQYNPISTKCSFLYVSEKLGNHNIRVTIKNEY
661 SKDSSYYFIIDFPIITVYGQSLFNAGWQMIDPTSTTIIPTPTPTSTSTSTSTSTPTTTNN
721 NQLVNDNNNKINELDGLKFQGGCIKTLGCQLSSNAKYVGGLPNVTASSTLNSLFSWGITS
781 DVQYDPVIIDLFINKSIATLQVQLFFTFYKPIDQYSSPLSVSIQKNPVLVMEPFAGDQPF
841 SKNLTFVYNNTQSLDFLNISFTSRGDIYLTSLAIFSVSDSLPQIIDPITPTLLPIESVKA
901 SK**PAILAIVLSIVLGSLALSIITILIV**KHRKRLSQFLSKSNKDIEYAQNNEIEIKVLPKI
961 TSHSSYPSISILDTISSDSIFNNQIPKNNNRYKFKNQSLNNNNYFNNNNNNNNSNNNNSN
1021 NIIYSNCNSNYSNSNSNNNNNNNNSNSNNNSNSNSNININSNSNSNSNSNGNNNYQIY
1081 SNKLESFKIDEISNDTIPIINSTFPDEFQTLEFQKLAFEILKREKRLDFSFRTTNDILTC
1141 CRHYQILKIFSNFPLRFNQSIITFGLINGKAKLGETYYDTLSITNDSTIRFTAFLILPMD
1201 NHSATFTSDHSSFDLGPGETFSIKFSITLHCTTRFFENFSIQINSNNIKEMYTLLKIKVE
1261 SESSTRLDFNDIHFQELIEKYSWEILYRGTVGDKNALLKLIKLKTKNCEEAYRELNIISR
1321 LKHQNILPLIGCVISKDYLCLAFEYPPLGSLDYIISKKKLKMSITQKIRILIDVAKGCKF
1381 LQQSSIIQKTLRARNIFLYDTNENAEVCAKVLDLTSSKTIKGLACNNYIERVDTPINLTR
1441 EISIIRDPKQNNDFNNSNNSNNNNNNNNNNNNNNNNSNNSNNSSSLKYNNHSFAVLSYE
1501 LLIDEILVGDTRKFGQEKPSIGLDKIDPNIKNFIHKCWNPIDGFTFNEILKTLKDFIESL
1561 N*

Figure 15b. Amino acid sequence of the *gdt1* gene product (the kinase domain is underlined; the two PKA sites are indicated by double underlining; the 4 transmembrane domains are shown in bold letters; the putative nuclear localisation signal "KHRKR" is part of the second PKA site; and the expressed D1 and D2 peptides are shown by dotted underlining). Several bio-informatic programs have been used to analyze the protein feature of gdt1. This protein is composed of 1561 amino acid residues, has a pI of 7.15, a calculated MW of 175292.79 Da, and contains 4 putative transmembrane domains in the direction of N-i-O-i-O-i-C (analyzed by EMBL TMpred program, see DISCUSSION).

6.2. gdt1 is an unusual receptor tyrosine kinase

The amino acid sequence of gdt1 was submitted to different databases for analysis. It was adjusted to the receptor protein kinase family by HMM consensus research (see http://genome.wustl.edu/Pfam/cgi-bin/hmm_align.cgi). Further analysis revealed that, gdt1 is most likely a tyrosine receptor kinase with unusual characters inside its kinase domain (see DISCUSSION). However, a serine/threonine kinase from *Entamoebae histolytica* (ENHPSTK_1) was chosen as the best homologue for gdt1 from the FASTA program. ENHPSTK_1 is a small cytoplasmic protein of 290 aa and belongs to the mos protein kinase family (Lohia and Samuelson, 1994). Since the gdt1 protein is a membrane-associated protein, it is clearly not a mos kinase homologue. The high homology rather comes from an unexpected conservation of the first 30 aa which are outside the conserved kinase domain (Fig. 16a).

Usually the core of a protein kinase domain consists about 270 aa and is characterized by 11 defined subdomains (see DISCUSSION). An additional sequence was found in gdt1, though in a not very conserved subdomain IX and severely disturbed the homology search. This sequence, ″NNSNNSNNNNNNNNNNNNNNNNNNNSNNSNNSSS″, is a typical asparagine-rich sequence found in many *Dictyostelium* proteins. During homology search this part of the sequence was always adjusted to the polyN region of various *Dictyostelium* proteins (but also to asparagine-rich proteins from other organisms) and gave non-specific alignments. Thus a new search with the FASTA program was performed without this polyN sequence (indicated as * in Fig. 16b), and homology was obtained along the entire kinase domain of gdt1. Both tyrosine and serine/threonine kinase homologues were found, among them was a *Drosophila melanogaster* neurotrophic receptor (receptor tyrosine kinase RTRK, Wilson *et al.*, 1993); murine Fer tyrosine kinase (MMU76762, Letwin and Pawson, unpublished data); rat TESK1B, a novel serine/threonine kinase in germ cells (Toshima *et al.*, 1995); mouse ECK, a receptor tyrosine kinase implicated in pattern formation (MMECK, Ganju *et al.*, 1994) and chicken eph-related tyrosine kinase (CEK4, Sajjadi *et al.*, 1991), etc. In *Dictyostelium discoideum*, so far, no receptor tyrosine kinase has been identified. However PYK2A, a non-receptor tyrosine kinase cloned from *Dictyostelium* (Tan and Spudich, 1990), was found to be a gdt1 homologue with the FASTA program. Thus this protein was also included in the homology analysis. Multiple alignments were performed between the kinase region of gdt1, PYK2A, RTRK, MMU76762, TESK1B, MMECK and CEK4 (Fig. 16b).

```
 ENHPSTK_1 protein serine/threonine kinase [Entamoebae (290 aa)

 initn:  50 init1:  50 opt: 300 Z-score: 369.7 expect() 2.3e-13

Smith-Waterman score: 300;  22.759% identity in 290 aa overlap


            1250      1260      1270      1280      1290
_gdt1  MYTLLKIKVESESSTRLDFNDIHFQELIEKYSWEILYRGTVGDKNALLKLIKLKTKNCE-
       :   . :..:.:..  :: ...  .. : . .. ..:.:    ... .: .: : ..
ENHPST MEFYVDINIETEQTCILDPDELIQKKKIGEGTFGVVYKGEFKGNSVAIKRMKPKINDNSS
              10        20        30        40        50        60

      1300      1310      1320      1330      1340       1350
_gdt1  --EAYRELNIISRLKHQNILPLIGCVISKDYLCLAFEYPPLGSLDYII-SKKKLKMSITQ
         :  .:.... ... . :. . : :: .:  :.. ::   ::.. .: :: . ..: .
ENHPST EIEFRKEVEMLEKFRCNYIIHFYGAVIIQDNKCMVTEYAKYGSVQKMIESKPSNSLSKSI
              70        80        90       100       110       120

       1360      1370      1380      1390      1400      1410
_gdt1  KIRILIDVAKGCKFLQQSSIIQKTLRARNIFLYDTNENAEVCAKVLDLTSSKTIKGLACN
       ::..:.:.:.: ..:...:... ..  :... . ...  : ::. :. :...:..:  :
ENHPST KIKMLLDIARGIEYLHNNGILHRDIKPDNMLITSLDNDIPVNAKLTDFGSARNINSLMTN
              130       140       150       160       170       180

       1420      1430      1440 (-35aa) 1490      1500          1510
_gdt1  -NYIERVDTPINLTREISIIRDPKQNNDFLKYNNHSFAVLSYELLIDEI----LVGDTRK
        .. . : ::  .. ::   .  :   :. ..   :... ..:  . :.    ...
ENHPST MTFTKGVGTPSFMAPEILKRKKYKTAADIYSFAI-SITLYDWETYLTEFEYPWVIATFVA
              190       200       210        220       230

           1520       1530       1540      1550
_gdt1  FGQEKPSIGLDKIDPN-IKNFIHKCW--NPIDGFTFNEILKTLKDFIESLN
        :...:. .:    :. . ..: .::  .: . ..... .: :. . .:..
ENHPST SGHRRPQNNL----PDYVYKLICECWCDEPTNRLNIEQTIKQLEIIQKSIQSKHH
     240           250       260       270       280       290
```

Figure 16a. Best alignments for the gdt1 protein with the FASTA programme. ENHPSTK-1 showed 22.8% identity to gdt1 in the kinase domain. The probability for fortuitous alignment was 2.3e-13. Only the kinase domain of gdt1 with deletion of the polyN stretch is shown.

```
                  10         20         30         40         50         60
                   |          |          |          |          |          |
MMU76762    DVS:LGELLGKGNFGEVYKGTLK-----D-K:TPVAIKTCKEDLPQ:ELKIKFLQEAKILKQY:DHPNIVKL
MMECK       CVA:RQKVIGAGEFGEVYKGTLKASSGKK-E:IPVAIKTLKAGYTE:KQRVDFLSEASIMGQF:SHHNIIPL
CEK4        NIS:IDKVVGAGEFGEVCSGRLKLPS-KK-E:ISVAIKTLKAGYTE:KQRRDFLGEASIMGQF:DHPNIIRL
TESK1B      DFD:CAEKIGAGFFSEVYKVRHR----QSGQ:VMV-LKMNKLPSN-:--RSNTLREVQLMNRL:RHPNILRF
PYK2A       DIQ:FIQKVGEGAFSEVWEGW-W----KGIH:VAI-KKLKIIGDEE:QFKERFIREVQNLKKG:NHQNIVMF
RTRK        DVE:FLEELGEGAFGKVYKGQL-LQPNKT-T:ITVAIKALKENASV:KTQQDFKREIELISDL:KHQNIVCI
GDT1        DIH:FQELIEKYSWEILYRGTV-------GD:KNALLKLIKL--KT:KNCEEAYRELNIISRL:KHQNILPL
Subdomain     :            I            :      II      :      III       :  IV

                 70         80         90        100        110        120        130
                  |          |          |          |          |          |          |
MMU76762    IGVCTQR:QPVYIIMELVPGGDFLTFLRKRKDE-----:LKLKQLVRFSLDVAAGMLYLESK:NCIHRDLA
MMECK       EGVVSKY:KPMMIITEYMENGALDKFLREKDGE-----:FSVLQLVGMLRGIASGMKYLANM:NYVHRDLA
CEK4        EGVVTKS:KPVMIVTEYMENGSLDSFLRKHDAQ-----:FTVIQLVGMLRGIASGMKYLSDM:GYVHRDLA
TESK1B      MGVCVHQ:GQLHALTEYMNGGTLEQLLSSPEP------:LSWPVRLHLALDIAQGLRYLHAK:GVFHRDLT
PYK2A       IGACYKP:ACI--ITEYMAGGSLYNILHNPNSSTPKVK:YSFPLVLKMATDMALGLLHLHSI:TIVHRDLT
RTRK        LGVVLNK:EPYCMLFEYMANGDLHEFLISNSPTEGK-S:LSQLEFLQIALQISEGMQYLSAH:HYVHRDLA
GDT1        IGCVISK:DYLCLAFEYPPLGSLDYIISKK-----KLK:MSITQKIRILIDVAKGCKFLQQS:SIIQKTLR
Subdomain     IV   :             V                :       VIA             :   VIB

                140        150        160        170        180        190        200
                  |          |          |          |          |          |          |
MMU76762    ARNCLV--:-GE--NNTLKISDFGMSR---QE:DGGVYSSSGLKQIPIKWTAPEALNYGR-YSSESDVWS
MMECK       ARNILV--:-NS--NLVCKVSDFGLSRVLEDD:PEATYTTSGGK-IPIRWTAPEAISYRK-FTSASDVWS
CEK4        ARNILI--:-NS--NLVCKVSDFGLSRVLEDD:PEAAYTTRGGK-IPIRWTSPEAIAYRK-FTSASDAWS
TESK1B      SKNCLV--:RREDGGFTAVVGDFGLAEKIPVY:REGARKEPLAVVGSPYWMAPEVLRG-ELYDEKADVFA
PYK2A       SQNILL--:--D-ELGNIKISDFGLSAEKS--:REGSMTMTNGGICNPRWRPPELTKNLGHYSEKVDVYC
RTRK        ARNCLV--:-NE--GLVVKISDFGLSR--DIY:SSDYYRVQSKSLLPVRWMPSESILYGK-FTTESDVWS
GDT1        ARNIFLYD:TNENAEVCAKVLDLTSSKTIKGL:ACNNY--IERVDTPINLTRREIS:II-RDPKQNNDFHS
Subdomain     VIB   :          VII             :          VIII          :    IX      *

                  210        220        230        240        250        260        270
                   |          |          |          |          |          |          |
MMU76762    FGILLWETFSLG:VCPYPGMTNQQAREQVE---RGYRMSAPQN:CPEEVFTIMMKCWDYKPENRPKFNDL
MMECK       YGIVMWEVMTYG:ERPYWELSNHEVMKAIN---DGFRLPTPMD:CPSAIYQLMMQCWQQERSRRPKFADI
CEK4        YGIVLWEVMSYG:ERPYWEMSFQDVIKAVD---EGYRLPPPMD:CPAALYQLMLDCWQKDRNNRPKFEQI
TESK1B      FGIVLCE-LI-A:RVP-ADPDYLPRTEDFGLDVPAFRTLVGND:CPLPFLLLAIHCCSMEPSARAPFTEI
PYK2A       FSLVVWEILT-G:EIPFSDLDGSQRSAQ--VAYAGLRPPIPEY:CDPELKLLLTQCWEADPNDRPPFTYI
RTRK        FGVVLWEIYSYG:MQPYYGFSNQEVINLIR---SRQLLSAPEN:CPTAVYSLMIECWHEQSVKRPTFTDI
GDT1        FAVLSYELLIDE:ILVGDTRKFGQEKPSIGLD----------K:IDPNIKNFIHKCW--NPIDGFTFNEI
Subdomain       IX      :              X              :             XI

                  280
                   |
MMU76762    HKEL:TVIKKMIT
MMECK       VSIL:DKLIRAPD
CEK4        VSIL:DKLIRNPS
TESK1B      TQHL:EQILEQLP
PYK2A       VNKL:KEISWNNP
RTRK        SNRL:KTWHEGHF
GDT1        LKTL:KDFIESLN
Subdomain   XI  :
```

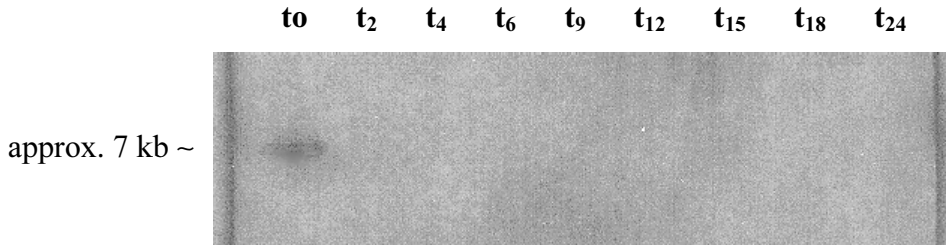
Figure 16b. Multiple alignments for the *gdt1* kinase domain with other protein kinases: a *Drosophila melanogaster* neurotrophic receptor tyrosine kinase RTRK (Wilson *et al.*, 1993), a murine Fer tyrosine kinase MMU76762 (Letwin and Pawson, unpublished data), a rat serine/threonine kinase TESK1B (Toshima *et al.*, 1995), a mouse receptor tyrosine kinase MMECK (Ganju *et al.*, 1994), a chicken eph-related tyrosine kinase CEK4 (Sajjadi *et al.*, 1991) and a *Dictyostelium* non-receptor tyrosine kinase PYK2A (Tan and Spudich, 1990). The polyN-stretch in gdt1 at the subdomain IX was deleted for better alignment. Identified amino acids are given in bold face, conservative exchanges in semi-bold. Subdomains of the kinase are indicated in the shaded area below the sequence and separated by colons.

7. Expression of *gdt1*

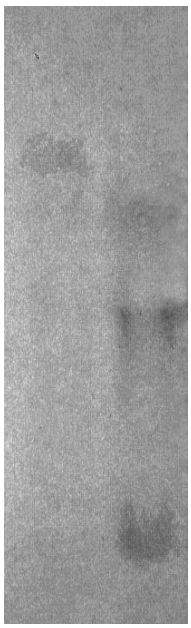
7.1. Transcription

The *gdt1* gene is very weakly expressed in *Dictyostelium,* in Northern-blots the signal was just above the detection level. The *gdt1* gene was transcribed to an approximately 7 kb mRNA during growth, and the expression was repressed when cells were set up for development. Figure 17a shows a Northern-blot during a developmental time-course of Ax2. A 7 kb band was only detected at t_0, when cells were just harvested from growth at the density of $1x10^6$ cells/ml. The same experiment was repeated with t_0 cells from the L8 mutant. As shown in Fig. 17b, the 7 kb mRNA was not expressed in L8 at t_0, but a smaller band at ca. 1.2 kb was detected, showing that in L8 a truncated mRNA of the *gdt1* gene was transcribed. As shown in Fig. 10, the insertion of the Bs^R cassette in L8 is at the *Xba*I site of *gdt1*, that is 826 bp after the start codon. Thus in the L8 mutant, transcription of *gdt1* goes into the act8 terminator of the Bs^R cassette and probably stops at a polyA signal shortly (159 bp) after the insertion site (data not shown). This resulted in an open reading frame of 984 bp. In addition, the polyA tail is usually around 100 bp in *Dictyostelium*, thus the start of the transcription for *gdt1* was predicted to be around 100 bp upstream of the start codon ATG. However, though 0.3 kb of the upstream genomic DNA was sequenced, the 5' end of the mRNA has not yet been determined.

Figure 17. Transcription of the *gdt1* gene



a. Ax2 cells were harvested from axenic growth at $2x10^6$, washed in phosphate buffer, then used for filter development. Cells were taken at the indicated time points of development and total RNA was prepared as described in METHODS 3.4. An equal amount of 25 μg total RNA was used for each lane, separated on a gel, blotted, and hybridised with the 3.7 kb *gdt1* gene probe. The blot was exposed for 4 days on a phosphor-imager plate.



b. Growing Ax2 and L8 cells were harvested from bacteria suspension at $1x10^6$ and washed free of bacteria by successive centrifugation. Total RNA was prepared as described. An equal amount of 25 µg total RNA was used for both Ax2 and L8, separated on a gel, blotted, and hybridized with the 3.7 kb *gdt1* gene probe. The blot was exposed to Kodak film for two weeks at -80°C. An approx. 7 kb band was detected in Ax2, but not in L8. Two bands at approx. 6 kb and 4.7 kb (corresponding to the position of the 26 S rRNA) most likely resulted from inefficient termination within the actin8 terminator. The predominant band at 1.2 kb indicated a truncated transcript of the *gdt1* gene (see text).

7.2. Generation of polyclonal antisera against gdt1

As described in METHODS 4.5, two different parts of the gdt1 protein were expressed as recombinant peptides for generating polyclonal antisera against gdt1.

7.2.1. Expression of Domain 1 and Domain 2 of gdt1 protein as 6xHis-tagged protein

Domain 1 (the part between the 2nd and the 3rd transmembrane domain) and Domain 2 (from the 4th transmembrane domain on) (see Fig. 15b) were expressed as His-tagged proteins as described. The expression vectors D1-pET15b and D2-pET15b were constructed by cloning the coding sequences for D1 and D2 into the pET15b vector (Novagen). The vectors D1-pET15b and D2-pET15b were transformed into BL21 cells (Novagen) and the transformants were selected on LB-Amp plates overnight. Recombinant proteins were induced in large-scale culture by adding 0.3 mM IPTG, extracted from the cells under denaturing conditions, and purified by Ni^{2+} affinity column (Pharmacia) with FPLC (Biologic system, Biorad). Since the stability and the effect on the growth of bacteria differs greatly for each protein, various conditions were tried for obtaining the maximum level of expression. Both recombinant D1

and D2 proteins (around the same size of 36 kDa) were purified under denaturing conditions on a Ni^{2+} column. However, the two proteins behaved very differently. D1 was induced with 4 hrs of IPTG treatment (Fig. 18a) but only weakly expressed with longer induction (Fig. 18b). The expression of D2 was not obtained with short IPTG treatment (data not shown) but was strongly induced after 16 hrs. This would argue for a weak stability for the D1 protein and a poor synthesis rate for D2. Since the proteins were eluted in 8M Urea, they were dialysed before using for the PKA assay (see METHODS 4.15). During dialysis, a strong precipitation was observed with D1, but not with D2, which remained soluble.

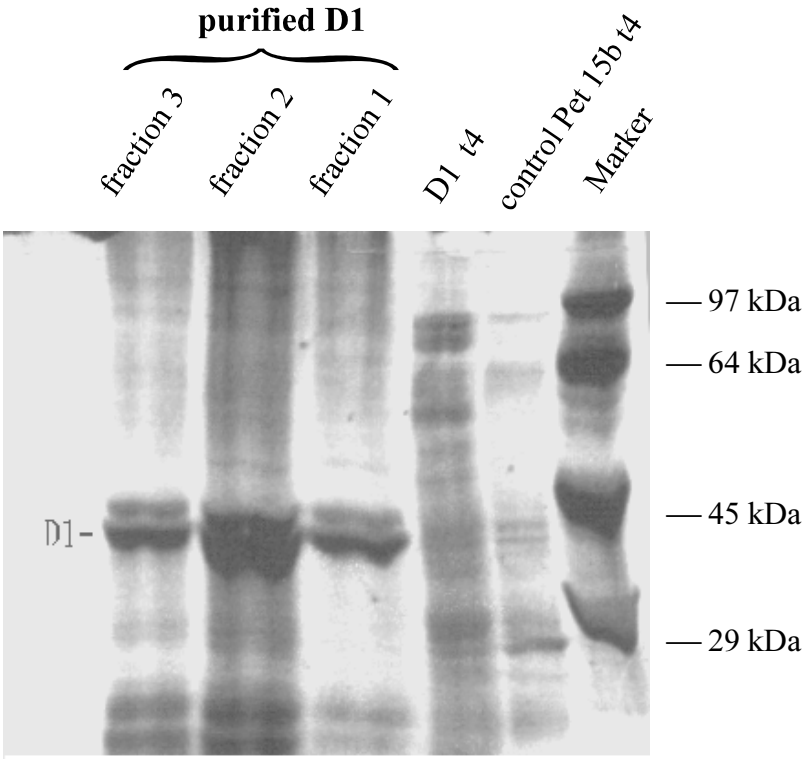


Figure 18a. The expression vector D1-pET15b was transformed into the BL21 strain and the recombinant His-tagged-D1 protein was induced by 4 hrs IPTG (0.3mM) treatment. The 36 kDa expressed D1 protein was purified under denaturing conditions as described (METHODS 4.6) by using the His-Trap™ kit (Pharmacia). Samples were taken from different fractions of the elution, an equal volume of each sample was separated on 12% SDS-PAGE gel and checked by Coomassie staining. Before purification, an aliquot of the sample of total protein extraction from t4 (4 hrs IPTG induction) was taken for D1-pET15b and the control pET15b vector transformants. Though there was no visible expression of D1 observed in the total protein fraction, the 36 kDa recombinant protein was purified at high level. Over 2 mg D1 protein were obtained from a 1 L large-scale culture. As for many recombinant proteins, two bands were obtained for the D1 peptide. Most likely, the smaller one resulted from incomplete translation of the transgene.

