

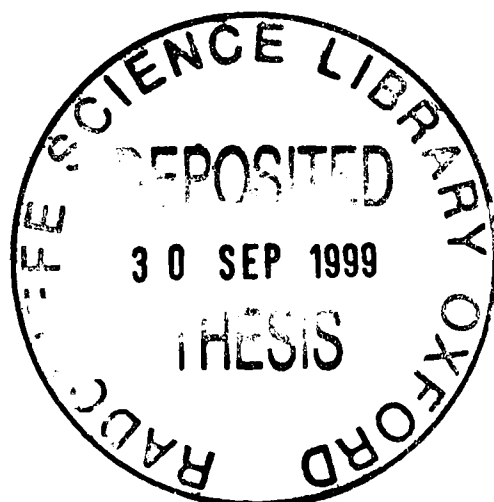
Development and Signal Transduction in *Dictyostelium*

Development and Signal Transduction in *Dictyostelium*

A thesis submitted to the Board of the Faculty of Biological Sciences,
University of Oxford, in partial fulfilment of the requirements for the degree
of Doctor of Philosophy

Hyun Ji Kim

February, 1999



Abstract

Abstract

Development and signal transduction in *Dictyostelium*

Hyun Ji Kim, St. Hugh's College

Submitted for the degree of Doctor of Philosophy
Hilary term, 1999

Dictyostelium, is a simple eukaryote that multiplies as separate amoebae. However when nutrients are no longer available it embarks on a developmental programme in which the amoebae collect together by chemotaxis and the resulting aggregates eventually transform into fruiting bodies consisting of a cluster of spores held up on a cellular stalk. The entire process of development normally takes about 24 hours. However there are mutants, termed rapidly developing mutants (rde) which complete development in about two-thirds of this time. RdeA null mutants have been reported to have elevated levels of cyclic AMP that may lead to increased activity of the enzyme, cAMP dependent protein kinase (PKA).

I started my work by measuring total cAMP levels in an rdeA mutant along with an aca-/rdeA- double mutant that is expected to have very low level of cAMP due to the absence of the adenylyl cyclase, ACA. Two *Dictyostelium* adenylyl cyclases were known at the beginning of my work; one is ACA the aggregative enzyme, and the other ACG, expressed only during spore germination. Contrary to expectation, I detected cAMP in aca-/rdeA cells. This raised the question of which enzyme was responsible for producing this cAMP. In collaboration with Dr. Pauline Schaap, I

discovered a novel adenylyl cyclase that I initially detected in *rdeA* and *regA* mutants but not in wild-type cells.

The product of the *rdeA* gene, RDEA was thought to be an H2-module histidine phosphotransferase of the kind acting in multi-step phosphorelays. Similarly REGA was believed to be a response regulator associated with a cAMP-phosphodiesterase. It had been proposed that RDEA phosphorylates REGA in a multi-step phosphorelay and it had been shown that it is the phosphorylated form of REGA that is active as a cAMP-PDE. I therefore thought that cAMP produced by the novel AC could be protected in *rdeA* mutants by the absence of the REGA cAMP-PDE activity and this idea was supported by my finding that the enzyme activity could also be detected in wild-type (*aca*-) cells when REGA-PDE was inhibited by IBMX.

In order to investigate further the proposed phosphorelay model, I tested for possible interaction between RDEA and REGA using the yeast two-hybrid system and also measured intracellular cAMP-phosphodiesterase activity in *rdeA* and *regA* mutants. I found that the interaction between RDEA and REGA appeared to be too transient to be detected in the two-hybrid system. In addition *rdeA* and *regA* mutants seemed to have levels of intracellular cAMP-phosphodiesterase activity similar to wild type. However REGA-PDE activity measured specifically by immuno-precipitation was completely absent in the *regA* mutant. It therefore appeared that there is another intracellular cAMP-phosphodiesterase, in addition to the REGA PDE, in *Dictyostelium* and that the latter cannot be easily detected in total cell lysates. One

possible explanation is that the novel adenylyl cyclase exists together with REGA in a complex (that may also include PKA) and that REGA PDE preferentially destroys the cAMP made by the novel adenylyl cyclase. I conclude that *rdeA* and *regA* mutants may develop rapidly due to high PKA activity induced by the accumulation of cAMP made by the novel AC when the REGA cAMP-PDE activity is absent.

Acknowledgements

Acknowledgements

My first and deepest gratitude goes to my supervisor, Prof. Julian D. Gross. Nothing can be comparable to his heart-warming encouragement and especially his enthusiastic and thorough supervision throughout the course of this project. I am also grateful to Prof. Peter C. Newell, my other supervisor for his consistent support. I thank Dr. Pauline Schaap in the University of Leiden for inviting me to her laboratory and for the exciting work with her. I also thank Dr. Peter Thomason in MRC, Cambridge for all his advice and gifts including anti-regA antibody. My thanks should also go to Jeanne Francois for her caring help during the two-hybrid work. I would also like to thank St. Hugh's College for a graduate scholarship and the British government for an ORS award.

I appreciate my colleagues in the first floor of the tower building ; Alison, Anna, Aruna, Brian, Bose, Calvin, Caroline, Cathy, David, Frances, Gareth, Gary, Gunilla, Hao-gen, Ji-Liang, Katalin, Kate, Kaushic, Kelly, Kei, Kunde, Li-Jun, Lynne, Matthew, Maud, Nahid, Phil, Rich, Rick, Sophia and Vivienne. I especially thank Katalin and Calvin for helping me at the beginning of my work. I would also like to thank with a nice smile the early birds in the Department - two Brians, Martin and Jeremy for their frequent help with the computing service, John for allowing me to have 30-fold my original quota in the home directory and finally Alex and OUCS for their fantastic job helping me with all kinds of presentations. I would also love to give my big hugs to my flatmates - Kat, Vivienne and Jonathan - you all made me feel so much at home all the time.

My love and greatest thanks go to my parents: Soo-Yong Kim and Jeong-Sook Seock
and to my brother Ikkie for their love and trust in me, and my final gratitude to my
lovingkind God for being always with me.

Abbreviations

Abbreviations

Ab	antibody
AC	adenylyl cyclase
ALC	anterior-like cells
8-Br-cAMP	8-bromo cyclic adenosine monophosphate
cAMP	cyclic adenosine monophosphate
[Ca ²⁺] _i	intracellular concentration of Ca ²⁺
DB	DNA binding domain
DTT	dithiothreitol
ecmA	extracellular matrix protein A
ecmB	extracellular matrix protein B
2H'-cAMP	2'-deoxyadenosine 3',5'-monophosphate
IBMX	3-isobutyl-1-methyl xanthine
IP	immuno-precipitation
LB	Luria Bertoni
LPS	lower pad solution
ml	millilitres
μl	microlitres
mM	millimolar

μM		micromolar
nM		nanomolar
NMR		nuclear magnetic resonance
o/n		overnight
PB		phosphate buffer
PKA		cAMP-dependent protein kinase
pstA	pstA	prestalk A
pstB	pstB	prestalk B
R_2C_2	R_2C_2	homodimers of the PKA regulatory subunit and the PKA catalytic subunit
TxAD		transcriptional activation domain
UAS		upstream activation sequence
X-gal		galactose attached to a chromophore (X) which is activated by cleavage from the sugar by β -galactosidase

Contents

Contents

Abstract	I
Acknowledgements	II
Abbreviations	III
Contents	IV

Chapter 1 - Introduction

1.1 How do life organisms age?	1
1.2 The life cycle of <i>Dictyostelium discoideum</i>	3
1.21 Vegetative growth	4
1.22 Aggregation	4
1.23 Late development	5
1.3 Regulation of the life cycle	6
1.31 Cyclic AMP	6
1.32 DIF	7
1.33 Ammonia	8
1.34 Calcium	8
1.4 Roles of PKA	9

1.5 Rapidly developing mutants of <i>Dictyostelium discoideum</i>	11
1.51 RdeA	11
1.52 RdeC	12
1.53 RegA	12
1.6 The two-component signalling system	13
1.61 The concept of two-component signalling systems	13
1.62 Multi-step phosphorelays as advanced two-component signalling systems	14
1.63 A possible multi-step phosphorelay in <i>Dictyostelium</i>	14
1.7 The aims of the thesis	17
 Chapter 2 - Materials and Methods	19
2.1 Cell stocks, growth and maintenance	19
2.11 Cell stocks and growth	19
2.111 AX2 (axenic strain)	19
2.112 AX2/rdeA	19
2.113 AX2/regA	19
2.114 DH1	20
2.115 DH1/rdeA	20
2.116 aca-	20

2.117 aca-/rdeA-	21
2.118 aca-/ACGact-15	21
2.12 Maintenance of strains	21
2.13 Media	22
2.2 Development of Dictyostelium discoideum	23
2.21 Normal development	23
2.211 Solutions	23
2.212 Development on filters	24
2.22 Development in monolayers	25
2.221 Solutions	25
2.222 Development in submerged monolayers	26
2.3 Adenylyl cyclase assay	27
2.31 Buffers and solutions	27
2.32 Assay of <i>in vivo</i> cAMP accumulation	27
2.33 Adenylyl cyclase assay	28
2.34 Isotope dilution assay	28
2.35 Methods employed for individual experiments	31
2.351 Measurement of “cyclic AMP relay” in 6 hour starved cells	31
2.352 Detection of cAMP accumulation in vegetative cells	32
2.353 Response of cAMP accumulation to increased osmolarity	33

2.354 Effect of the cAMP-PDE inhibitor, IBMX, on adenylyl cyclase activity	33
2.355 Testing G protein dependence of adenylyl cyclase activity	34
2.356 Effect of caffeine on adenylyl cyclase activity	34
2.357 Stimulation of adenylyl cyclase activity by Mg^{2+} and Mn^{2+}	35
2.358 Effect of pH on adenylyl cyclase activity	35
2.359 <i>In vivo</i> cAMP accumulation during starvation of <i>aca-/rdeA</i> cells	36
2.4 Yeast two hybrid system	36
2.41 Media for yeast growth	36
2.42 Molecular cloning for yeast two-hybrid test	38
2.421 The construction of <i>rdeA-pAS2</i>	38
2.422 The construction of <i>regA-pACT2</i>	39
2.423 The construction of <i>rdeA-pACT2</i>	40
2.424 The construction of <i>regA-pAS2</i>	40
2.425 Purification of DNA from agarose gel	40
2.426 <i>E.coli</i> transformation	41
2.427 Mini scale preparation of plasmids	42
2.428 Large scale preparation of plasmids	42
2.429 Buffers and solutions	44

2.43 Yeast transformation (LiAc transformation ; high efficiency)	46
2.431 Preparation of yeast competent cells	46
2.432 Heat-shock yeast transformation	47
2.433 Buffers and Solutions	47
2.44 Growing yeast transformants with selection	49
2.45 Colony-lift filter assay	49
2.451 Reagents and materials	49
2.452 Methods	50
2.46 Western Blotting	51
2.461 Purpose	51
2.462 Buffers and solutions	51
2.463 Procedures	55
2.5 Phosphodiesterase assay	58
2.51 Buffers and solutions	58
2.52 Total intracellular phosphodiesterase assay	60
2.53 Assay of the RegA phosphodiesterase	60
 Chapter 3 - The discovery of a novel adenylyl cyclase in the course of a physiological study of the rdeA mutant	 63
3.1 Introduction	63

3.11 PKA plays a key role controlling the rate of development.	63
3.12 Is high PKA activity responsible for the sporogenous phenotype of rapidly developing mutants?	63
3.13 Is the high PKA activity in rapidly-developing mutants due to elevated intracellular cAMP levels?	64
3.14 The two adenylyl cyclases known in <i>Dictyostelium</i>	65
3.2 Results	66
3.21 Measurement of total cAMP levels in the rdeA mutants	66
3.22 cAMP accumulation in 6 hour starved cells of the rdeA mutants	68
3.23 cAMP accumulation in vegetative cells of the rdeA mutants	71
3.24 Evidence that the new adenylyl cyclase activity is not ACG	73
3.25 Test of sporogenous phenotype	75
3.3 Discussion	76
3.31 Does high PKA activity cause rapid development?	76
3.32 Is the mechanism of rapid development of the rdeA mutant solved?	77
3.33 How are rapidly-developing mutants able to produce spores in submerged monolayers?	78
3.34 Does the novel adenylyl cyclase provide intracellular cAMP to activate PKA?	79
3.35 Another question may be answered by the novel adenylyl cyclase.	80

Chapter 4 - Biochemical characterisation of the novel adenylyl cyclase and evidence for the RDEA - REGA phosphorelay system in <i>Dictyostelium</i>	82
4.1 Introduction	82
4.11 How is the multi-step phosphorelay related to the new adenylyl cyclase?	82
4.12 Biochemical characteristics of the two known adenylyl cyclases, ACA and ACG	83
4.2 Results	84
4.21 Biochemical characterisation of the novel adenylyl cyclase	84
4.211 Naming the new adenylyl cyclase	84
4.212 Testing for G protein dependence of ACB	85
4.213 Mg ²⁺ and Mn ²⁺ dependence of ACB	87
4.214 ACB is very sensitive to caffeine.	89
4.215 pH dependence of ACB activity	91
4.22 ACB in relation to the multi-step phosphorelay in <i>Dictyostelium</i>	92
4.3 Discussion	93
4.31 cAMP produced by ACB is down-regulated by an RDEA-REGA phosphorelay.	93
4.32 ACB may provide intracellular cAMP to activate PKA.	94

4.33 What if ACB mediates ammonia depletion leading to culmination?	95
---	----

Chapter 5 - Testing possible interaction between the RDEA and the REGA proteins using the yeast two-hybrid system 97

5.1 Introduction 97

5.11 Why should the phosphorelay model be tested by the yeast two-hybrid system?	97
--	----

5.12 The yeast two hybrid system	98
----------------------------------	----

5.2 Results 100

5.21 Molecular cloning in order to express 4 fusion proteins.	100
---	-----

5.22 Test set 1	102
-----------------	-----

5.221 Colony-lift filter assays for Test set 1	102
--	-----

5.222 Western Blotting analysis of the Test set 1	105
---	-----

5.23 Test set 2	107
-----------------	-----

5.231 Colony-lift filter assays for Test set 2	107
--	-----

5.232 Western Blotting analysis of the Test set 2	108
---	-----

5.3 Discussion 110

5.31 The interaction between the RDEA and the REGA proteins is probably too transient.	110
--	-----

5.32 Upstream regulators of the RDEA phosphotransferase	112
---	-----

Chapter 6 - Further insight into the rdeA-regA multi-step phosphorelay	115
6.1 Introduction	115
6.11 A current view of how ACB was detected	115
6.12 An assumption underlies the current understanding.	115
6.13 Testing the assumption	116
6.2 Result	117
6.21 Measurement of total intracellular cAMP-PDE activity	117
6.22 Effect of IBMX and Rolipram on intracellular cAMP-PDEs	119
6.23 Immuno-precipitation of the REGA cAMP-PDE	120
6.231 Measurement of REGA-PDE activity at the vegetative stage	120
6.232 Comparison between REGA-PDE activity and total intracellular PDE activity during development	122
6.233 Comparison of the REGA-PDE activity between the rdeA mutant and the wild-type during development	124
6.234 A special relationship between ACB and REGA?	128
6.3 Discussion	130
6.31 The phosphorelay model could be far more complex than what	

we propose.	130
6.32 A possible complex of ACB-REGA	133
6.33 What if PKA is regulated by an ACB-REGA complex?	133
6.34 The presence of another intracellular cAMP-PDE	135
Chapter 7 - General Discussion	136
7.1 Summary	136
7.2 Possible roles of ACB	137
7.3 PKA regulation by an ACB-REGA complex	138
7.4 Mechanism of rapid development	139
7.5 A multi-step phosphorelay proposed	141
References	144
Publications	161

Chapter 1

Chapter 1

General Introduction

1.1 Rate of development and ageing.

Whenever life begins, it is followed by death. From the time we were born we have been growing, getting older and heading towards death. Each type of organism seems to have a characteristic life span. Human beings have one of the longest periods from birth to death and most of our life is spent as adults. Hence our life span is largely determined by how long we spend as adults. A question of some interest concerns whether there is a relation between rate of development and longevity. Some interesting evidence suggests that there is. Thus Yonemura et al. (1991) concluded that “longevity genes” of *Drosophila* that increase life span also appear to speed larval development. Furthermore in *C. elegans* mutations in the genes *clk1-3* and *gro-1* affect both rate of larval development and life span (Lakowski and Hekimi, 1996). Mutations in *clk-1* slow development and increase life span (see Jonassen et al, 1998). The *S. cerevisiae* homologue of *clk-1* is a mitochondrial membrane protein controlling synthesis of ubiquinone which is an essential electron transport component and a lipid antioxidant (Jonassen et al, 1998).

Thus the mutants of worm and fly clearly indicate that the system controlling ageing and rate of development can be deregulated by genetic means. This is true also in man. Werner’s syndrome is a striking yet sad example of this; it is a very rare disease that results in young adults in their twenties and thirties having already gone through the whole of the human life cycle and dying as very old-looking children (Martin, 1978). Bone and muscle developments do not seem to be dramatically accelerated but the internal organs and skin are exhausted as if they belonged to someone aged 70-80. Individual fibroblasts from patients with Werner’s syndrome have much shorter life spans as

expressed by population doubling levels. The gene affected in this disease is a recQ-type DNA helicase presumably involved in some aspect of the unwinding of DNA (Suzuki et al, 1997)

There is some remarkable evidence also of a link between components controlling periodicity study of circadian rhythms that have been studied in plants and many other organisms (Jacobshagen et al., 1996; Hicks et al., 1996; Kondo et al., 1997) and rate of development. The circadian rhythm regulating the flowering of plants appears to be connected to a system sensing the duration of the light source (Hicks et al., 1996; Somers et al., 1998; Wang and Tobin, 1998). Animal behaviour also indicates the presence of a biological clock that is at least partially independent of resetting by light. Examples of rhythms occurring in the absence of light can be found in the dormancy necessary for some animals to survive harsh environments or in prisoners in a completely dark cell. Nonetheless the vertebrate biological clock does not have a precise 24 hour periodicity and needs to be reset by exposure to cycles of light and dark (Kennaway, 1998). The evidence for a link between biological clocks and rate of development comes from studies of per mutants of *Drosophila*: those with shortened biological clocks develop faster from egg to adult than wild-type while those with abnormally long period develop more slowly (Kyriakou et al, 1990).

One situation where a biological rate mechanism is fairly well understood is that of growth rate in bacteria. This is controlled by the availability of nutrients and can be easily manipulated by altering the carbon source or availability of amino acids. It appears that the larger cells formed in richer media grow faster because the machinery for protein synthesis constitutes a greater proportion of their mass (Bremer and Dennis, 1987). Interestingly the same seems to hold true for the rate of multiplication of bacteriophage T4 in *E. coli* despite the fact that infection with this phage arrests host protein synthesis: the faster the growth rate of the bacteria at the time of infection the more

rapid the multiplication of T4 and the greater the burst size. This effect also seems to depend upon the greater content of the protein synthesizing system (Hadas et al, 1997).

Dictyostelium discoideum, the object of the current thesis, is a very simple eukaryotic organism that divides with a generation time of about 8 hours in axenic medium. The developmental cycle under study however takes place when the amoebae are starved of nutrients and is energised by the breakdown of endogenous RNA and proteins. *Dictyostelium* normally displays a 24 hour-developmental cycle. There exist rapidly developing mutants of *Dictyostelium* that can complete their cycle within two-thirds of the normal time. The mutants do not seem to show any defect in cell differentiation, but they produce unbalanced structures at the final stage of development. There has been accumulated some molecular understanding of these mutants and if aspects of the basic mechanism of ageing have been conserved through evolution, understanding the rapidly-developing mutants of *Dictyostelium* could provide some insight into these processes.

1.2 The life cycle of *Dictyostelium discoideum*

Dictyostelium discoideum was discovered by Raper in 1935 and it has become the most widely studied species among the slime molds. It has two alternative life cycles; sexual and asexual. The asexual life cycle has been studied extensively and can be easily manipulated in the laboratory. *Dictyostelium* undergoes an asexual developmental cycle that is completed within 24 hours after the onset of starvation. A sexual cycle is also possible, resulting from fusion of two sexually compatible strains or a single competent strain.

The hereditary information of *Dictyostelium* is carried on six chromosomes with 34 Mb of genomic DNA, 90 Kb of extrachromosomal elements and 55.5 Kb of mitochondrial genome. The DNA of

coding regions is composed of about 60 % adenosine and thymine (A+T), while the percentage A+T in non-coding regions can be higher than 85 %.

1.21 Vegetative growth

A single amoeba is capable of growing and dividing at the expense of nutrients to produce a large population of vegetative cells. They can grow on bacteria (e.g. *Klebsiella aerogenes* or *Escherichia coli*) on agar plates with a generation time of 3-4 hours while axenic strains can grow in a complex nutrient medium with a doubling time of 8 -9 hours (Franke and Kessin, 1977). *Dictyostelium* has no observable G1 phase; cells enter S phase immediately after mitosis; they undergo cytokinesis during S phase and then spend most of the cell cycle in G2 (Weijer et al., 1984).

1.22 Aggregation

Early in development a multicellular organism is formed by chemotactic aggregation of up to 10^5 cells in response to nanomolar pulses of cAMP (Chen et al., 1996). Aggregation involves the co-ordination activity of adenylyl cyclase controlling the relay of the cAMP signal from cell to cell, and of guanylyl cyclase controlling chemotaxis. These pathways can be activated by nanomolar cAMP pulses acting on the high affinity cell surface receptors cAR1 and/or cAR3 coupled to G proteins. During the early hours of starvation, a number of genes active during growth are shut down. At the same time cell surface receptors (cAR1, cAR3), an adenylyl cyclase (ACA) (reviewed Chen, 1996), extracellular cAMP-phosphodiesterase (PDE) (Lacombe et al. 1986) and a glycoprotein inhibitor of the PDE (PDI) (Wu and Franke, 1990) start to be expressed. An ACA-mediated signalling pathway is essential for aggregation since an *aca* null gene disruptant cannot aggregate (Pitt et al., 1992). Upon starvation, initiating cells start to emit cAMP. Neighbouring cells then respond to the cAMP by producing cAMP, releasing it and moving toward the source of

the signal (Ross et al., 1975). As soon as the maximal concentration has passed, the cells enter a refractory period (Gross, 1994). Therefore the cAMP signal is propagated outwards whereas chemotactic movement is inwards to the initiating centre.

1.23 Late development

After the first 8-10 hours of starvation the cells in aggregation streams start to make a mound at the centre and heterogeneity of cell types can then be recognised. Prespore and prestalk cells are distinguished by the expression of different mRNAs and they sort to separate prespore and prestalk regions as a tip is formed on the mound. The apical tip functions as an "organiser" that controls subsequent morphogenesis and is thought to be the source of a cAMP wave that moves toward the base of the aggregate (Siegert and Weijer, 1992). The prestalk cells sort to the apex (tip) while the rest of the gradually elongating mound is occupied by prespore cells and anterior-like cells (ALC). The tip on a mound gets progressively elongated during the several hours after tip formation. A finger-like structure erects on the substratum consisting of prestalk cells at its front and base and prespore cells in the remainder.

Three major cell types in the migrating slug have been observed; prespore cells, prestalk cells and anterior-like cells (ALC). Analysis of promoters for the prestalk genes, *ecmA* and *ecmB* has defined multiple classes of prestalk cells such as *pstA*, *pstO* and *pstAB* depending on the levels of expression of the *ecmA* and *ecmB* (Williams, 1997). The *ecmA* gene is expressed in all prestalk cells in the anterior region of the migrating slug (Jermyn et al., 1987 ; William et al., 1987). The *ecmB* gene is expressed in a subset of anterior prestalk cells that form a cone in the slug tip (Jermyn and Williams, 1991). A number of prestalk-like cells or ALC remain randomly scattered in the prespore zone and the posterior region of a slug is occupied mainly by the prespore cells.

The finger-like structure can fall over and crawl onto the substratum as a slug for a few hours to a few days in dark and humid conditions. Upon ammonia depletion, the slug enters culmination. At this time prestalk A cells enter a cone formed at the top of the stalk tube, transcription of a stalk cell-specific gene, *ecmB* is initiated and the cells differentiate into vacuolated stalk cells (Williams et al., 1993). The forming stalk tube elongates through the prespore mass, lifting it off the substratum. Simultaneously, the ALC population migrates to form the basal disc and the upper and lower cups of the maturing sorus. Meanwhile prespore cells begin to differentiate into heat and detergent-resistant spores.

1.3 Regulation of the life cycle

1.31 Cyclic AMP

Extracellular cAMP can be detected by four homologous cAMP receptors that are expressed at different developmental stages (Saxe et al., 1991). The cAMP receptors consist of seven-transmembrane-domains and are linked to heterotrimeric G proteins (Firtel et al., 1989; Hereld and Devreotes, 1992). The high affinity receptor cAR1 is maximally expressed during aggregation, and disruption of cAR1 prevents aggregation and abolishes the ability of cells to respond chemotactically to cyclic AMP (Sun and Devreotes, 1991).

cAR3 is another high affinity receptor, expressed a few hours after cAR1, and its expression is enriched in prespore cells (Johnson et al., 1993; Yu and Saxe, 1996). Disruption of the cAR3 gene does not cause any significant defect in developmental morphology (Johnson et al., 1993).

cAR2 and cAR4, low affinity cAMP receptors, are expressed when extracellular cAMP is at high levels. The cAR2 disruptant displays normal aggregation but its development is blocked at the tight mound stage (Saxe et al., 1993). The mRNA of cAR4 can be detected at low levels in tipped mounds and continues to accumulate through culmination (Louis et al., 1994).

A number of important developmental enzymes are activated by cAMP receptor-mediated signalling, among them adenylyl cyclase (ACA), guanylyl cyclase (GC) and phospholipase C (PLC) (Devreotes, 1994; Firtel, 1995). There were two known adenylyl cyclases in *Dictyostelium* when I started my work. Adenylyl cyclase A (ACA) is expressed at high levels during aggregation and at reduced levels during postaggregative development. ACA is periodically activated and displays adaptation, therefore generating periodic rises in intracellular cAMP concentration (Devreotes, 1982). Adenylyl cyclase G (ACG) is expressed only during spore germination. When ACG is stimulated by high osmolarity, the resulting increase in PKA activity inhibits germination. Guanylyl cyclase controls myosin phosphorylation and pseudopod extension in the chemotactic response (Ross and Newell, 1981; Liu and Newell, 1991; 1994; Liu et al., 1993). cAMP can raise intracellular Ca^{2+} levels either by opening Ca^{2+} channels in the plasma membrane (Coulkell, 1991) or possibly by activating a phospholipase C (PLC), resulting in diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (InsP3) production followed by InsP3-induced Ca^{2+} mobilisation (Bominaar et al., 1994).

1.32 DIF

Differentiation-inducing factor (DIF) was discovered in Dr. Julian Gross' laboratory, as a factor inducing stalk cell differentiation in submerged monolayers (Town et al., 1976; Kay et al., 1983). DIF is essentially undetectable at the vegetative stage but increases at the end of aggregation and reaches a peak in the slug stage (Brookman et al., 1982 ; Sobolewski et al., 1983). DIF-1 is not uniformly distributed in the slug; it is two-fold higher in the prespore region than in the prestalk (Brookman et al., 1987). This reverse gradient of DIF-1 might be explained by synthesis of DIF-1

by prespore cells, or by both cell types, and the localisation of DIF-1 dechlorinase in the prestalk zone.

DIFs are required for differentiation of prestalk cells *in vitro* and for prestalk gene expression (Jermyn et al., 1987 ; Williams et al., 1987). DIF-1 seems to antagonise prespore gene expressions (Early and Williams, 1988 ; Kay and Jermyn, 1983) and HM44, a mutant which produces abnormally low levels of DIF-1, forms tipless mounds that contain prespore but not prestalk cells (Kopachik et al., 1983 ; 1990).

1.33 Ammonia

Amoebae consume proteins and release large amounts of ammonia during development, and ammonia has been shown to regulate the entry into culmination (Newell et al., 1969; Schindler and Sussman, 1977). High concentrations of ammonia inhibit culmination and cause slugs to be maintained; depletion of ammonia induces slug migration to stop and initiates culmination and fruiting body formation (Schindler and Sussman, 1977).

The action of ammonia can be mimicked by a number of other weak bases that differ greatly in structure (Davies et al., 1993). This finding indicates that the effect of ammonia is not due to some unique chemical characteristic but to its weak base activity (Davies et al., 1993). It was further shown that exposure to biologically effective concentrations of weak bases caused a sustained increase in the pH of intracellular acidic compartments without any detectable effect on cytosolic pH, and it was therefore suggested that the target of weak-base action is some acidic compartment (Davies et al., 1993).

1.34 Calcium

Intracellular free calcium has been shown to play a key role in many signal transduction pathways in eukaryotes. In *Dictyostelium* various signals can provoke opening of calcium channels in the plasma membrane or in the membranes of intracellular compartments and cause rapid influx of Ca^{2+} into the cytosol, and elevation of $[\text{Ca}^{2+}]_i$ (Newell et al., 1995). After such stimulation Ca^{2+} ATPases located in the plasma membrane and in the membranes of intracellular stores rapidly return $[\text{Ca}^{2+}]_i$ to its basal level. This regulation of intracellular Ca^{2+} levels may mediate the effect of ammonia but calcium levels do not seem to affect the two adenylyl cyclases, ACA and ACG (Schaap et al., 1995).

1.4 Roles of PKA

cAMP is a second messenger for many hormones and all the effects of cAMP are believed to be mediated by cAMP-dependent protein kinase, PKA. Activated PKA can phosphorylate specific acceptor proteins and induce a variety of cellular functions. Most forms of PKA consist of two regulatory and two catalytic subunits. Each regulatory subunit has two binding sites for cAMP with different affinities. The R_2C_2 holoenzyme is inactive. When cAMP binds to the regulatory subunits, they dissociate from the catalytic subunits. The free C subunits are active and able to regulate other enzymes by phosphorylation. *Dictyostelium* PKA has rather a different structure. The holoenzyme has one regulatory subunit and one catalytic subunit and so exists as an RC heterodimer. The R subunit carries two binding sites for cAMP and the dissociation constant is 3 nM (de Gunzburg et al., 1984; Biondi et al., 1998).

cAMP-dependent protein kinase (PKA) plays an important role in many aspects of development as well as in learning and memory (Kandel and Abel, 1995). For instance, PKA mediates signalling in glial cell proliferation and differentiation, along with PKC and Ca^{2+} /Calmodulin dependent

protein kinase (Bhat et al., 1995). Yeast cAMP-dependent protein kinase (PKA) activity is essential for growth and cell cycle progression (Ward et al., 1995).

In *Dictyostelium*, it has been shown that PKA activity is required at many stages of development. When dominant-negative PKA R subunits were overexpressed under an actin 15 promoter, preaggregative genes, such as ACA, cAR1, csA and PDE were expressed at very reduced levels (Schulkes and Schaap, 1995). Expression of Discoidin I and dutA was completely blocked in a knockout mutant of the PKA catalytic subunit and expression could not be rescued by cAMP pulses (Kumimoto et al., 1996). The G-protein stimulated adenylyl cyclase, ACA, could not be activated in a null mutant of the PKA catalytic subunit (Mann et al. 1997). Overproduction of the PKA regulatory subunit prevented aggregation and early gene expression (Simon et al., 1989).

Spore maturation is highly dependent on PKA activity and addition of 8-bromo cAMP, a permeable cAMP analogue, enabled wild-type cells to produce spores in submerged monolayers. When dominant-negative PKA regulatory subunits were expressed under a prespore specific promoter, spore differentiation was inhibited.

PKA activity seems to be also essential for culmination (Harwood et al., 1992 a,b ; Kay, 1989).

When the PKA catalytic subunit was overexpressed, it caused accelerated development after aggregation (Anjard et al., 1992). Remarkably it has been reported that an aca- (PKA C) strain, that is one over-expressing the PKA catalytic subunit in the absence of ACA activity, produced fruiting bodies despite cAMP being undetectable throughout its development (Wang and Kuspa, 1997). It was therefore claimed that PKA activation could overcome all requirements for cAMP during development. However other interpretations are possible (see later).

1.5 Rapidly developing mutants of *Dictyostelium discoideum*

Three classes of rapidly developing mutants have been identified in *Dictyostelium*, rdeA, rdeC and regA. All of the mutants display the sporogenous phenotype: wild-type cells cannot make spores when they are incubated in submerged monolayers in the presence of cAMP, whereas sporogenous mutants, by definition, produce spores under the same conditions (Kay, 1989 ; Chang et al., 1998 ; Thomason et al., 1998). It is thought that high PKA activity may be responsible for this phenotype and I will discuss this further in chapter 3. The first described rde mutant, FR17, was reported in 1963 by Sonneborn et al. A set of rde mutants was classified into two loci: rdeA and rdeC, by genetic complementation experiments (Abe and Yanagisawa., 1983).

1.51 RdeA

Before the rdeA gene was isolated (Chang et al., 1998), there had been a great deal of previous study of the locus using chemical and radiation induced mutants. The time required for cell differentiation from vegetative cells to aggregation competent cells was found to be reduced. It was also reported that rdeA mutants showed precocious and excessive production of extracellular cAMP-phosphodiesterase (PDE) and of cAMP during the entire course of development (Abe and Yanagisawa, 1983). The final structures formed looked like tipped mounds shaped like pyramids and the mutants made numerous small aggregates without any sign of aggregation streams. It has been proposed that the rdeA gene encodes an H2- module histidine phosphotransferase active in a multi-step phosphorelay (Chang et al., 1998) and this aspect will be discussed in a later part of this chapter. The rdeA knockout mutant has been shown to make spores in submerged monolayers in the presence and absence of cAMP and this appears to suggest that there is high PKA activity in the rdeA cells (Chang et al., 1998).

1.52 RdeC

rdeC mutants were also much studied before the discovery of the responsible gene. It was again reported that the time required for spore and stalk cell differentiation following completion of aggregation is shortened. However in the case of these mutants aggregation itself was not advanced. Intracellular cAMP levels were shown to be extremely low and spore and stalk cell differentiation occurred without an apparent increase in these levels (Abe and Yanagisawa, 1983). The gene product of rdeC is the regulatory subunit of PKA, hence rdeC null mutants do not have a functional PKA R subunit (Simon et al., 1992). They therefore probably develop rapidly due to constitutive PKA catalytic activity, which of course occurs despite very low levels of intracellular cAMP. The mutants are sporogenous which also suggests high PKA activity (Kay, 1989).

1.53 RegA

The C-terminus of the regA gene product encodes a cyclic AMP phosphodiesterase, while the N-terminal region is homologous to the well-characterised response regulators of two-component signalling systems (Shaulsky et al, 1996; Loomis et al, 1997; Thomason et al, 1998). The cAMP-phosphodiesterase encoded by the regA is homologous to mammalian cAMP-PDEs and is quite different from the extracellular cAMP-PDE of *Dictyostelium* (Shaulsky et al, 1996 ; Thomason et al., 1998). RegA mutants presumably lack this intracellular cAMP-PDE activity and could therefore be thought to have high intracellular cAMP levels. They are sporogenous like rdeA and rdeC mutants (Thomason et al., 1998) presumably due to high PKA activity.

Sequence analysis of the *rdeA* and *regA* genes suggests the possibility that rapid development of the two mutants may be due to defects in a common signalling pathway. Although their rapid development may be due to high PKA activity as in *rdeC* mutants, in their cases this could be caused blocks in successive steps of a signalling pathway in which RDEA and REGA both participate.

1.6 The two-component signalling system

1.61 The concept of two-component signalling systems

Most prokaryotic organisms use two component signalling systems to respond to their environment (Parkinson, 1993 ; Hoch and Silhavy, 1995 ; Bourret et al, 1996). A two-component signalling system basically consists of a sensor kinase and a response regulator. A sensor kinase appears to have an input domain to receive environmental stimuli such as high osmolarity, nutrient starvation or host proximity, as well as a transmitter domain. The transmitter domain has a conserved histidine residue (H) which is autophosphorylated using ATP as substrate (Figure 1.1) and the phosphate on the histidine can then be transferred to an aspartic acid residue (D) in the receiver domain of the response regulator.

Phosphorylation of the aspartate generally controls an enzymatic or transcriptional activity of an output domain. The diagram in Figure 1.1 shows a prototype of two component signalling systems composed of two proteins. A response regulator is frequently found to exist in association with an output domain and the output domain can be the starting point of an enzyme cascade or transcriptional activity. Examples include the MAP kinase cascade in *S. cerevisiae* osmolarity regulation, transcriptional regulation in *B. subtilis* sporulation and control of *B. pertussis* virulence (Posas et al., 1996 ; Burbulys et al., 1991 ; Uhl and Miller, 1996).

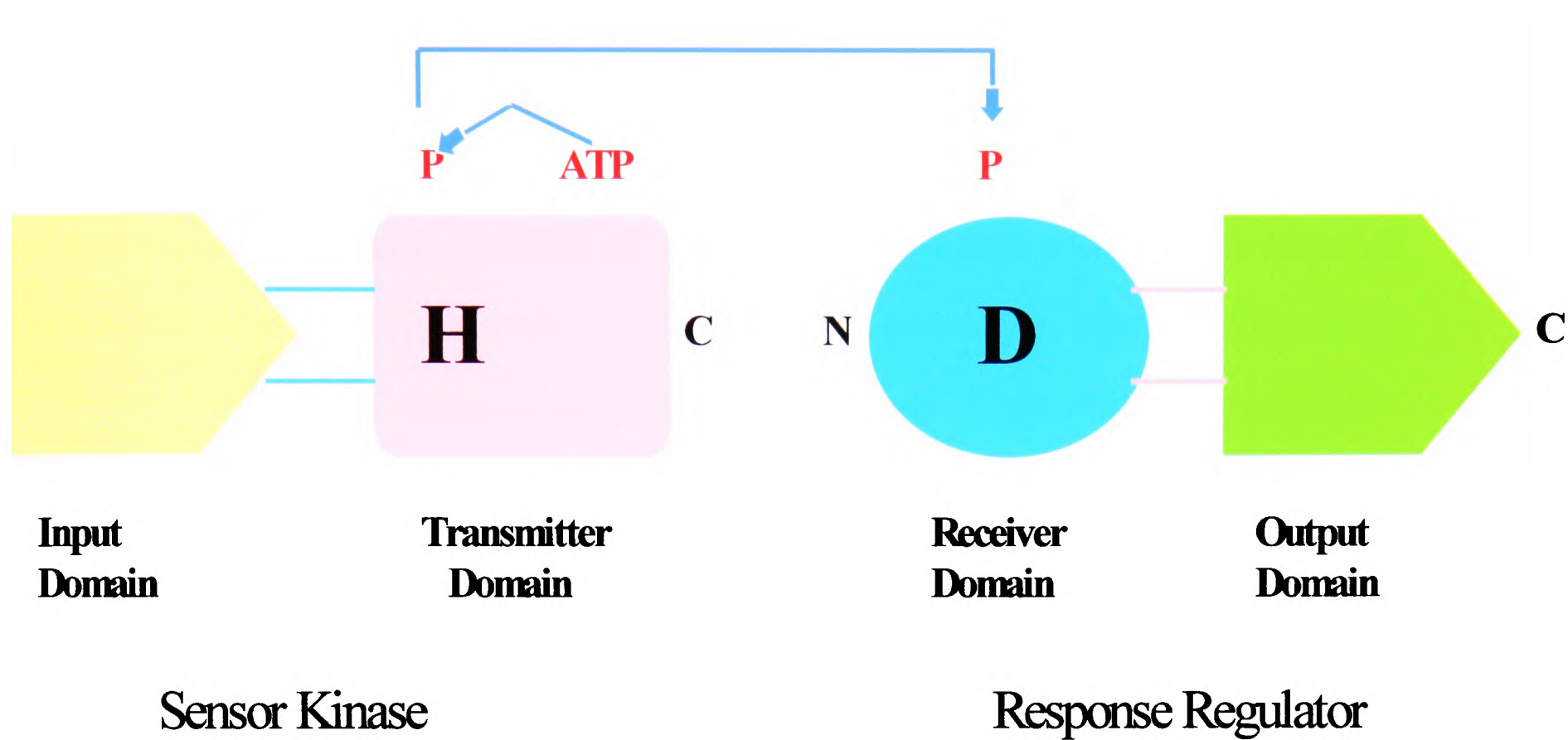


Figure 1.1 A diagram of a prototypical two-component signalling system
The diagram was slightly modified from Fig.1 of a published paper; Appleby J.L., Parkinson J.S. and Bourret R.B. *Cell*, 86, 845-848, 1996

1.62 Multi-step phosphorelays as complex two-component signalling systems

Two-component signalling systems have been reported in several eukaryotic organisms such as *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Neurospora crassa* and *Dictyostelium discoideum* (Posas et al., 1996 ; Chang et al., 1993 ; Alex et al., 1996). The eukaryotes seemed to use a more complex system having two pairs of His-Asp but it should be noted that the more complex system is also found in prokaryotic organisms (Burbulys et al., 1991). In the more complex system an intermediate phosphorylated response regulator transfers its phosphate to another HPt domain instead of directly controlling its own output (see upper panel of figure 1.4). There is thus an intermediate component containing a histidine receiver domain and an additional response regulator connected to its downstream. The system thus adopts an H1-D1-H2-D2 configuration (see upper panel of figure 1.4). In this figure SLN1 has an H1 and a D1 domain so that the protein can be autophosphorylated at the H1 and can then transfer the phosphate to the D1. YPD1 has an H2 domain receiving the phosphate from D1 and finally SSK1 has a D2 domain receiving the phosphate from the H2 domain. The phosphorylated form of SSK1 in turn controls a MAP kinase cascade (Posas et al., 1996). It is evident that it is somewhat misleading to call such a complex system a “two component system” and it is generally agreed that it is better to refer to it as a multi-step phosphorelay.

1.63 A possible multi-step phosphorelay in *Dictyostelium*

The RDEA protein as an H2-module histidine transferase

The *rdeA* gene was isolated by rescuing the plasmid and flanking regions from a DH1/*rdeA* mutant that was generated by restriction mediated plasmid integration (REMI) (Chang et al., 1998). The *rdeA* gene encodes a protein, 254 amino acid long and the corresponding mRNA could be detected from the vegetative to the slug stage (Chang et al., 1998). The RDEA protein did not seem to have

any enzymatic activity but its absence caused rapid development. The histidine residue, the 65th amino acid from the N terminus, appears to be the histidine common to H2 module histidine phosphotransferases (HPts) (see diagram in Figure 1.2 A). A simple multiple alignment of HPts was published in Chang et al., 1998 but did not include all the H2 phosphotransferases that I wanted to compare with the RDEA amino acid sequence. I therefore constructed the multiple alignment presented in Figure 1.2 B using the CLUSTAL W program (Thompson et al., 1994). The overall homology between the aligned sequences is very low and even the region very close to histidine (65) of RDEA has less than 30 % homology. When the full length RDEA amino acid sequence was submitted to several programs (DSC, PHD and Jprep) in order to predict secondary structure, none of the programs included any of the known H2 module histidine transferases in their multiple sequence alignments. This is because members of the H2 module histidine transferases are very divergent in terms of their functions, their overall amino-acid sequence and the number of subdomains they contain. They are much more like independent individuals sharing the same profession or occupation. However if a profile is made in the immediate vicinity of the active histidine residue (his65 in RDEA), members of the histidine transferases class of proteins (HPts) are recovered because of the concentration on this functional region.

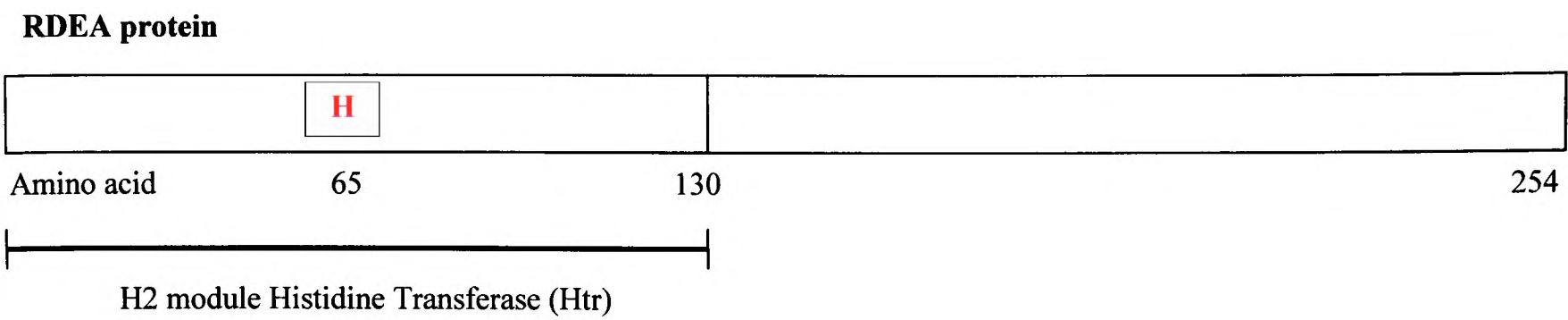
Thus figure 1.2 B shows that the region around histidine (65) of RDEA (marked in red) is indeed conserved in the various HPts. The proteins aligned are BarA of *Escherichia coli*, LemA of *Pseudomonas syringar*, EvgS of *Escherichia coli*, FrzE of *Myxococcus xanthus*, ArcB and CheA of *Escherichia coli*, BvgS of *Bordetella pertussis*, RcaC of cyanobacterium *Fremyella diplosiphon*, Ypd1 of *Saccharomyces cerevisiae* in blue and RdeA of *Dictyostelium discoideum* in red. This multiple sequence alignment was made using the ClustalW program (Thompson et al., 1994).

Figure 1.2 RDEA as a histidine phosphotransferase (Htr) in a multi-step phosphorelay.

A. A simple primary structure of the RDEA protein. The histidine (65) responsible for phosphate transfer is shown in red and the N-terminal region (1-130) is known to be functional.

B. A multiple sequence alignment was produced using Clustal W program. It shows the RDEA protein has a consensus histidine residue like many other histidine phosphotransferases.

A



B

	1	15	30	45	60	75	90
arA	--EVNEIVVNP	ATLDWQLALRQAAGKTD	LARDMLQMLLD	FLPEVRNKVVEEQLV	GENPE-GLVDLI	HKLHG	SCGYSGVPRMKNLCQLI
amA	-NEALEVHVGP	--LVLDHEEGLRLAAGKAD	LAADMLAMLLASLDAD	REAIRVARANQ	DVH-ALIERI	HRLHG	ATRYCGVPQLRSACQRA
rgS	---YQVAHIAPQYR	HLDIEALKNNTANDLQ	LMQEILMTFQHETHKDL	PAAFQALEAG	DNR-TFHQCI	HRIHG	AANILNLQKLINISHQL
rzE	-----	MDTEALKKSLKKF	----	QEV	TADRLQKIQLGVLDLEKETADQAAE	DVAREL	HTMKGEARMLGLAAIGQLAHAA
rcB	--TTEENSKSE	--ALLDIPMLEQYLELVGPKLITDGLAVFEKMM	PGYVSVLESNLTAQ	DDKK-GIVEEG	HKIKGAAGSVGLRHL	QQLGQQI	
leA	-----	MSMDISDFYQTFDEAD	----	ELLADMEQ	--HLLVLQPEAPDAEQLN	--AIFRAA	HSIKGGAGTFGFSVLQETTHLM
rgS	--AAPATHDATP	AAFSAESILALTQND	EALIRQLLEE	VIRTNRADVDQLQKLHQQADWP	KVSDMA	HRLAGGARVVDAKAMIDTALAL	
acC	-ESDRPQHLIP	---SDSQQQYLAF	LN	ETWTRTKPQSLDQMGILLQIVRDLQTNQLTPQQQT	QAQHV	HKLAGTLGIFGLTKTMHIARQL	
PD1	-MSTIPSEIIN	---WTILNEI	ISMDDDDSD	-FSKGLIIQFIDQAQTTFAQMQRQLDGEKNLT	ELDNLGH	FLKGSSAALGLQRIAWVCERI	
DEA	MISDYE	GPTPT--KKFDQDILFDYSEGEKE	-FTFELLDSYISSV	EEHLPELLNSSYIGCEA	-VRVLHS	HDIKGSSSYIGCEAVRYVSGKI	
	91	105	120	135			
arA	EQQL--RSGTKEEDLEPELLELLDEMDN	-VAR-EAS--KILG---					
amA	ETLL--KQNA-----	PHTEEALNDLDKAIIRLEAEA	-RVMA---				
rgS	EITP--VSDDSK----	PEILQLLNSVKEHIAELDQEI	-AVFCQKN				
rzE	EDVL--RAEREG----	KTATEVATDVLLRACDVLS	DLNEDLSGAN				
rcB	QSPD--LPAWEDN	-VGEWIEEMKEEWRHDVEVLKAWVAKATKK--					
leA	ENLL--DEARRGE--	MQLNTDIINLFLE	-TKDIMQEQLDAYKQSQ				
rgS	EKKA--QGQAGPS---	PEIDGLVRTLAAQSA	ALETQLRAWLEQRP				
acC	EYWL--GGRERLQP--	KHAPLMKTLVTALQQDIDHTT	LIQLSQI-				
PD1	QNLG--RKMEHFF---	PNKTEL	VNTLSDKSIINGINIDEDDEEIK				
DEA	EAYCKNDELEKAESFYPELKKEVEEVFKILSDFKKNWDKNHGEG-						

The REGA protein as a response regulator in association with a cAMP-phosphodiesterase

As mentioned the C-terminus of the *regA* gene product encodes a cyclic AMP phosphodiesterase, while the N-terminal region is homologous to the well-characterised response regulators of bacterial and eukaryotic two-component signalling systems (Shaulsky et al, 1996; Loomis et al, 1997; Thomason et al, 1998).

The response regulator domain has an aspartic acid at the position of amino acid 212 (in red, Figure 1.3 A) which receives a phosphate from an upstream histidine phosphotransferase, possibly the RDEA protein. The multiple sequence alignment shown in Figure 1.3 B also supported the idea that the N-terminal domain of the REGA protein is a response regulator in a multi-step phosphorelay system. The proteins aligned are RegA of *Dictyostelium discoideum*, Ssk1 of *Saccharomyces cerevisiae*, NtrC and CheY of *Escherichia coli* and PleD of *Caulobacter crescentus*. The *Dictyostelium* REGA aligned well with the *S.cerevisiae* SSK1 except for the loop of about 20 residues some 20 amino acid C-terminal to the aspartate, just as the RDEA domain aligned well with the YPD1 protein.

The C-terminus of the REGA protein encodes a cAMP-specific phosphodiesterase that shows good homology to other mammalian cAMP-phosphodiesterases but not to the *Dictyostelium* extracellular cAMP phosphodiesterases (Shaulsky et al, 1996). Figure 1.3 C shows that the REGA PDE, on the bottom line, has conserved amino acid residues found in a rat PDE, a human PDE and a bovine PDE. REGA was proven by Thomason et al., (1998) to be a cAMP-specific phosphodiesterase and not a cGMP-PDE. Therefore the REGA-PDE is considered to be homologous to mammalian cAMP-PDEs.

A proposed multi-step phosphorelay

To summarise, based on sequence analysis of the RDEA and REGA proteins and the similar phenotypes obtained when either of them was absent, it was proposed that RDEA phosphorylates REGA and activates the REGA cAMP-specific phosphodiesterase (Chang et al., 1998 ; Thomason et al., 1998). Chang et al. reported a multiple sequence alignment for the RDEA-related histidine phosphotransferases. Shaulsky et al. reported a multiple sequence alignment for the REGA-related response regulators. Both alignments were good but they were not sufficient for what I wanted to discuss in this chapter. Hence I made my own alignments using the ClustalW program (Thompson et al., 1994) though they gave essentially the same results as the published data for both proteins and they are presented in Figure 1.2 and 1.3.

1.7 The aims of the thesis

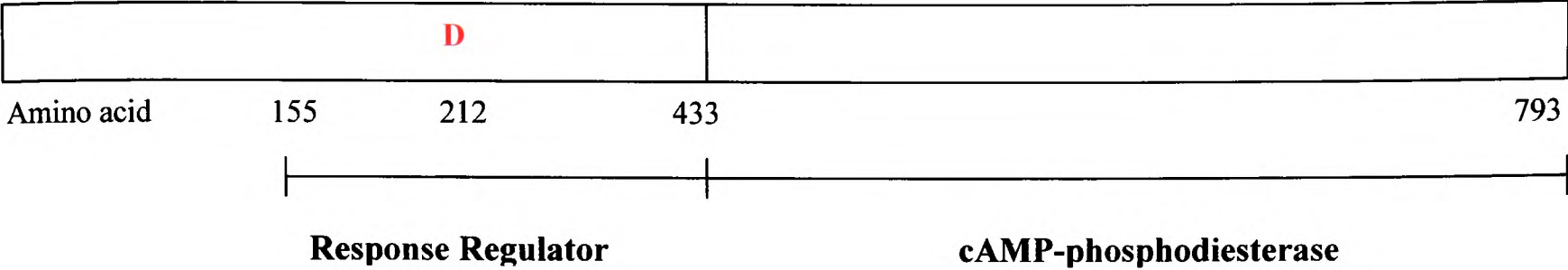
The aim of this thesis is to contribute to understanding the mechanism of the rapid development of *rdeA* mutants. I began by testing whether the *rdeA* mutants do have elevated cAMP levels as was reported by Abe and Yanagisawa (1983). This approach led me to discover a novel adenylyl cyclase and raised the question of how the *rdeA* mutants could have high PKA activity despite the absence of elevated intracellular cAMP levels. The gene products of *rdeA* and *regA* had been identified recently and sequence analysis pointed to the presence of a multi-step phosphorelay including the RDEA and REGA proteins (Shaulsky et al, 1996; Chang et al., 1998; Thomason et al., 1998). I wanted to know if the rapid development of the *rdeA* mutant is a consequence of the block in the proposed multi-step phosphorelay and tried obtaining experimental support for the phosphorelay model. I will conclude my thesis by discussing how PKA activity may be regulated in a complex with the REGA cAMP-phosphodiesterase and the novel adenylyl cyclase detected in rapidly developing mutants.