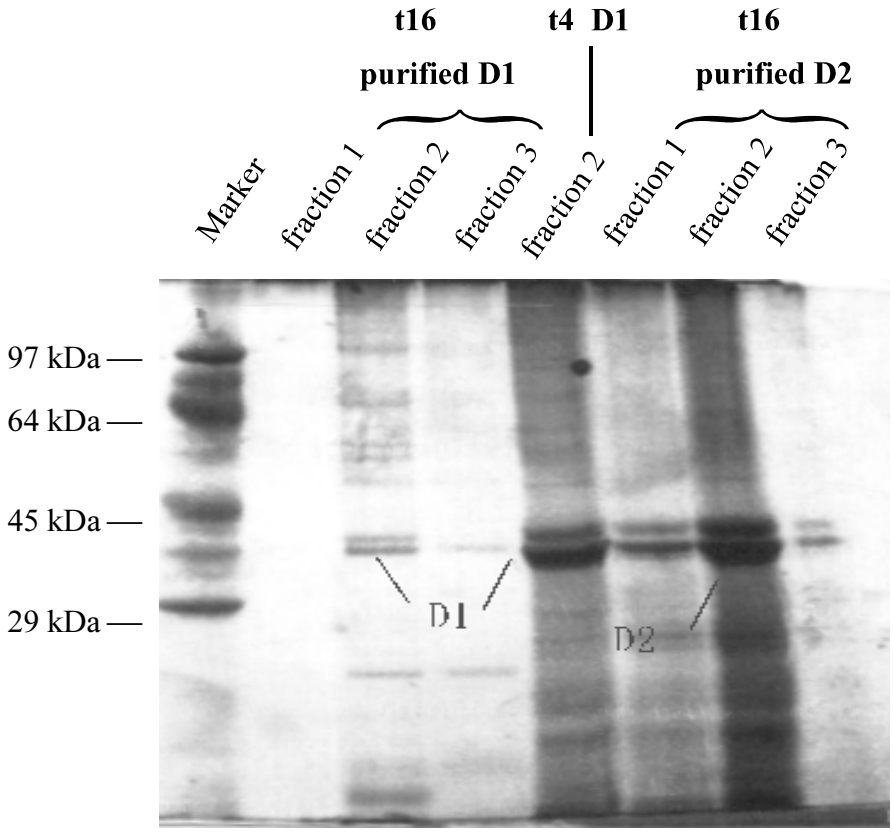


Figure 18b. D2 was cloned into the pET15b vector, expressed as His-tag protein and purified with Ni^{2+} column under the same conditions as D1. Though D2 was around the same size of 36 kDa as D1, it differed greatly from the D1 protein. For D1, induction with 4 hrs of IPTG (0.3 mM) treatment (lane t4 D1) was the strongest, while for D2 16 hrs induction were required. An equal volume of each fraction was analyzed by 12% SDS-PAGE and Coomassie staining.

7.2.2. Generation of the polyclonal antiserum for D1

The purified recombinant D1 and D2 protein were separated on a preparative SDS-PAGE and stained with Coomassie-blue. After destaining, the band of the recombinant proteins were cut out from the gel and prepared for immunization (see METHODS 4.16). Both bands of the doublets (Fig. 18a and 18b) were used. Polyclonal antiserum were generated against D1 and D2. Aliquots of the sera were used for affinity purification (METHODS 4.18) or purification with protein A-sepharose (METHODS 4.19). A specific antiserum was obtained for D1, but not for D2 (data not shown). However, after purification the activity of the D1 antiserum was reduced by a factor of 500 for affinity purification, and a factor of 200 for the proteinA-sepharose purification. In both case the final purified antibody was eluted with a low pH buffer (pH 2.5 acetate or pH 2.3 glycine), it was thus possible that the antibody was unstable at low pH. The non-purified antiserum directed against the D1 peptide was used for Western-

blots and it specifically recognized the recombinant D1 protein but did not recognize the recombinant D2 protein (Fig. 19).

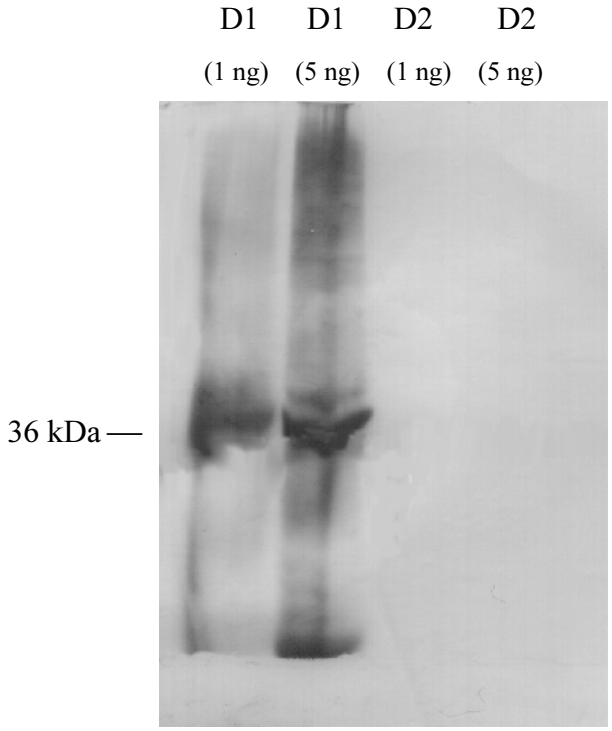


Figure 19. Polyclonal antiserum directed against D1 was generated as described. The antiserum was diluted by 1:10,000 for detection of recombinant D1 and D2 protein (both are 36 kDa) on a Western-blot. Only recombinant D1 was detected by the antibody, but no cross-reaction was observed with recombinant D2.

7.3. gdt1 is a 175 kDa membrane-associated protein

Several bio-informatic programs have been used for the analysis of the protein structure of gdt1. The 175 kDa protein is composed of 1561 amino acids and was predicted to be a membrane protein with 4 transmembrane domains (see Fig. 32, DISCUSSION). To determine the cellular localisation of the gdt1 protein, growing cells were fractionated into membrane and cytoplasm (METHODS 4.3), and analyzed by Western-blots with the polyclonal antiserum directed against the recombinant D1 peptide. No expression of gdt1 was detected in all cytoplasmic fractions (data not shown), but in the membrane fractions from Ax2 cells a band around 175 kDa was detected (Fig. 20). As this band was not present in L8, it can only be the specific signal from the gdt1 protein. A potential truncated translation product corresponding to the 1.2 kb mRNA (Fig. 17b) was not detected in L8 cells.

Nuclear proteins were also extracted from growing cells (METHODS 4.4) and examined for the expression of gdt1. Western-blot analysis was performed with the same antibody and no expression was detected (data not shown). This further confirms that the gdt1 protein is located on the cell surface. Further more, the cell-substratum attachment assays revealed a

strong difference between the L8 mutant and Ax2 (see Fig. 23 and Fig. 24), suggesting a function for the gdt1 protein as a cell-surface protein involved in cell adhesion. The antiserum was also used to detect the gdt1 protein in strain VI88, a mutant with a similar phenotype to the 2-9 mutant and initially supposed to carry a mutation in the *gdt1* gene (see DISCUSSION). However, as can be seen in Fig. 21, VI88 shows the same band of 175 kDa as Ax2.

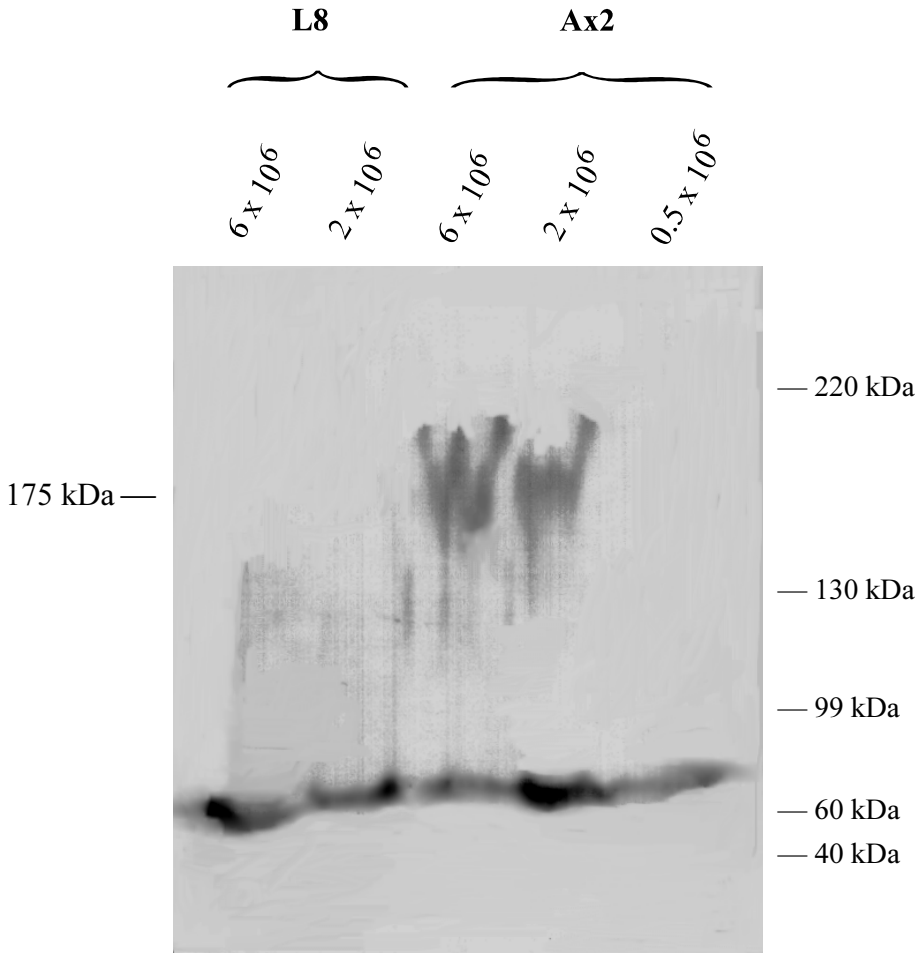


Figure 20. Ax2 and L8 cells were grown in bacteria suspension and harvested at the indicated cell densities. Cells were fractionated into cytoplasm and membrane proteins (METHODS 4.3), separated in 5-12% gradient SDS-PAGE, blotted overnight with "Tank transfer" and incubated with the anti-D1 polyclonal antiserum. The 175 kDa gdt1 protein was only detected in Ax2 growing cells at densities of $2x10^6$ and $6x10^6$, but not at the very low cell density of $0.5x10^6$. Smearing of the bands was due to high amounts of Triton X-114 in the membrane preparation. Even though a direct comparison with Fig. 21 is not possible, gdt1 protein seems to be strongly enriched in the membrane fraction.

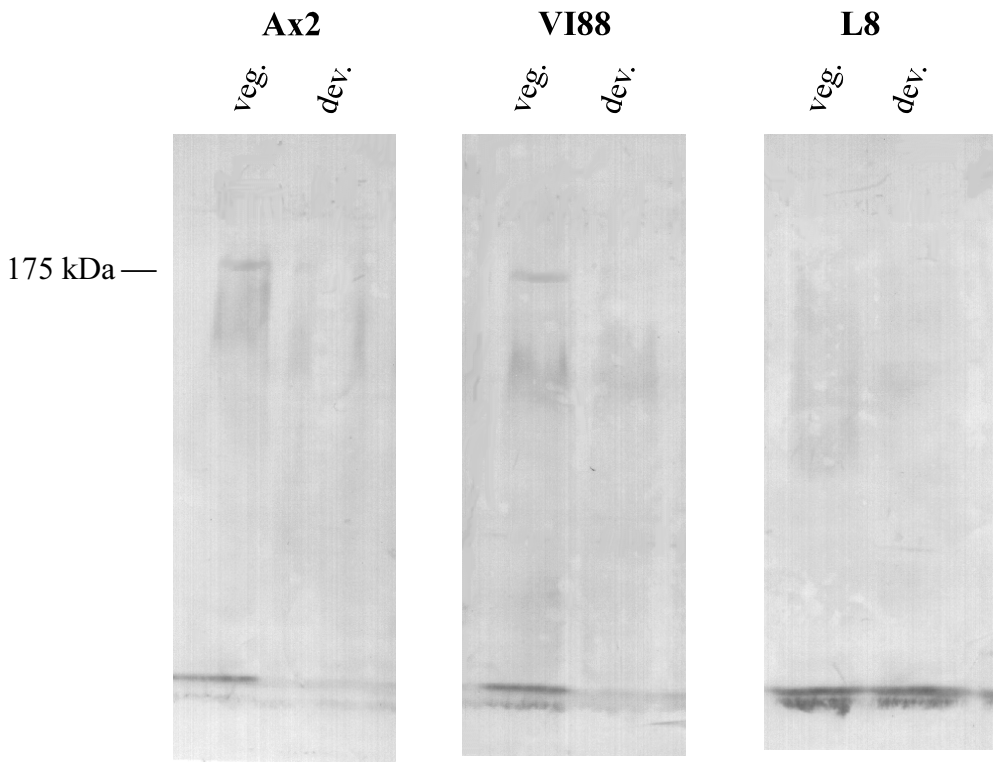


Figure 21. Ax2, VI88 and L8 cells were grown in bacteria suspension at $1x10^6$, then harvested, washed off bacteria, and starved for development as described (METHODS 2.3). 100 μg total protein were extracted from cells in vegetative growth (veg.) or after 5 hours of development (dev.). The samples were separated on 7.5% SDS-PAGE, blotted overnight with "Tank-transfer", and incubated with the polyclonal anti-D1 antiserum. A 175 kDa band was detected in veg. Ax2 and VI88 cells, but not in L8.

7.4. Kinase assay for the gdt1 protein

Since the sequence analysis revealed a kinase domain for the gdt1 protein, an autophosphorylation assay was performed to monitor the potential kinase activity of gdt1 (METHODS 4.15). The autophosphorylation assay was first tried for protein samples after immunprecipitation (METHODS 4.6) with the polyclonal anti-D1 antiserum but no clear result was obtained (data not shown). Then the same assay was performed for the membrane fractions (Fig. 22). Autophosphorylation of gdt1 should be observed as a band of 175 kDa in Ax2 but not in L8. Since the detergent Triton X-114 is solid above 20°C, reactions were performed at 4°C overnight instead of the normal 1 hr incubation at 30°C (the normal temperature for kinase assays). This technical problem could have influenced the kinase activity, since it has been observed that the low incubation temperature greatly reduced the kinase activity. For example, the cAMP dependent protein kinase (PKA) from *Dictyostelium* only displays 10% of its activity at 4°C (M. Veron, personal communication). Though no

autophosphorylation of gdt1 was seen in Fig. 22, the kinase activity of gdt1 may still be detected by another method. The kinase domain of gdt1 can be expressed as a recombinant protein and the activity could be obtained by the *in vitro* phosphorylation assays.

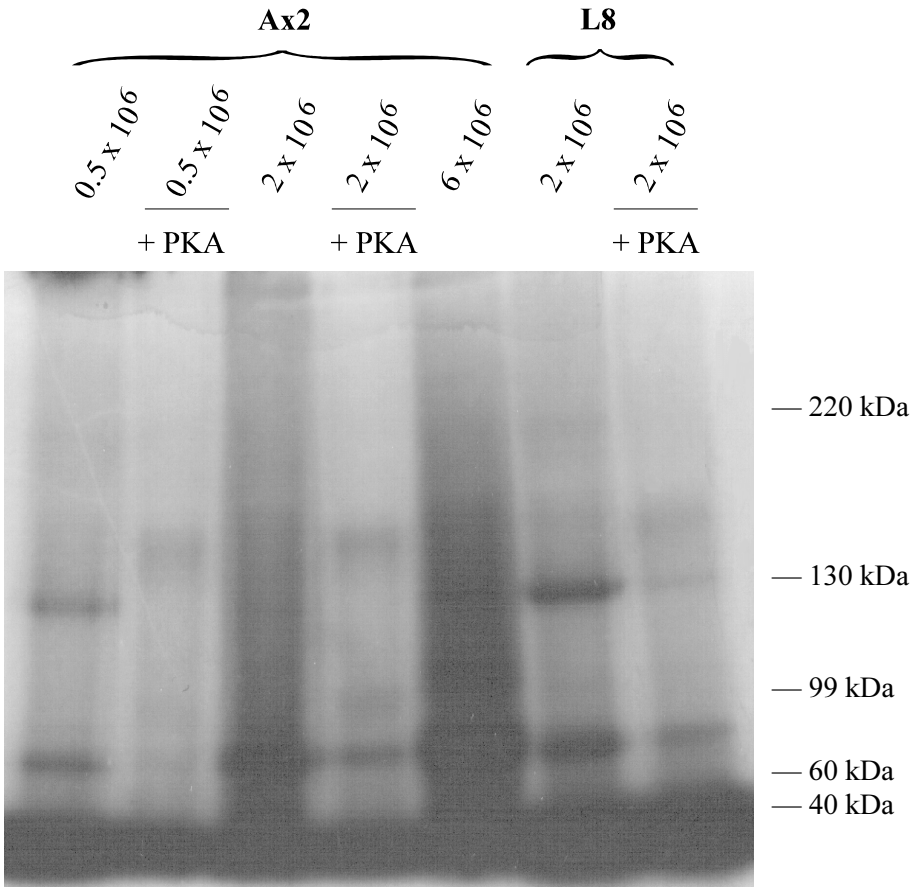
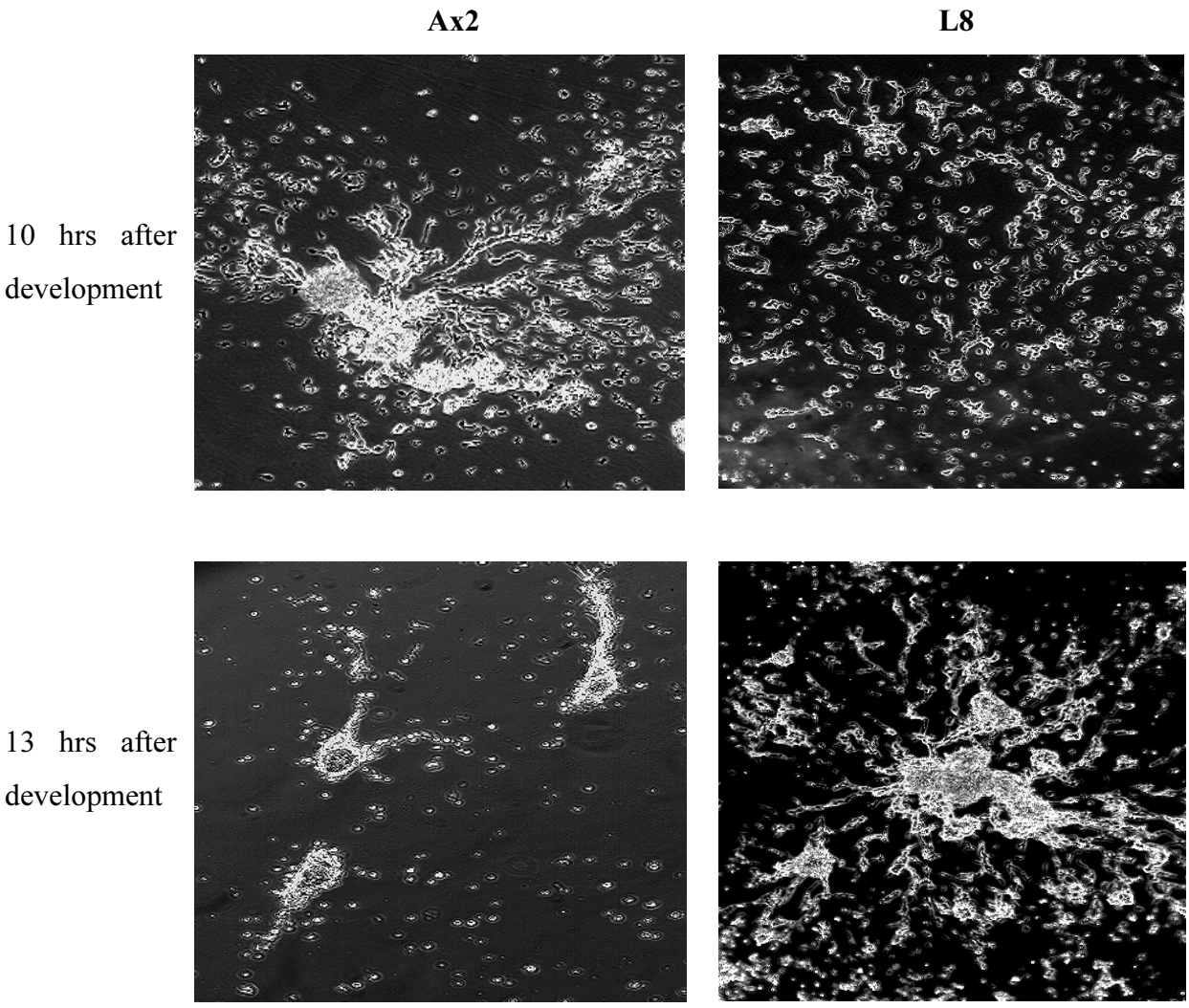


Figure 22. The same membrane fractions as indicated in Fig. 20 were used for autophosphorylation assays as described. Since the *in vitro* phosphorylation assay suggested that gdt1 protein could be a substrate of PKA (see Fig. 28), PKA was also added to part of the samples to see whether PKA phosphorylation could be observed under these experimental conditions. After overnight reaction at 4°C, samples were denatured and separated on 5-12% SDS-PAGE, then dried under vacuum at 80°C, and exposed to Kodak film at -80°C for 2 days.

8. The gdt1 protein may be involved in cell-substratum attachment or cell migration of *Dictyostelium discoideum*

The finding of a ″RGD″ (Arg-Gly-Asp) site in the protein sequence suggested another function for the gdt1 protein. RGD is the recognition site for fibronectin and vitronectin, and also constitutes the cell attachment sites of these proteins (Pierschbacher and Ruoslahti, 1984; Suzuki *et al.,* 1985). In *Dictyostelium*, discoidin I was found to have this RGD site. It has

been reported that a peptides containing the RGD site and the adjacent amino acids of discoidin blocked organised streaming during aggregation of *Dictyostelium* cells, and also blocked the cell attachment on a plastic surface (Springer *et al*., 1984). However, since discoidin is not expressed at the cell surface (Alexander *et al.*, 1992), the data indicated that there should be other membrane proteins on the cell surface of *Dictyostelium*, which contain the RGD sequence and are involved in cell-substratum attachment. Since the RGD site of the gdt1 protein is located in the extracellular domain (see Fig. 32 in DISCUSSION), it could be involved in cell attachment. The L8 mutant has a disruption in gdt1 before the RGD site, therefore it was examined in a streaming assay. In comparison to wild type cells, a 3 hours delay for streaming was observed in L8 cells (Fig. 23). Since the extracellular cAMP response appeared normally in the L8 mutant (Fig. 30), the delay for streaming suggested a defect in cell-cell attachment or cell-migration to form aggregates.