Figure 1.3 REGA as a response regulator in association with a cAMP-phosphodiesterase.

- A. A simple primary structure of the REGA protein. The aspartate (212) responsible for a phosphorelay is shown in red.
- B. A multiple sequence alignment was produced using Clustal W program. It shows the REGA response regulator domain has a consensus aspartate residue.
- C. A multiple sequence alignment was produced using Clustal W program. It indicates that REGA has a cAMP phosphodiesterase domain homologous to mammalian cAMP-phosphodiesterases.

A

REGA Protein



B

Alignment of a response regulator domain of the RegA

REGA	YSPSKVRILVADDDD	VQRKILNNLLKKFHY	-NVTLPNGEIAWEY	INKGQQKYDLVLT	DV	MMPHITGFDLLQRIN	
SK1	-FP-KINVLIVEDNV	INQAILGSFLRKHKI	-SYKLAKNGQEAVNI	WKEGGL--HLIF	MDL	QLPVLSGIEAAKQIR	
TRC	-MP-AGSILVADDDT	AIRTVLNQALS RAGY	-EVRLTGNAATLWRW	VSQGEG--DLVIT	DV	VMPDENAFDLLPRIK	
LED	---MSARILVVDDIE	ANVRLLEAKLTAEYY	-EVSTAMDGPTALAM	AARICP--TIILL	DV	MMPGMDGFTVCRKLK	
heY	MADKELKFLVVDDFS	TMRRIVRNLLKELG	NNVEEAEDGVDA	LNK	LQAGGY--GFVIS	DW	NMPNMDGLELLKTIR
;							
REGA	DHPVHR-----	-----	HIPVILMSG-TAVDY	KYANDTIKIGGQDFL	TKPIAKELLKKKIDT		
SK1	DFEKQNGIGIQKSLN	NSHSNLEKGTSKRFS	QAPVIIIVALTASNSQ	MDKRKALLSGCNDYL	TKPVNLHWLSKKITE		
TRC	KMRPN-----	-----	-LPVIVMS--AQNTF	MTAIRPSERGAYEYL	PKPFDLKEELITIVGR		
LED	DPTTR-----	-----	HIPVVLIT--ALDGR	GDRIQGLES GASDFL	TKPIDDVMLFARVRS		
heY	ADGAMS-----	-----	ALPVLMVT--AEAKK	ENIIAAAQAGASGYV	VKPFTPATLEEKLNK		
;							

Alignment of a cAMP-phosphodiesterase domain of the RegA

AT	WGLDVFKVAELSGNR	PLTAVIFRVLQERDL	LKTFQIPADTLLRYL	LTLEGHYHS-NVAYH	NSIHAADVQSAHVL	LGTPALEAVFTDLEV
JMAN	WGLNIFCVSDYAGGR	SLTCIMYMIFQERDL	LKKFRIPVDTMVTYM	LTLEDHYHA-DVAYH	NSLHAADVLOSTHVL	LATPALDAVFTDLEI
OVINE	WSFDVFALNEASGEH	SLKFMIYELFTRYDL	INRFKIPVSCLIAFA	EALEVGYSKYKNPYH	NLIHAADVQTQTVHYI	MLHTGIMHWLTELEI
[CTY	WEFDVFKYSEDD---	-LMPLLVD MFENFQL	PEIFKIPIEKLQRFI	MTVNALYRK-NNRYH	NFTHAFDVTQTVYTF	LTSFNAAQYLTHLDI
;						
AT	LAAIFACAIHDVDHP	GVSNQFLINTNSELA	LMYNDSSVLENHHLA	VGFKLLQGENCDIFQ	NLSTKQKLSLRMMVI	DMVLATDMSKHM SLL
JMAN	LAALFAAAIHDVDHP	GVSNQFLINTNSELA	LMYNDES VLENHHLA	VGFKLLQEDNCDIFQ	NLSKRQRQSLRKMVI	DMVLATDMSKHM TLL
OVINE	LAMVFAAAIHDYEHT	GTTNNFHIQTRSDVA	ILYNDRSVLENHHVS	AAYRLMQEEEMNVL I	NLSKDDWRDLRNLVI	EMVLSTDMSGHFQOI
[CTY	FALLISCMCHDLNHP	GFNNTFQVNAQTELS	LEYNDISVLENH HAM	LTFKILRNSECNILE	GLNEDQYKELRRSVV	QLILATDMQNHFEHT
;						
AT	ADLKT MVETKKVTSL	GVLLLDNYS DRIQVL	QSLVHCADLSNPAKP	LPLYRQWTERIMAEF	FQQGDRERESGLDIS	PMCDKHTASVEKSQV
JMAN	ADLKT MVETKKVTSS	GVLLLDNYS DRIQVL	RNMVHCADLSNPTKP	LELYRQWTD RIMAEF	FQQGDRERERGMEIS	PMCDKHTASVEKSQV
OVINE	KNIRNSLQQPE----	G---LD----KAKTM	SLILHAADISHPAKS	WKLHHRWTMALMEEF	FLQGDKEAELGLPFS	PLCDRKSTMVAQSQI
[CTY	NKFQHHLNNLPFDRN	KK--ED----RQMIL	NFLIKCGDISNIARP	WHLNFEWSLRVSDEF	FQQSHYETICGYPVT	PFMDKTKTTRARIAA
;						
AT	GFIDYIAHPLWETWA	DLVHPDAQELLD TLE	DNRE-WYQSRVPCSP	P-----HA	IGPDRFK-FELTLEE	TEEEEEEDERH--
JMAN	GFIDYIVHPLWETWA	DLVHPDAQEILD TLE	DNRD-WYYSAIRQSP	SPPPEEE-SRGP GHP	PLPDKFQ-FELTLE-	-----
OVINE	GFIDFIVEPTFSLLT	DSTEKIIIP LIEEDS	KTKT-PSYGASRRSN	MKGTTNDGTYS PDYS	LASVDLKSFKNSLVD	IIQQNK E-----
[CTY	DFIDFVASPLFQSMA	KFLK-ESQFLLKVIS	KNRENWQAYMELQKE	GK--CND----DDLQ	FMEDPTILVKS LPK	IDEEENRDKVSSS

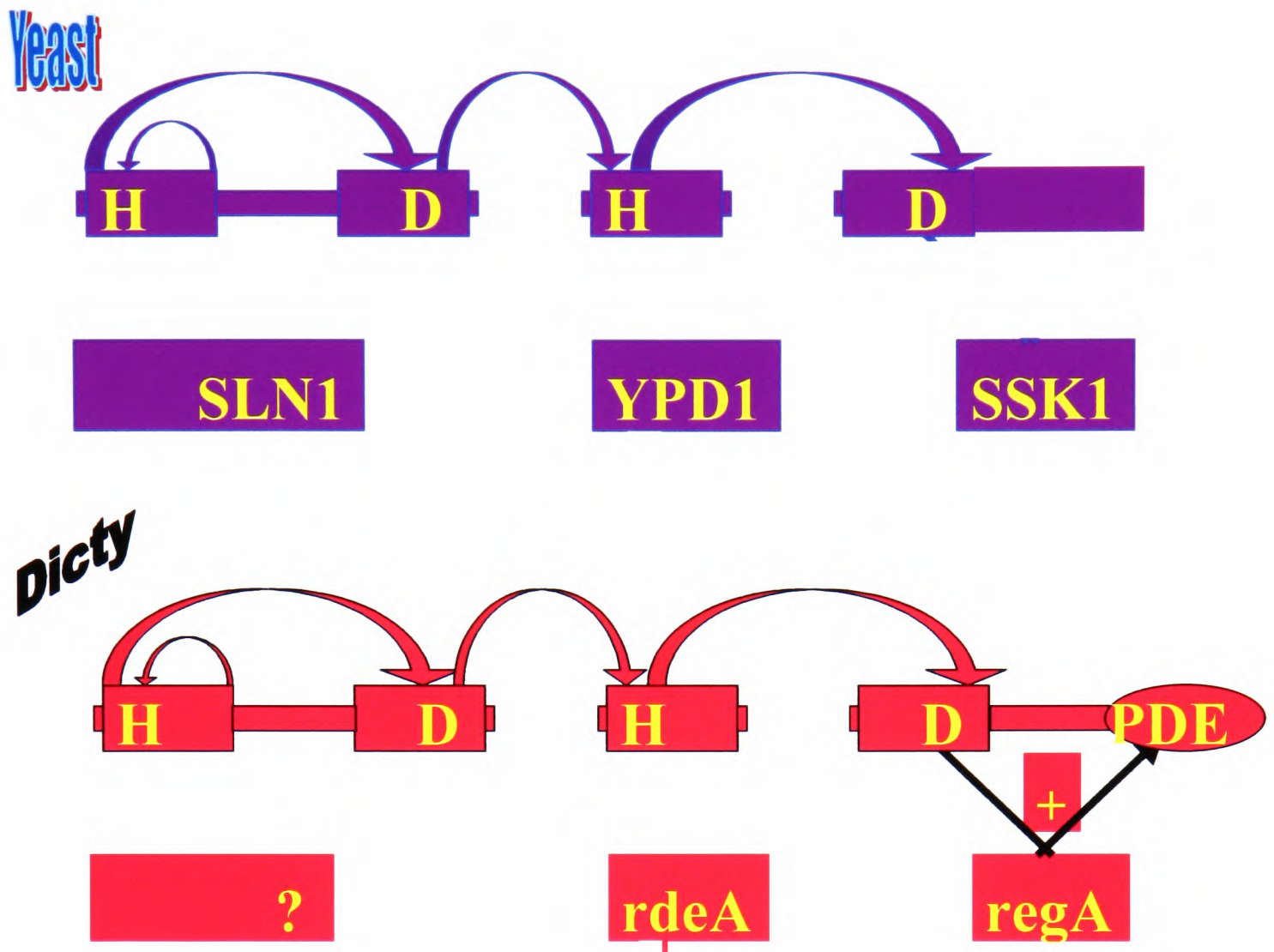


Figure 1.4 The proposed multi-step phosphorelay in *Dictyostelium*

The RDEA-REGA phosphorelay has been proposed by Chang et al. (1998) and Thomason et al. (1998). It is thought to be a similar system to that of *S.cerevisiae* presented in upper panel.

Chapter 2

Chapter 2

Materials and Methods

2.1 Cell stocks, growth and maintenance

2.11 Cell stocks and growth

2.111 AX2 (axenic strain)

Amoebae obtained from liquid nitrogen stocks were inoculated into Ashax medium (pH 6.5) and left stationary for 1 week at 22°C before being put onto a shaker (150-180 rpm). Cells grew with a doubling time of about 8 hours and were subcultured during the log phase of growth (2×10^6 cells/ml to 7×10^6 cells/ml). Fresh cultures were obtained periodically from liquid nitrogen stocks.

2.112 AX2/rdeA (Chang et al., 1998)

This strain was made by homologous recombination with an rdeA knock-out vector in the parental strain, AX2. The knockout vector carries the selectable marker Bsr^r inserted into a cDNA for the rdeA gene and was kindly given by Dr Wen-Tsan Chang. 5µg/ml of blasticidin was added to Ashax medium for axenic growth of this strain.

2.113 AX2/regA (Thomason et al., 1998)

This was made by electroporation of the pRegA knock-out vector into AX2 and the selection with 20 µg/ml blasticidin S. The strain designated HM1015 (AX2/regA) was kindly given by Dr Peter Thomason. 5 µg/ml of blasticidin was added to Ashax medium for axenic growth of HM1015.

2.114 DH1

The DdPYR5-6 mutant strain DH1 (lacking uridine monophosphate synthase activity: URA⁻), kindly provided by Prof. Peter Devreotes (Johns Hopkins University), was grown in axenic medium supplemented with uracil at 20 µg/ml.

2.115 DH1/rdeA (Chang et al., 1998)

The original REMI mutant, WTC10 was generated by Dr Wen-Tsan Chang (Chang et al., 1998). After he obtained a rescue plasmid with the inserted vector and surrounding DNA from WTC10, he recreated the mutant phenotype by homologous recombination. I used the recreated DH1/rdeA strain kindly provided by Dr Chang in my experiments. DH1/rdeA growth in Ashax medium did not require any selective marker since it was a URA⁺ transformant.

2.116 aca⁻ (Pitt et al., 1992)

The ACA gene was disrupted by gene targeting (Pitt et al., 1992). To achieve this a uracil auxotroph was transformed with a genomic fragment of ACA, pGSP2, in which a 2.0 Cla fragment had been replaced by the UMP-synthase gene (Pitt et al., 1992). This strain was kindly provided by Dr Peter Devreotes.

2.117 aca-/rdeA- (Chang et al., 1998)

This strain was made by homologous recombination with the Bsr^r rdeA knockout vector in the parental aca⁻ strain. The knockout vector carries the selectable marker Bsr^r inserted into a cDNA for the rdeA gene and was kindly given by Dr Wen-Tsan Chang. 5µg/ml of blasticidin was added to Ashax medium for axenic growth of this strain.

2.118 aca-/ACGact-15 (Pitt et al, 1992)

The strain was prepared by transformation of aca⁻ cells with pGSP1. The resulting cells were grown in HL-5 supplemented with 20 mg/ml G418. The aca-/ACG line harbours a fusion of the constitutive actin15 promoter and the entire coding sequence of the ACG gene, causing acg to be expressed during the entire course of development. It was kindly given by Dr Pauline Schaap.

2.12 Maintenance of strains

Dictyostelium discoideum strains were maintained on SM-agar plates at 22°C in the dark, in association with *Klebsiella aerogens*. In general URA⁺ transformants were selected in

FM medium lacking uracil. NEO^r transformants were selected and grown in axenic medium supplemented with 10 µg/ml of geneticin (G418) and Bsr^r transformants were selected and grown in axenic medium with 5 µg/ml of blasticidin S (Sutoh, 1993).

Long-Term Storage

Primary stocks of all strains were stored in liquid nitrogen as a suspension of amoebae in horse serum supplemented with 5% DMSO. New clonal cultures were initiated by spreading a thawed cell suspension onto SM-agar plates with *Klebsiella aerogens*.

2.13 Media

SM-agar (Sussman, 1987)

Glucose	40 g
Bacto Peptone (Difco)	40 g
Yeast Extract (Oxoid)	4.00 g
MgSO ₄ .7H ₂ O	4.00 g
KH ₂ PO ₄	8.80 g
K ₂ HPO ₄	4.00 g
Agar (Oxoid No.3)	48 g

dissolved in 4 Litre of dH₂O and autoclaved at 123°C for 15 min

Axenic medium (Ashax; Watts and Ashworth, 1970)

Bacto peptone, pH 6.3 (Oxoid)	14.30 g
Yeast Extract, pH 7.0 (Oxoid)	7.15 g
Na ₂ HPO ₄	0.50 g
KH ₂ PO ₄	0.48 g
Maltose (Sigma)	18.00 g
Dihydrostreptomycin sulphate	0.25 g

dissolved in dH₂O and adjusted to pH 6.5

final volume to 1 Litre and autoclaved at 123°C for 15 min

Bonner's Salt Solution

KCl	0.75 g
NaCl	0.60 g
CaCl ₂ .2H ₂ O	0.30 g

dissolved in 1 Litre of dH₂O and autoclaved at 123°C for 15 min

2.2 Development of *Dictyostelium discoideum*

2.21 Normal Development

2.211 Solutions

Lower Pad Solution (LPS; Newell et al., 1969)

KCl	1.50 g
KH ₂ PO ₄	5.45 g
MgCl ₂ .6H ₂ O	0.50 g
Dihydrostreptomycin sulphate	0.50 g

dissolved in dH₂O and adjusted pH to 7.3 with KOH

final volume made up to 1 litre and filter-sterilised

2.212 Development on filters

Preparation of filters;

Lower pads (Whatman No.17) and Millipore filters (GSWP 47mm, quartered if necessary) were boiled in deionized water for 2 minutes. They were placed inside a microflow hood and sterilised under UV light for 15 minutes. The dried filters were soaked in LPS for between 1 and 24 hours. Just before plating washed cells, a lower pad was placed onto a 90mm petri dish having removed excess liquid and a Millipore filter was placed on top of the lower pad.

Preparation of cells;

Cells in log phase (3×10^6 cells /ml to 7×10^6 cells /ml) were washed three times with LPS in a 50 ml Falcon tube. The speed of centrifugation could be controlled depending

on strains. For instance a pellet of AX2 cells in good conditioned was produced by spinning at 1200rpm to 1500 rpm for 2 min while pellets of AX2/rdeA, AX2/regA, DH1/rdeA, aca⁻ and aca⁻/ACG were obtained at 1500 rpm to 1700 rpm for 2 min. On the other hand aca⁻/rdeA cells could not be harvested by spinning at less than 2000rpm for 2 min since the cells were considerably smaller; otherwise most of the cell pellet was lost when the supernatant was discarded.

Plating cells on filter for development;

Washed cell pellets were resuspended at 10⁸ cells / ml in LPS and 400 µl, equivalent to 4 x 10⁷ cells, was plated evenly onto an intact Millipore filter. The plated cells were developed in the dark under well-moisturised conditions at 22°C.

2.22 Development in monolayers

2.221 Solutions

Spore induction medium (Kay, 1987)

MES	1.95 g (MES:2-[N-Morpholino]ethanesulfonic acid)
KCl	1.49 g
NaCl	1.17 g
CaCl2	0.15 g
Dihydrostreptomycin sulphate	0.2 g

dissolved in dH₂O and adjusted to pH 6.2; final volume made up to 1 Litre and autoclaved

2.222 Development in submerged monolayers

Preparation of cells;

Cells in log phase (3×10^6 cells / ml to 7×10^6 cells /ml) were washed three times with Spore induction buffer in a 50 ml Falcon tube. Washed cells prepared as above were resuspended at 10^6 cells / ml in spore induction buffer. For a standard assay, 2 ml of the suspension was combined with 2 ml of spore induction buffer in a 50 mm tissue culture dish so that the final cell density was 5×10^5 cells / ml unless stated otherwise. Caution was taken to cover the plate completely with cell suspension otherwise some cells could dry up because of surface tension.

Treatment with cAMP to induce spore formation;

cAMP powder was mixed with deionized water and the pH was brought to 7 with NaOH. The cAMP concentration was checked at the wavelength of 258 by spectrophotometer by comparing the OD with the OD given by a 1 M cAMP, pH 7 solution. For the standard sporogenous assay 5 mM cAMP was added at time 0 hour and samples were incubated for 48 hours at 22°C in dark and well-moisturised conditions. Mature spores and stalk cells were counted under a phase contrast microscope; a Leitz fluorescence microscope was employed on some occasions to examine cells stained by calcoflour which makes cellulose structures to fluoresce bright blue.

2.3 Adenylyl cyclase assay

2.31 Buffers and solutions

Lysis buffer 2 mM MgCl₂ in 10 mM Tris, (pH 8.0) 406 mg MgCl₂·6H₂O and

1.21 g Tris in 1 litre H₂O. Adjust pH to 8.0

0.1 M EDTA 3.72 g EDTA in 100 ml dH₂O and adjust pH to 8.0

0.5 M dithiothreitol 770 mg dithiothreitol (DTT) in 10 ml dH₂O and stored at -20°C as

0.5 ml aliquots.

0.1 M ATP 60.5 mg ATP in 1 ml dH₂O and stored at -20°C as 100ul aliquots

1 mM GTPγS 0.563 mg GTPγS;guanosine 5'-O-(3-thiotriphosphate) in 1 ml dH₂O

and stored at -20°C as 100μl aliquots

3-isobutyl-1-methyl xanthine (IBMX)

dissolved in DMSO and stored at -20°C

2'-deoxyadenosine 3',5'-monophosphate (2'H-cAMP)

dissolved in dH₂O and stored at -20°C

2.32 Assay of *in vivo* cAMP accumulation

Cells were harvested from growth medium, washed with 10 mM Na/K-phosphate buffer, pH 6.5 (PB) and either resuspended directly in PB at 10^8 cells/ml, or plated on PB agar at 2.5×10^6 cells/cm². The plated cells were starved for 6 hours at 22°C and subsequently collected and resuspended in PB at 10^8 cells/ml. The cell suspension was shaken gently for 5 min at 150 rpm to allow the cells to recover from centrifugation. 24-25 µl aliquots were incubated in microtitre plate wells with 3 µl of 10x concentrated variables, depending on the individual experiment. The plates were shaken at 22°C on a microtitre plate shaker. Reactions were started by addition of either 3 µl of 50 mM DTT or 3 µl of 50 µM 2'-H-cAMP in 50 mM DTT and terminated by addition of 30 µl of 3.5% perchloric acid. Lysates were neutralised with KHCO₃ and cAMP levels were determined by isotope dilution assay (van Haastert, 1984)

2.33 Adenylyl cyclase assay

Cells were harvested, washed once with PB, resuspended in ice-cold lysis buffer (2 mM MgCl₂ and 250 mM sucrose in 10 mM Tris, pH 8.0) and lysed through filters (pore size 3 µm). Aliquots of 10 µl of cell lysate were added to 5 µl of variables in microtitre plate wells and incubated at 0°C for 5 min. The reaction was started by adding 5 µl of assay mix (2mM ATP, 40 mM DTT in 2x-concentrated lysis buffer) and transferring the plates to a 22°C water-bath. The reaction was terminated by adding 10 µl of 0.2M EDTA, pH

8.0 and boiling the plates for 1 min. cAMP levels were assayed by isotope dilution assay.

2.34 Isotope dilution assay

Principle:

Unlabeled cAMP from unknown samples or standards competes with a known amount of ^3H -cAMP for binding to the regulatory subunit of cAMP dependent protein kinase. Unbound (^3H) cAMP is precipitated with activated charcoal and bound ^3H -cAMP is counted in the supernatant.

Solutions:

<u>cAMP assay buffer 1</u>	4 mM EDTA in 150 mM K-phosphate, pH 7.5
<u>cAMP assay buffer 2</u>	50 mg BSA in 25 ml of cAMP assay buffer 1 (0.2 % BSA)
<u>1:100 ^3H-cAMP stock</u>	5 μl equivalent to 40 Ci / mmol; 1 mCi/ml ^3H -cAMP + 500 μl H_2O
<u>cAMP standards</u>	make 8×10^{-7} M cAMP in H_2O (16 pmol/20 μl) ; make dilutions to 8, 4, 2, 1, 0.5, 0.25 pmol/20 μl
<u>cAMP binding protein</u>	Bovine Heart cAMP protein kinase purified by Dr. Pauline Schaap
<u>Activated charcoal</u>	1.25 g charcoal and 0.5 g BSA in 25 ml cAMP assay

solution 1. Stir gently for 3 hours on ice. Store at 4-6 °C and stir before use on ice for 15-30 min.

Assay:

Make directly before use: ^3H -cAMP binding mix: 40 μl 1:100 stock + 1ml cAMP assay solution 1. (Enough for 50 samples; scale up as required)

Add together: 20 μl ^3H -cAMP binding mix

20 μl cAMP standard or unknown sample

20 μl binding protein

For assay blanks, add 20 μl cAMP assay solution 2 instead of binding protein.

Incubate for 90 min or longer on ice. Add 40- μl charcoal to as many tubes as can be processed at once. Shake for a few seconds and incubate for 1 min at 0°C. Centrifuge for 2 min at 9000xG (Eppendorf centrifuge) or for 10 min at 3000 rpm (microtitre plate centrifuge) in the cold room. Transfer 50 μl supernatant to 1.5 ml cocktail.

Calculation:

Subtract assays blank from all samples and make standard curve.

Abscissa: pmol cAMP (standards), ordinate: C_0/C_x

C_0 : cpm 3H-Blank (no cAMP added)

C_x : cpm 3H-Blank standards or unknowns

Determine slope (tangent) = α

Unknowns: pmol cAMP/ assay = $[(C_0/C_x)-1] / \alpha$

Notes:

1. Buffer: The assay is rather sensitive to pH and is unreliable above pH 8 or below pH 6. Tris does not buffer well around pH 7 and cAMP is furthermore not completely stable in Tris; therefore phosphate buffer is used.
2. cAMP binding protein should not be added before the ^3H cAMP and sample. Otherwise since one of the binding sites has a very low off-rate, the new equilibrium is formed too slowly.
3. Because of the sensitivity of the assay system to pH, neutralisation of samples in perchloric acid should be done accurately. I did it as follows: add e.g. 50 μl of 3.5 % perchloric acid (v/v) to 50 μl of cell suspension. Mix well and neutralise with 25 μl 50 % saturated KHCO_3 (made as 100 % saturated solution at room temperature and then diluted 1:1 with water). Spin down the perchloric precipitate and use the supernatant in the assay.
4. Treatment of the charcoal / BSA suspension should be done carefully. It has to be stirred for a long time to equilibrate and to get rid of lumps. But stirring should not be fast otherwise the absorbing capacity of the charcoal drops for some reason.

2.35 Methods employed for individual experiments

2.351 Measurement of “cyclic AMP relay” in 6 hour starved cells

100ml of cultures of AX2, AX2/rdeA, AX2/regA, DH1/rdeA, aca- and aca-/rdeA- was harvested from growth medium, washed with 10 mM Na/K-phosphate buffer, pH 6.5 (PB). Washed cells were plated on PB agar at 2.5×10^6 cells/cm², starved for 6h at 22°C and subsequently collected and resuspended in PB at 10^8 cells/ml. The cell suspension was shaken for 5 min at 150 rpm to allow the cells to recover from centrifugation. 25µl aliquots of cell suspension were incubated in microtitre plate wells and the microtitre plate was shaken at 22°C on a microtitre plate shaker. Reactions were started by adding either 5 µl of 30 mM DTT (control) or 5 µl of 30 uM 2'H-cAMP in 30 mM DTT, and terminated by adding 30 µl of 3.5% perchloric acid. For T₀ samples 30 µl of 3.5% perchloric acid had been added to the microtitre plate wells before cell aliquots were added. Lysates were neutralised with 15 µl 50 % saturated KHCO₃, and 75 µl cAMP assay buffer 1 was added to render the cAMP concentration in a range best suited for the assay system. The data represent the means and s.e.m. (standard error means) of two independent experiments performed in triplicate.