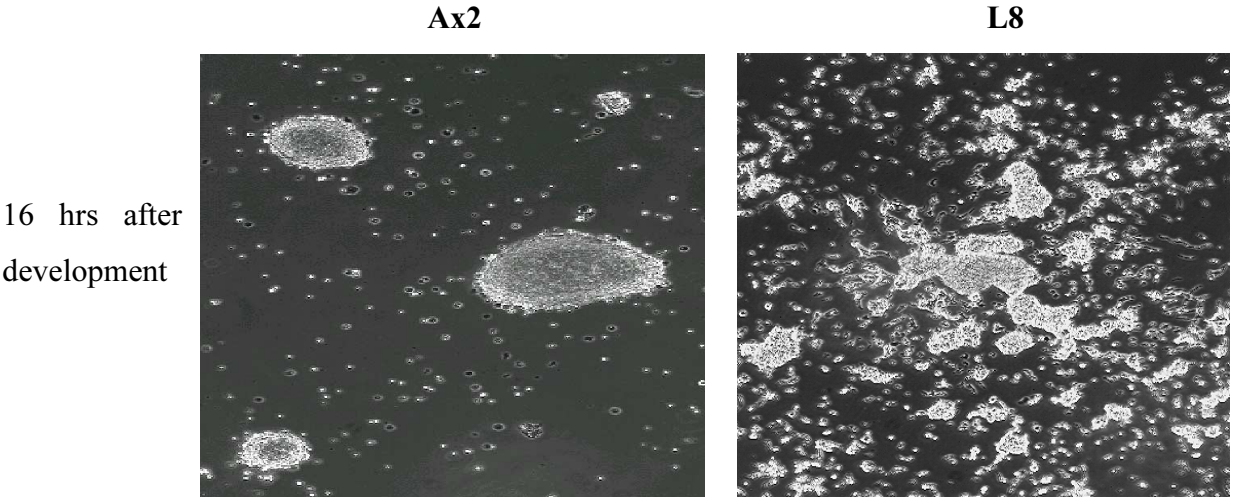


Figure 23. The streaming assay on a plastic surface for Ax2 and L8 cells. Ax2 and L8 cells were collected at $1x10^6$ after axenic growth and washed once with phosphate buffer. $2x10^6$ cells were suspended in 2 ml phosphate buffer and plated on a 25 mm diameter petri dish (Greiner). In comparison to the wild type Ax2 cells, a 3 hours delay for aggregation was found for the L8 cells, and the aggregates were very loose in comparison to Ax2.

The phenotype of L8 would suggest that the gdt1 protein presents an additional function for adhering to the bacterial food source bacteria. As described previously (section 1, 2, 3, and 4), the L8 (2-9) mutant displayed a premature developmental phenotype during bacterial growth. It has been reported that the food source bacteria inhibits *Dictyostelium* cells to develop by regulating the expression of some early developmental genes (Clarke *et al.*, 1988). Other direct assays were performed to investigate the adhesion of cells to carbohydrates which are abundantly expressed on the bacterial cell surface, and to examine the morphogenesis and differentiation of *Dictyostelium* cells on immobilized sugars. *Dictyostelium* cells were able to adhered to some special immobilized carbohydrates (the carbohydrates were linked to polyacrylamide), e.g. glucose, maltose and cellobiose (Bozzaro and Roseman, 1988). Furthermore, the multicellular morphogenesis of submerged *Dictyostelium* cells was inhibited when they were bound to glucoside immobilized on polyacrylamide gels: the aggregates repeatedly disperse and re-aggregate (Gambino *et al.*, 1992). The L8 mutant was examined in the morphogenesis assay of cells binding to glucoside-polyacrylamide gels (METHODS 2.4). Hexanol coupled gels was a negative control since wild type cells can aggregate normally. Surprisingly, L8 cells did not aggregate on both glucoside-linked gels and the hexanol control, while Ax2 cells formed aggregates on hexanol-gels and delayed or imperfect aggregates on the glucoside-gels (Fig. 24). The results support the assumption that gdt1 is

involved in cell-substratum and/or cell-cell adhesion as suggested by the streaming assay (Fig. 23).

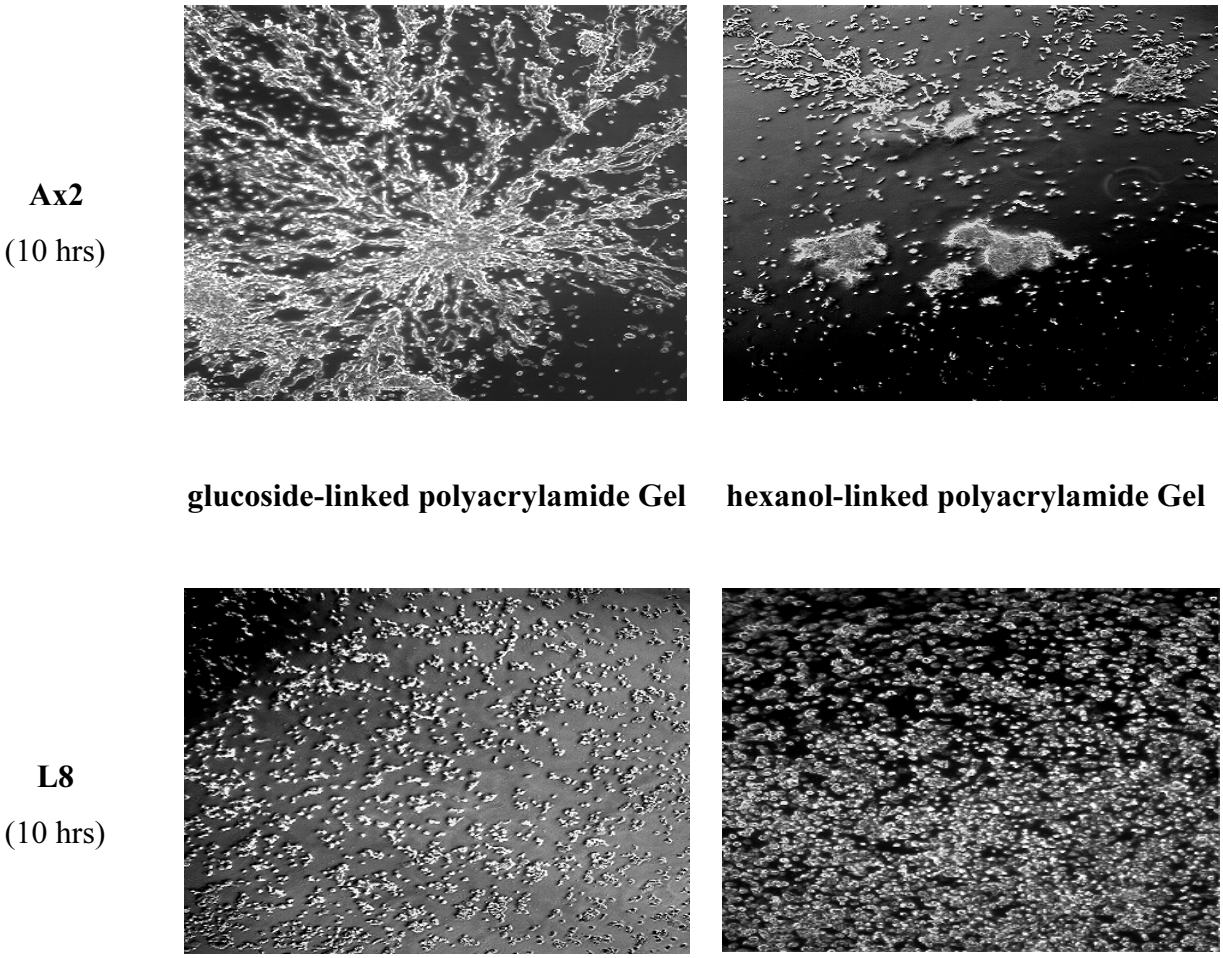


Figure 24. Multicellular morphogenesis on glucoside-polyacrylamide gel. Ax2 and L8 cells were harvested from axenic growth at density of $1x10^6$ and washed with phosphate buffer. The glucoside- and hexanol-polyacrylamide gels were prepared for this assay as described (METHODS 2.4). L8 cells were unable to aggregate on both glucoside- and hexanol-gels, while Ax2 cells formed aggregates on hexanol-gel and delayed aggregates on the glucoside-gel.

9. The signalling pathway of gdt1 and its regulatory network

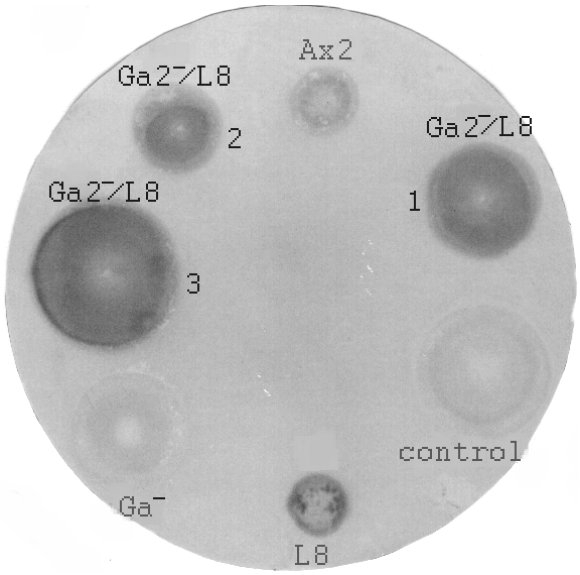
9.1. The PKA signalling pathway influences the phenotype of the gdt1 mutants

From the above data it could be concluded, that gdt1 is most likely a receptor kinase which generates a signalling pathway for the repression of discoidin expression. Disruption of gdt1 will result in an overexpression of discoidin and the premature GDT.

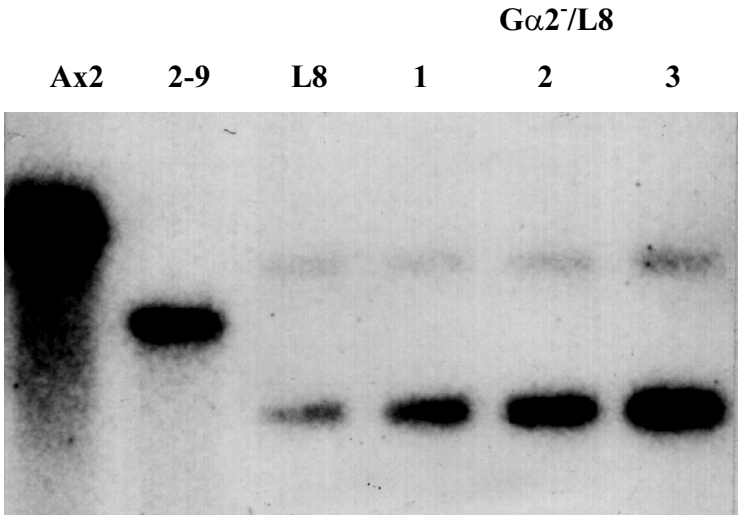
An intracellular signalling pathway for the induction of discoidin has been proposed (see INTRODUCTION 4, Endl *et al.*, 1996). This pathway is generated by a so far unknown extracellular factor, an unknown receptor, the receptor-associated G protein α2 subunit, CRAC, and cAMP dependent protein kinase (PKA). Knock-out mutants in either of the components (e.g. Gα2$^-$, CRAC$^-$ and PKA C$^-$ or Rm for PKA mutants) in this pathway all showed a complete loss of discoidin expression and the disability to aggregate and to undergo further developmental processes. Expression of a mutated R subunit (Rm) which can no longer bind cAMP, causes permanent repression of PKA activity. Consequently, *Dictyostelium* cells do not enter the developmental cycle (Harwood *et al.*, 1992) and, in the case of discoidin, very low expression is observed (Endl *et al.*, 1996). The similar result was obtained from the PKA C$^-$ mutant, a real knock out of the C subunit and with the complete loss of PKA activity (Mann and Firtel, 1991). This suggested that the PKA pathway could have link(s) to the gdt1 pathway and that somehow PKA controls the activation of gdt1. The disruption of PKA could result in a constitutively active gdt1 and thus the constant repression of discoidin expression. We started out from the hypothesis that PKA inactivates gdt1 resulting in discoidin induction.

To determine epistatic relationships between the PKA pathway and gdt1, double mutants Gα2$^-$/L8, Rm/L8 and PKA C$^-$/L8 were generated. In all constructions, the linearized vector 2-9-BsR-XbaI was transformed into the parent strain (Gα2$^-$, Rm and PKA C$^-$). More than 50% of the transformants had the *gdt1* gene disrupted by homologous recombination, and resulted in a discoidin^{over} phenotype (Fig. 25, 26). Like their parent strain Gα2$^-$, Rm, or PKA C$^-$, the double mutants Gα2$^-$/L8, Rm/L8 and PKA C$^-$/L8 did not aggregate and had a regular colony shape (no ragged edges). The gene disruptants were confirmed by Southern-blots (Fig. 25b for Gα2$^-$/L8, the same disruption pattern for Rm/L8 and PKA C$^-$/L8 but data not shown).

Figure 25. Disruption of *gdt1* in Gα2⁻ resulted in a strong induction of discoidin while the aggregation⁻ phenotype of the parent Gα2⁻ strain was maintained.



a. Colony-blots with monoclonal anti-discoidin antibody for Gα2⁻/L8 mutants. After transformation with the linearized vector 2-9-BsR-XbaI into the Ga2⁻ strain, the transformants were selected in medium containing Blasticidine, then cloned on *Klebsiella* plates. To select the real gene disruption mutants which should contain a stable BsR cassette, the clones were reselected in Blasticidine medium. Half of these transformants died after a few days of reselection while the others were stable. 3 independent cell lines (Gα2⁻/L8 1-3) from the stable clones and one of the unstable transformants (control) were randomly selected and checked in a colony-blot with the monoclonal anti-discoidin antibody. All Gα2⁻/L8 cell lines showed the same discoidin^{over} phenotype as the L8 mutant, while the control clone showed discoidin null like the parent strain Gα2⁻. Southern-Blot analysis (see Fig. 25b below) further proved that the Gα2⁻/L8 cell lines were identical double mutants and that they were generated by gene disruption by homologous recombination.



b. Genomic DNA from Ax2, 2-9, L8 and the Ga2⁻/L8 double mutants (1-3) was prepared, digested with *Hind*III, performed for Southern-blot analysis and hybridized with a ^{32}P-labelled 3.7 kb *gdt1* gene probe. A same pattern of two bands (see Fig. 10) was found for L8 and all the Ga2⁻/L8 double mutants and proved that all the double mutants carried the expected disruption of the *gdt1* gene.

Discoidin expression in double mutants Rm/L8 and PKA C⁻/L8 was examined by Western-blots. As shown in Fig. 26, induction of discoidin was detected in both double mutants, but at a lower level compared to the L8 parent strain. During growth, discoidin expression was considerably reduced in comparison to L8 but still significantly higher than in the parent PKA mutant (Rm or PKA C⁻ strain) or even in wild type Ax2 cells. At high cell density where cells starved, inactivation of PKA caused only a minor reduction of the elevated discoidin levels found in L8. Taken together, these results suggest that PKA has a dual effect on the growth-differentiation-transition (see DISCUSSION): one which is independent of gdt1 results in morphological development and induction of discoidin expression; and a second one which is mediated by the gdt1 gene product. PKA is essential for discoidin induction but this requirement could be partially by-passed by a gdt1 disruption.

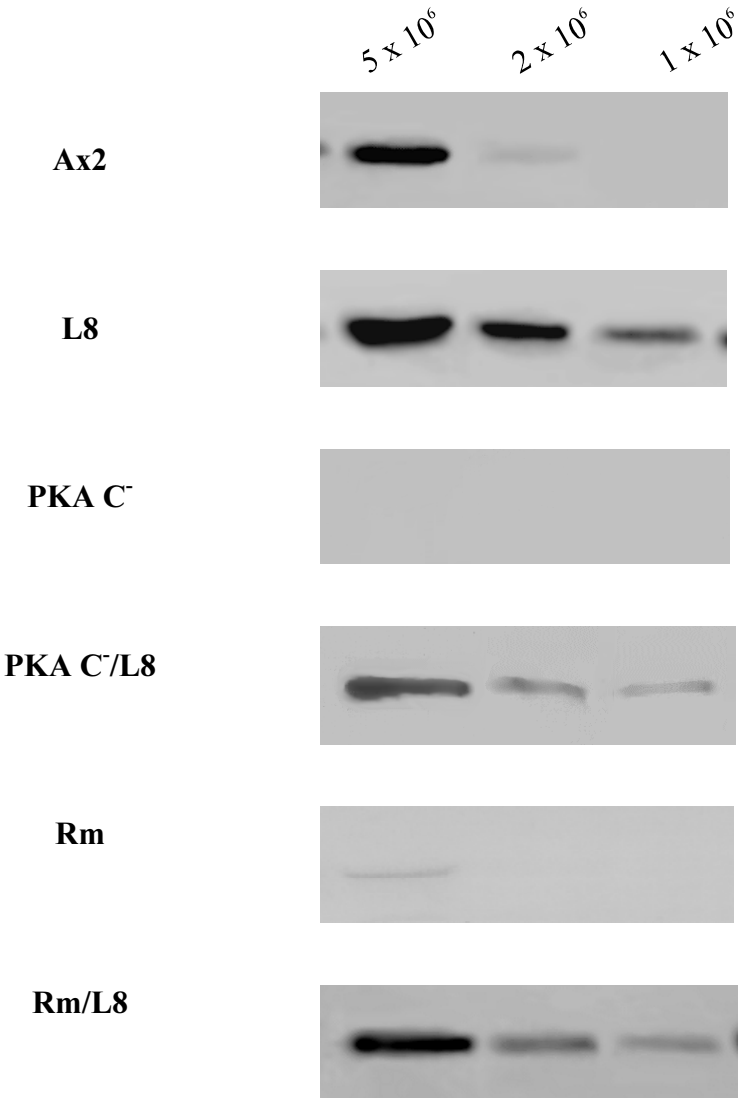


Figure 26. Discoidin^{over} expression in the Rm/L8 and PKA C$^-$/L8 double mutants. Cells were harvested from bacterial suspension at the indicated cell densities. Western-blots were performed with 80-52-13 monoclonal anti-discoidin antibody and a peroxidase coupled secondary goat-anti-mouse antibody. The double mutants displayed discoidin overexpression, especially at low densities in comparison to Ax2 wild type cells. Compared to the L8 parent, discoidin protein was reduced in the double mutants, especially at low cell densities. The very low expression of discoidin in the Rm mutant was probably due to insufficient overexpression of the dominant negative mutant form of the PKA regulatory subunit.

9.2. The regulation of gdt1 by PKA is confirmed by the PKA inhibitor H89

H89 (N-(2-(p-Bromocynnamylamine)ethyl)-5-isoquinoline-sulfoamide, Calbiochem) is a specific inhibitor of the C subunit of PKA, and has been used for generating inhibition of PKA in wild type cells (Anjard *et al*., 1997). H89 binds to the ATP binding site of the PKA C

subunit and thus completely blocks the activity of PKA. An experiment was carried out by adding H89 to the starved Ax2, L8 and VI88 cells, a mutant strain which shows a similar discoidin^{over} phenotype as L8 (see DISCUSSION). The cells were first grown in KA suspension up to $1x10^6$ cells/ml, then harvested and suspended in phosphate buffer at $2x10^7$ cells/ml density. Cells were developed for 5 hours, with and without 50 mM H89 (final concentration) respectively. Figure 27 shows a Western-blot developed with the anti-discoidin mAb. Discoidin expression was strongly repressed in Ax2 by H89, while no effect was found for L8 and VI88.

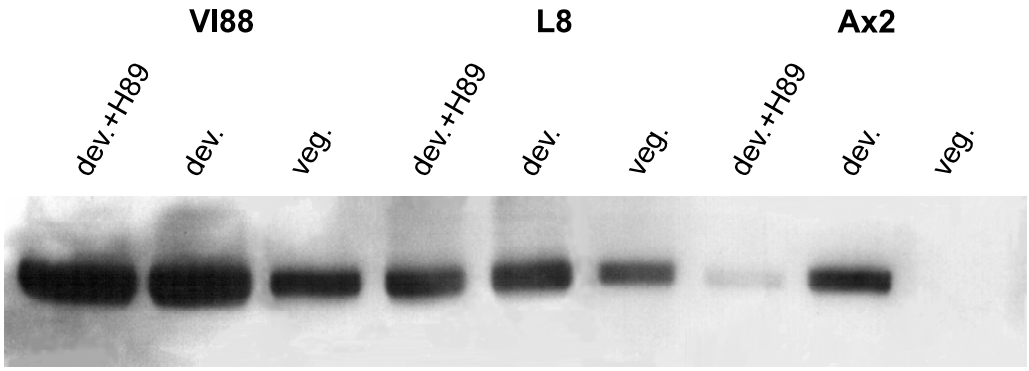


Figure 27. Growing cells from Ax2, L8 and VI88 were harvested from KA suspension and developed for 5 hours at 21°C. H89 was added to cells at the onset of development at a final concentration of 50 mM. Discoidin expression was strongly repressed by H89 in Ax2, while no effect was observed for L8 and VI88 cells. The data confirmed that a gdt1 disruption in L8 could by-pass the requirement for PKA for discoidin expression.

9.3. gdt1 is a PKA substrate *in vitro*

The gdt1 protein contains two putative phosphorylation sites for PKA, ″RFRNSL″ and ″KHRKRLSQ″ (indicated in Fig. 15b). The consensus phosphorylation site of PKA is ″RR(A/G)S*L″, where the serine residue is phosphorylated but the adjacent residues are also required for phosphorylation by PKA. Thus the second site was more conserved than the first one. The recombinant peptides of D1 and D2 (D1 containing ″RFRNSL″ and D2 containing ″KHRKRLSQ″, see Fig. 15b) were prepared for PKA assays (METHODS 4.15). The recombinant C subunit of *Dictyostelium* PKA, a kind gift of M. Veron, was used for the phosphorylation assays. Two different recombinant PKA C subunits were generated: C309 (42 kDa), which had only the kinase part of PKA C and high catalytic activity *in vitro*; and

the complete C subunit (70 kDa), which was less stable thus had low activity *in vitro* (Etchebehere *et al.*, 1997). Both PKA C recombinant proteins were tested, and they all phosphorylated the recombinant D1 protein (Fig. 28 for C309, data not shown for complete C). Surprisingly, the recombinant D2 protein was not phosphorylated, though the phosphorylation site seemed closer to the consensus. The data suggests that gdt1 may be regulated *in vivo* by PKA phosphorylation.