2.352 Detection of cAMP accumulation in vegetative cells

40 ml culture of AX2, AX2/rdeA, AX2/regA, DH1, DH1/rdeA, aca- and aca-/rdeA- were harvested from growth medium and resuspended in PB at 10^8 cells/ml. The cell

suspension was shaken for 5 min at 150 rpm to allow the cells to recover from centrifugation. 25 µl aliquots of cell suspension were incubated in microtitre plate wells and the microtitre plate was shaken at 22°C on a shaker. Reactions were started by adding either 5 µl of 30 mM DTT (control) or 5 µl of 0.6 M NaCl in 30 mM DTT and terminated by adding 30 µl of 3.5% perchloric acid. For T₀ samples 30 µl of 3.5% perchloric acid had been added to the microtitre plate wells before the cell aliquots were added. Lysates were neutralised with 15 µl 50 % saturated KHCO₃ and 75 µl cAMP assay buffer 1 was added as before.

2.353 Response of cAMP accumulation to increased osmolarity

100 ml cultures of AX2/rdeA, AX2/regA, DH1/rdeA and aca-/rdeA- were harvested as before. 3 µl of 50 mM DTT together with 3 µl of either 0M, 0.1M, 0.2M, 0.5M, 1M, 2M, 3M and 5M NaCl was added to microtitre plate wells in advance. As a T₀ control 3 µl of 50 mM DTT with 3 µl of 0M, 1M and 5M concentrations of NaCl was added to microtitre plate wells together with 30 µl of 3.5% perchloric acid. Reactions were started by adding 24 µl aliquots of cell suspension and terminated by adding 30 µl of 3.5% perchloric acid after 10 min. Lysates were neutralised with 15 µl 50 % saturated KHCO₃ and 75 µl cAMP assay buffer 1 was added to to render the cAMP concentration in the range best suited for the assay system. The data represent the means and s.e.m. (standard error means) of two independent experiments performed in triplicate.

2.354 Effect of the cAMP-PDE inhibitor, IBMX, on adenylyl cyclase activity

aca- and aca-/rdeA cells were harvested from growth medium, washed once with PB and resuspended in ice-cold lysis buffer (2 mM MgCl₂ and 250 mM sucrose in 10 mM Tris, pH 8.0) at 10⁸ cells/ml. The cell suspensions were lysed through filters (pore size 3 μm). 10 μl aliquots of cell lysate were added to 10 μl assay mix (1mM ATP, 20mM DTT in lysis buffer) in the presence or absence of 0.2mM IBMX in microtitre plate wells and incubated at 0°C for 5 min. Reactions were started by transferring the microtitre plates to a 22°C water-bath. For t₀ samples 10 μl of 0.2M EDTA, pH 8.0 had been added to microtitre plate wells before the cell lysate was added. The reaction was terminated by adding 10 μl of 0.2M EDTA, pH 8.0 at the indicated time periods and finally the microtitre plate was boiled for 1 min. cAMP levels were determined as above.

2.355 Testing G protein dependence of adenylyl cyclase activity

aca-/rdeA cells lysates were prepared in the presence and absence of 30 μM GTPγ S or 30 μM GDPβ S. Aliquots of 10 μl of cell lysate were then assayed as described.

2.356 Effect of caffeine on adenylyl cyclase activity

aca-/rdeA- cell lysates were prepared as before and assayed with 3 μl of 50 mM DTT

together with 3 μ l of 0 M, 2 M, 5 M, 10 M, 20 M and 50 M concentrations of caffeine added to microtitre plate wells in advance. For T_0 samples 3 μ l of 50 mM DTT with 3 μ l of 50 M of caffeine was added to microtitre plate wells along with 30 μ l of 3.5% perchloric acid beforehand. Reactions were started by adding 24 μ l aliquots of cell suspension and terminated by adding 30 μ l of 3.5% perchloric acid after 20 min. Lysates were neutralised with 15 μ l of 50 % saturated KHCO_3 and 75 μ l cAMP assay buffer 1 was added to the neutralised solution to produce a cAMP range best fitted for assay.

2.357 Stimulation of adenylyl cyclase activity by Mg^{2+} and Mn^{2+}

aca-/rdeA cells were harvested from growth medium, washed once with PB and resuspended in ice-cold lysis buffer without MgCl_2 (250 mM sucrose in 10 mM Tris, pH 8.0) at 10^8 cells/ml. The cell suspension was lysed through filters. 0 mM, 1 mM, 2 mM, 4 mM, 10 mM, 20 mM and 40 mM concentrations of MgCl_2 and MnCl_2 were prepared in H_2O . 2x concentrated assay mix was made to 2 mM ATP, 40 mM DTT in 2x-lysis buffer without MgCl_2 (500 mM sucrose in 20 mM Tris, pH 8.0). 5 μ l of variables and 5 μ l of 2x-concentrated assay mix were added to microtitre plate wells beforehand on ice. 10 μ l aliquots of cell lysate were added to the prepared microtitre plate and incubated at 0°C for 5 min. Reactions were started by transferring the microtitre plate to 22°C water-bath. For T_0 samples 10 μ l of 0.2M EDTA, pH 8.0 had been added to microtitre-plate wells before cell lysate was added. The reaction was terminated by adding 10 μ l of 0.2M EDTA, pH 8.0 after 20 minutes followed by boiling the microtitre-plate for 1 min. cAMP levels were determined by isotope dilution assay (van Haastert, 1984). The data represent the means

and s.e.m. (standard error means) of two independent experiments performed in triplicate.

2.358 Effect of pH on adenylyl cyclase activity

aca-/rdeA cells were harvested from growth medium, washed once with PB and resuspended in modified lysis buffer (2 mM MgCl₂ and 250 mM sucrose in 2 mM Tris, pH 7.5) at 10⁸ cells/ml. The cell suspension was lysed through filters (pore size 3 µm). 0.5 M Tris-maleate of pH 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 9.0 was prepared respectively. 5 µl of 0.5 M Tris-maleate of the indicated pH as well as 5 µl of modified assay mix (2 mM ATP, 40 mM DTT in 4 mM MgCl₂) was added to microtitre-plate wells on ice in advance. 10 µl aliquots of cell lysate were added to the prepared microtitre-plate and incubated at 0°C for 5 min. The reaction was terminated by adding 10 µl of 0.2M EDTA, pH 8.0 after 10 minutes and finally the microtitre plate was boiled for 1 min.

2.359 In vivo cAMP accumulation during starvation of aca-/rdeA cells

aca-/rdeA cells were either used directly from growth medium (T₀) or first plated on PB agar and incubated for 1, 2, 3, 4, 5, or 6 hours at 22°C. Subsequently cells were resuspended in PB to 10⁸ cells/ml, exposed to 5mM DTT for 0, 2, 5, or 10 minute and assayed for total cAMP levels. Data were standardised on the protein level of the cell suspensions and expressed as percentage of cAMP accumulated after 10 minutes in cells at T₀. Means and s.e.m.(standard error means) of three experiments performed in triplicate are presented.

2.4 Yeast two-hybrid system

2.41 Media for yeast growth

YPD medium

Bacto yeast extract 10g/L

Bacto peptone 20g/L

Glucose	20g/L
---------	-------

adjust pH 5.8 and autoclave at 121°C for no more than 15 min

to prepare plates, add Bacto agar (20 g/ L)

SD medium (Synthetic Dropout)

Yeast nitrogen base without amino acids 6.7 g/L

Bacto agar 20 g/L (for plates only)

100 ml of the appropriate sterile 10X dropout solution

add water up to 1 L

adjust pH to 5.8 and autoclave

10X drop out supplement

SD-Leu, SD-Trp and SD-Leu-Trp were required and an easy way to prepare them was as follows,

	10X conc.
A) L-Isoleucine	300mg/L
B) L-Valine	1500mg/L
C) L-Adenine Hemisulfate Salt	200mg/L
D)L-Arginine HCl	200mg/L
E)L-Histidine HCl monohydrate	200mg/L
F)L-Leucine	1000mg/L
G)L-Lysine HCl	300mg/L
H)L-Methionine	200mg/L
I) L-Phenylalanine	500mg/L
J) L-Threonine	2000mg/L
K) L-Tryptophan	200mg/L
L) L-Tyrosine	300mg/L
M) L-Uracil	200mg/L

2.42 Molecular cloning for yeast two-hybrid test

2.421 The construction of *rdeA*- *pAS2*

The cDNA of the *rdeA* gene was obtained by PCR amplification and digested with Nco1(

Promega) and BamH1 (Promega) overnight. The double digested *rdeA* gene was purified from a 1% agarose gel using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). The plasmid pAS2 was digested with Nco1 and BamH1 at 37°C for 2 hours and purified from 1% agarose using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). The pAS2 vector fragment was ligated to the *rdeA* gene fragment at 16°C overnight. The ligated product was transformed into *E.coli* and the transformants were incubated on LB (Luria Bertoni)-ampicillin agar at 37°C overnight. Several colonies were picked and inoculated for making mini-preps. They were cultured in a shaking incubator at 37°C overnight and the plasmid was purified by mini-prep and digested with Nco1 and BamH1. The digested product was analysed on a 1% agarose gel and the expected ligation product was confirmed. 500 µl of *E.coli* culture containing the ligated vector was reinoculated into 500 ml of LB (Luria Bertoni) broth with 100 µg/ml ampicillin for large-scale preparation of the plasmid. After large-scale preparation the DNA was concentrated to 1µg/µl

2.422 The construction of *regA-pACT2*

RegA cDNA gene was obtained by PCR amplification and digested with EcoR1 and Xho1 (both from Promega) overnight. The double digested *regA* gene was purified from a 1% agarose gel using Gene Clean. The plasmid pACT2 was digested with EcoR1 and Xho1 at 37°C for 2 hours and purified from 1% agarose using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). The pACT2 vector fragment was ligated to the *regA* gene fragment at 16°C overnight. The following procedures were the same as in section 2.431 “The construction of *rdeA- pAS2*” except EcoR1 and Xho1, the replaced restriction

enzymes.

2.423 The construction of *rdeA*- *pACT2*

The *rdeA*-*pAS2* plasmid was digested with *Nco*I and *Bam*H1 and the *rdeA* gene fragment was purified from 1% agarose gel using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). A plasmid *pACT2* was digested with *Nco*I and *Bam*H1 at 37°C for 2 hours and purified from 1% agarose gel using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). The *pACT2* vector cut was ligated to the *rdeA* gene cut at 16°C for overnight. The following procedures were same as 2.431 “The construction of *rdeA*- *pAS2*”.

2.424 The construction of *regA*- *pAS2*

The *regA*-*pACT2* plasmid was digested with *Eco*R1 and *Xho*I and the *regA* gene fragment was purified from 1% agarose gel using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). A plasmid *pAS2* was digested with *Eco*R1 and *Xho*I at 37°C for 2 hours and purified from 1% agarose gel using GeneClean II Kit (BIO 101, Inc. Cat #1001-400). The *pAS2* vector cut was ligated to the *regA* gene cut at 16°C for overnight. The following procedures were same as 2.431 “The construction of *rdeA*- *pAS2*” except *Eco*R1 and *Xho*I, the replaced restriction enzymes.

2.425 Purification of DNA from agarose gels

[=How to use GeneClean II Kit (BIO 101, Inc. Cat #1001-400)]

DNA was run on 1 % agarose gels at 80 - 110 Voltage in 1x TAE and bands were excised from the gel using very weak UV light so as not to damage the DNA. The gel fragments contained in 1.5 ml Eppendorf tubes were weighed and 3 times volume NaI (volume / weight) of the weight was added to the tube. The tube was incubated at 50 °C for 5 minutes to 15 minutes until the gel was completely melted in the NaI. Glassmilk was mixed very thoroughly for 2-3 minutes by being vortexed horizontally, before 10 µl to 12 µl aliquots were taken out. After an aliquot of glassmilk had been added to the tube containing the DNA fragment, it was mixed by inverting and incubated on ice for 5 minutes, then centrifuged at 1,000 rpm, room temperature for 5 seconds and the supernatant transferred to a fresh tube. The pellet was mixed well with 800 µl New Wash solution kept at -20 °C and centrifuged under the same conditions as in the previous step. The washing step was repeated three times and any beads left on the wall of the tube were removed using a Pasteur pipette attached to a vacuum line. 40 µl to 60 µl of TE buffer was added to the pellet and it was incubated at 50 °C for 1 minute to get the pellet dissolved. To remove any glassmilk bead remaining in the tube it was centrifuged at 12000 rpm, room temperature for 30 seconds and the supernatant transferred to a fresh tube. This step was repeated three times and I generally obtained 31 µl to 51 µl of final DNA solution. Finally the purified DNA was checked on a 1 % agarose gel before any further step.

2.426 *E.coli* transformation

JM109 high efficiency competent cells were purchased from Promega. In some

experiments competent *E. coli* cells were kindly supplied by Dr W-T. Chang. Competent cells kept at -70 °C were thawed on ice and gently mixed with 0.5 µg of the plasmid to be transformed. The tube was incubated on ice for 20 minutes to 30 minutes and heat shocked at 42°C for 45 to 90 seconds. Immediately after heat shock the tube was incubated on ice for another 2 minutes. 1 ml LB (Luria Bertoni) broth or SOC medium was added, mixed well and incubated at 37°C in a water bath for 1 hour. 100µl to 200 µl of the transformation mix was plated on LB (Luria Bertoni) -amp agar and incubated at 37°C overnight.

2.427 Mini scale preparation of plasmids

Cells were pelleted from 1-3 ml culture and resuspended in 200 µl of cell suspension. Cells were then lysed by adding 200 µl cell lysis solution and 200 µl neutralisation solution was added. Centrifugation for 5 minutes at 12000 rpm at room temperature was performed to clear the lysate. The supernatant was transferred to a fresh tube and mixed thoroughly with 1 ml Wizard Miniprep Resin (from Promega). The mixture was then transferred to a Wizard Minicolumn and washed once with 2-ml column wash solution. 50 µl of deionized water or TE buffer was added and DNA eluted after 1 minute by micro-centrifugation for 20 seconds.

2.428 Large scale preparation of plasmids

Purification of DNA pellets from a large amount of E.coli culture

500 ml *E. coli* transformants cultured in LB broth was harvested by centrifugation at 5000 rpm, 4°C for 10 minutes. The supernatant was removed completely by vacuum with a Pasteur pipette. The cell pellet was resuspended in 20 ml solution 1, mixed gently and incubated on ice for 5 minutes. 40 ml of solution 2 was freshly prepared just before being added to the cell suspension in solution 1. It was mixed completely by inverting the tube slowly and gently. At this step the cell suspension was very sticky, and it was incubated at room temperature for 10 minutes. 20 ml of solution 3 was added and mixed thoroughly for a few minutes for neutralisation. This step should be done very carefully to obtain a good protein pellet and to clear the very sticky solution. The solution was incubated on ice for a further 10 minutes and centrifuged at 5000 rpm, 4°C for 20 minutes in a Sorvall GS3 rotor. If the pellet was not well formed, the centrifugation step was repeated either at 5000 rpm, 4°C for 20 minutes or 5500 rpm, 4°C for 10 minutes. The supernatant was transferred to a fresh autoclaved tube and 50 ml isopropanol was added and mixed slowly by inverting the tube. It was incubated at room temperature for 5 to 10 minutes and centrifuged at 5000 rpm, room temperature for 30 minutes. The supernatant was carefully removed and the DNA pellet rinsed once with 70 % EtOH. Any beads of liquid that adhered to the wall of the tube were removed using a Pasteur pipette attached to a vacuum line. The DNA pellet was dried on the bench in the inverted tube for 45 minutes. The air-dried pellet was dissolved in 4.1 ml TE (pH 8.0) and the desired plasmid DNA was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient.

Purification of plasmid DNA from a DNA pellet

4.6 g CsCl was added to 4.1-ml nucleic acid solution along with 0.5 ml 10 mg/μl EtBr. It

was mixed by vortexing for seconds and the red solution was transferred into a Quick-seal centrifuge tube (4.6 ml capacity) via a Pasteur pipette. The tube was centrifuged at 45,000 rpm, 20°C in a Vti 65 rotor for more than 16 hours with vacuum on. Since the velocity is so high, balance should be checked to the –2 digit level. The plasmid band was collected into a 1.5 ml Eppendorf tube using a 19G needle and syringe. EtBr was removed by extracting with isoamyl alcohol 6 or 7 times. The isoamyl alcohol could be replaced by 1-butanol or isobutyl alcohol. The plasmid DNA was then precipitated by addition of 3 volumes of water followed by 2 volumes of ethanol. After incubation at -20°C overnight it was centrifuged at 10000 rpm for 15 minutes at 4°C. The pellet was rinsed with 95% ethanol, air dried and dissolved in TE buffer. DNA concentration was measured at 260nm using a spectrophotometer, and diluted to 1 µg/µl.

2.429 Buffers and solutions

Solution 1 for large scale preparation

50 mM Glucose

25 mM Tris.Cl (pH 8.0)

10 mM EDTA (pH 8.0)

This can be prepared in batches of 100 ml, autoclaved and stored at 4 °C

Solution 2 for large scale preparation

Stock solutions;

10 N NaOH

20 % SDS

Solution 2 should be freshly prepared as follows.

0.8 ml 10 N NaOH

2 ml 20 % SDS

Add water up to 40 ml and mix by vortexing.

Solution 3 for large scale preparation

5 mM potassium acetate	60 ml
------------------------	-------

Glacial acetic acid	11.5 ml
---------------------	---------

H ₂ O	28.5 ml
------------------	---------

total volume	100 ml
--------------	--------

Stored at 4°C

Ampicillin

Stock solution was made to 50 mg/ml in H₂O and filtered. 1ml aliquots were kept at -20°C and diluted to 50 µg/ml or 100 µg/ml (for strong selection) for selection.

LB (Luria-Bertoni) Broth or (LB agar Plates)

Bacto trypton	10 g
Bacto yeast extract	5 g
NaCl	10 g
(Bacto agar	15 g)

dissolved in dH₂O and adjusted to pH 7.0 with NaOH

final volume to 1 Litre and autoclaved at 123°C for 15 min

6x agarose gel loading buffer

0.25 % Bromophenol blue

0.25 % Xylene cyanol FF

30 % Glycerol in H₂O

filtered and stored at room temperature.

2.43 Yeast transformation (LiAc transformation; high efficiency)

2.431 Preparation of yeast competent cells

A few large colonies were inoculated into 10 ml of YPD medium in a Falcon tube (preculture) at 30 °C overnight (doubling time: 4hrs). The precultured cells (5ml-10ml) were reinoculated into 50 ml or 100 ml YPD medium. OD value was measured after 4 hours (desirable OD 0.6-0.8; cells should be in log phase for efficient transformation). Cells were harvested in 50 ml falcon tubes and washed in 10 ml of dd H₂O and spun

down. 50 ml of cell culture was resuspended in 500µl - 1.0ml of freshly made 0.1 M LiAc. The cells were when I originally started with . The cell suspension in 0.1 M LiAc was incubated at 30 °C in a heat block for 30 min-1 hour and meanwhile plasmids were pipetted in 1.5 ml Eppendorf tubes.

2.432 Heat-shock yeast transformation

Each transformation required

50 µg of carrier DNA (5µg of 10 µg/ul herring sperm DNA),

500 ng-1 µg of each plasmid to be transformed and 50µl-100 µl of cells.

Fresh 40 % PEG (polyethylene glycol) was made just before use. 600 µl of 40 % PEG was added to each tube prepared above for yeast transformation, mixed by pipetting and then incubated at 30 °C (heat block or water bath) for 30 min. This step could be done overnight but then the number of transformants would be reduced. 70 µl of 100 % DMSO was added to make the final concentration 10 % DMSO and heat shock was given at 42°C for 15-20 min. Cells were spun down at 12000-13000rpm for 3-4 minutes at room temperature and the supernatant was discarded. The cell pellet was washed by pipetting with 1 ml of sterile H₂O and then spun down again at 12000-13000rpm for 3-4 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 1ml of dH₂O (deionised water). 100 µl of the transformants from each tube was plated on the appropriate plate depending on selective conditions.

2.433 Buffers and Solutions

Herring testes carrier DNA (10 mg/ml)

Sonicated, herring testes carrier DNA in solution was purchased separately.

(Sigma #K 1606-A)

PEG/LiAc solution (polyethylene glycol/lithium acetate)

Final conc. To prepare 10 ml of Solution

PEG 4000 40% 8ml of 50% PEG

TE buffer 1X 1ml of 10X TE

LiAc 1X 1ml of 10X LiAc

50% PEG 4000 (Polyethylene glycol, average molecular weight =3,350 ;

Sigma #p-3640) Filter-sterilise or autoclave.

100% DMSO (Dimethyl sulfoxide; Sigma #D-8779)

10X TE buffer

0.1 M Tris-Hcl, 10 mM EDTA, pH 7.5. Autoclave

10X LiAc

1 M lithium acetate. Adjust pH to 7.5 with diluted acetic acid and autoclave

PBS

KH_2PO_4 0.144g

NaCl 9g

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 0.795g

dissolved in 1 litre water

2.44 Growing yeast transformants with selection

Transformants were grown at 30°C for 2 to 5 days until the colonies were 2-3 mm in diameter. The degree of ventilation should be watched to avoid the agar plates' being dried out before colonies had grown properly. Transformants were selected on SD-agar plates containing all amino acids except that omitted for selection.

2.45 Colony-lift filter assay

2.451 Reagents and materials

Materials

Whatman #5 or VWR Grade 410 paper filters, sterile

90 mm petri dishes

Forceps for handling the filters

Liquid nitrogen and its container

Reagents:

Z buffer

Na ₂ HPO ₄ .7H ₂ O	16.1 g/L
NaH ₂ PO ₄ .H ₂ O	5.50 g/L
KCl	0.75 g/L
MgSO ₄ .7H ₂ O	0.246 g/L

Adjusted to pH 7.0, autoclaved and stored at room temperature for up to 1 year.

X-gal stock solution

Dissolve 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-GAL ; #8060-1) in N,N-dimethylformamide (DMF) or DMSO at 20mg/ml. Stored in the dark at -20 °C

Z buffer / X-gal solution

Z buffer	100 ml
β-mercaptoethanol (β-ME ; Sigma #M-6250)	0.27 ml
X-gal stock solution	1.67 ml

Mixed well; 2.5 - 5 ml was aliquoted onto each plate to be assayed.

2.452 Methods

Fresh colonies (for instance, those grown at 30 °C for 2-4 days), of 1-3 mm in diameter were used for best results. For each plate of transformants to be assayed, a sterile Whatman #5 filter was presoaked by being placed in 2.5 - 5 ml of Z buffer / X-gal solution in a clean 90 mm petri dish. A clean and dry filter was placed over the surface of the plate of colonies to be assayed using forceps. The filter was gently rubbed with the side of the forceps to help the colonies cling to the filter. When the filter has been evenly wetted, it was carefully lifted off with forceps and transferred (colonies facing up) into a pool of liquid nitrogen. The filter was submerged completely for 10 seconds with forceps and removed once frozen. The frozen filter was thawed at room temperature to permeabilize the cells. The filter, colony side up was placed on the presoaked filter and incubated at 30 °C for 30 minutes to 6 hours. The filter was checked periodically for the appearance of blue colonies.

2.46 Western Blotting

2.461 Purpose

In order to check whether the fusion proteins were expressed as expected.

As described in chapter 5, two sets of western blots had to be performed and the experimental procedures were the same for each set. Only the test for set 2 will be described out.

2.462 Buffers and solutions

Protease inhibitor cocktail

70 µl per 1 ml of cracking buffer

Types of proteases inhibited

Pepstatin A	66 µl	carboxyl proteases
Leupeptine	2µl	thiol & serine proteases
Benzamidine	500 µl	trypsin, plasmin, thrombin
Aprotinin	120 µl	some serine proteases

Total 688 µl

Blocking solution

Milk (Co op)	10 g
10 X PBS	10 ml
Tween 20	100 µl

Add water to 100 ml

Washing solution

Milk (Co op)	10 g
10 X PBS	200 ml
Tween 20	2 ml

Add water up to 2L

Cracking buffer stock solution

To prepare 100ml		
Urea	8M	48g
SDS	5% w/v	5g
Tris-HCl (pH6.8)	40 mM	4ml of a 1M stock solution
EDTA	0.1mM	20µl of a 0.5M stock solution
Bromophenol Blue	0.4mg/ml	40 mg
Deionized H ₂ O		To a final volume of 100 ml

SDS-PAGE gels

Running gel (10 %)

30 % / 0.8 % Stock	3.3 ml
(30 % w/v Acrylamide / 0.8 % w/v Bis acrylamide ratio 37.5:1 from Scotland Easigel)	
1 M Tris pH 8.8	3.75 ml
20 % SDS	50 µl
10 % APS (ammonium persulphate)	100 µl
TEMED	10 µl

Water	2.9 ml
-------	--------

total volume	10 ml
--------------	-------

Stacking gel

30 % / 0.8 % Stock	1.6 ml
--------------------	--------

(30 % w/v Acrylamide / 0.8 % w/v Bis acrylamide ratio 37.5:1 from Scotland Easigel)

1 M Tris pH 6.8	1.25 ml
-----------------	---------

20 % SDS	50 µl
----------	-------

10 % APS (ammonium persulphate)	165 µl
---------------------------------	--------

TEMED	33 µl
-------	-------

Water	7.1 ml
-------	--------

total volume	10 ml
--------------	-------

5x Sample buffer

DTT	0.77 g
-----	--------

SDS	1.0 g
-----	-------

1 M Tris 6.8	4.0 ml
--------------	--------

Glycerol	5.0 ml
----------	--------

Bromophenol Blue	300 µl (0.2 % in EtOH)
------------------	-------------------------

Add water up to 20 ml.

Running buffer (5x stock solution)

Tris base	15.1 g
Glycine	94 g
20 % SDS	25 ml
dd H ₂ O	300 ml

Adjust pH to 8.3 and make up to 1 Litre with dd H₂O.

2.463 Procedures

Preparation of protein extracts

2) Test set II (RDEA: Tx AD & REGA: DNA BD)

Transformants were inoculated into selective medium as follows.

Transformants harbouring the pAS2 vector alone (control) in SD-Trp.

Transformants harbouring the pACT2 vector alone (control) in SD-Leu.

Transformants harbouring the pAS2-regA plasmid (control) in SD-Trp.

Transformants harbouring the pACT2-rdeA plasmid (control) in SD-Leu.

Transformants harbouring pAS2-regA and pACT2-rdeA in SD-Trp-Leu.

For the abbreviations of SD, Leu and Trp, please refer to page 37-38.

Five different transformants were inoculated into each selective medium and incubated overnight. The precultures (10ml of each cell culture) were reinoculated into 10ml of YPD. (Harvest the precultured cells by centrifugation and add 10 ml of YPD to the cell pellet.) To produce more protein they were grown for another 4 hours. The cells were harvested by centrifugation at 3700 rpm, for 3-5 mins, at room temperature and resuspended in 10 ml of water for washing. The cell suspension was spun down again at 3700 rpm, for 3-5 mins at room temperature. Cracking buffer was freshly prepared as follows.

Prewarm the stock for 5 min at 60 °C

3 ml stock (blue)

30 µl βmercaptoethanol

210 µl cocktail of protease inhibitors

150 µl PMSF (serine protease inhibitor); As the $t/2$ of PMSF is 7 min, PMSF was

added every 10 min.