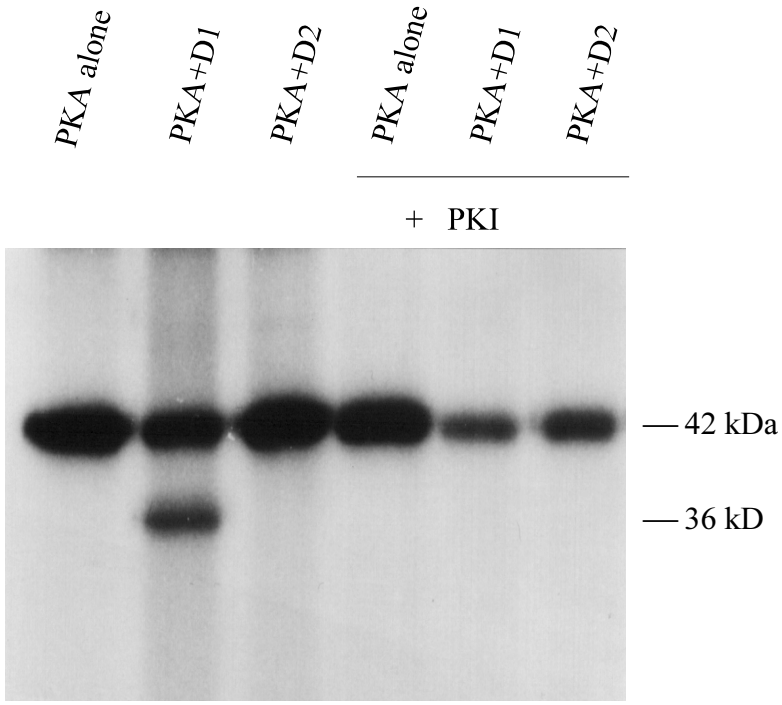


Figure 28. ***In vitro*** **phosphorylation of gdt1 by PKA C309.** Samples after PKA assay were separated on 12% SDS-PAGE, dried at 80°C under vacuum and exposed to X-ray film. PKA assays were performed with PKA C309 and the recombinant D1 and D2 proteins at the indicated conditions. Though PKA C309 was first saturated by cold ATP to reduce the background, the 42 kDa band from PKA C309 was still detected since PKA C309 had a strong binding to free ATP. PKA C309 phosphorylated the 36 kDa recombinant D1 protein, and this phosphorylation was specifically inhibited by PKI, a specific inhibitor of PKA.

10. The repressing signalling of folate is altered in L8

From previous work (Blusch *et al.*, 1995, Endl *et al.*,1996, Wetterauer *et al.*, 1995) it is known that the regulation of discoidin expression is different in axenic growth and in cells grown on bacteria. When cells are grown on bacteria, the discoidin genes are induced by cell-density-sensing factors before the food supply is exhausted, and expression increases continuously thereafter. In axenic medium, discoidin is induced at a considerably lower cell

density, but is no longer expressed in stationary phase. It has been shown that the food source bacteria can produce factors (e.g., ammonia, folate, glucoside), which act as extracellular signals and regulate the growth-differentiation-transition (GDT) of *Dictyostelium* cells. Usually, these factors act as repressors of discoidin expression. However, non of these factors has been proven to be responsible for the phenomenon during axenic growth, not even the cAMP signal, which is secreted by *Dictyostelium* cells and represses discoidin expression in late development (Fig. 3 in INTRODUCTION). Since the gdt1 pathway is also a negative regulator for the GDT and discoidin expression, experiments have been designed to investigate the interaction between gdt1 and the known repressors, folate and cAMP, in bacteria and in axenic growth.

Ax2 and L8 cells were harvested from bacterial suspension at the cell density of $1x10^6$, and washed free of bacteria with phosphate buffer. Cells were then set up for development at a density of $2x10^7$ cells/ml as previously described (Endl *et al*., 1996). cAMP (final concentration of 0.15 mM) or folate (final conc. of 1mM) were added at the onset of development and cells were harvested after 5 hours of development. A sample from the stationary phase (a few hours after the food source was depleted) was taken as a positive control. Repression effect on discoidin transcription was detected by Northern-blot (Fig. 29). However, no repressing effect was detected in L8 for both cAMP and folate, whereas both signalling responses were observed in wild type Ax2. The result demonstrated that when L8 cell were grown in bacteria, they lost the ability to respond to both cAMP and folate signalling.

The same experiment was performed with cells grown in axenic medium. Total protein was prepared and used for Western-blots with the monoclonal anti-discoidin antibody (Fig. 30). An obvious repression by cAMP was found in L8, but the inhibition by folate was seriously affected. The same result was also obtained by Northern-blots hybridized with an *in vitro* transcript of the discoidin Iγ gene (data not shown). The data from axenic growth confirmed that the L8 mutant did not respond to folate signalling. However, it also showed that when free of bacterial disturbance, the cAMP response could be normally displayed by the L8 cells.

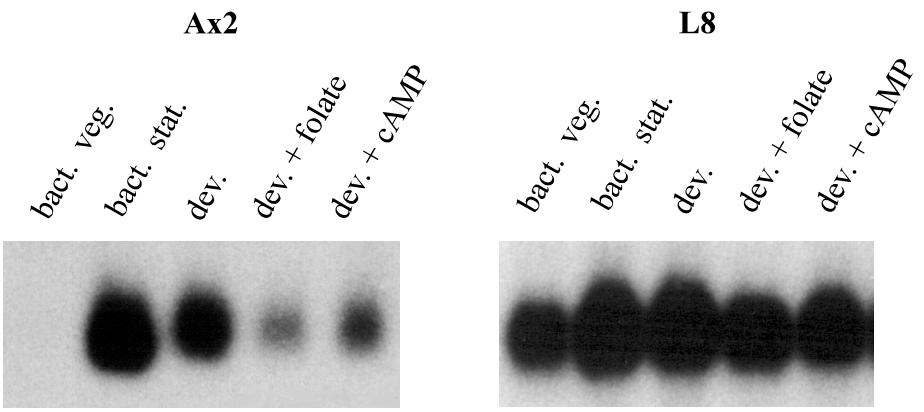


Figure 29. Growing cells of Ax2 and L8 were harvested from bacteria suspension and developed in phosphate buffer for 5 hours. cAMP and folate were added at the onset of development, with a final concentration of 0.15 mM for cAMP, and 1 mM for folate. Total RNA was prepared as described, an equal amount of 10 µg for each lane was loaded on the same gel, blotted and hybridized with an *in vitro* transcript of the discoidin Iγ gene.

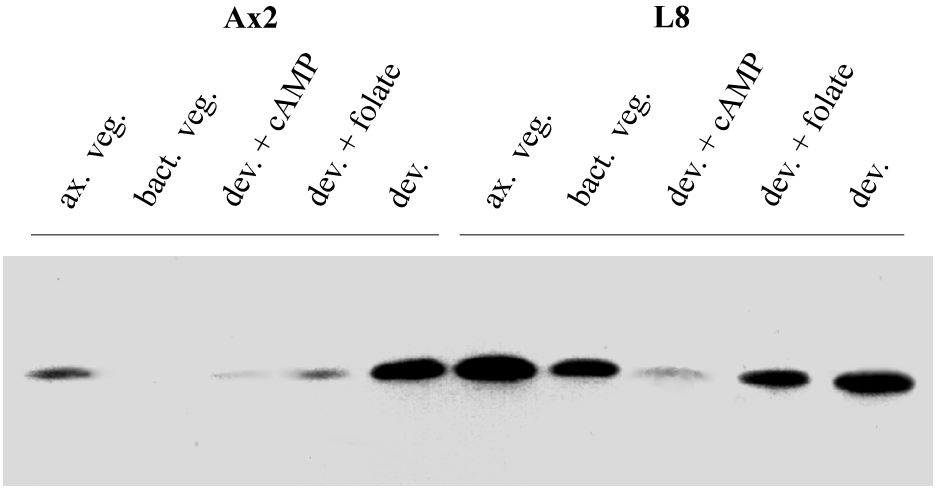


Figure 30. Growing cells of Ax2 and L8 were harvested at $1x10^6$ from axenic culture. For development, cells were resuspended in phosphate buffer to a density of $1x10^7$, and developed at 21°C for 5 hours. cAMP and folate were added at the onset of development (with a final concentration of 0.15 mM for cAMP, and 1 mM for folate). Total proteins were prepared and an equal amount of 15 µg protein for each sample was used for Western-blot analysis with the monoclonal anti-discoidin antibody. A sample of vegetative cells growing in bacteria suspension (same probe as in Fig. 29) was used to compare the discoidin expression in axenic medium and in bacterial suspension. Overexpression of discoidin and the response to cAMP were observed in L8 cells, but the response to folate was strongly reduced in comparison to wild type cells.

V. DISCUSSION

1. Isolation of the REMI 2-9 mutant opens the way to the identification of regulatory genes which control the growth-differentiation-transition.

In an attempt to identify signal transduction components regulating the transition from vegetative growth to development in *Dictyostelium*, discoidin I gene expression was used as a marker for screening mutants affected in the growth-differentiation-transition (GDT). The discoidin I gene family consists of 3 major polypeptides that are coordinately regulated (Devine *et al.*, 1982). Analysis of the linkage of this gene family by use of restriction fragment length polymorphism (RFLP) revealed that all the discoidin I genes are located on linkage group II (Welker, 1988). Thus, it is not possible that a discoidin null mutant can be generated by disruption of all discoidin I genes at the same time, and the mutations in discoidin mis-expression strains are most likely in genes involved in the regulation of this multigene family (Alexander *et al.*, 1983). Several mutants with defects in discoidin regulation have been previously described and analysed (Alexander *et al.*, 1983; 1986; Wetterauer *et al.*, 1993). Among them are the VI88 (Wetterauer *et al.*, 1993) and drsA (Alexander *et al.*, 1983) strains which display a similar discoidin^{over} phenotype, the disB and disA strains (Alexander *et al.*, 1986) and the motA and daxA strains (Alexander *et al.*, 1983) which show very low level expression of discoidin. By using parasexual analysis (Welker *et al*., 1985) the mutations in these strains have been assigned to different linkage groups: VI88 and disB alleles were mapped to linkage group III (Alexander *et al.*, 1986; Primpke and Wetterauer, personal communication); drsA was defined to linkage group IV (Alexander *et al.*, 1986); and disA, motA and daxA alleles were found to be linked to the discoidin I gene family loci in chromosome II (Welker, 1988). Interestingly, the drsA locus (a disc$^-$ allele) is epistatic to the disc$^+$ allele disB and results in the restoration of discoidin I expression in cells carrying the disB mutation (Alexander *et al.*, 1986). This indicates that normal expression of the discoidin I genes is dependent on the sequential action of the disB$^+$, drs$^+$ and disA$^+$ gene products. However, all previously defined mutants were generated by chemical mutagenesis, and it has not yet been possible to identify the molecular basis of the affected genes.

To further elucidate the signal transduction pathways involved in the GDT, new techniques had to be established to identify the components in the regulatory network leading to discoidin I gene expression. REMI is a method for generating insertional mutants by restriction enzyme

mediated integration (Kuspa and Loomis, 1992) and allows for the tagging and subsequent isolation of a disrupted gene from a mutant of interest. Many mutants with morphological alterations in development have been identified, and led to the isolation of many new genes which are involved in developmental regulations (Segall *et al.*, 1995; Wang *et al.*, 1996; Chang *et al.*, 1996). We have applied REMI mutagenesis to isolate mutants and genes involved in the GDT pathway by screening for mis-expression of discoidin. Among others, we detected a discoidin overexpression mutant, denominated 2-9, which expressed high levels of discoidin during growth and development. In addition, the mutant cells developed more rapidly, and aggregated in the bacterial lawn. The affected gene, denominated *gdt1*, was isolated from the 2-9 mutant by plasmid rescue, and the phenotype was reconstructed in wild type cells (mutant L8) by targeted gene disruption. As discussed below, gdt1 appears to be the first component in a new signal transduction pathway in *Dictyostelium* which is interconnected to other previously described pathways. The analysis of other GDT mutants should reveal further components in this pathway and help to unravel this signalling network.

2. Comparison of the two discoidin^{over} strains L8 and VI88

Since the mutant VI88 (Wetterauer *et al.*, 1993) has a similar phenotype as the 2-9 mutant, a comparative characterization was carried out to elucidate if the mutants were affected in the same gene. VI88 was directly generated from wild type Ax2, thus the reconstructed L8 strain (in Ax2 genetic background) was used instead of the original REMI 2-9 mutant (in DH1 genetic back ground, see METHODS 3.2) for the comparison. Parasexual genetic analysis was performed using the marker strain HU1628 to generate diploid cells with both strains, L8 and VI88 (Primpke *et al.*, unpublished results). Both mutations revealed to be recessive, thus any potential transcript of *gdt1* (a truncated mRNA was observed in the L8 strain, see Fig. 17) could neither cause a gain of function nor a dominant negative mutation. Segregation analysis revealed that both mutations were localised on chromosome III, but it could not be determined whether they mapped to the same locus. To determine whether VI88 and L8 were affected in the same gene, VI88/L8 diploids were generated. The complementation test did not result in any wild type progeny (Primpke and Wetterauer, personal communication), strongly suggesting that both mutants were affected in the same gene. The conclusion was supported by the H89 experiment (Fig. 26), in which specific inhibition of cAMP dependent protein kinase (PKA) led to a lack of discoidin expression in wild type strain Ax2, but not in the L8 and VI88 strains. Both mutants L8 and VI88 were indistinguishable in the ability to by-pass

the effect of PKA inactivation on discoidin expression. There was, however, a stronger phenotype observed in VI88, in that the discoidin I genes were expressed at higher levels than in the L8 mutant (Fig. 27). This could indicate different disruptions of the *gdt1* gene in both strains: chemical mutagenesis in VI88 may have introduced an early stop codon or a mis-sense mutation which inactivates its function, whereas in the L8 mutant the truncated mRNA may still be expressed to a partially functional protein. Therefore the *gdt1* gene product was detected by Western-blots with a polyclonal antibody generated against D1 (Fig. 21). The VI88 showed a wild type expression of the 175 kDa gdt1 protein, whereas in the L8 strain no expression of gdt1 was detected. Thus it could be assumed that the mutation in the VI88 mutant was most likely a point mutation or a very short deletion within the coding region of the *gdt1* gene, and that the mutation did not result in an early stop codon but a complete functional inactivation of the gdt1 protein. A further discrepancy was observed when the expression of early developmental genes was monitored in the two mutants: the gene for the cell adhesion protein CsA (Noegel *et al.*, 1986) was overexpressed in L8 but not in VI88, and the cAMP receptor cARI (Klein *et al.*, 1988) was reduced in L8 but normal in VI88 (Fig. 8 for L8, data not shown for VI88).

Taken together, the complementation test strongly suggested that VI88 and L8 were affected in the same gene. The different disruptions in *gdt1* indicated that they all resulted in a complete loss of function since they all displayed the same phenotype. Diploids of VI88 and L8 with a marker strain showed that both mutants did not play a dominant negative effect or a gain of function. Nevertheless, VI88 displayed a stronger phenotype in respect to discoidin overexpression and different effects on the expression of other early developmental genes than L8. It is thus not yet possible to clearly state if VI88 carries a functional mutation in the *gdt1* gene. The Wetterauer lab is currently sequencing the *gdt1* gene in the VI88 mutant to solve this problem.

3. Disruption of the *gdt1* gene results in premature entry into GDT but the developmental process itself is not affected

Disruption of the *gdt1* gene caused early entry into development, as demonstrated by the premature expression of discoidin I (Fig. 7) and the early-aggregation pattern in morphological development (Fig. 5). Since it has been previously defined that overexpression of the PKA catalytic subunit results in accelerated development and premature expression of

discoidin, a PKA C overexpressing strain (KP4, Anjard *et al.*, 1992) was used as a control to define the 2-9 phenotype. In comparison to KP4 and wild type Ax2, L8 cells started to aggregate earlier but the further developmental process was not accelerated: L8 cells formed multicellular aggregates as early as 7 hours after starvation, but afterwards the intervals between developmental stages were normal. This indicated that the *gdt1* gene was only involved in the growth-differentiation-transition (GDT) pathway, but was not substantial for late development. In the L8 strain, although discoidin I expression was clearly detectable in vegetative cells, expression was not constitutive, but increased with cell density (Fig. 7). Mis-regulation patterns were also observed for other developmental genes. *csA* and *pde* (Faure *et al.*, 1986) were prematurely expressed or overexpressed in L8, but the cAMP receptor cARI was clearly down-regulated in comparison to wild type cells (Fig. 8). This leads to the interpretation that the *gdt1* gene participates in an interacting network which controls the developmental expression of various genes by different pathways during morphogenesis of *Dictyostelium*.

The premature developmental phenotype of L8 especially showed in the colony morphology of cells feeding on bacteria: L8 cells formed aggregates in the bacterial lawn (Fig. 4b). The irregular colony shape and the aggregation in the bacterial lawn were probably consequences of the premature developmental competence. This may be explained by the assumption that in the gdt1 mutants the “prestarvation response“ (e.g. PSF induced growth-differentiation-transition at high PSF/bacteria ratios, see INTRODUCTION) was constitutively active. This might render cells more susceptible to biochemical cues triggering aggregation and result in aggregates close to the growing edge of the colony. This was, however, unlikely since the prestarvation response normally results in very low discoidin expression levels (Blusch *et al.*, 1995), but the gdt1 mutants showed high levels of discoidin. An alternative explanation would be that the gdt1 mutants showed a premature starvation response long before the food supply was depleted. This would be in agreement with the premature aggregation competence and the spreading of cells out of the colony to reach more abundant food resources. Since a gdt1 disruption affects discoidin expression in both axenic and bacterial growth (Fig. 29 and 30), the premature developmental competence may result from both a “prestarvation response“ (generated by *Dictyostelium* cells themselves) and from the “mis-perception“ of the food supply.

4. mRNA stability of the *gdt1* gene may be regulated by structural determinants within its 3'-untranslated region

The *gdt1* gene was isolated by different approaches (Fig. 15a), analyzed and used for the reconstruction of the 2-9 phenotype. Interestingly, though the detected mRNA size is nearly 7 kb, the coding region of the *gdt1* gene comprises only 4.7 kb, in agreement with the observed 175 kDa protein. The truncated mRNA from the L8 mutant indicates a transcriptional start located around 150 bp upstream to the initiation codon. Thus *gdt1* mRNA contains most likely almost 2 kb untranslated sequence at the 3' end. In higher eukaryotes, 3' untranslated regions were shown to be involved in the regulation of mRMA stability (Ross, 1996; Russell and Liebhaber 1996), but long untranslated mRNA sequences are unusual in *Dictyostelium* (Manrow *et al.*, 1988). The potential 3' untranslated region of the *gdt1* gene shows some interesting features: a potential structural hairpin formed by inverted repeats $_{4922}$UUGGGAC-$_{4948}$GUCCCAA directly after the end of the ORF, a relatively G-C rich tract, and several UUAUUUAU and CCAA (or UUGG) repeats (see APPENDIX 3). UUAUUUAU repeats in 3'-untranslated regions have been shown to confer mRNA lability in many cytokine genes (Henics *et al.*, 1994), and also to preclude mRNA translation (Kruys *et al.*, 1989). In addition to the UUAUUUAU repeats, a structural hairpin, called SLDE (Stem-Loop-Destabilizing Element) was found in the interleukin 2, interleukin 6 and granulocyte colony-stimulating factor mRNAs. The SLDE is functionally distinct from the UUAUUUAU elements but also destabilizes mRNA (Brown *et al.*, 1996). These structural determinants inside the 3'-untranslated region suggest a high instability of the *gdt1* mRNA. Preliminary data (Fig. 17b) confirms this assumption: 3' truncated *gdt1* mRNA in the L8 mutant accumulates to higher levels and appears to be more stable.

As observed from the Northern-blot (Fig.17a), the *gdt1* gene was only transcribed during growth and rapidly switched off as soon as cells started to develop. After 2 hours of development, no signal was detected until the end of the developmental life cycle. The rapid decay of the mRNA also supported the hypothesis for a regulation of mRNA stability of the *gdt1* gene.

5. The gdt1 protein contains 4 transmembrane domains and is most likely positioned on the cytoplasmic membrane

The hydrophobicity plot for gdt1 predicted 7 possible transmembrane domains (TM) as defined by scores over 500 in the hydrophobicity quantification (Fig. 31). As indicated, for each helix the hydrophobic score changed with the direction of the helix, e.g., from outside-inside (O-i) or from inside-outside (i-O). For some helices this could result in a complete loss of hydrophobicity and result in different models. For example, helix IV, V and VI are all strong in one direction (i-O) but weaker than the base line in the other (O-i), therefore only one of the three helices could actually form a transmembrane structure.

Based on the significant TM-segments, 2 models were predicted for the topology assignment of gdt1 (Table 5). Model 1 contains 5 transmembrane helices I, II, III, IV and VII, with a total score of 6354. With this model, the kinase domain will be inside the cytoplasmic membrane but the putative PKA site will be outside. Model 2 contains 4 transmembrane helices I, II, IV and VII, with a total score of 5958. With this model, both the kinase domain and the putative PKA site would be inside the cytoplasmic membrane. Remarkably, the hydrophobic scores of helix II change strongly in the two models, from 799 (direction i-O in model 1) to a very strong score of 1056 (direction O-i in model 2). Also, in model 1, the 3rd transmembrane domain helix III has a score of 558, which is just above the 500 base line and not very significant. Since only the scores above 1000 are certain, model 2 is the most probable structure for gdt1 (Fig. 32).

However, though the model with 4 transmembrane domains seems more likely, further experiments are required to discriminate both possibilities. Antibodies will be generated against the C-terminus (the putative protein kinase domain) which is predicted to be intracellular. Whole cells will be treated with proteinase K such that only the extracellular part of gdt1 will be degraded. Western-blot on extracts from the treated cells will be performed. If there are 4 transmembrane domains, anti-D1 antibodies against the second intracellular part (between TM2 and TM3) should reveal a truncated protein of about 40 kDa (Fig. 33) whereas no band will be observed in the second hypothesis. Antibodies against the kinase domain should detect a cleaved product of around 80 kDa in both cases.

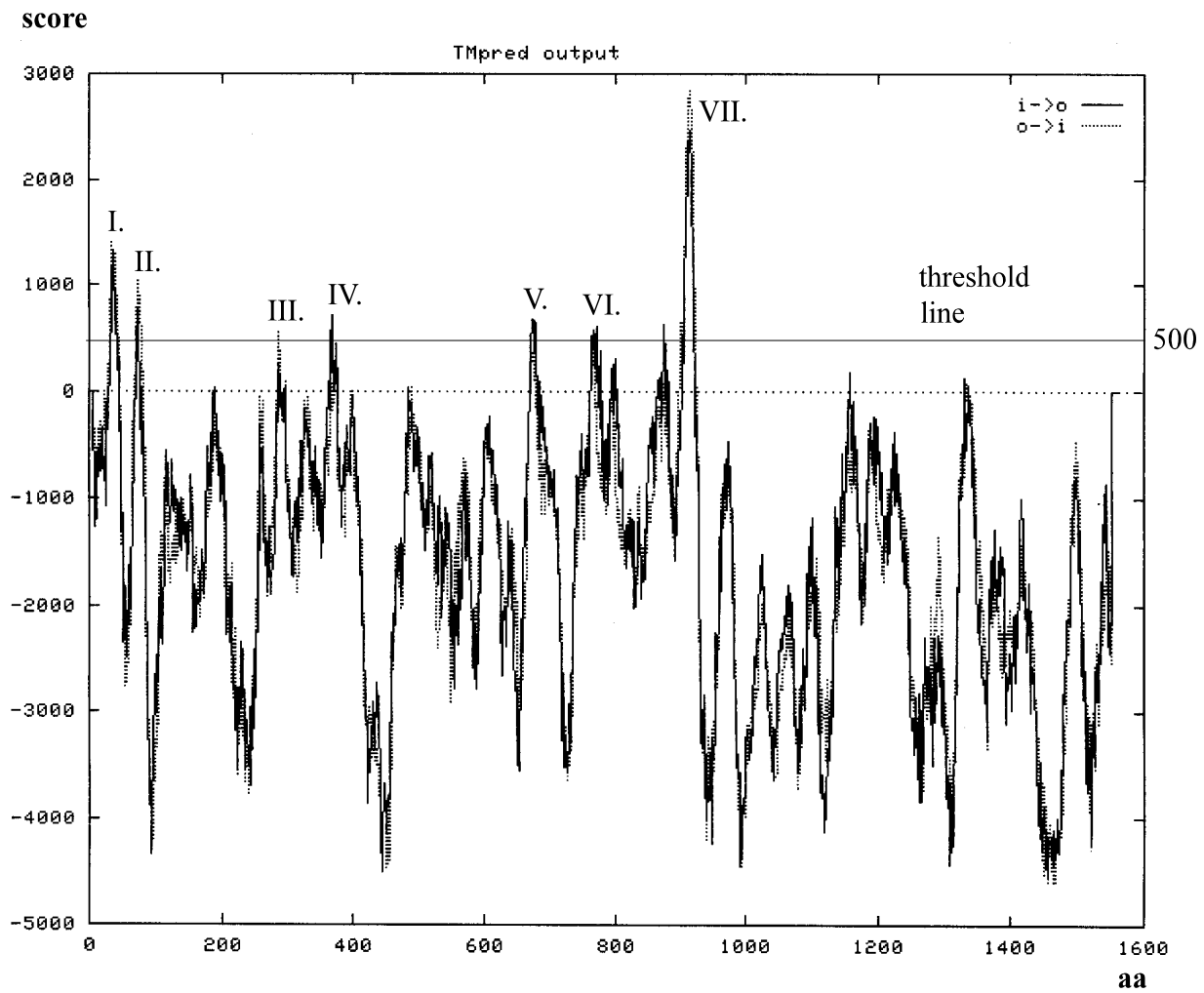


Figure 31. Prediction graphic for possible transmembrane domains in the gdt1 protein. The results were generated with EMBL TMpred program. There are 7 possible transmembrane helices in gdt1 protein. The score for each helix differs with its direction, that from O->i (outside to inside, dotted line) and i->O (inside to outside, solid line) the hydrophobic quantification could vary strongly, and only the scores above 500 are considered as significant. The helices I, II, and VII are significant for both directions, but IV, V and VI are only strong in the i-O direction. Helix III has a score of 558 with the direction of O-i, but only 288 from i-O. Based on this analysis, a 4 transmembrane-domain model was chosen for gdt1, which contains helix I, II, IV and VII.

Table 5. Two models were predicted for the gdt1 protein with the TMpred program

Model I: *5 strong transmembrane helices*						**Model II:** *4 strong transmembrane helices*					
#	*from*	*to*	*length*	*score*	*orientation*	*#*	*from*	*to*	*length*	*score*	*orientation*
I	*26*	*46*	*(21)*	*1412*	*O-i*	*I*	*26*	*46*	*(21)*	*1317*	*i-O*
II	*66*	*84*	*(19)*	*799*	*i-O*	*II*	*66*	*84*	*(19)*	*1056*	*O-i*
III	*280*	*296*	*(17)*	*558*	*O-i*						
IV	*359*	*379*	*(21)*	*726*	*i-O*	*IV*	*359*	*379*	*(21)*	*726*	*i-O*
VII	*904*	*927*	*(24)*	*2859*	*O-i*	*VII*	*904*	*927*	*(24)*	*2859*	*O-i*

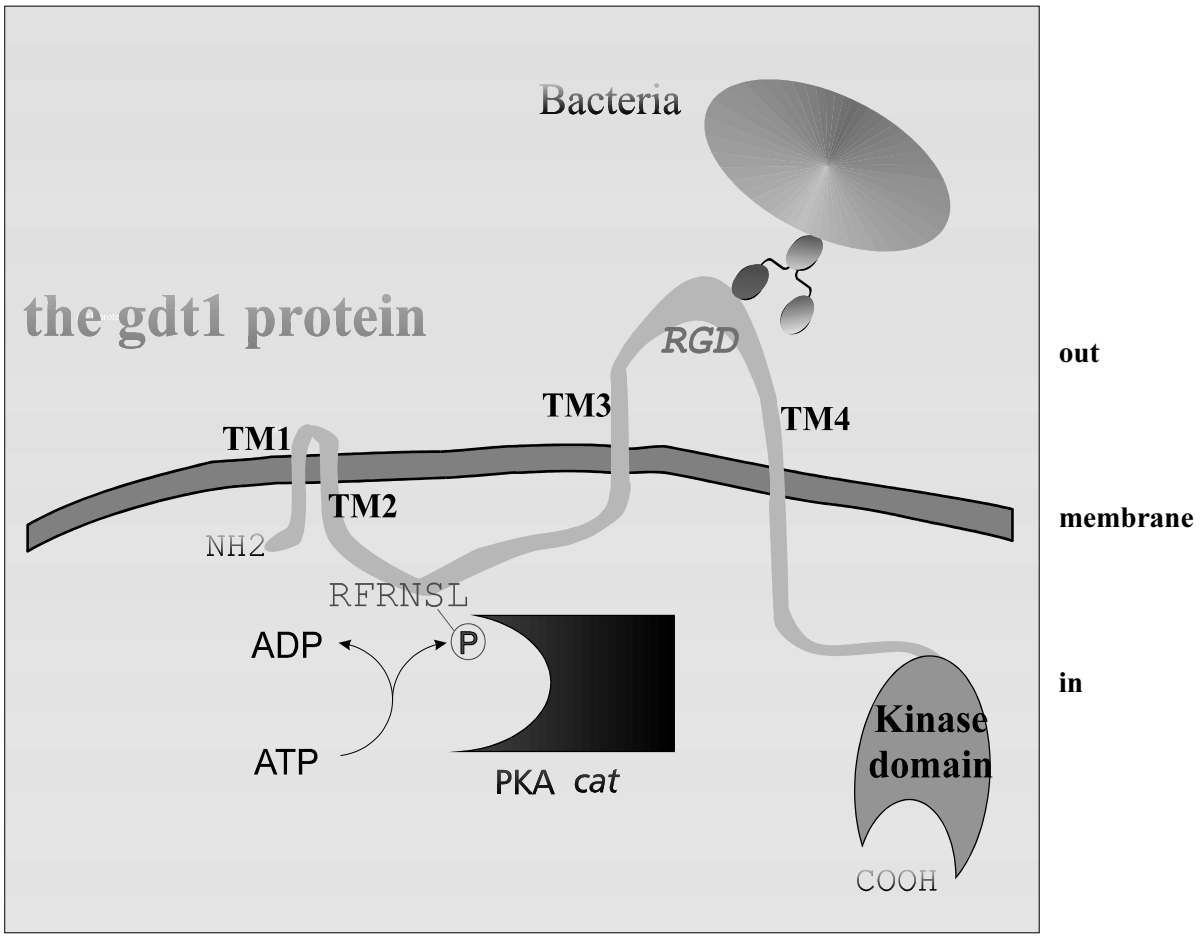


Figure 32. Model for the transmembrane structure of the gdt1 protein. The 4 putative transmembrane domains are indicated as TM1-4. The putative cell adhesion site "RGD", the kinase domain and the putative PKA site "RFRNSL" are shown at the expected positions according to the model.

In addition to the 4 transmembrane domains, there are some other functional domains (sites) in the amino acid sequence (Fig. 15b). According to the model, the kinase domain is located at the C-terminus inside the cytoplasm and is completely hydrophilic. The putative PKA phosphorylation site D1 is inside the cytoplasm, and the ″RGD″ site which was implicated in the cell-substratum adhesion is located at the large extracellular domain (Fig. 33). The association of gdt1 with the membrane was shown by Western-blots of Triton X-114 extracts (Fig. 20). Since the expression was not detected in nuclear extracts, the gdt1 protein is clearly not localised on the nuclear membrane. There was no consensus sequence found for the localisation signals to intracellular membranes, like the transmembrane Golgi retention signal

(Hobman *et al.*, 1995; Graham and Krasnov 1995). Thus, the gdt1 protein is most likely located on the cytoplasmic membrane.

6. The gdt1 protein is a putative receptor tyrosine kinase

Though the sequence comparison revealed no significant homology at the N-terminus, the C-terminus included characteristics which proposed the specificity of a tyrosine kinase (see below). The protein kinase super-family is up to now composed of 4000 members and new ones are found everyday (reviewed by Hanks and Hunter, 1995). Most of these protein kinases are defined as such based only on sequence homology, but usually not on protein kinase activity by biochemical analysis. Protein kinases are core components in signal transduction pathways. They usually exist in eukaryotes or viruses infecting eukaryotes, even though homologues were also found recently in some prokaryotes such as *Myxococcus xanthus* (Udo *et al.*, 1996) and *Yersinia pseudotuberculosis* (Hakansson *et al.*, 1996). In *Dictyostelium*, so far approx. 30 protein kinases have been cloned, but none of them has been identified to be a receptor tyrosine kinase. The protein kinase domain of gdt1 was defined by using multiple sequence alignments with some other protein kinases from various higher eukaryotic organisms (Fig. 16b). A weak but significant similarity (22% identity and approximately 67% homology along 290 aa) was observed at the consensus subdomains of the analysed protein kinases.

The universal structure for all protein kinases was established by intensive site directed mutagenesis, alignments of over 300 kinases and crystallography (Hanks and Hunter, 1995). The core of a protein kinase is about 270 aa long and is characterised by a defined series of conserved motifs. Structural data and sequence conservation were used to discriminate 11 different subdomains. Because of its relative simplicity, the catalytic subunit of mouse PKA is now used as the basic structure for comparison and nomenclature for all the protein kinase superfamily (Fig. 31 from Zheng *et al.*, 1992).

There may be some insertions or deletions, usually located between the conserved motives, but all the members of the family are along the same basic lines. The catalytic subunit of a kinase folds up into a two lobed structure. The upper lobe includes domains I to IV, and is linked to the lower lobe by domain V. The catalytic reaction takes place in the cleft between both lobes (Zheng *et al.*, 1992). Two main families of protein kinases were defined based on the choice of amino-acid substrates: serine/threonine protein kinases and tyrosine kinases.

True tyrosine kinases were found only in higher eukaryotes and are often involved in intercellular signalling. Though there are some dual protein kinases which can phosphorylate serine, threonine and tyrosine, they do not harbour any specific motifs and thus do not form a family. Sub-families were defined as serine/threonine and tyrosine kinases based on specific sequence and biochemical properties.

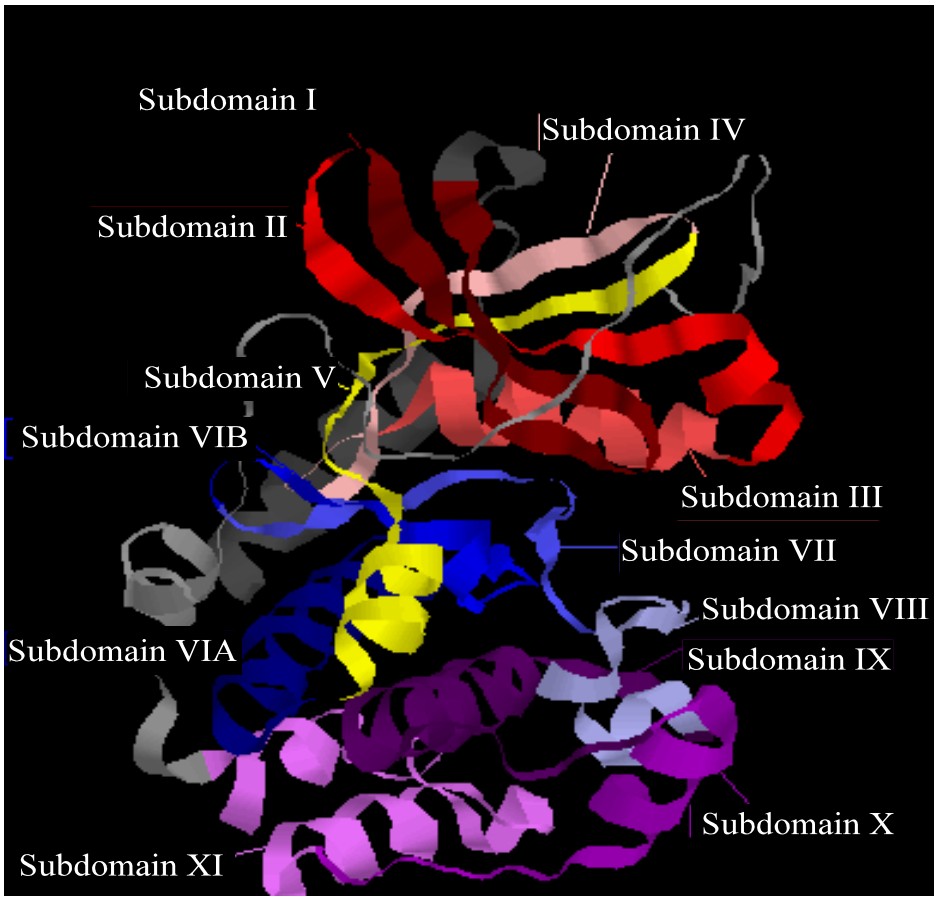


Figure 33. 3D structure of protein kinase A-catalytic subunit (from the crystallography of mouse PKA, Zheng *et al.*, 1992)**.** The subdomain descriptions use the convention of naming the beta sheets as numbers, starting with I at the N-terminus of the kinase domain. The alpha helices are named in a similar manner, using Roman letters, starting with A.

The gdt1 protein presents an overall homology in its protein kinase domain of about 300 aa, with the exception of a polyN-repeat insertion inside subdomain IX (Fig. 16b). Such polyN-repeats are very common in *Dictyostelium* sequences but do not present any conserved motif. The polyN repeats always disrupt the correct homology frame and result in false matching with many asparagine-rich sequences in the database. Thus the multiple alignments were performed with the deletion of 37 aa (see RESULTS 6.2), and all the universally conserved amino-acids were found at their expected location (see below).

The first domain (subdomain I) contains a 12 aa “glycine rich loop” which is involved in ATP binding. This subdomain is located in the upper lobe and is exposed to the surface of the cleft. gdt1 presents the following variation with the known consensus:

```
      aa Nr.      1.    2.- 5.    6.     7.   8. 9.        10.-12.
Consensus: (LIV)GxGx(FYW)(GSA)x(LIVCAT)xx(GSTACLIVMFY)
Gdt1:         I  ekys   W   e  i L      yr G
```

The amino acids indicated in brackets correspond to the different variations at the given position, while x indicates any amino acid. The two first glycines are quite conserved but absent in gdt1, but the surrounding hydrophobic amino acids are well conserved. Viral protein kinases also present weak homology in this area (Bischoff and Slavicek, 1994). The crystal structure of this region shows that some of conserved hydrophobic amino-acids are buried inside the loop to stabilize the structure, whereas the hydrophilic amino acids (including the glycines) are exposed to the surface for interacting with the Mg^{2+}-ATP complex. The underlined hydrophobic amino-acids which are important for sequestering the adenine ring of ATP are conserved in gdt1.

The subdomain II can be of variable size but usually contains the following motif located about 5 to 18 amino-acids after the “glycine rich loop”:

```
aa Nr.                    1.          2.       3.          4.
Consensus: (LIVMFYWCSTAR)(AIVP)(LIVMFAGCKR)K
Gdt1                      A    I    L          K
```

The lysine residue (universally conserved for all kinases, in bold) at the end of the motif that was shown to interact with the α and β phosphate of ATP (Hanks and Hunter, 1995) is present in gdt1. This lysine forms a salt bridge with an invariant glutamic acid located about 17 amino acids downstream in subdomain III. In gdt1 the glutamic acid is found 15 amino acids after the lysine.

Subdomain IV plays a structural role and is poorly conserved among other protein kinases. However, gdt1 presents good homology in this domain with receptor tyrosine kinases, especially with the neurotrophic receptor from *Drosophila* (Fig. 16b).

The domain V is characterized by an invariant glutamic acid (in bold) surrounded by hydrophobic residues. This motif is perfectly conserved in gdt1:

aa Nr.	1.-3.	4.5.	6. - 9.	10.
Consensus:	3 hydrophobic	**E**Y	2 to 4 hydrophobic	hydrophilic
gdt1:	LAF	**E**Y	PPLG	S

The conserved glutamic acid forms hydrogen bounds with the ribose of the ATP while the hydrophilic residue after the second hydrophobic stretch is involved in substrate binding and therefore variable since protein kinases have different substrate specificities.

The gdt1 protein presents good overall homology with all selected kinases in the subdomain VIA (see figure 16b). Since this subdomain has a conformational function, the highly conserved consensus suggests an important feature for this group of protein kinases. Subdomain VIB contains the “catalytic loop” required for the phosphotransfer. This sequence is located on the surface of the protein kinase and on the cleft to face the glycine loop. Two different consensus sequences were established for this catalytic loop, consensus 1 is specific for serine/threonine kinases and consensus 2 is specific for tyrosine kinases. They are usually distinguished by the presence of a conserved lysine for serine/threonine kinase or arginine (Nr. 9) for most tyrosine kinase.

aa Nr.	1.	2.	3.	4.5.	6.	7. -10.	11.-13.
Consensus 1:	(LIVMFYC)x	(HY)x	D	(LIVMFY)xx	k**N**	(3 times	LIVMFYCT)
Consensus 2:	(LIVMFYC)x	(HY)x	D	(LIVMFY)xx	R**N**	(3 times	LIVMFYC)
gdt1:	I	i	q	kt L	raR**N**		IFL

Since gdt1 contains an arginine in position 9, it might belong to the tyrosine kinase family or to dual specific serine/threonine/tyrosine kinases. The hydrophobic residues from this consensus are especially well conserved in gdt1 but the non-conservation of both Nr 5 aspartic acid (D) and Nr 3 histidine/tyrosine (HY) is quite unusual. Thus gdt1 is more close to the tyrosine kinase consensus. Like the glycine loop, the catalytic loop contains mostly hydrophilic residues and is bordered by hydrophobic residues. gdt1 shows a good conservation of all hydrophobic residues defining the limits of the catalytic loop. But at the

highly conserved hydrophilic residues, gdt1 exhibits replacements with other hydrophilic residues, suggesting that catalysis might occur through a special mechanism.

Subdomain VII usually contains the well conserved triplet DFG in serine/threonine kinases, but this is absent in some membrane spanning receptor kinases (Hanks and Quinn, 1991). The aspartic acid present in gdt1 is an invariant residue which is required to orient the gamma phosphate of Mg^{2+}ATP for transfer to the substrate. A conserved R/K residue located 5 aa after the aspartic acid residue which plays an important conformational role is also found in gdt1.

aa Nr.	1.- 3.	4.	5.	6.-	9.
Consensus:	K x x	**D**	**F**	**G** x x	**K**
gdt1:	K v l	**D**	l t	s s	**K**

Subdomain VIII forms a crooked chain that faces the cleft between the large and small lobes. It contains the APE motif which is conserved in serine/threonine kinase but less in tyrosine kinase. For this motif, only the invariant glutamic acid is found in gdt1. The E residues is involved in stabilization of the large lobe. The activity of most protein kinases is regulated by phosphorylation on the serine, threonine or tyrosine in this subdomain VIII. These S, T or Y residues are located few amino-acids after the beginning of subdomain VIII and about 11-15 amino acids before the conserved glutamic acid. In gdt1, the only phosphorylatable amino acid in this area is a tyrosine lying 4 amino acids after the first amino acid of subdomain VIII and 13 amino acid before the E residue. Phosphorylation at this Y site can correspond to autophosphorylation of gdt1 or to a phosphorylation by another protein kinase. In the case of PKA, autophosphorylation occurs at threonine 197, it is usually stable and not sensitive to phosphatases. Some protein kinases like the MAP kinase family are regulated by phosphorylation/dephosphorylation processes on residues of this subdomain. Using monoclonal antibodies against phospho-tyrosine might allowed to detect the presence of a phospho-tyrosine in gdt1.

aa Nr.	1.-	4.	5.	6.-	15.	(+4)	16.	17.	18.
Consensus:	xxxx	Y	xxxxxxxxxx		(xxxx)		(AS)	P	**E**
gdt1:	acnn	Y	---iervdtp		inlt		-	r	**E**

Subdomain IX has a large alpha helix containing an invariant aspartic acid residue. This D residue is present in gdt1 (2 amino acids before the 38 aa insertion), and the adjacent residues present some homology with the receptor tyrosine kinases (see Fig. 16b). The aspartic acid

residue is important for both the stabilization of the catalytic loop and the recognition of substrate. gdt1 contains an insertion of 38 amino acids, mostly an asparagine repeats directly after the conserved aspartic acid. An insertion at this position is unusual and might reduce the enzyme stability. However, after the polyN insertion gdt1 presents again good homology for subdomain IX.

aa Nr.	1. -	9.	10.
Consensus:	x x x x x x x x x		**D**
gdt1:	i i - r d p k q n n		**D**

The two last domains are often poorly conserved and may contain big insertions or deletions. The subdomain X has the structure of a small alpha helix sitting at the base of the large lobe, towards the outside. The sequence from this region is not well conserved, and of unknown function. No clear conserved residues are found between gdt1 and the analysed protein kinases (Fig. 16b). The most important feature of subdomain XI is an almost invariant R residue, that is however, not found in gdt1. On the other hand, gdt1 presents two other motifs, CW and TF which are present in many tyrosine kinases but not very common in serine-threonine kinases. Usually the R residue in subdomain X interacts with the conserved E residue from subdomain VIII to stabilize the structure of the lower lobe. However, the requirement for the R residue to stabilize the protein structure could be replaced by another mechanism. The fact that gdt1 contains a large polyN insertion in subdomain IX may play a role in interaction with other residues. It has been reported that asparagine-rich (polyN) sequences are involved in protein-protein interactions, especially in *Plasmodium falciparum* antigen-host antibody recognition (Franzen *et al.*, 1989; Wahlgren *et al.*, 1991).

Among all protein kinases, gdt1 displayed the best homology with a non-receptor serine-threonine kinase in Entamobae (ENHPSTK-1), a small protein which contains only 290 aa (see Fig. 16a). It is, however, unlikely that the membrane-associated gdt1 protein is a direct homologue of ENHPSTK-1. The high homology score came mostly from the sequences before the “Glycine rich loop”, which were not conserved for all protein kinases but were highly conserved between gdt1 and ENHPTK-1. The other well conserved region was the CW sequence in the last subdomain, which was rather unusual for the serine/threonine kinase ENHPSTK-1.

In conclusion, gdt1 is probably a receptor tyrosine kinase or a dual specificity kinase but also presents unique differences suggesting that it might correspond to a new family. The unusual features in both “the glycine rich loop” (subdomain I) and the “catalytic loop” (subdomain VI) suggest that gdt1 might catalyze phosphotransfer through an unusual mechanism. The presence of an insert close to the D residue which is important for the protein stability, and the absence of the conserved R from subdomain XI might result in an unstable enzyme activity with a partially unfolded lower lobe. On the other hand, poly-asparagine, like poly-glutamine can be involved in protein-protein interactions, or may be used by other proteins to modulate the kinase activity of gdt1.