The above reagents were resuspended in a 50 ml Falcon tube and 320 µl of glass beads was put into 1.5 ml Eppendorf tubes (prepare 6 tubes). The cells resuspended in cracking buffer (total volume; about 400 µl) were transferred into the 1.5 ml Eppendorf tubes containing the glass beads and mixed by pipetting. The tubes were incubated for 10 mins at 70°C, vortexed for 1 min and then spun down at 14000 rpm, for 5 mins, at 4°C. The supernatant was taken out and kept on crushed ice. 40 µl of cracking buffer was added to the pellet (10 % of the original volume of the cracking buffer) to facilitate resuspension. The tubes were boiled for 5 min at 100°C (This was done in an attempt to solubilise

membrane proteins or other insoluble proteins not collected at the previous step).

Each tube was vortexed again for 1 min and spun down. The supernatant was added to the previous tubes and mixed. Protein extracts were then ready to be loaded on a gel.

From SDS-PAGE to detection

Loading;

Protein samples were boiled for 1-2 min just before being loaded. 10% SDS protein gel was prepared according to the recipes described in 2.462 "Buffers and solutions".

10 µl of marker and 20 µl of the samples were loaded and the SDS gel was run for 1 hr at 100 V. In the meantime, blocking solution, washing solution and filter were prepared.

How to prepare filters for transfer;

Cut one nitro-cellulose filter and three pieces of 3MM paper to the size required.

Soak the filter in methanol and then wash it with water to remove the methanol.

Soak in transfer buffer.

How to make blotting sandwiches;

All the proteins in a SDS gel are denatured and negatively charged. The gel was placed to face the negative pole and the filter towards the positive pole so that all the proteins could be transferred to the filter. Blotting was carried out at 100 V for 1 hour with a dry-ice bag placed in the tank to maintain the temperature low. After transfer the filter was removed carefully, washed and incubated in blocking buffer.

Treatment of primary and secondary antibodies;

Dilution of primary Abs;

DNA BD antibody	TX AD antibody	
2.5µg/µl	2.1µg/µl	(Stock)
1/2000	1/5000	(dilution constant)
1 µl of stock in 5 ml of washing sol.		
0.5µg/ml	0.4 µg/ ml	(working conc.)

Before adding 1st Ab, the filter was washed 3 times with washing buffer (15 min for 1st and 2nd, 5 min for 3rd). The washed filter was placed in a plastic bag, which was sealed on three sides. 5 ml of 1st Ab was added in the bag and one side of the bag was sealed. After 1 hr incubation at room temperature the filter was taken out and washed 3 times. 5ml of 2nd Ab (anti mouse 2nd Ab 1/5000 dilution) was treated as for the 1st Ab.

Detection:

Mix 3 ml of substrate 1 and 3ml of substrate 2 (Amersham ECL detection system, RPN 2105). The filter was incubated for 1 min at room temperature in the mixed substrate solution. Before the filter was soaked in the substrate solution, excess solution was removed.

Cut the filter and wrap it up with cling film. Finally the filter was placed in a cassette and developed in dark room.

2.5 Phosphodiesterase assay

2.51 Buffers and solutions

Lysis buffer 1 (for total intracellular phosphodiesterase assay); (Henderson, 1975)

50 mM Tris-HCl

12.5 mM MgSO₄

250 mM Sucrose

Adjust pH to 7.5

Lysis buffer 2 (for REGA phosphodiesterase assay); (Thomason et al., 1998)

50 mM Tris-HCl

50 mM KCl

5 mM MgCl₂

10 % Glycerol

Adjust pH to 8.0

IP buffer

50 mM K₁/K₂PO₄

1 mM MgCl₂

1 % BSA (Bovine Serum Albumin)

10 % glycerol

Adjust pH to 7.3

Alkaline Phosphatase from calf intestine, 1 $\mu\text{g}/\mu\text{l}$ and stored at 4°C

(Boehringer Mannheim)

0.5 M dithiothreitol 770 mg dithiothreitol (DTT) in 10 ml dH_2O stored at -20°C as

0.5 ml aliquots.

IBMX, 3-isobutyl-1-methyl xanthine

dissolved in DMSO and

stored at -20°C (SIGMA # I-7018)

Rolipram, 4-[3-(Cyclopentyloxy)-4-methoxy-phenyl]-2-pyrrolidinone

dissolved in H_2O and stored at -20°C

(SIGMA #R-6520)

2.52 Total intracellular phosphodiesterase assay

Cells were harvested, washed once with PB and resuspended in lysis buffer 1 (50 mM Tris-HCl, 12.5 mM MgSO_4 , 250 mM Sucrose, pH 7.5) at 10^8 cells/ml. The cell suspension was lysed either through nuclepore filters (25mm, pore size 3 μm) or by being frozen in dry ice followed by thawing on ice. Aliquots of 50 μl of cell lysate were added to 50 μl of assay mixture prepared in microtitre-plate wells and incubated at 30°C for 10, 20 or 30 minutes. The microtitre-plates were prewarmed in a 30°C water-bath and the reaction was started by adding aliquots of 50 μl of cell lysate to the 50 μl of assay mix [0.6 μM of ^3H -cAMP, 20 mM DTT and alkaline phosphatase (0.1 unit per reaction, 1 unit

= 1 μ mol/min, 25°C) in lysis buffer 1]. Reactions were terminated by transferring 100 μ l-reaction mixture to 900 μ l of Dowex in a disposable scintillation vial. (The Dowex was base- and acid- washed before equilibration in absolute ethanol, and the final slurry contains 6 g of Dowex/20 ml of ethanol.) The slurry was then well mixed with 9 volumes of toluene/PPO/POPOP scintillant and the free adenosine determined by scintillation counting. Dowex quenches the radiation of the adsorbed cAMP.

2.53 Assay of the RegA phosphodiesterase

Immunoprecipitation;

Frozen pellets of 10^8 cells were lysed at 1.1×10^8 /ml on ice in IP buffer containing protease inhibitors and precleared by centrifugation at 13,000 rpm in a micro-centrifuge for 40 seconds. The supernatant was added to a fresh tube containing the equivalent of 25 mg (dry weight of protein A Sepharose CL-4B [Pharmacia]/ 10^8 cells and 45 μ l (or 90 μ l of serum stored in 50 % glycerol) of R1/2F or pre-immune serum. After agitation for 1 hour at 4°C the Sepharose beads were pelleted by centrifugation at 13,000 rpm in a micro-centrifuge for 8 seconds. The supernatant was removed and the pellet washed 6 times in the equivalent of 8 bed volumes (or 6 x 0.9 ml) of IP buffer (minus the BSA). The beads can then be assayed for PDase activity or boiled in SDS-PAGE sample buffer for Western Blotting.

Notes

- The protein A Sepharose beads were rehydrated and washed several times in MilliQ

dH₂O before being equilibrated and stored in IP buffer at 4°C.

- The precipitate from the equivalent of 2×10^7 cells was used for each test (using 9 µl of R1/2F antiserum and 5 mg dry weight of protein A Sepharose) since this amount of starting material contains sufficient regA protein to be easily detected by both Western Blotting and by PDase assay.

The REGA phosphodiesterase assay

The immunoprecipitate was resuspended in lysis buffer 2 (50 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 10 % Glycerol, pH 8.0) at 10^8 cells/ml. Aliquots of 50 µl of the resuspended immunoprecipitate were added to 50 µl of assay mixture prepared in microtitre-plate wells and incubated at 25°C for 30 or 60 minutes. The microtitre-plates were prewarmed in a 5°C water-bath and the reaction was started by adding aliquots of 50 µl of the immunoprecipitate to the 50 µl of assay mix [0.6 µM of ³H-cAMP, 20 mM DTT and alkaline phosphatase (0.1 unit per reaction, 1 unit = 1 µmol/min, 25°C) in lysis buffer 2). Reactions were terminated and counted as above.

Chapter 3

Chapter 3

The discovery of a novel adenylyl cyclase in the course of a physiological study of the *rdeA* mutant

3.1 Introduction

3.11 PKA plays a key role in controlling the rate of development.

Mutants exhibiting altered rate of development can provide useful insight into rate limiting events in development (Ambros and Moss, 1994). As mentioned in Chapter 1 there are three classes of rapidly developing mutants in *Dictyostelium*, *rdeA*, *rdeC* and *regA*. *rdeC* mutants lack a functional regulatory subunit of cAMP-dependent protein kinase (PKA) so that PKA activity is constitutive. PKA plays an important role in many aspects of development as well as in learning and memory (Kandel and Abel, 1995) and in *Dictyostelium* genetic manipulation has demonstrated that it is essential for gene expression throughout development (Schulkes and Schaap, 1995 ; Mann et al, 1997). Thus the level of PKA activity appears to have a profound influence on the rate of development.

3.12 Is high PKA activity responsible for the sporogenous phenotype of rapidly developing mutants?

The previously isolated *rdeA* mutant HTY 507, produced by chemical mutagenesis, (Abe et al, 1981) and *rdeC* mutants were known to be sporogenous (Kay, 1989) i.e. they can produce spores in submerged monolayers provided cAMP is present. The

regA mutant I have been using (HM1015 from Dr. Peter Thomason) was originally found when sporogenous mutants were identified in a pool of REMI mutants. We still do not exactly understand how sporogenous mutants are able to produce spores in submerged monolayers when given 5 mM cAMP, since this is a concentration that should be high enough to cause ACA (Adenylyl Cyclase responsible for Aggregation) to adapt. The reason wild type cells can only make prespore cells could be that their levels of PKA activity is not high enough to induce spores since the main adenylyl cyclase (ACA) is adapted. Whatever the exact explanation, it is generally believed that sporogenous mutants have higher PKA activity in submerged monolayers than wild type cells and that this increase PKA activity is responsible for spore induction.

3.13 Is the high PKA activity in rapidly developing mutants due to elevated intracellular cAMP levels?

As I pointed out earlier, rdeA mutants were reported to have elevated intracellular cAMP levels during vegetative growth and during development (Abe and Yanagisawa, 1983; Coukell and Chang, 1980). I measured total cAMP levels in the AX2/rdeA mutant generated by REMI and in aca-/rdeA cells during vegetative growth and development to check whether the AX2/rdeA strain had elevated cAMP levels, as had been reported for the chemically induced rdeA mutants. Although AX2/rdeA cells did show rapid development, I did not find that total cAMP levels were elevated in AX2/rdeA cells (see Figure 3.1). It seems unlikely that the absence of elevated cAMP levels in AX2/rdeA cells is due to their genetic lesion having been induced by a different method than the previously studies rdeA mutants. On the other hand it is not easy to understand how AX2/rdeA cells are rapidly developing, but do

not have elevated cAMP levels, if we believe that elevated intracellular cAMP levels are the fundamental reason for high PKA activity and that this in turn produces the rapid development phenotype. One possibility is that even if we assume that the basic mechanism for rapid development is high PKA activity, rdeA cells might not necessarily need to have markedly elevated intracellular cAMP levels since the PKA regulatory subunit has extremely high affinity for cAMP (de Gunzburg et al., 1984). It seemed likely that studying the biochemical physiology of AX2/rdeA cells in depth could provide insight into how PKA activity is regulated to induce rapid development. I approached this question by measuring cAMP accumulation by intact rdeA and wild type cells. Since total cAMP is a complex function of adenylyl cyclase activities, rates of destruction of intracellular and extracellular cAMP and the rate of secretion of cAMP from the cell it seemed more informative to measure "instantaneous" rates of cAMP accumulation by intact cells at various times during development.

3.14 The two adenylyl cyclases known in *Dictyostelium*

As outlined in Chapter 1, two adenylyl cyclases, ACA and ACG, were known in *Dictyostelium discoideum* when I started my work. One, ACA was thought to be responsible for all cAMP produced during development and is maximally expressed around aggregation but shows negligible activity at the vegetative stage. ACA is coupled to cAMP receptors in the plasma membrane as well as to G proteins (Pitt et al., 1992). The mechanism activating ACA would appear to be as follows:

extracellular cAMP binds to cAMP receptors and the activated cAMP receptors cause the G protein $\beta\gamma$ subunit to separate from the GTP-bound G protein α subunit. The dissociated G protein $\beta\gamma$ subunit interacts in the cytoplasm with CRAC (cytosolic

regulator of adenylyl cyclase) and the complex approaches the plasma membrane and activates ACA (Theibert and Devreotes, 1986 ; Lilly and Devreotes, 1995 ; Insall et al., 1994). The other adenylyl cyclase, ACG, is expressed only during germination and is a single transmembrane-spanning form unlike ACA, a form with 12 transmembrane spans (Pitt et al., 1992). The structure of ACG is thus closer to a guanylyl cyclase than to conventional adenylyl cyclases. ACG is coupled neither to cAMP receptors nor to a G protein so it is not activated by extracellular cAMP (Pitt et al., 1992). It is known to be an osmosensor and its activity is stimulated by osmolarity in the range of 1 mM to 100 mM NaCl and is optimally active at 100 mM NaCl (van-Es et al., 1996). When high osmotic pressure is detected in the environment, ACG is activated and the high concentration of cAMP produced by ACG prevents *Dictyostelium* spores from germinating (van Es et al., 1996). Hence ACG as an osmosensor facilitates *Dictyostelium* survival by ensuring that germination occurs under optimal conditions.

Below I report the results of an examination to determine if there is any difference in cAMP accumulation between *rdeA*- and the *regA*- mutants on the one hand, and wild-type cells on the other.

3.2 Results

3.21 Measurement of total cAMP levels in the *rdeA* mutants

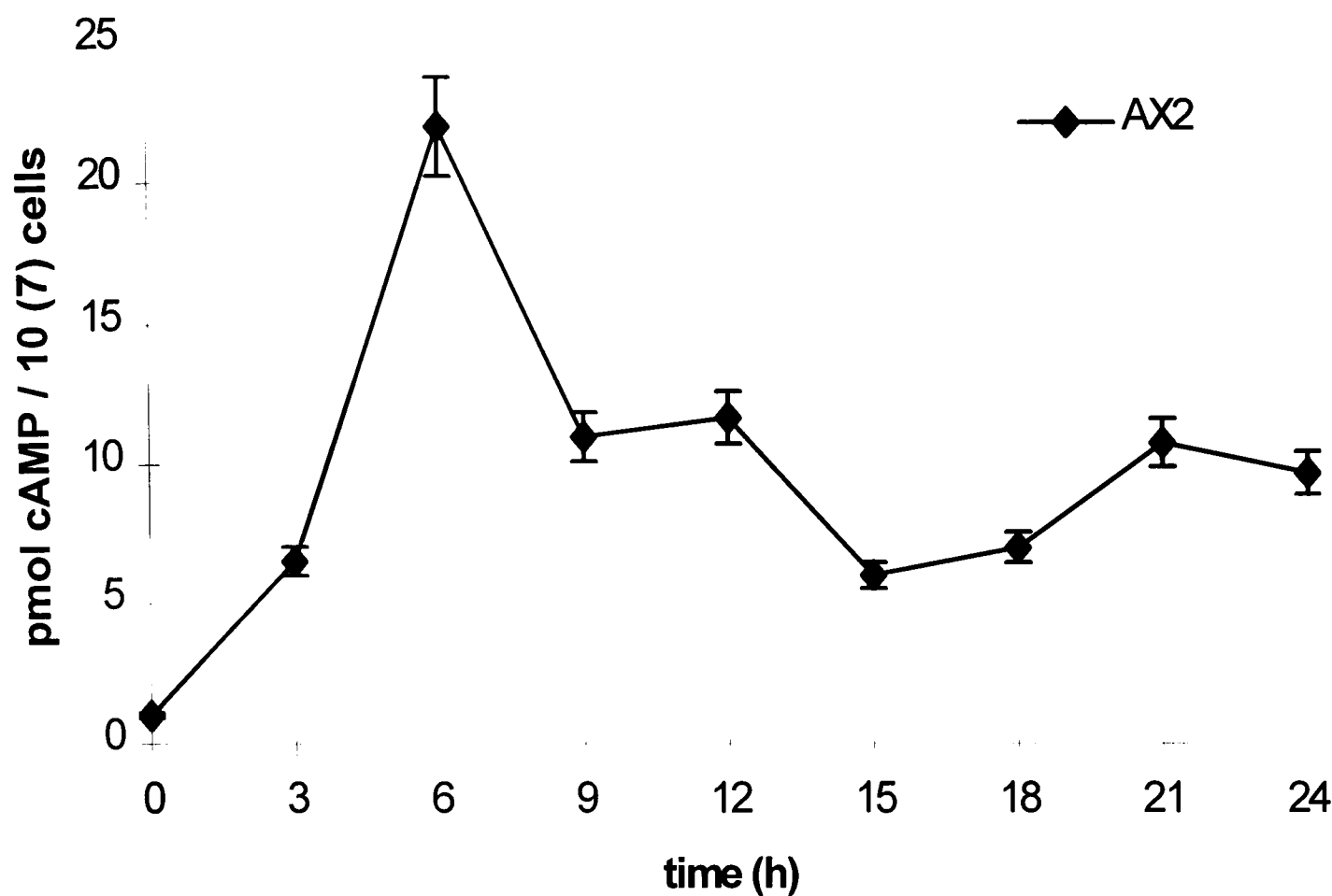
It was previously claimed that *rdeA* mutants accumulate elevated intracellular cAMP levels and high levels of total cAMP (intracellular and extracellular) at the vegetative stage as well as during development (Abe and Yanagisawa, 1983 ; Coukell and Chan, 1980). I used the REMI-induced AX2/*rdeA* strain, along with an *aca*-/*rdeA* double

mutant (see sections 2.112 and 2.117 in chapter 2; Chang et al., 1998) and measured total cAMP levels in these strains together with their parental strains throughout development. Cells in log phase were harvested from growth medium, washed and resuspended in LPS (Lower Pad Solution) at 10^8 cells / ml. Approximately 4×10^7 cells were plated onto the whole of a Millipore filter (dark grey, 45 mm in diameter) evenly. The cells were developed at 22°C for the indicated time periods in dark and humid condition. A filter was then transferred to the bottom of a 10 ml centrifuge tube and 1 ml of 1.75 % perchloric acid was added and vortexed for 10 seconds. After the samples were neutralised by adding 0.25 ml of 50 % saturated KHCO_3 , cAMP levels were determined by the isotope dilution assay (van Haastert, 1984; see Chapter 2). No dithiothreitol (DTT) was added during the experiment and so the cAMP levels obtained were a function of the amount of cAMP produced by adenylyl cyclase(s) together with hydrolysis of cAMP by any extracellular or intracellular cAMP-phosphodiesterases that might be present. This protocol was based on the methods of measurement of total cAMP described by Abe and Yanagisawa, 1983.

Figure 3.1 shows that AX2/rdeA cells do not seem to have significantly elevated cAMP levels compared to AX2 cells. The pattern of total cAMP levels during the development of the AX2 looked similar to what was previously reported, with a peak around aggregation and a decline followed by a further slight increase during culmination (Abe and Yanagisawa, 1983 ; Brenner, 1978). The results I obtained with AX2/rdeA cells were rather variable as implied by the large error bars. It was however clear based on several repeated experiments that the cAMP levels in AX2/rdeA were not much higher than in AX2.

Total cAMP measurement during development

A



B

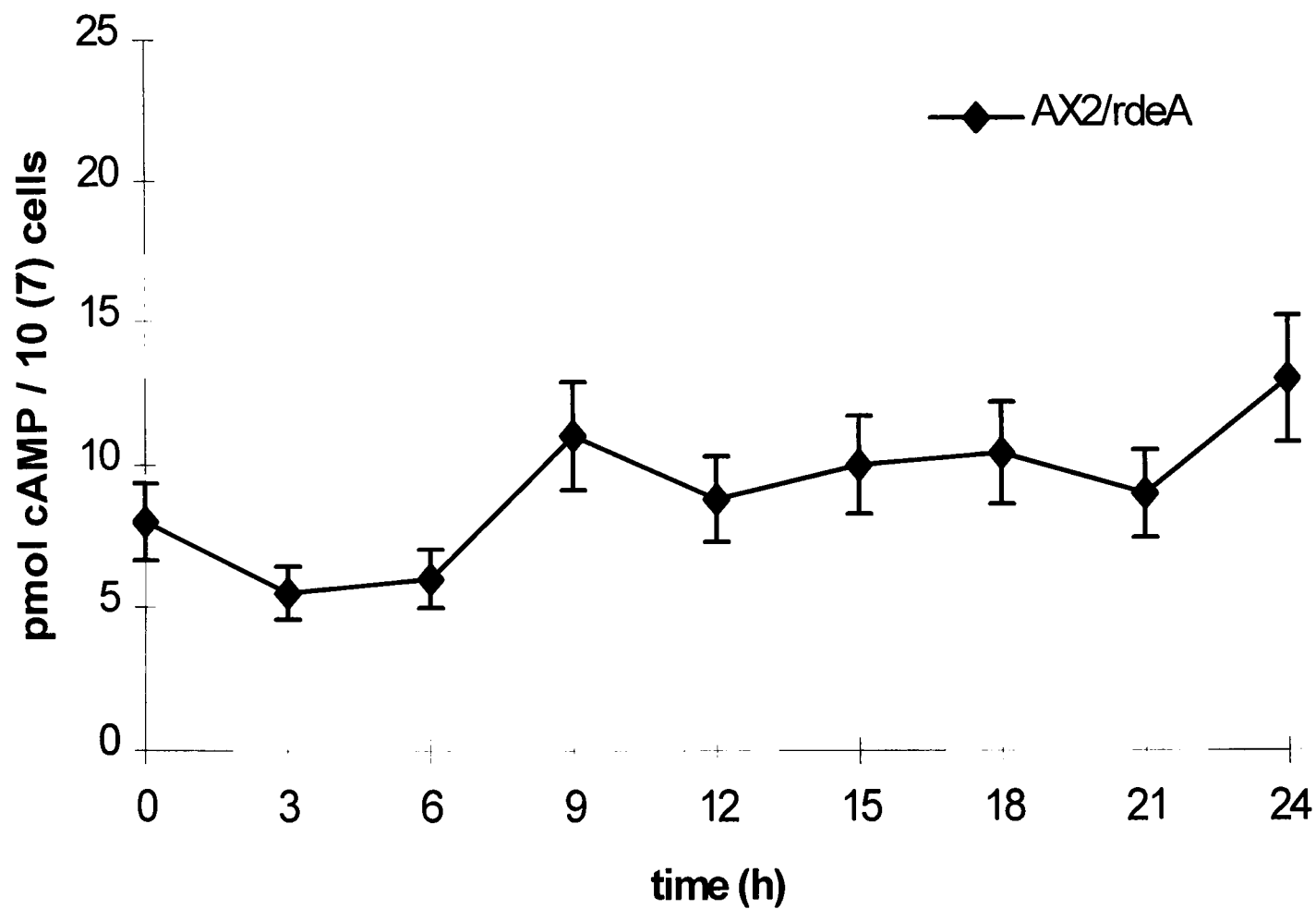


Figure 3.1 Total cAMP measurement in AX2 and AX2/rdeA strains
Experiments were repeated 3-4 times and standard error bars are given.

Total cAMP measurement

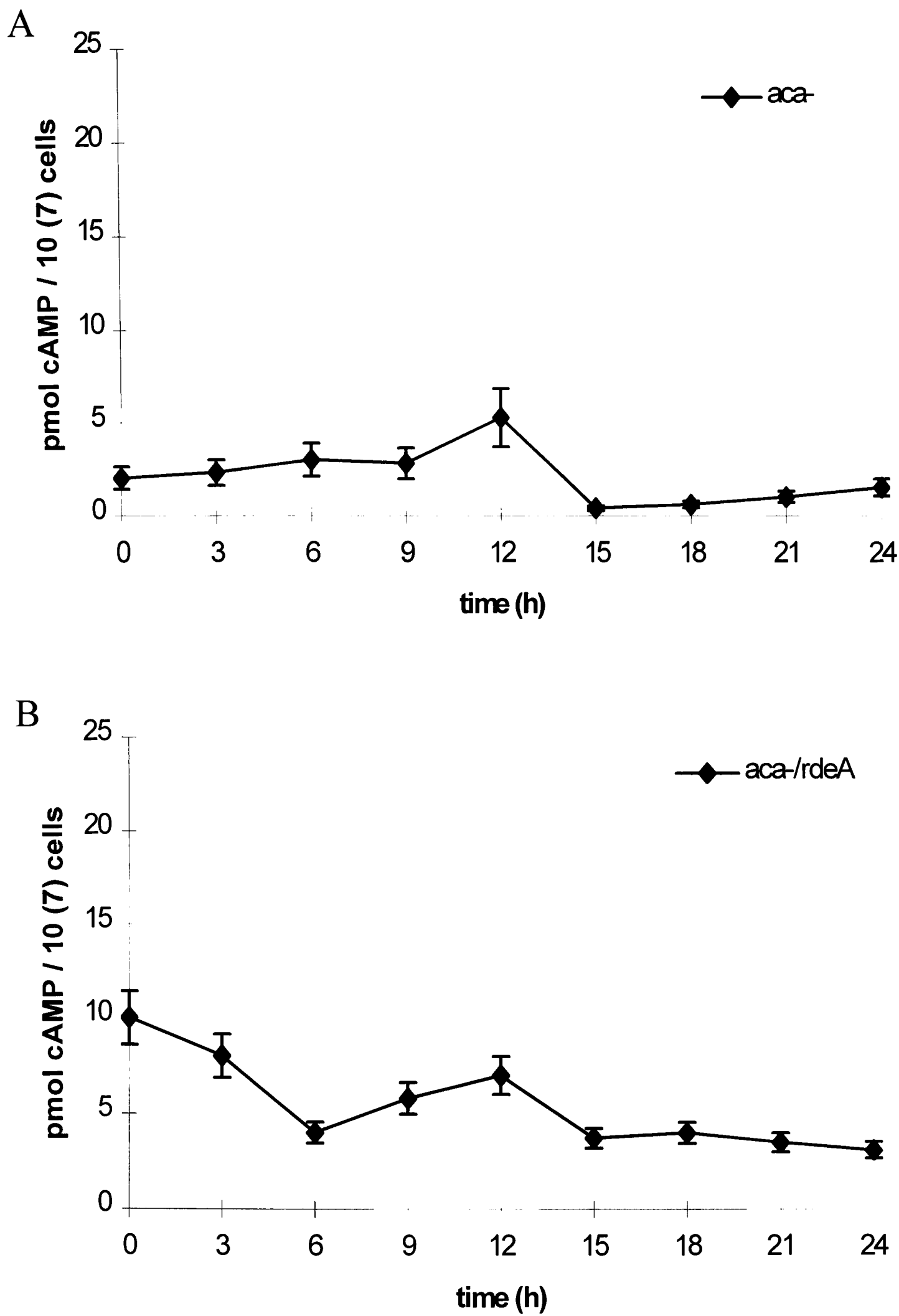


Figure 3.2 Total cAMP measurement in *aca-* and *aca-/rdeA* strains
Experiments were repeated 3-4 times and standard error bars are given.