On the way to determine the complete sequence of the *gdt1* gene, several additional gene disruptions (indicated in Fig. 10) were generated. In the original REMI mutant, gdt1 was disrupted inside the second transmembrane domain TM2. The L8 mutant, the D and X series mutants carried the same disruption in the intracellular loop between TM2 and TM3, but before the putative PKA phosphorylation site “RFRNSL”. In the K series mutants, an insertion was introduced at 3.9 kb after the ATG codon, leading to the possible expression of a truncated gdt1 protein which contains all the 4 transmembrane domains but with the kinase domain at the C-terminus deleted. All disruptants displayed the same phenotype. This supported the conclusion that the whole 1561 aa long ORF represented a single gene. Further more, since the most 5’ as well as the most 3’ disruption showed no differences in phenotype, one had to assume that none of the mutants expressed a partially functional protein, though a small mRNA transcript of 1.2 kb was detected in L8 (see Fig. 17b). The kinase domain is located at the very C-terminus, thus its kinase activity appears to be crucial for gdt1 function.

The fact that gdt1 is a cytoplasmic membrane associated protein supports the assumption that it belongs to the receptor kinase family. Since ligands for receptor kinases are variable, the extracellular regions of receptor kinases are usually not conserved. This could explain why the large N-terminal part of gdt1 reveals no homology to any sequences in the databases.

7. The RGD site suggests a cell-substratum adhesion function for the gdt1 protein

In addition to the receptor kinase feature, another motif suggests further functions of the gdt1 protein. The RGD (Arg-Gly-Asp) sequence is the minimal structure in the adhesive glycoprotein fibronectin which can be recognized by mammalian cells and promotes cell-attachment (Suzuki *et al.*, 1986). The conservative substitutions of Lys for Arg, Ala for Gly, or Glu for Asp each resulted in abrogation of the cell attachment activity. This site was also found in other proteins which were known to interact with the cell surface, like vitronectin (Pierschbacher and Ruoslahti, 1984), various types of collagens (Pfaff *et al.*, 1993) and thrombin (Bar-Shavit *et al.*, 1996). Using a synthetic peptide containing the RGD sequence prevented gastrulation of *Drosophila* embryos (Naidet *et al.*, 1987), indicating that the RGD sequence might also be used by invertebrates to mediate cell-attachment phenomena. More recently, a native bone sialoprotein (Stubbs *et al.*, 1997) was characterized which facilitated the adhesion of several cell types via its integrin binding RGD site, presenting a direct proof for the *in vivo* function of the RGD tripeptide sequence. In the lower eukaryotic organism *Dictyostelium*, treatment of cells with a synthetic peptide containing the RGD motif blocked cell attachment and developmental morphogenesis (Springer *et al.*, 1984). This suggested that RGD could be part of a universal cell adhesion system. However, the RGD motif does not only exist in cell-surface proteins, but also appears to be involved in the interaction of cytoskeletal proteins with the cell matrix (Ohno, 1995). In *Dictyostelium,* the discoidin proteins may be an example: they have a conserved RGD sequence and are required for cytoskeletal organization in cell elongation during aggregation (Alexander *et al.*, 1992). In the gdt1 protein, the RGD site is located at the large potential extracellular domain of 548 amino acids, and could therefore play a role for ligand binding. Further more, if the gdt1 protein is on the cell-surface, it may also be involved in the cell-cell attachment between *Dictyostelium* cells, or in the adhesion of bacteria to *Dictyostelium* cells.

In the streaming assay (Fig. 23), cells were plated at low density to form a single layer by attachment to the plastic surface. Under such conditions, *Dictyostelium* cells had to migrate over a long distance to form classical streams in liquid, but could not further differentiate unless placed at an air-water interface (Brodie *et al.*, 1983). Thus the conditions were different from the standard developmental assay and revealed defects in chemotaxis motility and cell adhesion. It has been shown that rapid-development mutants also failed to stream normally in this assay (Abe *et al.*, 1983). An obvious delay for streaming and the looser formation of aggregates was observed in L8, indicating that a disruption of gdt1 resulted in

mis-regulation of cell-cell attachment and/or cell-migration. These data fit well with the observations made after treatment with RGD peptides (Springer *et al.*, 1984), supporting the assumption that gdt1 is located on the cell surface and involved in the interaction of cells with each other and/or their environment (e.g. substratum). So far some glycoproteins, e.g. csA (contact site A) and gp24 have been identified to mediate direct cell-cell adhesion in *Dictyostelium,* but no "RGD" site is contained in these proteins (Siu *et al.*, 1988, Stadler *et al.*, 1989, Desbarats *et al.*, 1992, Loomis and Fuller, 1990). Interestingly, a slight overexpression of *csA* was observed in L8 (Fig. 7), which should rather increase cell-cell adhesion.

It is, however, also possible that the defects in streaming and aggregation in the mutant are secondary effects mediated by the signal transduction function of gdt1 (see below).

8. gdt1 is a new component in the signalling pathway of the GDT.

The finding that gdt1 is a putative receptor tyrosine kinase supports the assumption that it is a signal transduction component. Most receptor tyrosine kinases, e.g. insulin receptors and nerve growth factor receptors, are activated by autophosphorylation upon ligand binding, and activation is reversed by dephosphorylation via a tyrosine phosphatase (reviewed in Zhang *et al.*, 1993; Lee and Pilch, 1994). gdt1 is a negative regulator of differentiation. As a receptor it most likely senses nutrients or bacteria and mediates an intracellular signal which represses the growth-differentiation-transition and discoidin expression. As expected, gdt1 mutants entered the developmental cycle more rapidly, but were normal in the following steps of differentiation (see Fig. 5). This was in contrast to other rapid development mutants (like PKA-C overexpressing strains) which were accelerated in later stages of development.

The finding that gdt1 is a negative regulator of discoidin expression suggests that the discoidin gene family is repressed during growth, and that expression is rather mediated by a release of this repression than by an active induction. The expression pattern of gdt1 supports this suggestion. gdt1 is only expressed during growth, but then quickly down-regulated after the onset of development (Fig. 17a, 21). This agrees with the regulation pattern of discoidin, which is expressed at very low levels (maintained by a repressing factor) during growth but strongly induced (release of the repression) after the onset of development. Most probably when cells are grown in plenty of bacteria, gdt1 is bound by its ligand and the intracellular repressing-pathway is activated; when cells are deprived of their food source, gdt1 may loose

the ligand and release repression. In agreement with this, discoidin expression is predominantly under negative control. This conclusion is supported by a number of further REMI mutants: the frequency of discoidin overexpression strains by far exceeds the discoidin minus phenotype (our own unpublished observations, and Wetterauer, personal communication). Since most REMI mutants should carry loss of function mutations, proteins in repressing pathways appear to be more frequent than activating components.

gdt1 disruptants expressed discoidin at lower cell densities, i.e. prematurely, the final maximal level was, however, similar to that in wild type cells (Fig. 7 and 8). The gdt1 protein appeared not to be expressed in wild type cells at very low densities (Fig. 20), when cells did not express discoidin. Under these conditions, a different pathway probably prevented the onset of differentiation. Apparently, *gdt1* transcription is regulated by a density sensing factor like PSF. The data suggested that gdt1 expression was continuously increasing with cell density to inhibit the gradually increasing competence of cells to enter the GDT. Due to the low expression of gdt1 mRNA and protein, this could unfortunately not be shown directly (but see Fig. 20). Moreover, as shown in Fig. 7, disruption of *gdt1* did not only result in overexpression of discoidin, but also mis-regulation of other developmental genes (e.g., induction of *csA*, premature expression of *pde*, and repression of *carA*). Both PDE and cARI are signalling molecules involved in the cAMP pathway, which generates repression of discoidin expression (see Fig. 3 in INTRODUCTION). Thus the gdt1 signalling component is most likely involved in multiple events.

There is also evidence that the regulation by bacteria is affected in the gdt1 mutants. This is observed in the L8 mutant, which displays a normal growth in axenic medium but grows much slower than the wild type Ax2 strain when grown in bacteria (Table 2). However, the potential bacterial ligand for gdt1 is not yet discovered. Folate is a good candidate since it is a repressing signalling molecule for *Dictyostelium* and is secreted by bacteria. Furthermore, the response to folate is impaired in L8 (Fig. 30). Folate is, however, not the main repressing signal: autoclaved bacteria (where folate was destroyed) were used for culturing Ax2 cells and the same repression of discoidin was obtained (Clarke and Gomer, 1995). This suggests that the bacterial signal molecule comes from the bacteria themselves, most likely from the bacterial cell wall or the surface-glycoproteins. Probably, bacteria adhere to *Dictyostelium* cells at specific binding sites and thus generate intracellular signalling pathways which finally repress the onset of development. Bozzaro and co-workers have shown that linked-glucosides could repress development in *Dictyostelium* (Gambino *et al.,* 1992), supporting the “bacteria-

repression" hypothesis. Since the bacterial sugars are the main contents of the bacterial cell wall, the regulatory capacity of glucoside was tested for the L8 mutant (Fig. 24). However, the L8 cells failed to form any multicellular morphology on both glucoside-polyacrylamide and the control hexanol-polyacrylamide gel, suggesting that the defect in the L8 mutant was not related to sugar binding. Further analysis of the L8 mutant is performed at the Bozzaro lab, to test whether the abnormal morphogenesis is a direct consequence of substratum binding, and to identify the specific ligand for gdt1 protein.

Slower growth in bacterial suspension culture and the wide spreading of cells on a bacterial lawn also suggested that gdt1 mutants could be impaired in phagocytosis. This was, however, not the case: L8 cells rather displayed better phagocytosis in the standard yeast cell assay (Maniak *et al*., 1995) than wild type cells (Maniak, personal communication).

9. gdt1 and PKA function in two parallel but interacting pathways

The cAMP dependent protein kinase (PKA) is known to influence discoidin expression: inactivation of PKA blocks morphological development (Mann *et al.*, 1992; Simon *et al.*, 1989; Harwood *et al.*, 1992) and leads to strongly reduced discoidin expression in wild type strains (Fig. 26). Furthermore, the Gα2$^-$ mutant has a similar phenotype as both the PKA C$^-$ and PKA Rm mutants (Burding and Clarke, 1995; Endl *et al.* 1996). Thus the PKA pathway activates discoidin expression and any lesion in this positive pathway will lead to a block in morphological development and complete loss of discoidin.

In order to establish the relationship between the gdt1 pathway and the signalling chain of PKA, double mutants (Gα2$^-$/L8, PKA C$^-$/L8 and Rm/L8) were investigated. The result of these experiments were complex but suggested a regulatory network of two parallel signalling cascades:

(1) In both PKA C$^-$/L8 and Rm/L8 mutant strains, discoidin I was remarkably accumulated during growth, i.e., when wild type cells did not express any detectable discoidin. This showed that the gdt1 mutant phenotype was detectable in a functional PKA$^-$ background and would argue for a dominant function of the gdt1 pathway on discoidin regulation. Furthermore, since the double mutants still maintained the aggregation deficient phenotype of the PKA$^-$ parents, the gdt1 pathway was clearly not involved in the later developmental process.

(2) Although discoidin I was still overexpressed in both the PKA C$^-$/L8 and the Rm/L8 strain in comparison to wild type cells, the amount of the protein was reduced in comparison to the L8 single mutant strain. This would argue for an independent effect of PKA on the regulation of discoidin expression, which should be in parallel with the gdt1 pathway.

(3) Complete knock-out of the PKA activity (the PKA C$^-$ mutant) resulted in a complete loss of the discoidin expression (Fig. 26). Even in stationary cells (i.e. at cell density of $5x10^6$) no discoidin expression was detected. This would suggest an absolute requirement of PKA for discoidin expression: either PKA is downstream of gdt1 in the gdt1 repressing pathway, or the inducing pathway via PKA is dominant. However, both assumption cannot be true, since the phenotype of the double mutants (PKA C$^-$/L8 and Rm/L8) clearly demonstrated that the gdt1 and PKA pathways must be in parallel, and that the gdt1 pathway was dominant on discoidin regulation.

(4) Taken together, the only explanation for the mis-expression pattern of discoidin in all the mutants would be that gdt1 and PKA function in two parallel pathways but interact with each other: the gdt1 pathway plays a dominant role during growth, but is negatively regulated by PKA at the growth-differentiation-transition (GDT). Thus, knocking out PKA in wild type cells will result in a constantly active gdt1 protein and no discoidin expression, whereas in the L8 mutant the repressing pathway of gdt1 is completely lost and the PKA pathway is active. In the double mutant, disruption of gdt1 allows discoidin expression during growth but the induction by PKA is missing. Therefore an intermediate phenotype is obtained. This interpretation was supported by the experiment with the specific inhibitor of PKA, H89 (Fig. 27): when H89 was added to L8 cells at the onset of development, it did not result in any change of the discoidin expression. The data suggest different contributions of PKA to discoidin induction in growing and stationary phase cells, which may be explained by the involvement of PKA in two parallel pathways (both gdt1 and PKA pathways) leading to discoidin regulation.

(5) The observation that all L8, Rm/L8 and PKA C$^-$/L8 mutants displayed cell density dependent accumulation of discoidin argued for a third pathway which was independent of both the gdt1 and the PKA pathway. Cell density dependent expression of discoidin during growth could also be shown in wild type cells, when sensitive reporter genes were used (Wetterauer *et al.*, 1993). The fact that the L8 mutant also overexpressed discoidin in axenic medium supports the assumption that this third pathway is independent of gdt1 and that it is generated by factor(s) secreted from *Dictyostelium* cells. There are two factors, PSF (prestarvation factor) which is continuously secreted by *Dictyostelium* cells to measure the food source/cell density ratio, and a second factor CMF (conditioned medium factor) which is

released upon starvation and activates the developmental process. Surprisingly, the CMF knock-out mutant ΔCMF showed a normal pattern of discoidin expression (Fig. 7) but the morphological development was blocked. This argues that CMF is not involved in the regulation of discoidin, but only in morphological development. Thus the PSF factor remains to be the candidate for the third pathway which senses cell density and leads to induction of discoidin expression.

The data demonstrate that discoidin transcription is regulated by different signalling pathways. In fact, this has been suggested previously by the identification of multiple regulatory elements in the discoidin Iγ promoter (Vauti *et al*., 1990). The interpretation of all the data is summarized in figure 34. At least four pathways contribute to discoidin I regulation (Fig. 34):

I. A positive cell density control: independent of gdt1 and PKA (see Fig. 7, 26).

II. A negative gdt1 control: regulated in part by PKA and cell density (see Fig. 21, 26).

III. The identified positive Gα2-CRAC-ACX-PKA pathway (ACX is a newly identified adenylyl cyclase, Schaap and Gross, personal communication): feeding into gdt1 control by interaction of gdt1; indirectly required for cell density control in the presence of gdt1 (see Fig. 26); and leading to morphological development.

IV. The known negative pathway via extracellular cAMP /Ca^{2+} influx signalling: repressing pathway in development (Endl *et al.*, 1996); also leading to morphological development.

Pathway II is mostly dominant and acts via precocious activation of gdt1, but does not contribute to morphological development. Pathway III is required for morphological development and at the same time induces discoidin expression during early development. This may in part occur by phosphorylation (inactivation) of gdt1 in pathway II by PKA. Consequently, a PKA minus mutant could not inactivate the gdt1 kinase and should display a discoidin null phenotype. Though this is in fact the case, the level of discoidin expression in PKA^-/L8 double mutants clearly shows that PKA is involved in an additional pathway regulating discoidin expression. Apparently, gdt1 has no negative influence on morphological development, therefore the gdt1 disruption could not rescue the morphological phenotype ($aggregation^-$) of mutants in pathway III (Ga2-CRAC-PKA). On the other hand, it could rescue the discoidin null expression in a PKA^- mutant and even result in discoidin overexpression compared to wild type cells. However, disruption of gdt1 in a PKA^-

background did not lead to discoidin expression levels as found in the L8 mutant. This clearly showed that gdt1 was not simply a downstream component of the PKA pathway (pathway III).

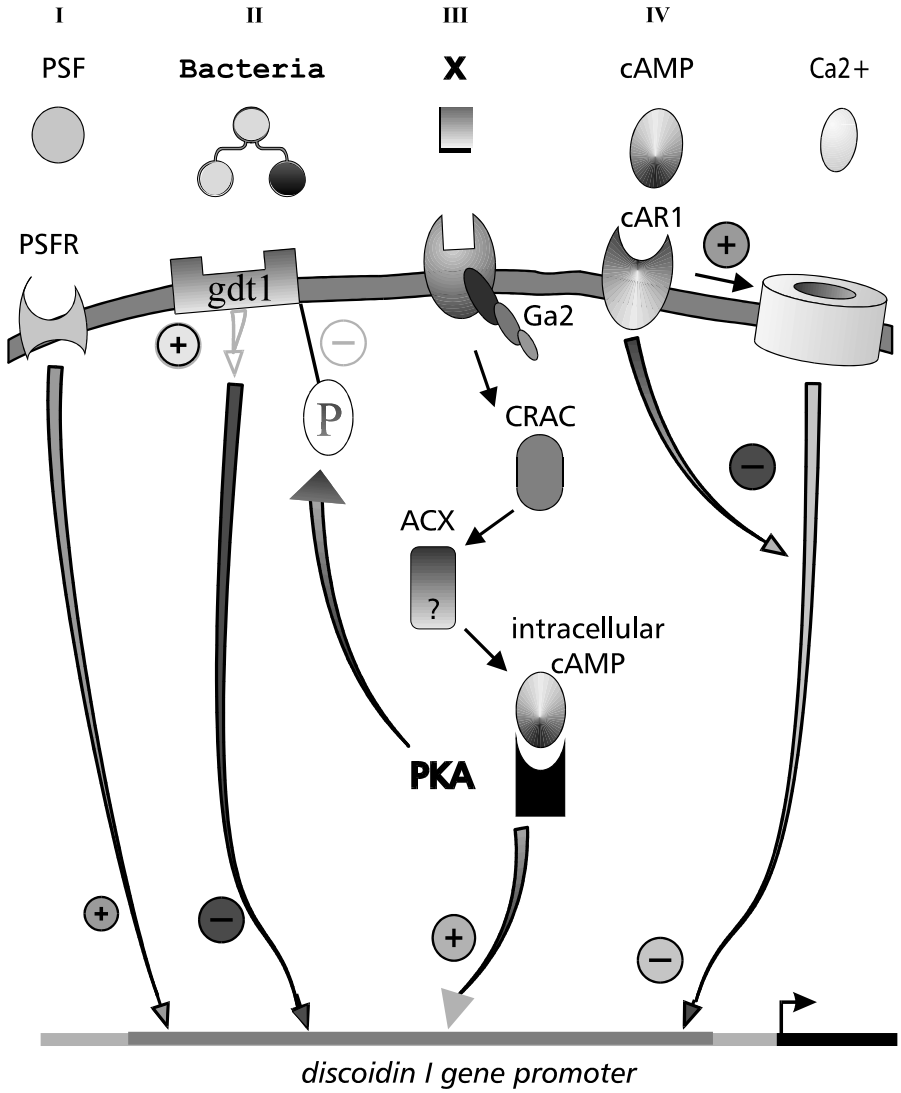


Figure 34. Regulation of discoidin transcription. See text for description.

The pathway IV from cAMP and Ca^{2+} remains unclear. So far it is known that the cARI receptor is involved in this signalling and generates repression on discoidin transcription during development. The result that the L8 mutant responded to cAMP showed that this pathway was independent of gdt1 (Figure 30). L8 did, however, not respond to folate, suggesting that it was also involved in this pathway. It has been found so far that Gα4, Gβ (Blusch and Nellen, 1994) and ERK2 (Maeda *et al.*, 1997) are necessary components in the folate response. Thus it is suggested that the gdt1 pathway connects to a common component in the folate signalling cascade. Since both gdt1 and ERK2 are protein kinases, the common

component is most likely regulated by phosphorylation through either of the kinases. However, since folate is not the main repressing signal from bacteria, this pathway is not indicated in the model.

The assumption that PKA regulates gdt1 was shown by in vitro phosphorylation assays. Both the recombinant D1 and D2 contain a putative PKA target site but only D1 could be phosphorylated by PKA in vitro (see Fig. 28). However, since the second PKA site "KHRKRLSQ" is directly after the very hydrophobic transmembrane domain TM4 (Fig. 15b), it could be protected from phosphorylation *in vitro*.

10. Future experiments to unravel events downstream of gdt1

Interestingly, the "KHRKR" sequence inside the second putative PKA site is a conserved basic loop which can serve as a NLS (nuclear localisation signal) for many nuclear proteins (Soullam and Worman 1993; Moroianu *et al.*, 1996). However, all data so far showed that gdt1 is a membrane-associated protein, and there is no indication for cleavage on a tyrosine receptor kinases or transport of the kinase domain into the nucleus. The JAK kinase family (see section 6 in INTRODUCTION) consists of intracellular protein kinases which are associated with cytokine receptors. Upon ligand binding, the JAKs autophosphorylate and are transported to the nucleus. There they further phosphorylate and activate STAT transcription factors which finally induce transcription of multiple targets. Interestingly, a family of STAT homologues was recently cloned from *Dictyostelium* and was shown to be expressed in vegetative growth and to bind to DNA elements upon tyrosine phosphorylation (Kawata *et al.*, 1997). Though there is so far no precedent for such a mechanism, it would be interesting to see if the gdt1 kinase domain is transferred to the nucleus. This would require an antibody against the kinase domain which is not yet available.

Recently, a new REMI mutant has been isolated in B.Wetterauer's lab in LMU München which has a similar phenotype as L8. The affected gene was isolated and identified as a metallo-protease (Wetterauer, personal communication). Though no direct experiment has been done, it would be interesting to investigate if this protease may be involved in the cleavage of the *gdt1* gene product.

Since the similarity of gdt1 with other receptor tyrosine kinases is low, the function of gdt1 still needs further proof. The experimental approach to use the membrane fraction for

autophosphorylation assays is difficult because of the extreme hydrophobic conditions. There is so far no technique to solve this problem. A good solution would be to express the kinase domain as a recombinant protein and perform a phosphorylation assays. The overexpression of the kinase domain in *Dictyostelium* should result in a permanently active kinase which requires no induction by bacteria and could not be down-regulated by PKA. Consequently, one would expect a discoidin null phenotype but no effect on morphological development (except for a potential delay in the onset of development). Immunofluorescence assays should also be performed to detect the localization of this gdt1 kinase and answer the question of potential nuclear translocation.

11. Conclusions

The REMI technique and the use of discoidin as a marker for molecular analysis of the GDT has proven to be successful: a screen reveals signal transduction components which influence the transition from growth to differentiation, and displays the expected phenotypes in early development, i.e., premature aggregation in the L8 mutant. The cloned *gdt1* gene is likely to belong to the receptor tyrosine kinase family. Receptor tyrosine kinases are supposed to have arisen during evolution with the appearance of multicellular organisms; no true tyrosine kinase was found in the completely sequenced yeast genome (Darnell, 1997). The function of receptor tyrosine kinases has been defined as a transducer of intercellular communication, which regulates cell proliferation and differentiation. gdt1 perfectly fulfils this definition since it is a negative regulator of the growth-differentiation-transition in *Dictyostelium*. The presented data confirm another previous suggestion that the first step of differentiation, can occur in the absence of any visible morphological development (Endl *et al*., 1996). Further analysis of the gdt1 mutants and other mutants will reveal new components involved in the cellular decision between growth and differentiation. Since *Dictyostelium* is a facultative multicellular organism, the gdt1 signalling pathway could therefore present new signalling components relevant for higher eukaryotes.

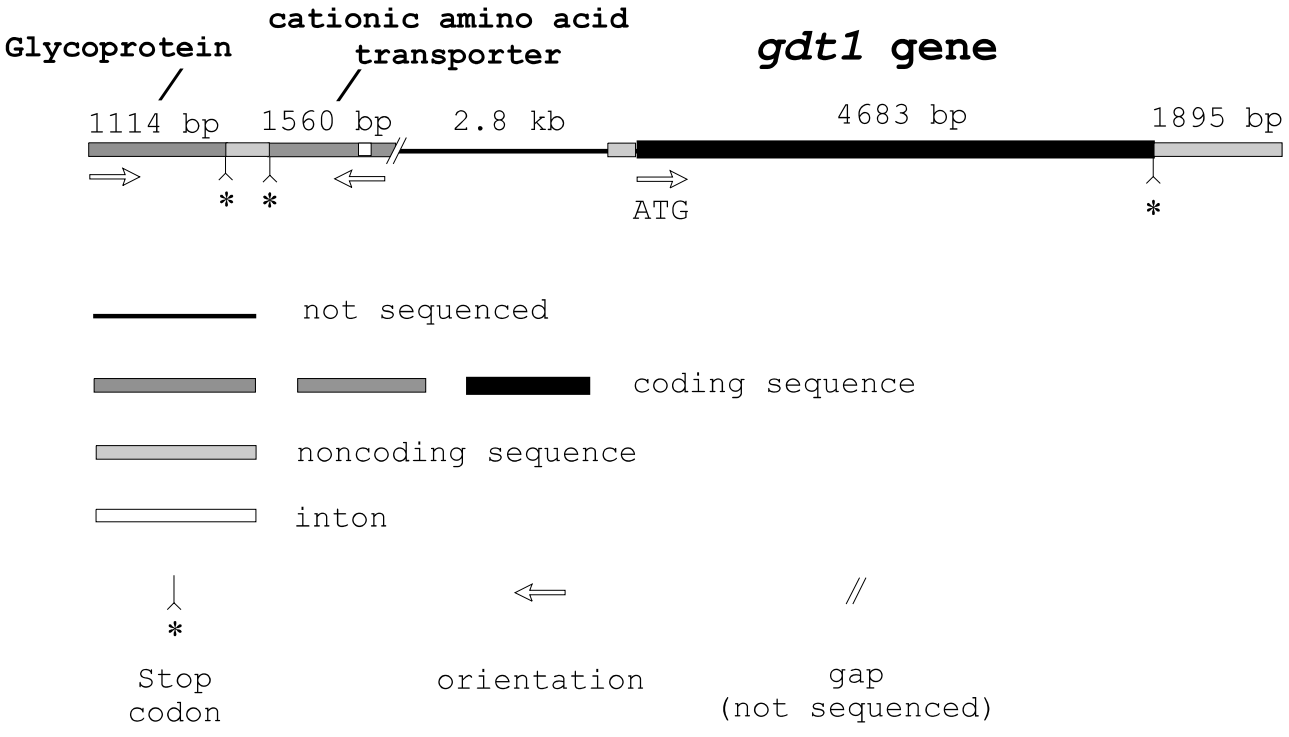
VI APPENDIX

Sequence of the *gdt1* gene

Nearly 12 kb genomic sequence have been isolated on the way to clone the *gdt1* gene. The sequences were separated into two parts, with a possible gap of about 0.5 kb resulting from spontaneous deletion during the REMI mutagenesis. The right part contains the 3' terminus of an unknown glycoprotein (ORF in the same direction as *gdt1*) and the 3' terminus of a cationic amino acid transporter homologue with an ORF opposite to *gdt1*. This 2943 bp fragment was completely sequenced and the sequence was submitted to the Dicty data base. The large left part is a 9.2 kb genomic structure containing the locus of the *gdt1* gene. However, the sequence was not completed for this part, about 2.5 kb of the upstream sequence before the ″ATG″ (at 114 bp) of the *gdt1* gene is still missing. The identified sequence includes the complete coding region of the *gdt1* gene (a continuous open reading frame for 4683 bp) and 1895 bp non-coding sequence after the stop codon ″TAA″ (at 4796 bp). This 6691 bp sequence was submitted to the EMBL database (accession number AJ000992).

Appendix 1.

Genomic structure of the *gdt1* gene locus



Appendix 2.

The 2949 bp sequence from the upstream genomic structure of the *gdt1* gene
(Only the ORF is shown. The coding sequences are marked in bold letters)

the unknown glycoprotein → ORF

```
   ATCGATTATTTAAATGTTTATATTAATGGTAATTTTACAAATGAAAATACTGGTGGTGGT
 1 ---------+---------+---------+---------+---------+---------+ 60
   TAGCTAATAAATTTACAAATATAATTACCATTAAAATGTTTACTTTTATGACCACCACCA

   I  D  Y  L  N  V  Y  I  N  G  N  F  T  N  E  N  T  G  G  G  -

   AATGGTAAAAAGAATGAAAATATGATAATCAATTTCAATGGTAATGAAATCAAGGATTAT
61 ---------+---------+---------+---------+---------+---------+ 120
   TTACCATTTTTCTTACTTTTATACTATTAGTTAAAGTTACCATTACTTTAGTTCCTAATA

   N  G  K  K  N  E  N  M  I  I  N  F  N  G  N  E  I  K  D  Y  -

   AAATATCAACATGATGCTAAAAATGGTTCAATCAGTACAGATTTAATTTTATTTAGTTTA
121 ---------+---------+---------+---------+---------+---------+ 180
   TTTATAGTTGTACTACGATTTTTACCAAGTTAGTCATGTCTAAATTAAAATAAATCAAAT

   K  Y  Q  H  D  A  K  N  G  S  I  S  T  D  L  I  L  F  S  L  -

   TATGGTGATAATATTAAATCTGGTAATGTTTCAATCACCTATGGTAATGGTTTAAAGAGT
181 ---------+---------+---------+---------+---------+---------+ 240
   ATACCACTATTATAATTTAGACCATTACAAAGTTAGTGGATACCATTACCAAATTTCTCA

   Y  G  D  N  I  K  S  G  N  V  S  I  T  Y  G  N  G  L  K  S  -

   AATGAAATGTCATTCCAATTGAAATCATTTGTATCTAGAATTAAAAAACCAAATACAACT
241 ---------+---------+---------+---------+---------+---------+ 300
   TTACTTTACAGTAAGGTTAACTTTAGTAAACATAGATCTTAATTTTTTGGTTTATGTTGA

   N  E  M  S  F  Q  L  K  S  F  V  S  R  I  K  K  P  N  T  T  -

   GGTGGTTTATTAGAATTAATTGGTAATTTTTATTTTTTAAATGATTCAAGTAGAGTTAAT
301 ---------+---------+---------+---------+---------+---------+ 360
   CCACCAAATAATCTTAATTAACCATTAAAAATAAAAAATTTACTAAGTTCATCTCAATTA

   G  G  L  L  E  L  I  G  N  F  Y  F  L  N  D  S  S  R  V  N  -

   TCTAAAGTTTTCATTACCACTTCAAAACAACAACAACAACACCAACAACAAGAAAAAAAA
361 ---------+---------+---------+---------+---------+---------+ 420
   AGATTTCAAAAGTAATGGTGAAGTTTTGTTGTTGTTGTTGTGGTTGTTGTTCTTTTTTTT

   S  K  V  F  I  T  T  S  K  Q  Q  Q  Q  H  Q  Q  Q  E  K  K  -

   GAAGAATGTCAAATTATATCAATTGATAATTCAAAAATTAAATGCTTTATTGATTCAAAT
421 ---------+---------+---------+---------+---------+---------+ 480
   CTTCTTACAGTTTAATATAGTTAACTATTAAGTTTTTAATTTACGAAATAACTAAGTTTA

   E  E  C  Q  I  I  S  I  D  N  S  K  I  K  C  F  I  D  S  N  -
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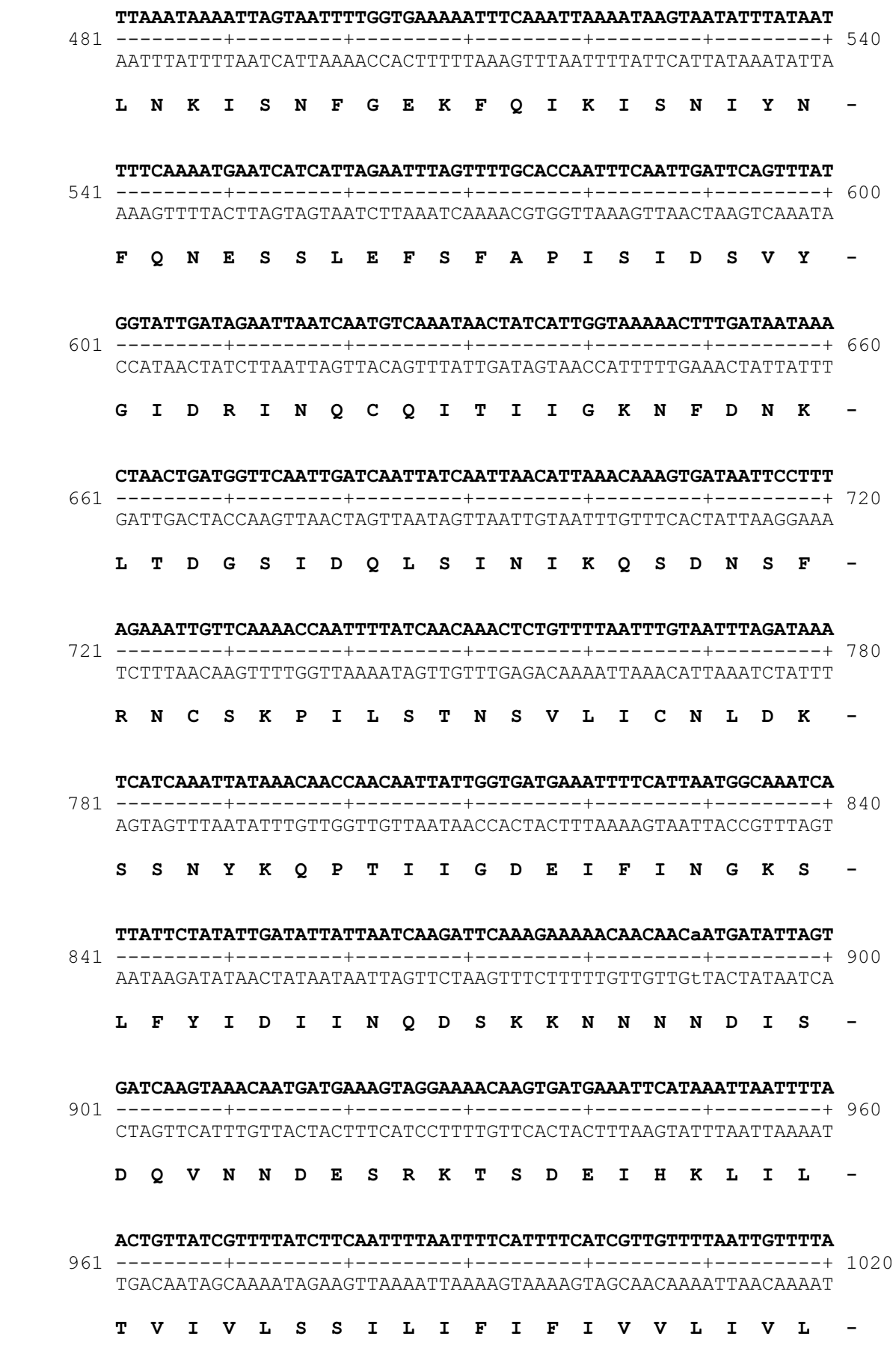
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L N K I S N F G E K F Q I K I S N I Y N -
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541 ---------+---------+---------+---------+---------+---------+ 600
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F Q N E S S L E F S F A P I S I D S V Y -
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601 ---------+---------+---------+---------+---------+---------+ 660
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G I D R I N Q C Q I T I I G K N F D N K -
CTAACTGATGGTTCAATTGATCAATTATCAATTAACATTAAACAAAGTGATAATTCCTTT
661 ---------+---------+---------+---------+---------+---------+ 720
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L T D G S I D Q L S I N I K Q S D N S F -
AGAAATTGTTCAAAACCAATTTTATCAACAAACTCTGTTTTAATTTGTAATTTAGATAAA
721 ---------+---------+---------+---------+---------+---------+ 780
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R N C S K P I L S T N S V L I C N L D K -
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781 ---------+---------+---------+---------+---------+---------+ 840
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S S N Y K Q P T I I G D E I F I N G K S -
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841 ---------+---------+---------+---------+---------+---------+ 900
AATAAGATATAACTATAATAATTAGTTCTAAGTTTCTTTTTGTTGTTGtTACTATAATCA
L F Y I D I I N Q D S K K N N N N D I S -
GATCAAGTAAACAATGATGAAAGTAGGAAAACAAGTGATGAAATTCATAAATTAATTTTA
901 ---------+---------+---------+---------+---------+---------+ 960
CTAGTTCATTTGTTACTACTTTCATCCTTTTGTTCACTACTTTAAGTATTTAATTAAAAT
D Q V N N D E S R K T S D E I H K L I L -
ACTGTTATCGTTTTATCTTCAATTTTAATTTTCATTTTCATCGTTGTTTTAATTGTTTTA
961 ---------+---------+---------+---------+---------+---------+ 1020
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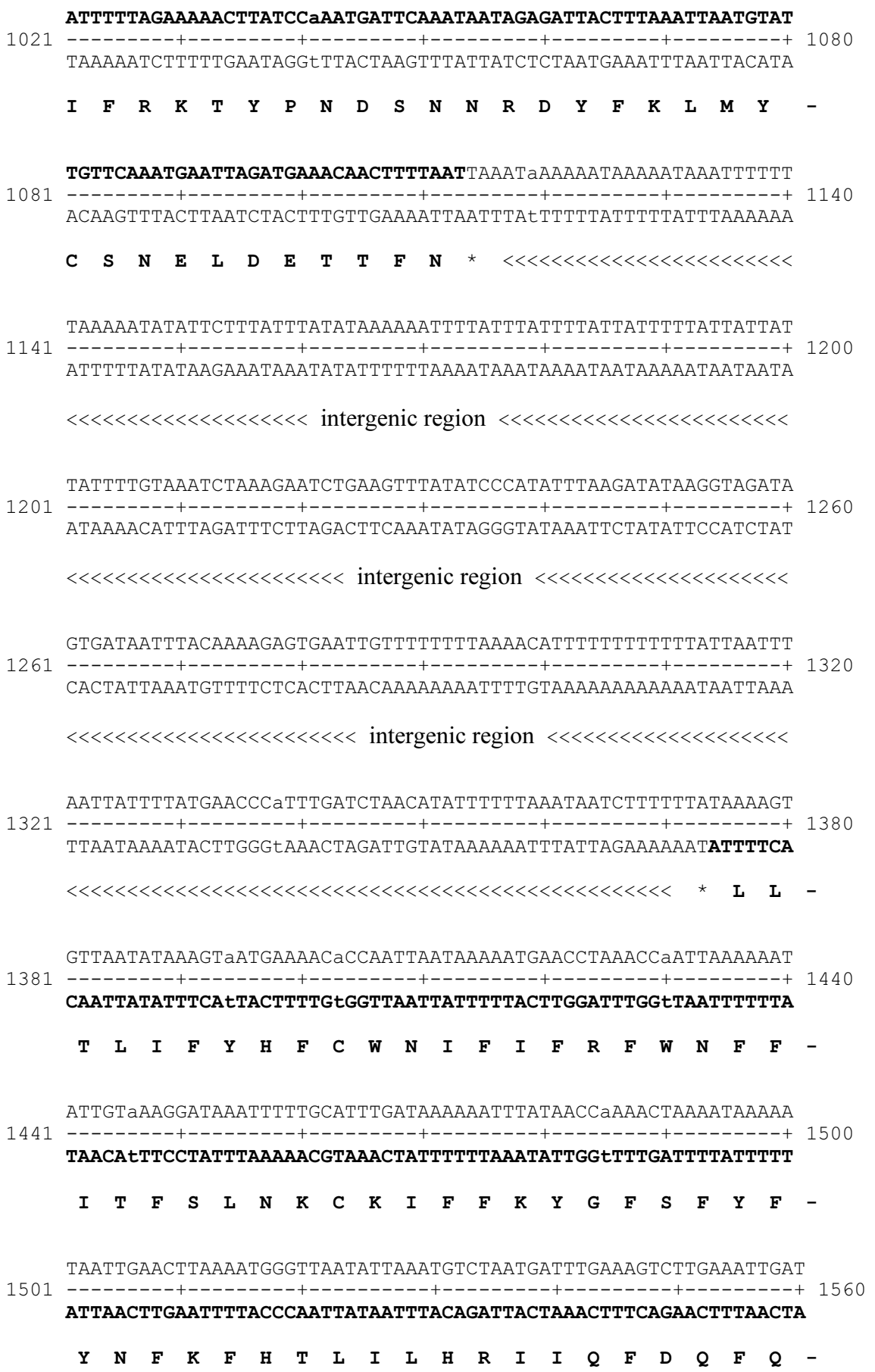
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I F R K T Y P N D S N N R D Y F K L M Y -
TGTTCAAATGAATTAGATGAAACAACTTTTAATTAAATaAAAAATAAAAATAAATTTTTT
1081 ---------+---------+---------+---------+---------+---------+ 1140
ACAAGTTTACTTAATCTACTTTGTTGAAAATTAATTTAtTTTTTATTTTTATTTAAAAAA
C S N E L D E T T F N * <<<<<<<<<<<<<<<<<<<<<<<<
TAAAAATATATTCTTTATTTATATAAAAAATTTTATTTATTTTATTATTTTTATTATTAT
1141 ---------+---------+---------+---------+---------+---------+ 1200
ATTTTTATATAAGAAATAAATATATTTTTTAAAATAAATAAAATAATAAAAATAATAATA
<<<<<<<<<<<<<<<<<<<< intergenic region <<<<<<<<<<<<<<<<<<<<<<<<
TATTTTGTAAATCTAAAGAATCTGAAGTTTATATCCCATATTTAAGATATAAGGTAGATA
1201 ---------+---------+---------+---------+---------+---------+ 1260
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<<<<<<<<<<<<<<<<<<<<<<<< intergenic region <<<<<<<<<<<<<<<<<<<<<<
GTGATAATTTACAAAAGAGTGAATTGTTTTTTTTAAAACATTTTTTTTTTTTTATTAATTT
1261 ---------+---------+---------+---------+---------+---------+ 1320
CACTATTAAATGTTTTCTCACTTAACAAAAAAAATTTTGTAAAAAAAAAAAATAATTAAA
<<<<<<<<<<<<<<<<<<<<<<<<<< intergenic region <<<<<<<<<<<<<<<<<<<<
AATTATTTTATGAACCCaTTTGATCTAACATATTTTTTAAATAATCTTTTTTATAAAAGT
1321 ---------+---------+---------+---------+---------+---------+ 1380
TTAATAAAATACTTGGGtAAACTAGATTGTATAAAAAATTTATTAGAAAAAATATTTTCA
<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<< * L L -
GTTAATATAAAGTaATGAAAACaCCAATTAATAAAAATGAACCTAAACCaATTAAAAAAT
1381 ---------+---------+---------+---------+---------+---------+ 1440
CAATTATATTTCAtTACTTTTGtGGTTAATTATTTTTACTTGGATTTGGtTAATTTTTTA
T L I F Y H F C W N I F I F R F W N F F -
ATTGTaAAGGATAAATTTTTGCATTTGATAAAAAATTTATAACCaAAACTAAAATAAAAA
1441 ---------+---------+---------+---------+---------+---------+ 1500
TAACAtTTCCTATTTAAAAACGTAAACTATTTTTTAAATATTGGtTTTGATTTTATTTTT
I T F S L N K C K I F F K Y G F S F Y F -
TAATTGAACTTAAAATGGGTTAATATTAAATGTCTAATGATTTGAAAGTCTTGAAATTGAT
1501 ---------+---------+---------+---------+---------+---------+ 1560
ATTAACTTGAATTTTACCCAATTATAATTTACAGATTACTAAACTTTCAGAACTTTAACTA
Y N F K F H T L I L H R I I Q F D Q F Q -

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1561 ---------+---------+---------+---------+---------+---------+ 1620
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     N  L  R  V  Y  R  I  V  A  I  N  T  I  I  F  I  I  I  I  P  -

     TTGTACCTGTAAAACTTAAATAATTATAAACTTCCCAACAAGATTTTGCATAAATATTTT
1621 ---------+---------+---------+---------+---------+---------+ 1680
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     I  T  G  T  F  S  L  Y  N  Y  V  E  W  C  S  K  A  Y  I  N  -

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1681 ---------+---------+---------+---------+---------+---------+ 1740
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     K  T  S  G  V  I  L  G  I  M  L  I  I  F  G  I  L  S  I  I  -

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1741 ---------+---------+---------+---------+---------+---------+ 1800
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     A  F  H  P  T  K  F  R  K  N  T  K  G  L  N  S  I  V  G  S  -

     CTTTAGTTTTACCAAACGAATAAATTATTCTAAAAACAATATTAAACCAACTACTAACTA
1801 ---------+---------+---------+---------+---------+---------+ 1860
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     E  K  T  K  G  F  S  Y  I  I  R  F  V  I  N  F  W  S  S  V  -

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1861 ---------+---------+---------+---------+---------+---------+ 1920
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     V  G  F  G  S  V  V  G  A  I  D  V  I  I  S  F  W  Y  G  V  -

     ATCTATTTGCATATAGTTGAATGGGTGATTGATTAACATTTATTGAAAATATATTTGAAG
1921 ---------+---------+---------+---------+---------+---------+ 1980
     TAGATAAACGTATATCAACTTACCCACTAACTAATTGTAAATAACTTTTATATAAACTTC

     F  R  N  A  Y  L  Q  I  P  S  Q  N  V  N  I  S  F  I  N  S  -

     GTCCAACAGCAACAACCAATGAATACATTGCCCATATAAACCAAGTTGCAGCAAATGCAA
1981 ---------+---------+---------+---------+---------+---------+ 2040
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     P  G  V  A  V  V  L  S  Y  M  A  W  I  F  W  T  A  A  F  A  -

     TTGAACCATAAACTGCAATTGGTATATTCTTTTTTGGATTTCTAGTTTCTTGACCTAACG
2041 ---------+---------+---------+---------+---------+---------+ 2100
     AACTTGGTATTTGACGTTAACCATATAAGAAAAAACCTAAAGATCAAAGAACTGGATTGC

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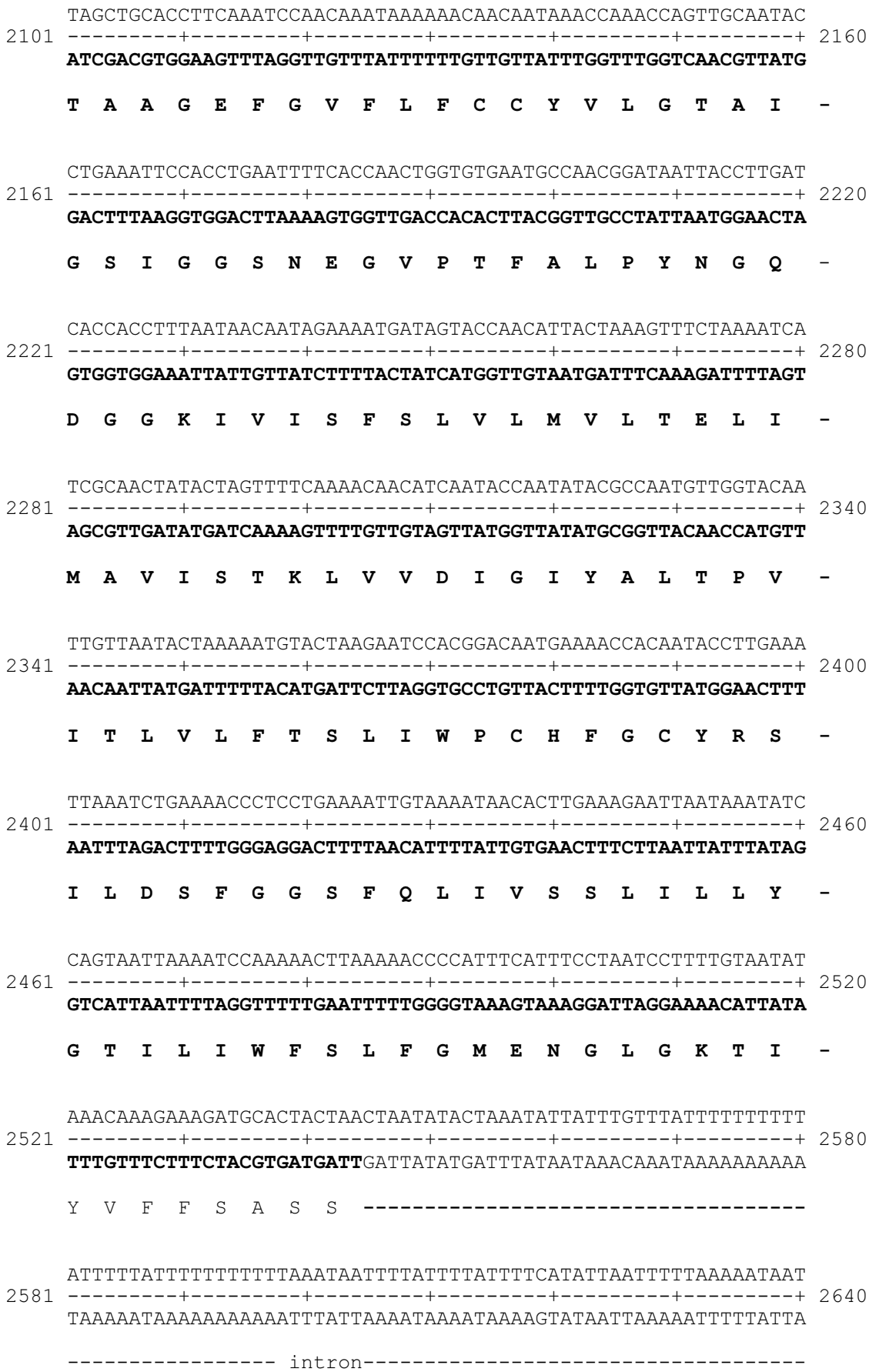
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T A A G E F G V F L F C C Y V L G T A I -
CTGAAATTCCACCTGAATTTTCACCAACTGGTGTGAATGCCAACGGATAATTACCTTGAT
2161 ---------+---------+---------+---------+---------+---------+ 2220
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G S I G G S N E G V P T F A L P Y N G Q -
CACCACCTTTAATAACAATAGAAAATGATAGTACCAACATTACTAAAGTTTCTAAAATCA
2221 ---------+---------+---------+---------+---------+---------+ 2280
GTGGTGGAAATTATTGTTATCTTTTACTATCATGGTTGTAATGATTTCAAAGATTTTAGT
D G G K I V I S F S L V L M V L T E L I -
TCGCAACTATACTAGTTTTCAAAACAACATCAATACCAATATACGCCAATGTTGGTACAA
2281 ---------+---------+---------+---------+---------+---------+ 2340
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M A V I S T K L V V D I G I Y A L T P V -
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2341 ---------+---------+---------+---------+---------+---------+ 2400
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I T L V L F T S L I W P C H F G C Y R S -
TTAAATCTGAAAACCCTCCTGAAAATTGTAAAATAACACTTGAAAGAATTAATAAATATC
2401 ---------+---------+---------+---------+---------+---------+ 2460
AATTTAGACTTTTGGGAGGACTTTTAACATTTTATTGTGAACTTTCTTAATTATTTATAG
I L D S F G G S F Q L I V S S L I L L Y -
CAGTAATTAAAATCCAAAAACTTAAAAACCCCATTTCATTTCCTAATCCTTTTGTAATAT
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G T I L I W F S L F G M E N G L G K T I -
AAACAAAGAAAGATGCACTACTAACTAATATACTAAATATTATTTGTTTATTTTTTTTTT
2521 ---------+---------+---------+---------+---------+---------+ 2580
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Y V F F S A S S ----------
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2581 ---------+---------+---------+---------+---------+---------+ 2640
TAAAAATAAAAAAAAAAATTTATTAAAATAAAATAAAAGTATAATTAAAAATTTTTATTA
---------- intron----------