Figure 3.2 shows that there seems to be cAMP present in the early hours of starvation of *aca-/rdeA* cells. Quite high levels of total cAMP were detected at the onset of starvation and the level decreased gradually and disappeared by 8 to 12 hours after starvation. It was of interest to enquire further about this cAMP made in the *aca-/rdeA* cells. The next step was therefore to check whether any adenylyl cyclase activity could be detected in vegetative *aca-/rdeA* cells and if so whether this was ACA.

3.22 cAMP accumulation in 6 hour starved cells of the *rdeA* mutants

In the same set of experiments, I measured the cAMP relay response of aggregation competent *rdeA* cells to see whether their rapid development was due to high adenylyl cyclase A activity or to a defect in adaptation resulting in abnormally high cAMP accumulation. The increase in cAMP levels resulting from whatever cause might not have been detected when measuring total cAMP levels because of hydrolysis by cAMP phosphodiesterases.

Cells were developed on non nutrient agar plates at 22°C for 6 hours or given cAMP pulses at 22°C for 4-5 hours to let them reach the aggregation competent stage. Since ACA is coupled to cAMP receptors, 2H'-cAMP which is a non-permeable cAMP analogue was given to stimulate ACA activity. Once adenylyl cyclase starts to produce cAMP, it is important to minimise hydrolysis by cAMP-phosphodiesterases. For this reason 5 mM DTT that is known to inhibit the extracellular cAMP phosphodiesterase of *Dictyostelium* (Henderson, 1975) was always added from the start of the measurements.

AX2/rdeA cells

Figure 3.3 shows the cAMP relay response of AX2/rdeA cells along with that of the parental strain, AX2. Aggregation-competent cells were stimulated with the cAMP receptor agonist 2'-deoxyadenosine 3', 5'-monophosphate (2H'-cAMP) in the presence of 5 mM dithiothreitol (DTT). In the AX2 cells (Figure 3.3 A) activity was increased by addition of 5 μ M 2H'-cAMP. AX2/rdeA cells (Figure 3.3 B) seemed to show a slightly more rapid cAMP accumulation in the 2 minute samples but the total activity did not look unusually high. Activity was also stimulated by 2H'-cAMP and appeared neither to be high nor to be abnormal in terms of adaptation.

DH1/rdeA and AX2/regA cells

The DH1/rdeA strain is the original REMI mutant described in 2.115 that showed rapid development, reaching its final developmental stage by 17-18 hours after starvation. The AX2/regA strain is the other class of rapidly developing mutant with a similar phenotype described in 2.113 (Thomason et al., 1998). In order to get a broader idea of the adenylyl cyclase activities in rapidly developing strains, the cAMP relay responses of DH1/rdeA cells and AX2/regA cells were also examined. Figure 3.4 shows that aggregation competent cells of the DH1/rdeA line also did not produce abnormally high levels of cAMP. Activity was again stimulated by 2H'-cAMP and reached a maximum after about 4 minutes. Therefore ACA in DH1/rdeA cells seemed to behave like that of AX2/rdeA cells. Figure 3.4 B shows that aggregation competent AX2/regA also did not have high adenylyl cyclase activity and showed abnormal adaptation.

Figure 3.3 cAMP accumulation in 6 hour starved AX2/rdeA and AX2 cells.

A parental strain AX2 and an rdeA mutant strain AX2/rdeA were starved on non-nutrient agar plates at 22°C for 6 hours. cAMP relay response in aggregation competent cells was measured with 5 uM 2'H-cAMP and/or 5mM DTT. Solid circles represent 2'H-cAMP and DTT and open circles represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the diameter of circles, it is not shown.

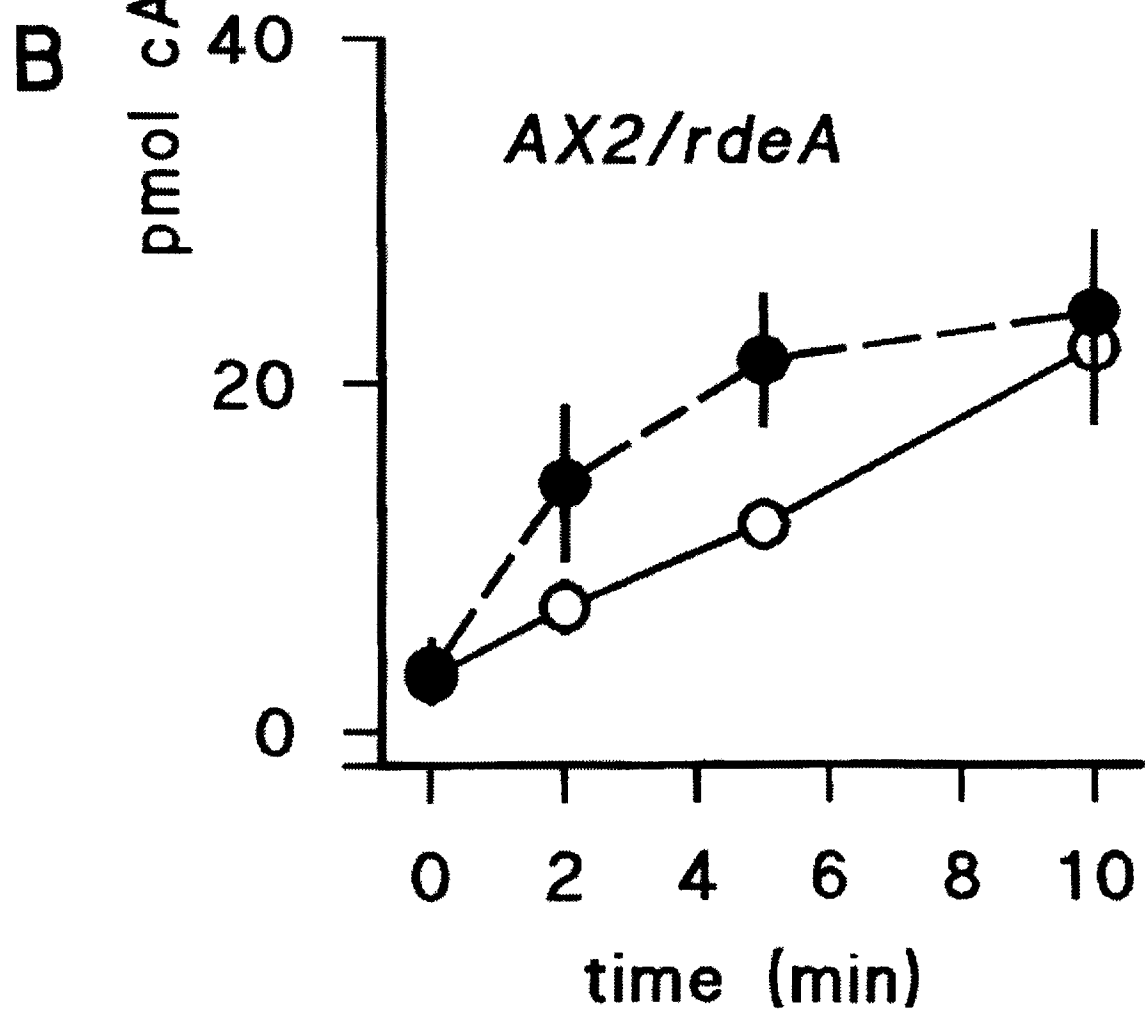
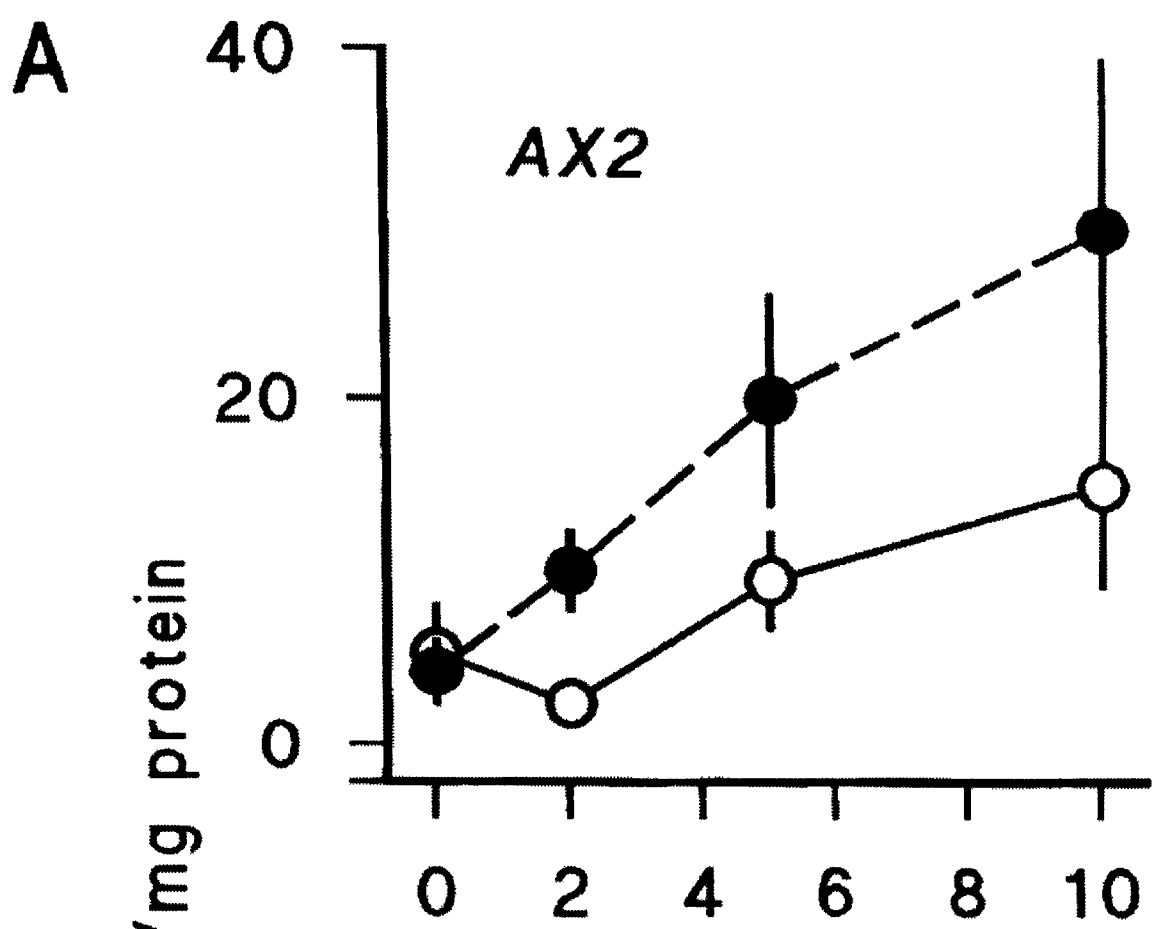
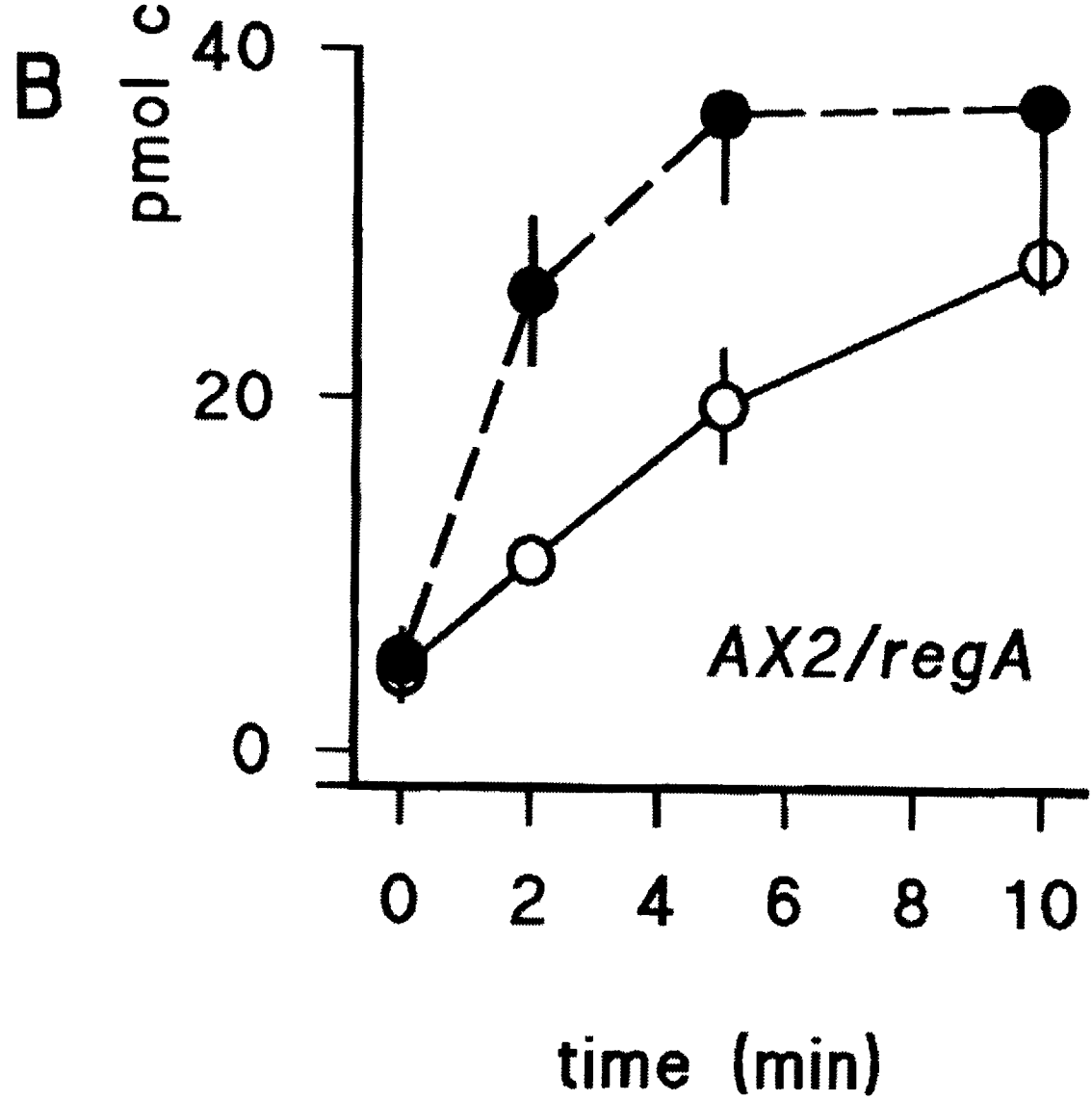
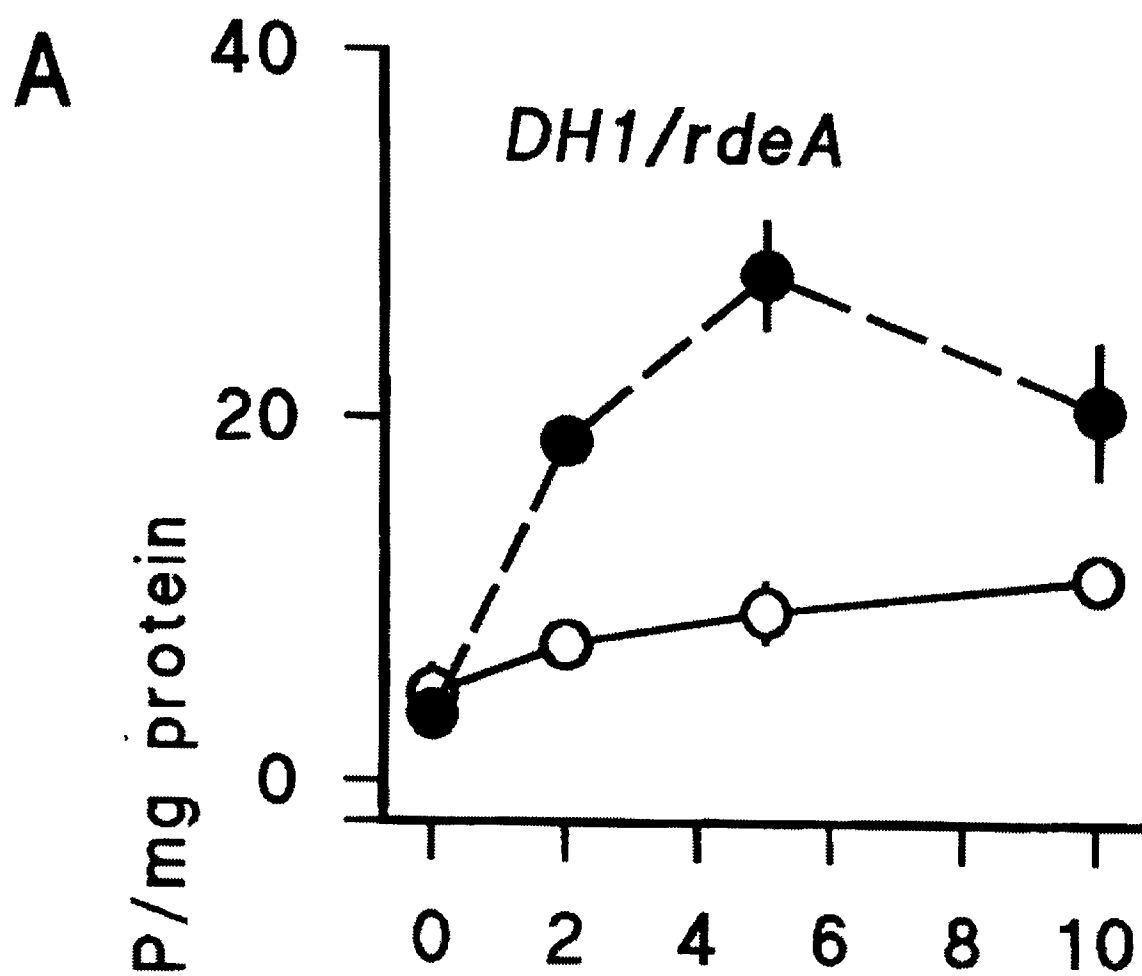


Figure 3.4 cAMP accumulation in 6 hour starved DH1/rdeA and AX2/regA cells.

DH1/rdeA and AX2/regA cells were starved on non-nutrient agar plates at 22°C for 6 hours. cAMP relay response in aggregation competent cells was measured with 5 uM 2'H-cAMP and/or 5mM DTT. Solid circles represent 2'H-cAMP and DTT and open circles represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the diameter of circles, it is not shown.



Hints of a novel adenylyl cyclase

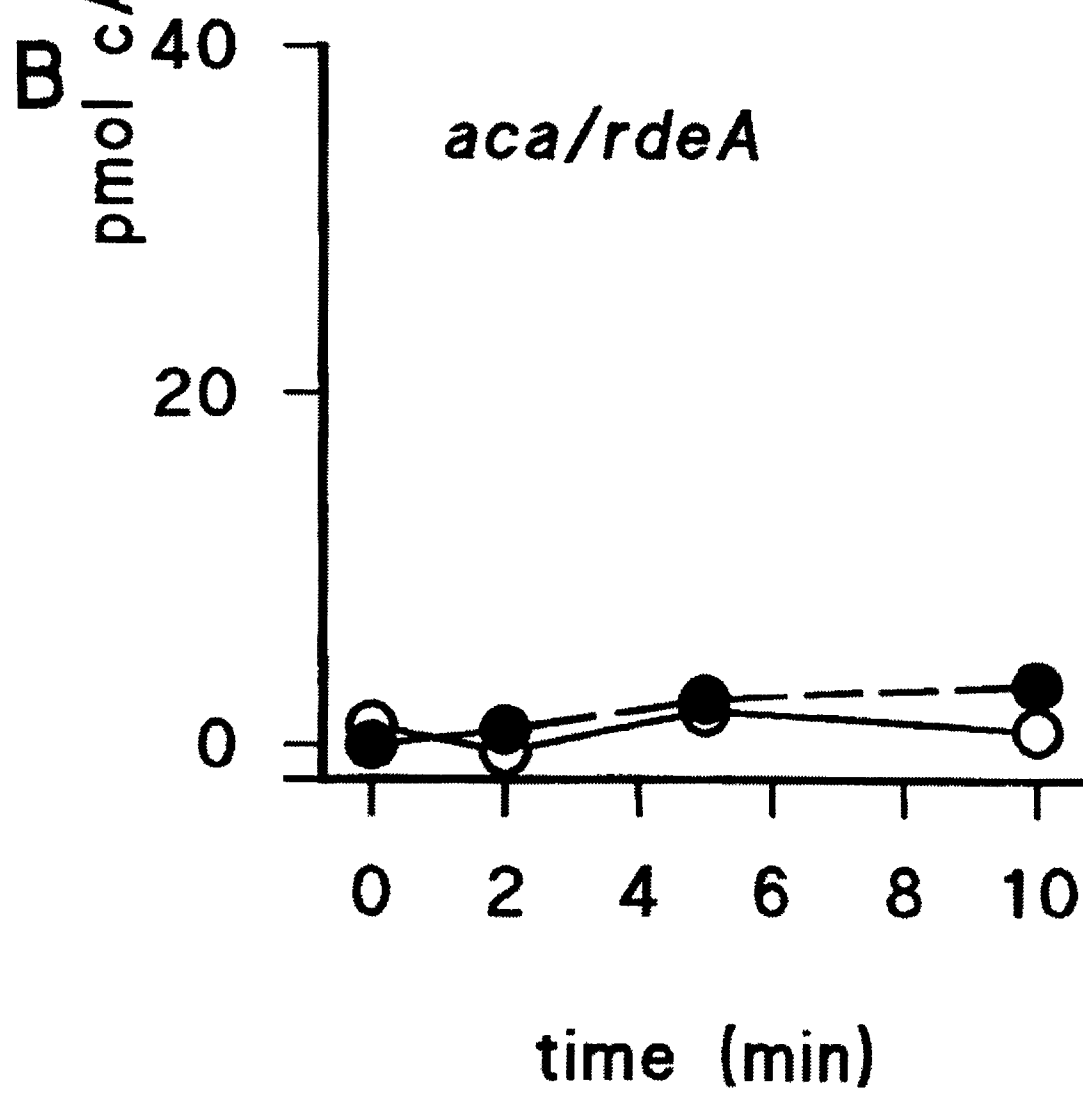
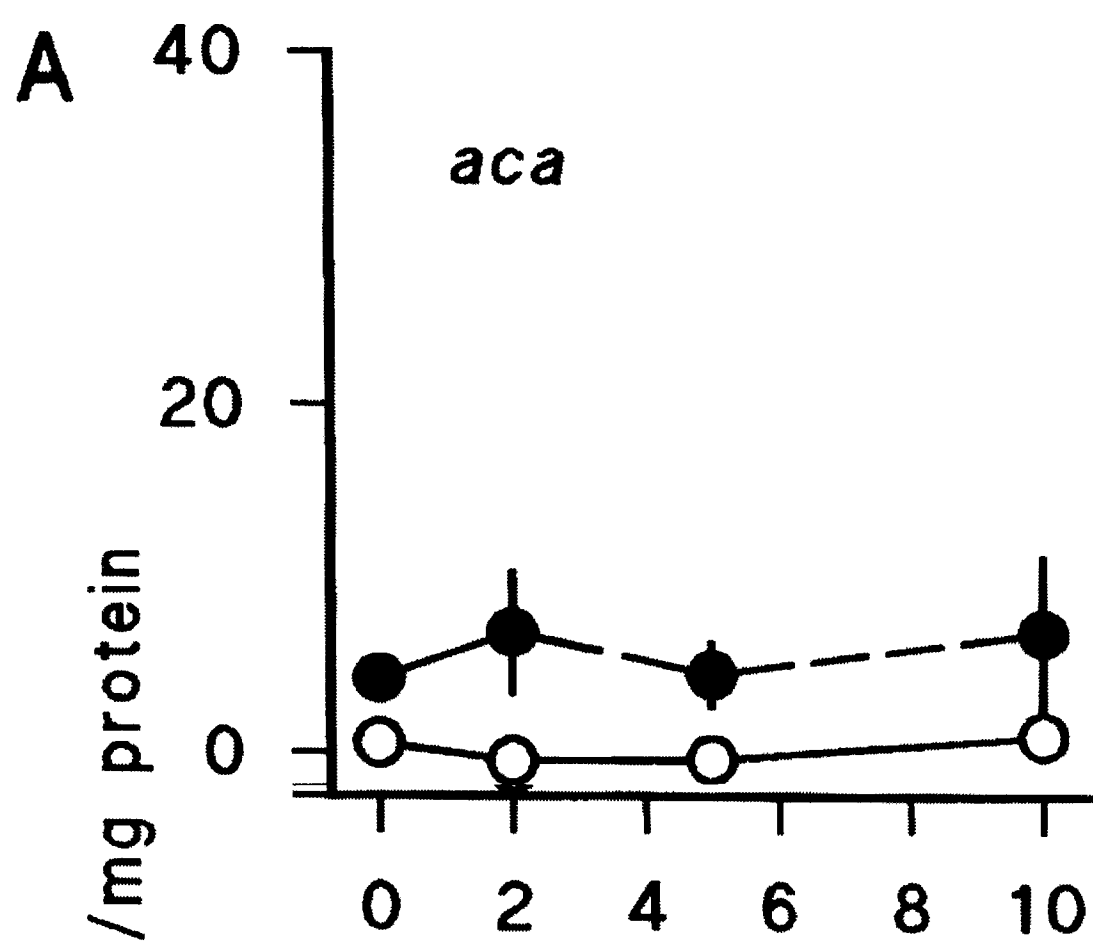
An *aca-/rdeA* double mutant was also included in the measurements of cAMP accumulation by 6 hour-starved cells. The *aca-/rdeA* strain was constructed by homologous recombination using an *rdeA* knock-out vector transformed into the *aca-* strain (Chang et al., 1998 ; see also 2.117). Like the *aca-* strain, it cannot aggregate but the double mutant cells were able to make spores in submerged monolayers in the presence of 5 mM cAMP (Figure 3.15). When I measured total cAMP levels in the *aca-/rdeA* cells during the first 24 hours of starvation, quite significant amounts of cAMP were detected at the vegetative stage as well as early in starvation (Figure 3.2 B). The data appeared to suggest the presence of a new adenylyl cyclase although the possibility of ACG had also to be considered. Naturally the cAMP relay response was measured in the *aca-/rdeA* strain as the next step. *aca-/rdeA* cells were starved on non-nutrient agar plates at 22°C for 6 hours, the same as the other strains (Figure 3.3 and 3.4) and *aca-* cells, the parental strain of the *aca-/rdeA* double mutant were treated in the same way as a control.

Figure 3.5 A shows a very low level of cAMP accumulation in *aca-* cells as expected, though I can not say that there was no cAMP accumulation at all. There seemed to be slightly higher activity in the presence of 2H'-cAMP but this might not be significant considering the large standard errors. Figure 3.5 B shows that there was no cAMP accumulation in 6 hour starved *aca-/rdeA* cells. This result was disappointing but it was premature to conclude that *aca-/rdeA* cells do not have any adenylyl cyclase activity after examining only one time point.

In summary Figure 3.3 to 3.5 indicate that aggregation competent *rdeA* and *regA* mutants do not seem to have elevated adenylyl cyclase activity that could account for their rapid development. In addition the 6 hour starved *aca-/rdeA* cells did not behave

Figure 3.5 cAMP accumulation in 6 hour starved *aca*- and *aca*-/*rdeA*- cells.

A parental strain *aca*- and an *rdeA* double mutant strain *aca*-/*rdeA*- were starved on non-nutrient agar plates at 22°C for 6 hours. cAMP relay response in 6 hour starved cells was measured with 5 uM 2'H-cAMP and/or 5mM DTT. Solid circles represent 2'H-cAMP and DTT and open circles represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the diameter of circles, it is not shown.



differently from *aca-* cells.

3.23 cAMP accumulation in vegetative cells of the *rdeA* mutants

Total cAMP measurement in *aca-/rdeA* cells mentioned in the latter part of 3.21 indicated that there could be a novel adenylyl cyclase activity in the *aca-/rdeA* at the vegetative stage and early in starvation. I next examined cAMP accumulation in vegetative cells of *rdeA* and *regA* strains. In order to detect any ACG (Adenylyl Cyclase for Germination) activity *in vivo*, 100 mM NaCl was added to duplicate assay samples along with 5 mM DTT. ACG is not expected to be active at the vegetative stage of wild type cells but if ACG were activated at the vegetative stage in *rdeA* mutants its activity should be increased by the optimal osmolarity, 100 mM NaCl. Cells were harvested from growth medium and resuspended in PB (phosphate buffer) at 10^8 cells /ml. Aliquots of cell suspension were then incubated with 5 mM DTT in the presence or absence of 100 mM NaCl.

AX2/*rdeA* cells

Figure 3.6 indicates that vegetative AX2/*rdeA* cells had some adenylyl cyclase activity while the parental strain AX2 cells did not. The activity detected in the AX2/*rdeA* cells was not stimulated by high osmolarity (Figure 3.6 B). Thus AC activity in the presence of 100 mM NaCl (solid squares) was lower than the activity without 100 mM NaCl indicating that the adenylyl cyclase activity detected in vegetative AX2/*rdeA* cells is inhibited by high osmolarity. If the activity was due to ACG, it should have been stimulated by 100 mM NaCl. This result suggested the exciting possibility that I might be detecting a new adenylyl cyclase since ACA is not

expressed at the vegetative stage and the adenylyl cyclase activity detected in AX2/rdeA was inhibited rather than stimulated by high osmolarity.

DH1/rdeA cells

The next question was if the new adenylyl cyclase activity is consistently detected when there is a genetic lesion of the rdeA gene. To answer that question I measured cAMP accumulation at the vegetative stage of DH1/rdeA cells, the original REMI mutant (see 2.115). Figure 3.7 shows that vegetative DH/rdeA cells indeed also displayed an adenylyl cyclase activity whereas the parental strain, DH1 did not. The activity was inhibited by 100 mM NaCl as in AX2/rdeA cells.

AX2/regA cells

A further question was if the new adenylyl cyclase activity could also be detected in regA mutants that are in the other phenotypically similar class of rapidly developing mutants. Figure 3.8 shows that vegetative AX2/regA cells also displayed an adenylyl cyclase activity, unlike the parental strain, AX2 vegetative cells. The activity was again inhibited by 100 mM NaCl (Figure 3.8 B). This result suggested that there is a close relationship between rdeA mutants and regA mutants and that the RDEA protein and the REGA protein might in some sense both “control” the new adenylyl cyclase activity.

aca-/rdeA cells

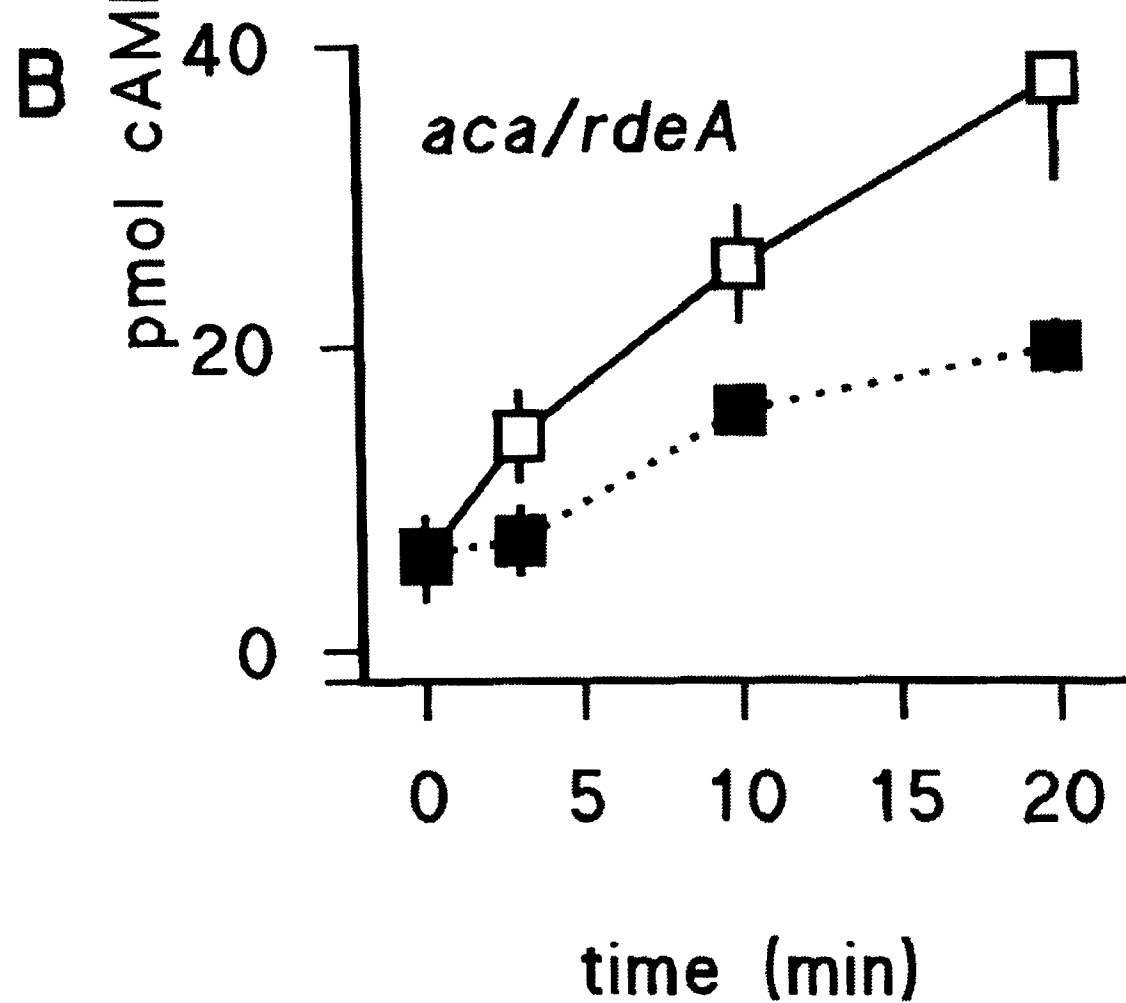
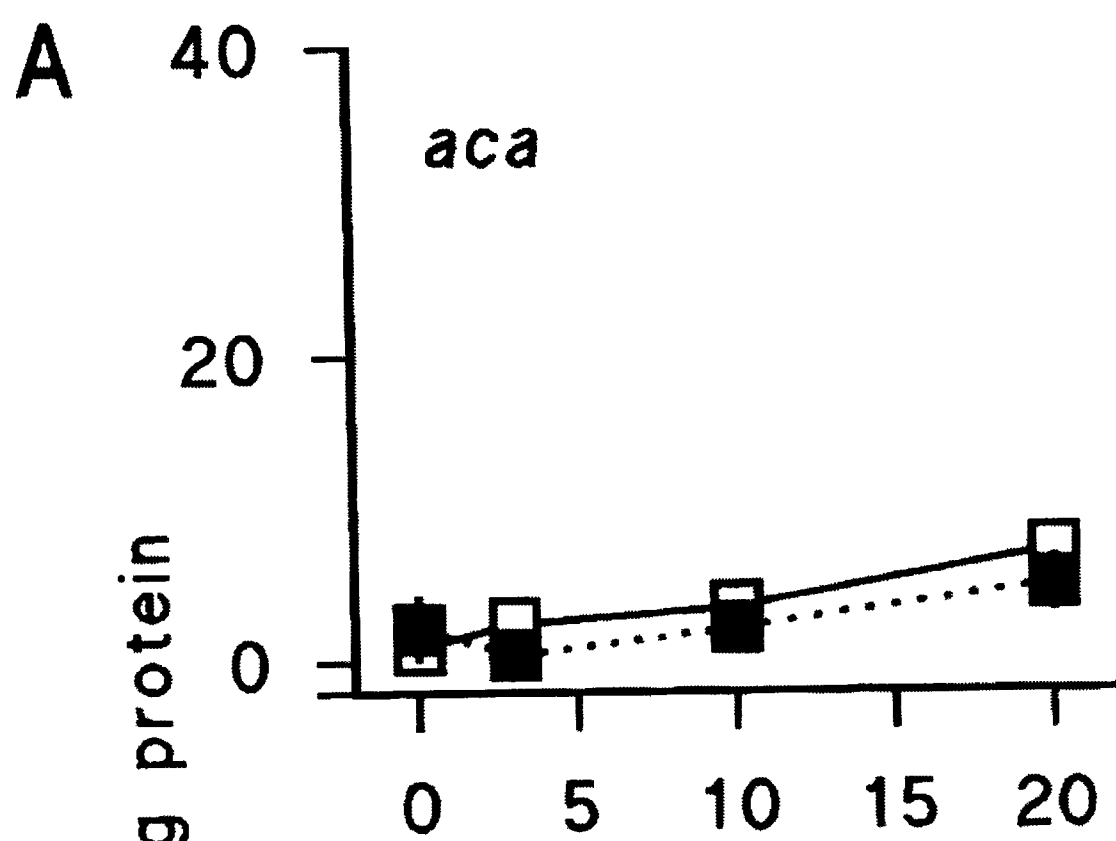


Figure 3.9 cAMP accumulation in vegetative *aca*- and *aca*-/rdeA- cells.

A parental strain *aca*- and an *rdeA* double mutant strain *aca*-/rdeA were harvested from growth medium and resuspended in PB. cAMP accumulation was measured in the presence of 100 mM NaCl and/or 5mM DTT. Solid squares represent NaCl and DTT and open squares represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the length of squares, it is not shown.

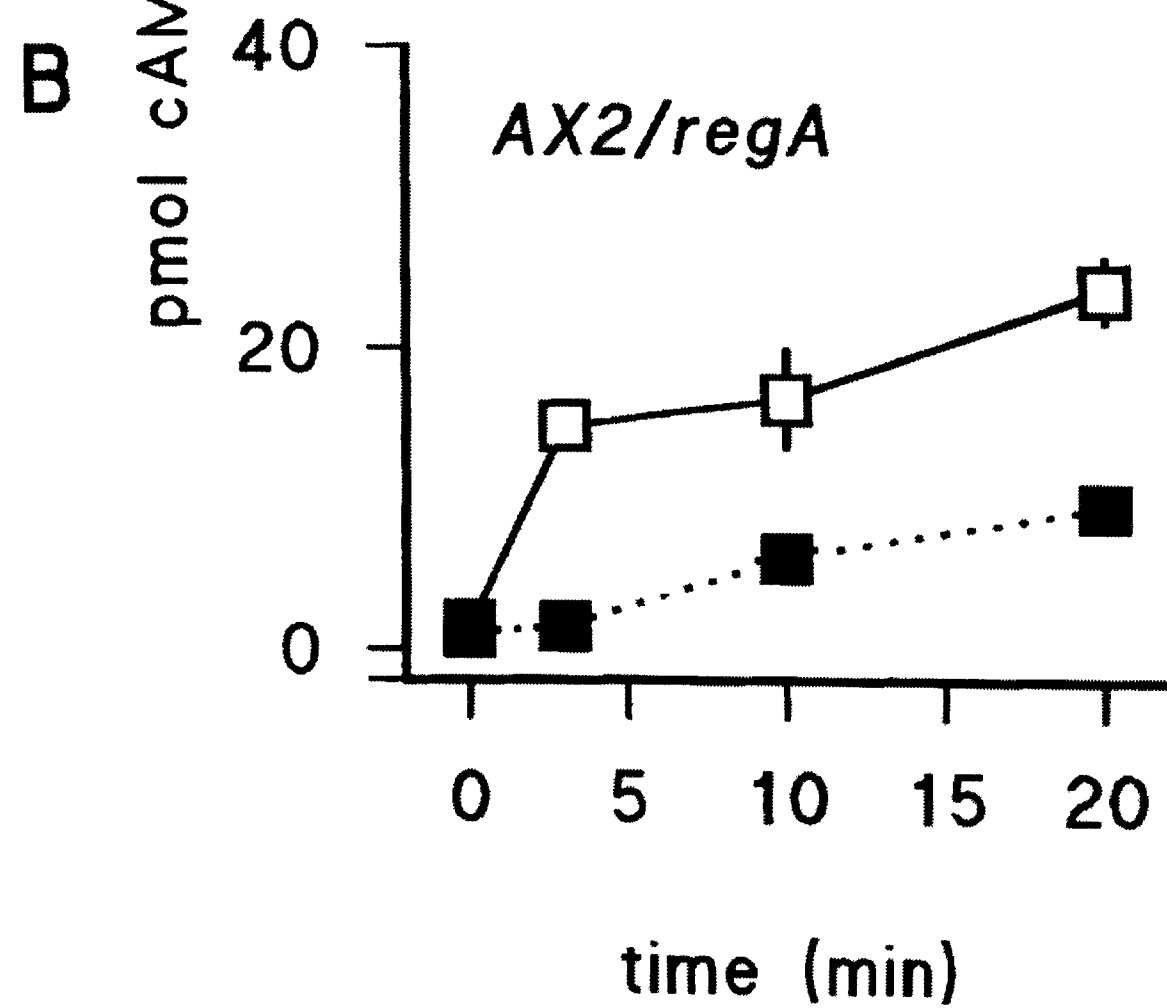
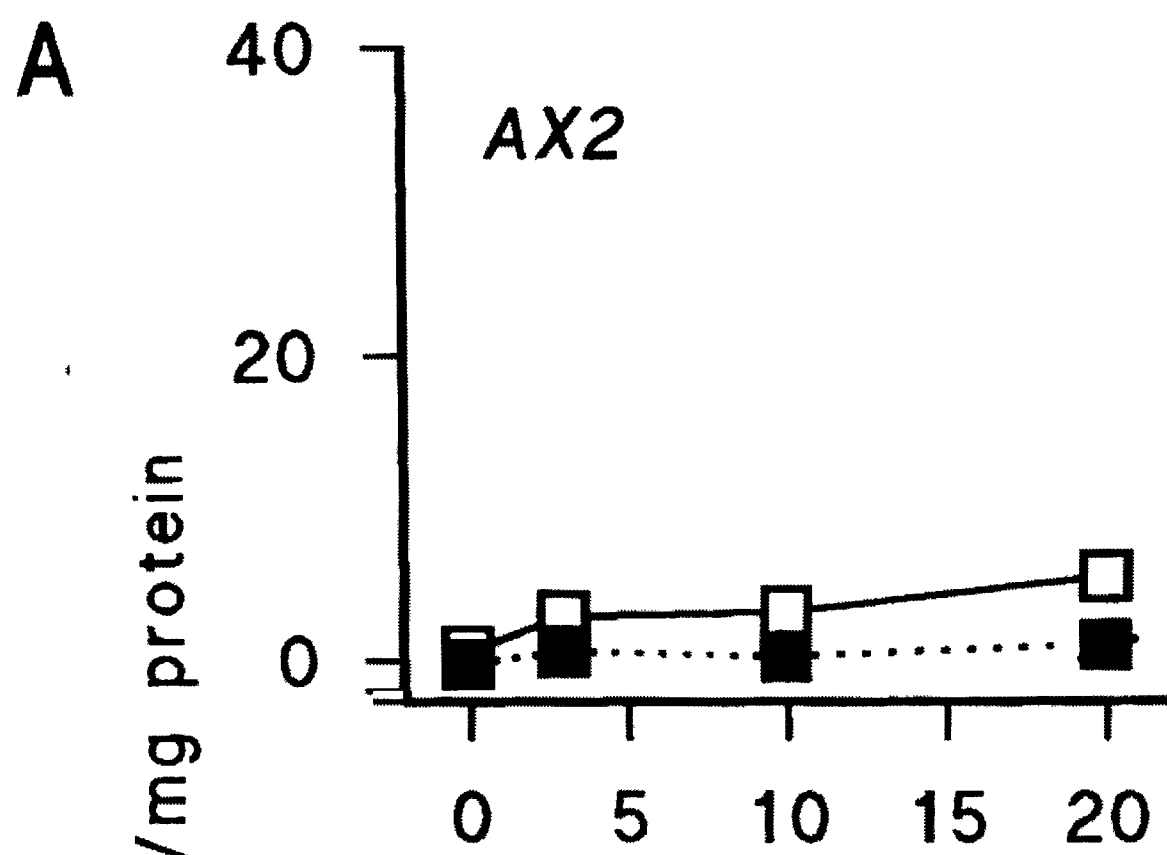


Figure 3.8 cAMP accumulation in vegetative AX2/regA and AX2 cells.

A parental strain AX2 and a regA mutant strain AX2/regA were harvested from growth medium and resuspended in PB. cAMP accumulation was measured in the presence of 100 mM NaCl and/or 5mM DTT. Solid squares represent NaCl and DTT and open squares represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the length of squares, it is not shown on the graph.

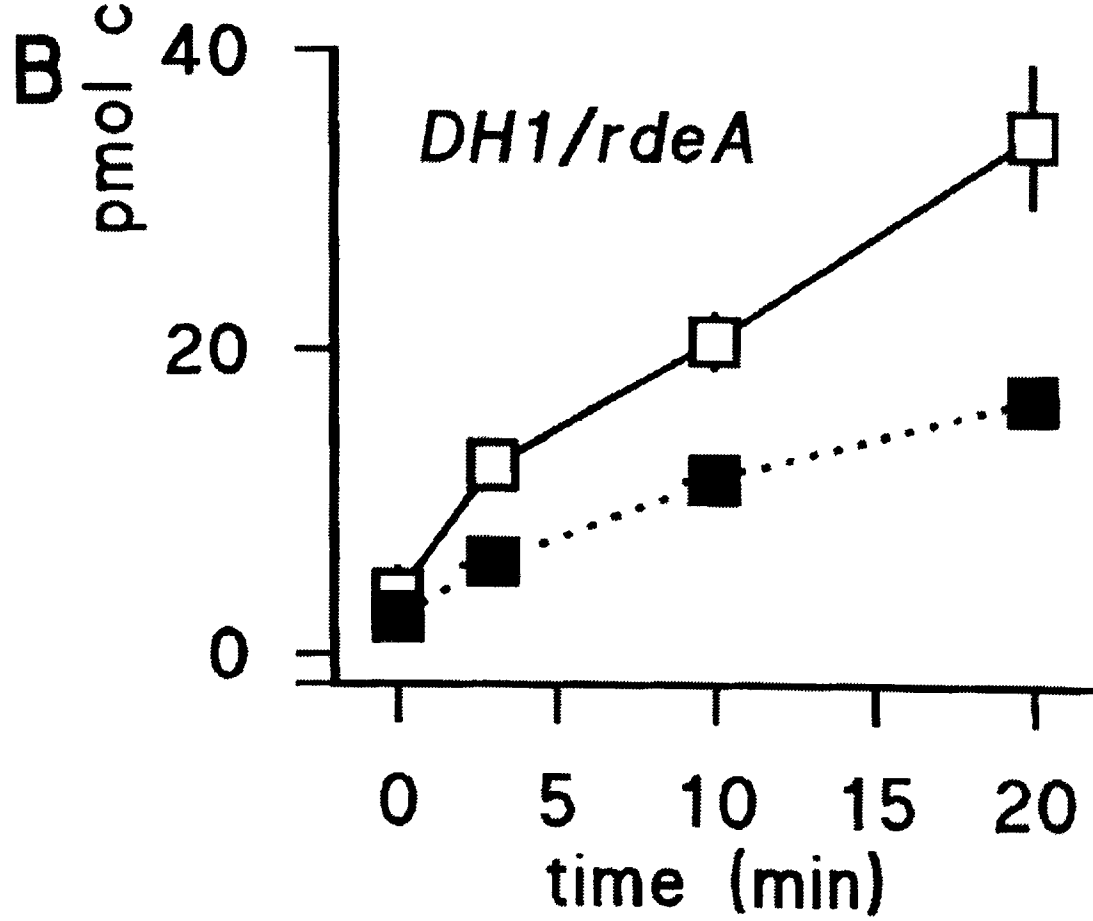
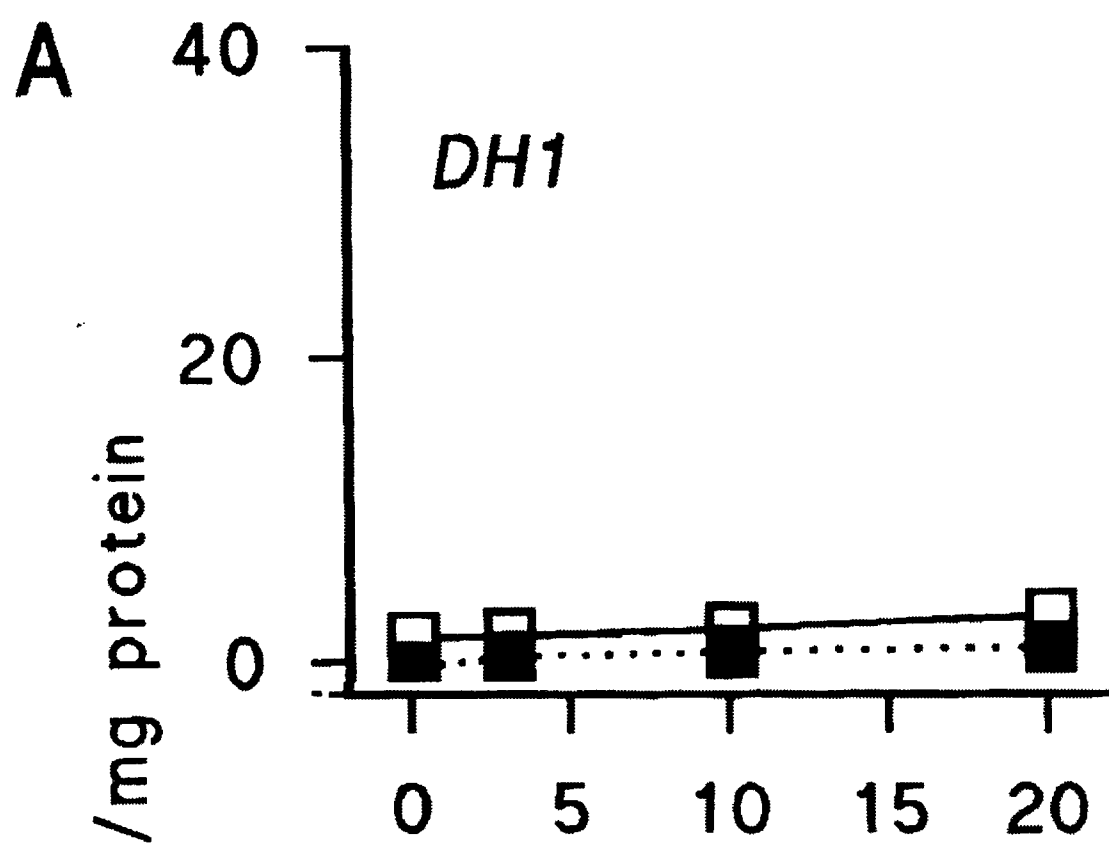


Figure 3.7 cAMP accumulation in vegetative DH1/rdeA and DH1 cells.

A parental strain DH1 and an rdeA mutant strain DH1/rdeA were harvested from growth medium and resuspended in PB. cAMP accumulation was measured in the presence of 100 mM NaCl and/or 5mM DTT. Solid squares represent NaCl and DTT and open squares represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the length of squares, it is not shown.

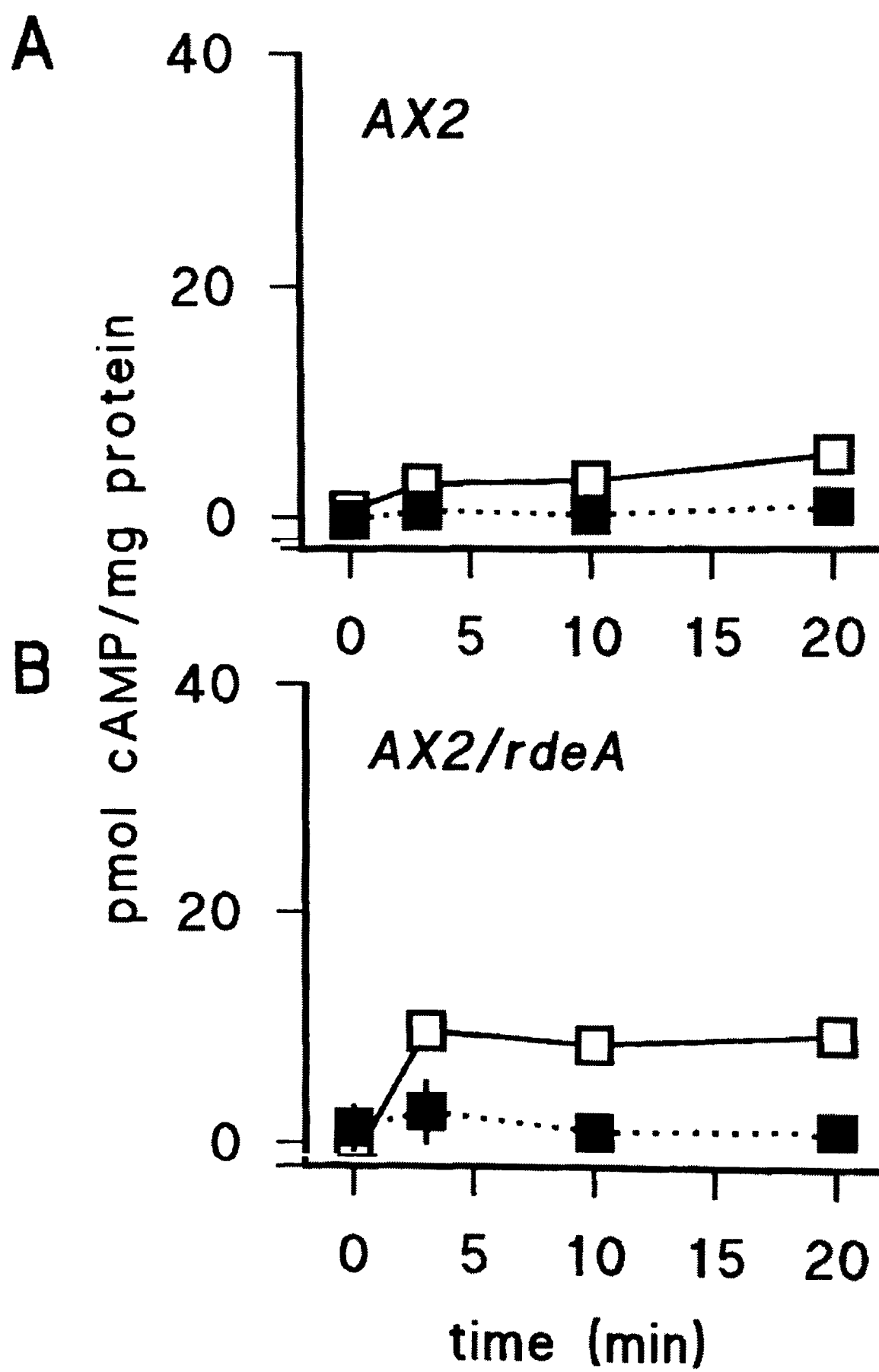


Figure 3.6 cAMP accumulation in vegetative AX2/rdeA and AX2 cells.