```
    ACTACATACAGTTCCAAATATACTAATTGTATTTGCAATTGATAAACAACATAATGTACC
2641 ---------+---------+---------+---------+---------+---------+ 2700
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    --   C V T G F I S I T N A I S L C C L T G -

    AATAAAAACTGTAAATGGTACACTCGTACCTGCTGATGCTGCAATATTTGGAAAAACAAA
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      I F V T F P V S T G A S A A I N P F V F -

    AAAAGTTGCTGCACTTAATCCAATACCACCAACTGATAATGCAATACATTCAATTACACC
2761 ---------+---------+---------+---------+---------+---------+ 2820
    TTTTCAACGACGTGAATTAGGTTATGGTGGTTGACTATTACGTTATGTAAGTTAATGTGG

      F T A A S L G I G G V S L A I C E I V G -

    AATAGTATTTTTTTTTAATGTATTAAATTCATCATCAACTGTTATTCCACTAGTACAACT
2821 ---------+---------+---------+---------+---------+---------+ 2880
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      I T N K K L T N F E D D V T I G S T C S -

    ATCAAAACTTGAATTTGTACTTAAATCACTACTTTTACAATTTATTTTTTCTAATTCCAT
2881 ---------+---------+---------+---------+---------+---------+ 2940
    TAGTTTTGAACTTAAACATGAATTTAGTGATGAAAATGTTAAATAAAAAAGATTAAGGTA

      D F S S N T S L D S S K C N I K E L E M -

    ACTGGATCC
2941 ---------+---------+---------+---------+---------+---------+ 3000
    TGACCTAGG
      S S G-
```

ORF ← **the cationic amino acid transporter**

Appendix 3.

The 6691 bp sequence containing the complete coding region of the *gdt1* gene
(the complete coding sequence for the *gdt1* gene is shown is black and other noncoding sequences are shown in grey. All the primers used for analysis are indicated in the sequence. The start codon ATG, the stop codon TAA and the putative polyA signal AATAAA for *gdt1* mRNA are indicated in bold letters.)

```
1    AAAAAAAAAA CTATGAAGGT TCAAAATAAC TAATCAAAAA AAAAAAAAAA AAAAAAAAAA

                     Primer 183 ⟶TG ATAATTATTA AATAGGGAC
61   AAAAAAAAAA AAAAAAAAAA AAAAAAAATG ATAATTATTA AATAGGGACA AAA**ATG**AAAT

121  ACAGAGTTGG TAATGGTGGA ATACCAATTT ATAAAATTAA TGTATTATGT GTTGAAGGAA

181  CATTAGAAAT TCCAGAAGGT ATATCATTTT TTAATGTTGG AGCTTTATTT ATATTACCAG

241  GTGGTGTATT AAATAGTAAA TCAAGTATCA GATTTACAGA TTTAGATCCT TATAATTCAA

                       Primer 5′-FPC ⟶(TTCATA) GGGAGGATCA TTATCATTG
301  AAATGGATCC ATTTAATTTC TTTCCAGGTA TGATGGTTTT GGGAGGATCA TTATCATTGA
                                                CCCTCCTAGT AATAGTAACT

361  TTGGAGAGAA AAAAAGAATT TTCCAAGCCA CTAGAATTGA TGATTATCAA TTACAAATTG
     AACC⟵ Primer 166

421  AAGATTTCAA AAAAATAGGA TCATTGACAA ACAATATATA TTTAGGTTCA AAAGTTACAA

481  TTTATAGTCA ACAAATTTCA GAAGGTCAAA CATGTTCATT TTCATTTGGA GCAAGTAATG

541  ATAAAATTAA TTTAACATCA TCATCATCGT CATCATTAAG AGGAACCAAT TGTTTACCAA

601  TTTCAAAAAA TGATAAAAAT ATAATAGTTC ATTTTAATTG TAAATATTTA TTAGGTGGTT

661  CAAGTGAATC ATTTATAGAT TCTACATCGA CTGTTGGATC ATCTATATAT ATAACTGGTG

721  ATTCACAAGT TCAAATTGAA AATTTTACAT TGGATTCAAT AGGTAAAACC ACCAATAAAC

781  TTTATAATGA TACGAAATTA ATTTTCTCAA ATGATAAACC AAATCAAGTG ATCGATATAA

841  TAAAGGGTGA AAATCAAAGA TTTAGAAACT CATTGTATAT AGAATTTTCA AATAGTGTAG

                         Primer FPC-seq1 ⟶GAATC TAGAGCACCA CTCA
901  TTATAAAAGG TTGTGCTATC ATTGATAGAG TTAAAGAATC TAGAGCACCA CTCATTTTCG

961  TTAGTTCAAA TGTATCATTA TCAGAGAGTT TAATAGTTAG TAAATCAGGT TCAAATTTAA

1021 TCGCTCAATA TGGCACTGAA TTTATAAAAT CAAAATTAAA TCATTACTTT TTAATACCAC
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1141 ATTATGGATT TGAAGGTAAT GGAATCTATT CATTATCTCC AAATGTTCAA TCAATAAATG

1201 ATACTTTTAT TTCTCAATTG ATTGCTCTTA ATTTTAATTT CATTGGTAAT AGGTCCATTA
←Primer$_1$(3′) GTAACCATTA TCCAGGT

1261 TAACAGGTTT TGATAATGAT TGTTACTCAC CTTGTACAAA TACTTCAATT TTATTTTCAA

1321 ATTTAATTCA ATATCCTGTT GATTTTAAAA TTAATAATTC AACTTATTTT TATAAATTAA

1381 ATAATAATAA TAATGATAAT AATAATAATA CAAACAATTT TAACTTATTA AATATTAATA

1441 ATGAAAATAA TAATAATGAT GATAATAATA ATAATAATAA TTCACAAAAT CATTATCTTT

1501 TAAATATTAA TAATAATGGT AATTCACCAG GAAGATTTTT TACAATTAAA GATTTAGTAG

1561 CATCAGATAC AGTTTCAGCT TATTTACCAA ATAGTGCATT AGTATTTAAT AATTTAAATG

1621 CAACCGAAAA TTTTTCATTC AATGGTACAG TTTCAAGATT AGATTTTATA AATTCAAGTT

1681 TTAATACAAA CTTAACTTCA ACACAAAAGT TTTTTGATGA AACAACAACT ACAGTCACAA

1741 ACTTTCAAAA TACTTATATT TATGGGTCAC CAGAAACAAC TGAACCACCT GAAAAAGTAT

⟶TGGGTCATC GATTACTCCA Primer seq2
1801 TTGGGTCATC GATTACTCCA ATTTATTATT TTAGTAAAAG TGTTTTGGAT TCATTAAAAG

1861 TTGAAACTGT TTATCCAAAT GAACCTCATA AAATGTTTTT CAATTCTTCA GTTTCTCTTT

1921 CAGTTAAATT GAATAATATG GGTTTAAAAA CTCCAATCAT TTGTATTTCC TCATCCAATG

1981 GTATGGAATC AAAAGAGGTT CAATATAATC CAATCTCAAC AAAATGTAGT TTCCTTTATG

2041 TATCTGAAAA ATTAGGTAAT CATAATATTC GTGTTACAAT TAAAAATGAA TATTCTAAAG

2101 ATTCAAGTTA TTATTTTATA ATTGATTTTC CAATTATAAC TGTTTATGGA CAATCTTTAT

2161 TTAATGCTGG TTGGCAAATG ATTGATCCAA CATCAACAAC AATAATACCA ACTCCAACAC

2221 CAACATCGAC ATCAACATCA ACATCAACAT CAACACCAAC AACAACAAAT AATAATCAAT

2281 TAGTTAATGA TAATAATAAT AAAATAAATG AATTGGATGG TTTAAAATTT CAAGGTGGTT

2341 GTATAAAAAC TTTAGGTTGC CAACTTTCAA GTAATGCAAA ATATGTTGGT GGTTTACCAA

2401 ATGTTACAGC CTCATCAACA TTAAATAGTT TATTTTCTTG GGGAATTACA TCAGATGTTC

2461 AATATGATCC AGTTATAATT GATTTATTTA TTAATAAATC AATAGCAACT TTACAAGTTC

2521 AATTATTTTT CACTTTTTAT AAACCAATCG ATCAATATTC AAGTCCATTA TCTGTATCTA

Primer$_2$5′ ⟶ATGGAAC CATTCGCTGG CGA
2581 TTCAAAAAAA TCCAGTTTTA GTGATGGAAC CATTCGCTGG CGATCAACCT TTTTCAAAAA

2641 ATTTAACATT TGTTTATAAT AACACACAAT CACTCGATTT TTTAAATATT TCATTCACAA

2701 GTCGTGGTGA TATTTATTTA ACATCATTGG CAATCTTTTC AGTTAGTGAT TCATTACCTC

2761 AAATTATTGA TCCAATAACA CCAACCTTAT TACCCATTGA AAGTGTTAAA GCTAGTAAAC

2821 CAGCTATTTT GGCAATTGTT TTATCAATAG TTTTAGGATC TTTGGCTTTA TCAATCATTA

2881 CAATTTTAAT AGTTAAACAT AGAAAAAGAT TATCTCAATT CCTCTCAAAA TCAAATAAAG

2941 ATATTGAATA TGCCCAAAAT AATGAAATTG AAATTAAAGT TTTACCAAAA ATTACAAGTC

3001 ATTCATCTTA TCCATCAATA TCAATATTAG ATACAATATC ATCAGATTCA ATATTTAATA

3061 ATCAAATACC AAAAAATAAT AATAGATATA AATTTAAAAA TCAAAGTTTA AATAATAATA

3121 ATTATTTTAA TAATAATAAT AATAATAATA ATAGTAATAA TAATAATAGT AATAATATTA

3181 TTTATAGTAA TTGTAATAGT AATTATAGTA ATAGTAATAG TAATAATAAT AATAATAATA

3241 ATAATAGTAA CAGTAATAAT AATAGTAATA GTAATAGTAA TATTAATATT AATAGTAATA

3301 GTAATAGTAA TAGTAATAGT AACAGTAATG GTAATAATAA TTATCAAATT TATTCAAATA

3361 AATTAGAAAG TTTTAAAATT GATGAAATTA GTAATGATAC AATACCAATT ATAAATAGTA

3421 CATTTCCAGA TGAATTTCAA ACTTTAGAAT TTCAAAAATT AGCATTTGAA ATATTAAAAA

3481 GAGAAAAGAG ATTAGATTTT TCATTTAGAA CAACAAATGA TATATTGACA TGTTGCCGCC
ACTGT ACAACGGCGG

3541 ATTATCAGAT TTTAAAGATT TTTCCAAATT TCCCATTAAG GTTTAATCAA AGTATAATAA
T⟵ Primer$_2$3′

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3661 TTACAAATGA TTCAACAATA AGATTTACAG CATTTTTAAT TTTACCAATG GATAATCATT

3721 CAGCCACTTT TACATCAGAT CATTCATCAT TTGATCTTGG TCCAGGTGAA ACATTTTCAA

3781 TAAAATTTTC AATTACTTTA CATTGTACAA CAAGGTTTTT TGAAAATTTT TCAATTCAAA

Primer 167 ⟶AAAGTGAAT
3841 TCAATTCAAA TAATATTAAA GAAATGTATA CTCTACTCAA AATAAAGGTT GAAAGTGAAT

CCTCGACAAG
3901 CCTCGACAAG ATTGGATTTT AATGATATTC ATTTCCAAGA GTTGATAGAG AAATATTCAT

3961 GGGAGATATT ATATAGAGGA ACAGTTGGAG ATAAAAATGC TTTACTTAAA TTGATAAAGT

4021 TAAAAACAAA AAATTGTGAA GAAGCTTATA GAGAATTAAA TATTATTAGT AGATTAAAAC

4081 ATCAAAATAT TTTACCATTG ATTGGTTGTG TAATTTCAAA GGATTATTTA TGTTTAGCCT

4141 TTGAATATCC ACCCCTTGGA AGTTTGGATT ATATAATTTC AAAAAAGAAA CTAAAAATGT
AGG TGGGGAACCT TCAAACC⟵——— Primer 184

4201 CAATCACTCA AAAGATTAGA ATTCTTATTG ATGTTGCCAA AGGTTGTAAA TTCCTTCAAC

4261 AAAGTTCAAT CATTCAAAAA ACTCTAAGAG CTAGAAATAT TTTCCTTTAT GATACCAATG
Primer FPC_{BglII}⟶CCAATG

4321 AAAATGCAGA GGTTTGTGCT AAAGTATTAG ATCTAACATC AAGTAAAACT ATTAAAGGAT
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4381 TAGCTTGTAA TAATTATATA GAAAGAGTTG ATACACCAAT TAATTTAACC AGAGAAATTT

4441 CAATTATAAG AGATCCAAAA CAAAACAATG ATTTTAATAA CAGTAATAAT AGTAATAATA

4501 ATAATAATAA CAACAACAAT AATAATAATA ATAATAATAA TAATAGTAAT AATAGTAATA

4561 ATAGTAGTTC ACTAAAGTAT AATAATCATT CATTTGCAGT CTTATCTTAT GAATTACTAA

4621 TTGATGAAAT TTTAGTTGGT GATACTCGCA AATTTGGCCA AGAAAAACCA TCAATAGGTT

4681 TAGATAAAAT TGATCCAAAC ATTAAAAACT TTATTCATAA ATGTTGGAAT CCAATCGATG

4741 GTTTCACTTT TAATGAAATT TTAAAAACTT TAAAAGATTT TATTGAAAGT TTAAAT**TAA**A

4801 TTCTTTACAA TAATATGTTT TTTTAAAAAA TAAAAGTTTA ATTTATTTAT TTATCTTTTA

4861 ATTAATTATT ATTATTTTTA TTTATTTTTA TTGTTATCTA AGGTATTCTA AAAATATATT

4921 CTTGGGACCA TTTGTACTGA TGATAGAGTC CCAATCTGAA ATTGAAAAAG CATTTTTGAA

4981 ATTATTTATG TTATTTGTCT CTAAATTCTT GGAAATTTCA AAAAGTTGAT TTTCACCAAT

5041 TGGTAATTTA TTTACCCAAG TAACCAAATA GTTATTGAGA CCACTATCAA TAATTTGTTG

5101 TTTGGATAAA TTATTAAAAT TATTTATATC TTTAATAATT TCAAATTGGT TATTATTATA

5161 AATAATATTA TTATTTATAT TATTCGTATT GTTATTGTTA TTATTTTTAT TTTTATAAAT

5221 TATTAAAATT GAATTTCTTT TTTCATAATC CAAAAAATCA TTTCTATTAA TATGATATAT

5281 GTCCACTCGT ACATCTAATT GCTTTATTTT CAATTTGATT AATATGAATT GAACTTTTTT

5341 CACCATTTGA CATAATGATT GAATGTAATT GATTATTTAA TGGAATTGGA TTACCAATAA

5401 AACTAAAATC ACCAATACTT ATTTTTTCAA TTAAATTGAA TGATTGGAAT GAAATTTCAC

5461 CATTTTTTAC TCTATCAATA GAGATTAATA AATGTGATGG AATATTATTT AAAGATAGTC

5521 TTCCAATATT ATCTTTTTTA CAATTTTTAC AAAATTCAAT TTGACCAGTT TACTTGTATA

5581 AACTATTTTC AACACAACTT TGTATATCAT TTGATCGATT TACTGAAAAT TCAAATATTA

5641 TTGGATTTGA TTTAGTTAGA GTATTACAAA CTGTACAAAA AACTTGAAAA TTTGAAGTAA

5701 AAATTGGTTC TTCAATTGAT TCAGAAATTA TTAGAAATTG ATTTAAGTAA TTAACTAAAA

5761 TTTTTGATGG TACTATTGAA GATGGTCCAA CCATTTGAAT TTAATAAACT GGAATTAATT

5821 TTTAAAAAAA AAAAAAAAGA ACAATTAATA AATAAAGATT ATTATTATTA TTTTATTTTA

5881 TCATGTATTA AACTTACACT AATAAAGGCT CTGCATTATA ATGGTTTAGC GAAAAATTGT

5941 TAAAAAATTT TGGAAAATTG TTTTTTAAAA TAATGAAAGA CTGATTGTAA TTTTTAAAAA

6001 AATCTGGTAA TAAATCATTT TTAAAATGAT CAAATAAATT GAACCCAAGT TTTTTTGAGA

6061 GAAAAATGGA TAATAAGACC TTGAAATCCA AAAAAATTGG ATAATCATTA CCATTAACAA

6121 AGGATTTCAA TGTTTGGAAG GTTTTTGATT TTTTAATAGT ACCAAATCTA TGAACTTATA

6181 ATAAACGAAG TAATTTATCT ATTATGTTAA AATTCCTATT TTCAGACTTG ACAAATCTAT

6241 CAAAAAGATT ATTTAAAACT AATTCTCGAT TACACAAATA TGAAGATGAA AATAATCTAT

6301 AAAACAAAGA TTGATTATCT AAACTTTCTT TAAGGAATAA ATCTATTACC AAATCTACAT

6361 CATATTCTCT TAATTTAGGT TTTAATCCAA AGAATAAATA AAAAATTGAT TTTGGTGATT

6421 GTTGATTTTT **AATAAA**TTTT TTGAAGAAAA ATTCATATAT ACCATTTGAA AATTCTGAAG

6481 AAGGAAATGG ATTTGAAGTA TTTGATTTTA TTAAAACATC AATTATTTCA CTAAAAACTG

6541 AATCTATATA TGAATAATTA TTCCTTATTT GTCTTTTAAT AATGAATTAA TTCTTTTGTT

6601 AGCTTATAAA ATACCTTTGT CAATTTTTTG ATTTGATATA CCTACCTGTA GGTTGTGATA

6661 TAAAACCAAA TTGATTTTGA TGGATATTTT T

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Ich versichere, die vorliegende Arbeit eigenständig und mit den angegebenen Mitteln angefertigt zu haben.

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Changjiang ZENG

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