A parental strain AX2 and an rdeA mutant strain AX2/rdeA were harvested from growth medium and resuspended in PB. cAMP accumulation was measured in the presence of 100 mM NaCl and/or 5mM DTT. Solid squares represent NaCl and DTT and open squares represent DTT only. Standard error means are given as bar and when s.e.m. is smaller than the length of squares, it is not shown.

I next examined if vegetative *aca-/rdeA-* cells displayed the adenylyl cyclase activity. The parental *aca-* cells did not show significant cAMP accumulation (Figure 3.9 A) though I can not say with confidence that there was no adenylyl cyclase activity at all. In contrast vegetative *aca-/rdeA* cells displayed quite high adenylyl cyclase activity and again the activity was inhibited by 100 mM NaCl (Figure 3.9 A). With this result it was clearly concluded that the activity was not due to ACA.

The high adenylyl cyclase activity in vegetative *aca-/rdeA* cells seemed to agree well with my previous unexpected finding of cAMP in measurement of total cAMP in the *aca-/rdeA* at the vegetative stage and during the early hours of starvation (Figure 3.2 B). Although the adenylyl cyclase activity detected in vegetative *rdeA* and *regA* cells seemed to be a novel activity based on its being inhibited by 100 mM NaCl, a more thorough examination of the response to high osmolarity was required to be sure that it was not due to ACG. The following section addresses this question.

3.24 Evidence that the new adenylyl cyclase activity is not ACG

As described, *in vivo* ACG activity is stimulated by osmolarity in the range of 1mM up to 100 mM NaCl and higher concentrations inhibit its activity. ACA activity on the other hand is inhibited by increased osmolarity over this same range (Schaap et al, 1995). If the activity was due to ACG, it should increase gradually with increasing osmolarity up to 100 mM NaCl.

This was tested experimentally as follows. All four strains that showed the new activity were harvested from growth medium, washed once and resuspended in PB at 10^8 cells / ml. Aliquots of the cell suspensions were then incubated with 5 mM DTT

along with the indicated concentrations of NaCl at 22°C for 10 minutes. The response of ACG activity to increased osmolarity is presented for comparison (Figure 3.10, derived from Kim et al., 1998).

AX2/rdeA cells

Figure 3.11 shows that the activity in vegetative AX2/rdeA cells was maximal in the absence of NaCl (the solid square at 0 mM NaCl). It was considerably inhibited by 5 mM NaCl and gradually decreased further with increasing concentration of NaCl. The activity at 250 mM NaCl was less than 20 % of the maximal activity observed at 0 mM NaCl. This result made it clear that the adenylyl cyclase activity detected in vegetative AX2/rdeA cells was not due to ACG.

DH1/rdeA cells

Figure 3.12 shows that the adenylyl cyclase activity in vegetative DH1/rdeA cells also had maximal activity in the absence of NaCl. The activity at 0 mM NaCl was not as high as the activity in AX2/rdeA cells (Figure 3.11) and the inhibition did not seem to be so dramatic either. Nevertheless the activity in DH1/rdeA cells did decrease gradually with the increased concentrations of NaCl. Thus despite the relatively low adenylyl cyclase activity it was clear that the activity was inhibited rather than stimulated by increased osmolarity.

AX2/regA cells

Figure 3.13 shows that the adenylyl cyclase activity in vegetative AX2/regA cells also

had maximal activity in the absence of NaCl (the solid square at 0 mM NaCl). Activity was rather variable but nevertheless it was again clear that it was not stimulated by increased osmolarity.

aca-/rdeA cells

Figure 3.14 shows the adenylyl cyclase activity in vegetative aca-/rdeA cells had maximal activity in the absence of NaCl. Although in this experiment it did not seem to be significantly inhibited by low osmolarity (up to 50 mM NaCl) it was strongly inhibited by high osmolarity (100 mM to 250 mM NaCl). Thus the activity in vegetative aca-/rdeA cells can be neither due to ACG nor to ACA. The results presented in Figure 3.11 - 3.14 together demonstrate that the activity detected in rdeA and regA mutants is a novel activity.

It should also be noted that detection of this new activity seemed to be strictly dependent on the presence of a lesion in the rdeA or regA genes, supporting the idea that the RDEA and REGA products function in a common signalling pathway.

3.25 Test of sporogenous phenotype

As mentioned briefly above I tested if aca-/rdeA cells could make spores in submerged monolayers, and I examined AX2/rdeA, AX2 and aca-/ACG strains at the same time. The aca-/ACG strain harbours a construct expressing ACG under the control of the actin 15 promoter (and no ACA activity) (see 2.118: aca-/ACG). All strains were grown in shaking culture at 22°C. Cells in log phase (3×10^6 cells / ml to 7×10^6 cells /ml) were washed three times with spore induction buffer and washed cells were resuspended at 10^6 cells / ml. For a standard assay, 2 ml of the suspension

Osmoregulation of ACG

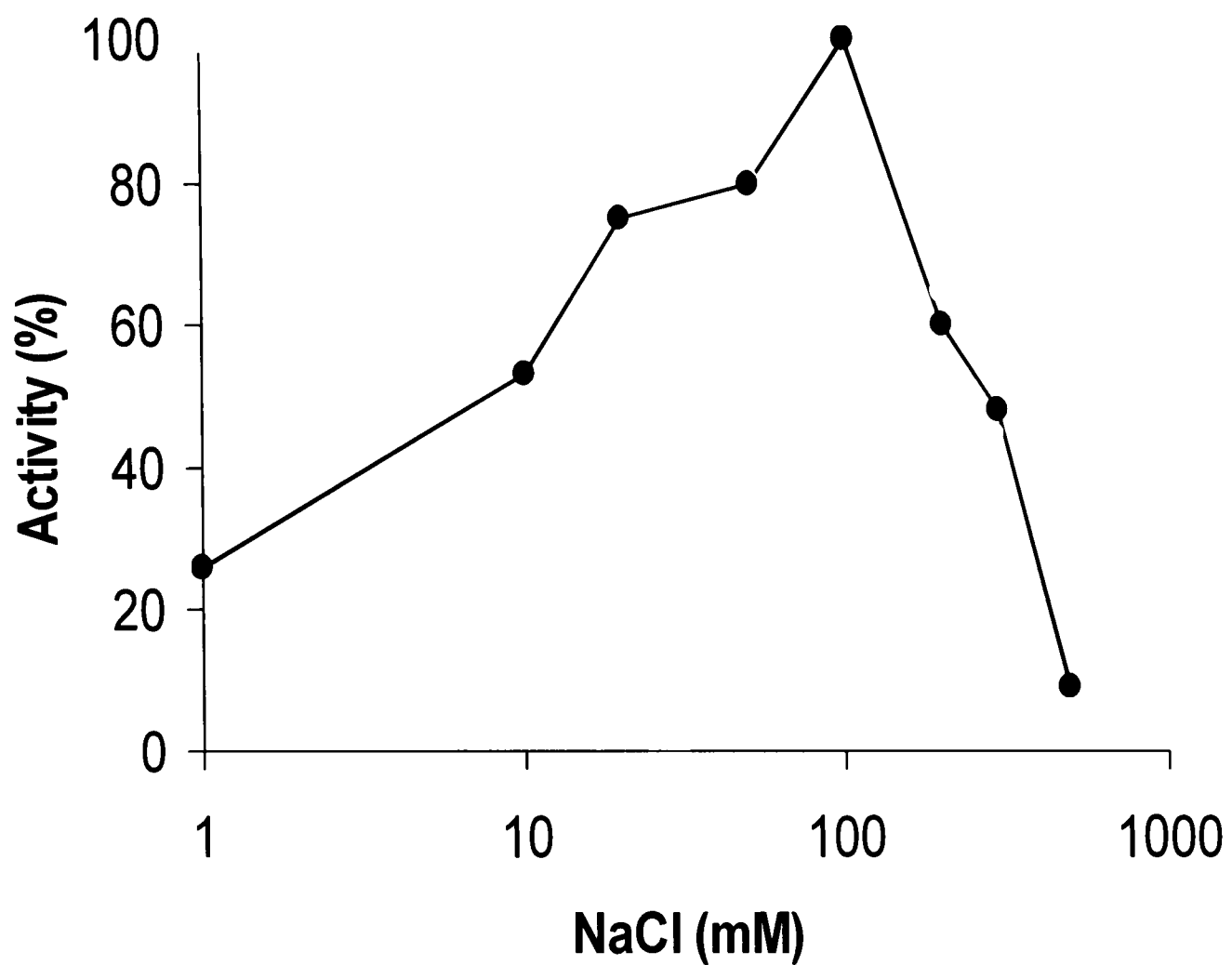


Figure 3.10 Effect of osmolarity on ACG

The data are retrieved from Kim et al., A novel adenylyl cyclase detected in rapidly developing mutants in *Dictyostelium*, Vol. 273, No.47, pp. 30859-30862, 1998, Journal of Biological Chemistry

Figure 3.11 Effect of osmolarity on cAMP accumulation of vegetative AX2/rdeA cells.

Vegetative AX2/rdeA cells were washed once, resuspended in PB and then incubated with 5 mM DTT in the presence of indicated concentrations of NaCl. Solid squares represent cAMP accumulation after incubation for 10 minutes and open squares represent cAMP accumulation at time 0 min. Data are presented in semi-logarithmic scale and standard error means are given as bar. When s.e.m. is smaller than the length of squares, it is not shown.

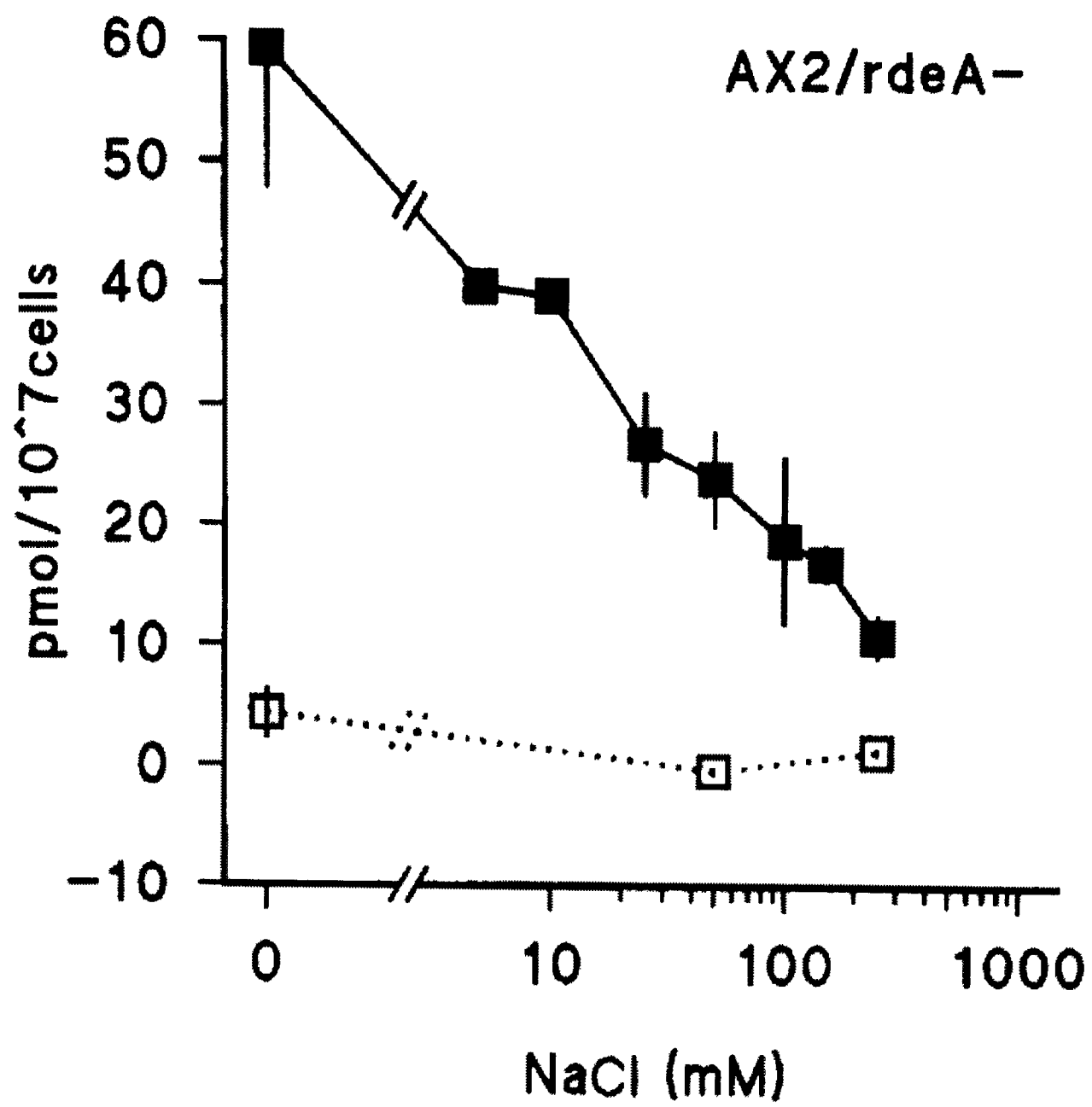


Figure 3.12 Effect of osmolarity on cAMP accumulation of vegetative DH1/rdeA cells.

Vegetative DH1/rdeA cells were washed once, resuspended in PB and then incubated with 5 mM DTT in the presence of indicated concentrations of NaCl. Solid squares represent cAMP accumulation after incubation for 10 minutes and open squares represent cAMP accumulation at time 0 min. Data are presented in semi-logarithmic scale and standard error means are given as bar. When s.e.m. is smaller than the length of squares, it is not shown.

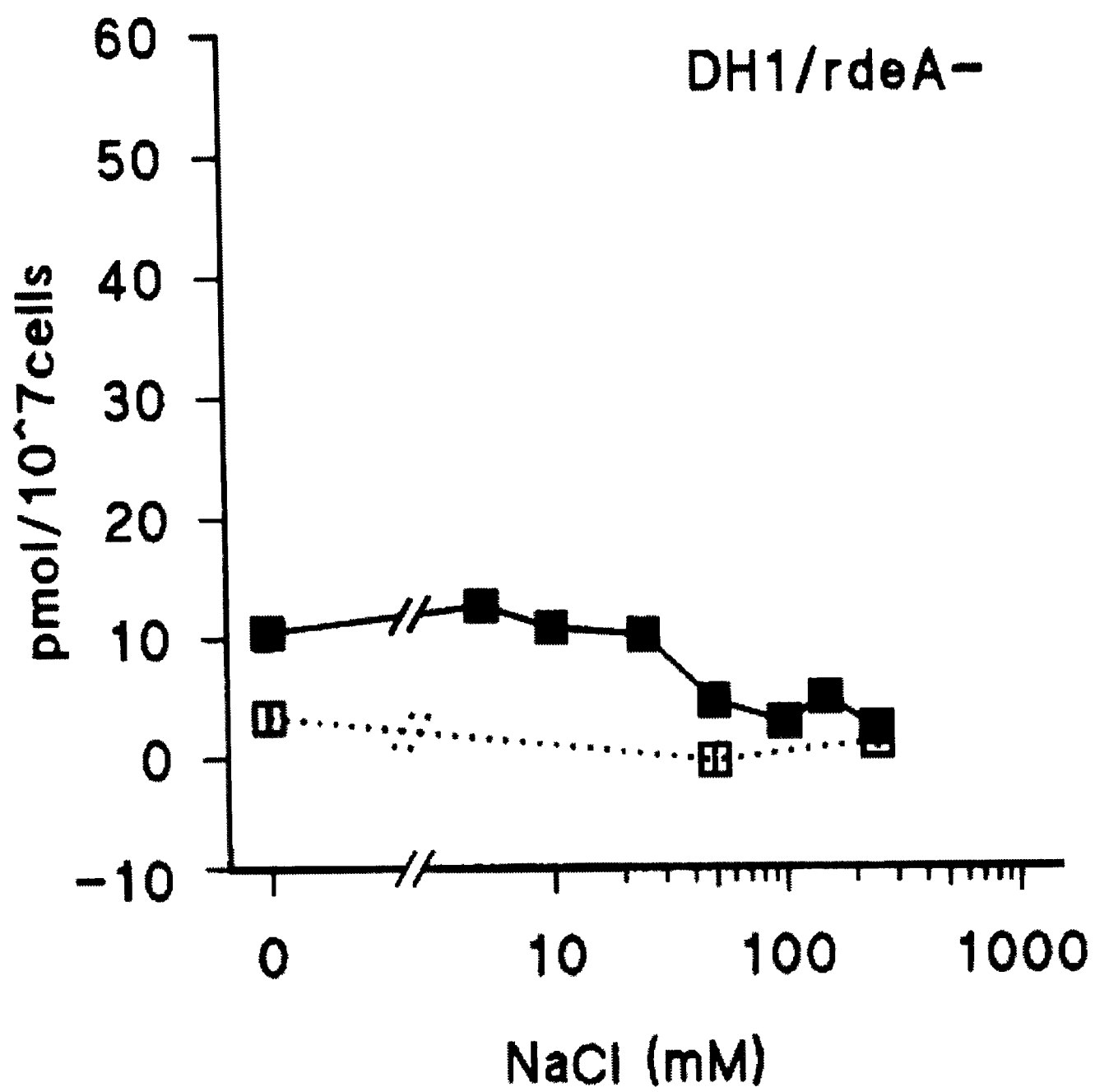


Figure 3.13 Effect of osmolarity on cAMP accumulation of vegetative AX2/regA cells.

Vegetative AX2/regA cells were washed once, resuspended in PB and then incubated with 5 mM DTT in the presence of indicated concentrations of NaCl. Solid squares represent cAMP accumulation after incubation for 10 minutes and open squares represent cAMP accumulation at time 0 min. Data are presented in semi-logarithmic scale and standard error means are given as bar. When s.e.m. is smaller than the length of squares, it is not shown.

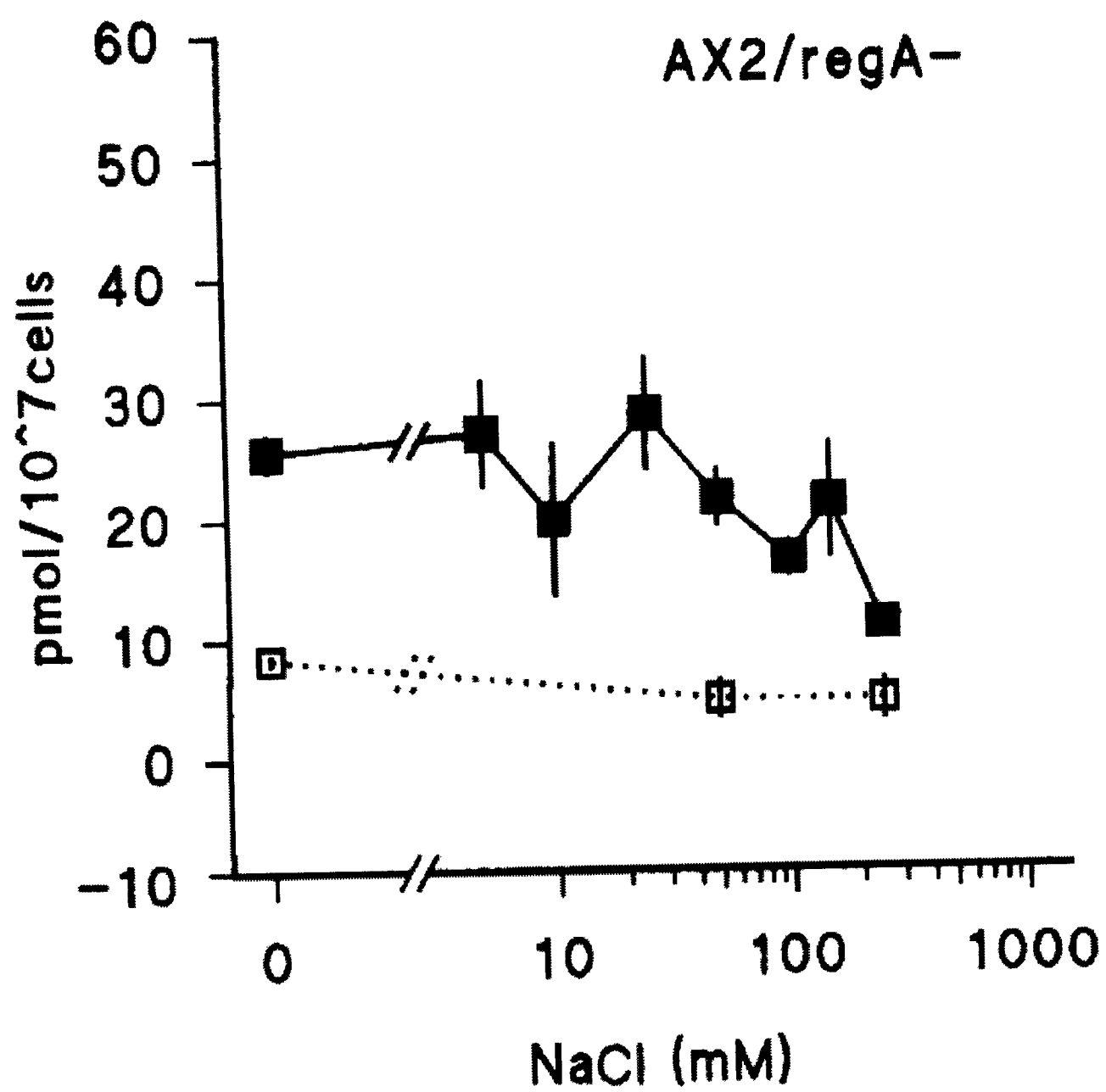
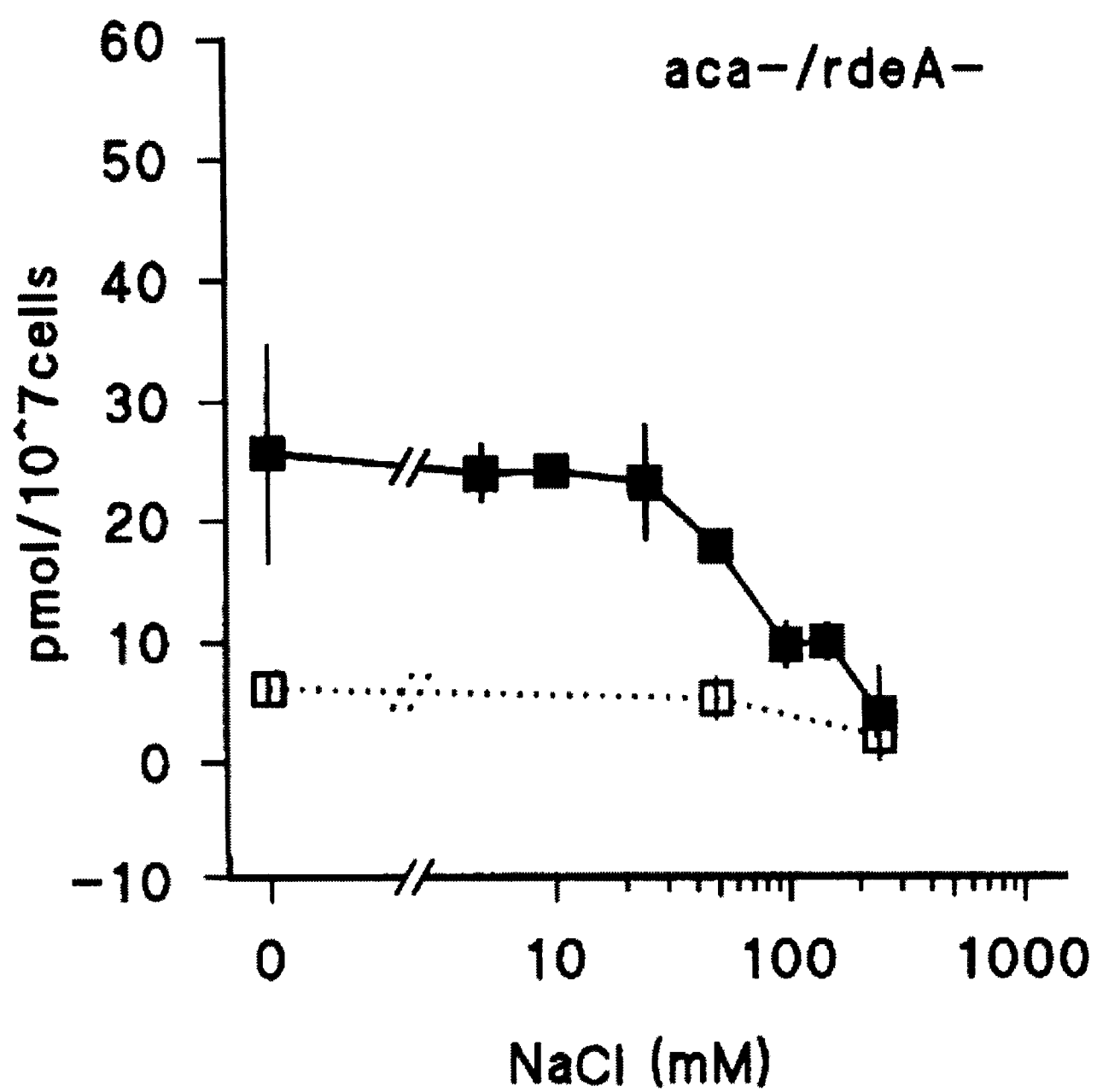


Figure 3.14 Effect of osmolarity on cAMP accumulation of vegetative *aca-/rdeA* cells.

Vegetative *aca-/rdeA* cells were washed once, resuspended in PB and then incubated with 5 mM DTT in the presence of indicated concentrations of NaCl. Solid squares represent cAMP accumulation after incubation for 10 minutes and open squares represent cAMP accumulation at time 0 min. Data are presented in semi-logarithmic scale and standard error means are given as bar. When s.e.m. is smaller than the length of squares, it is not shown.



Sporogenous assay in submerged monolayer

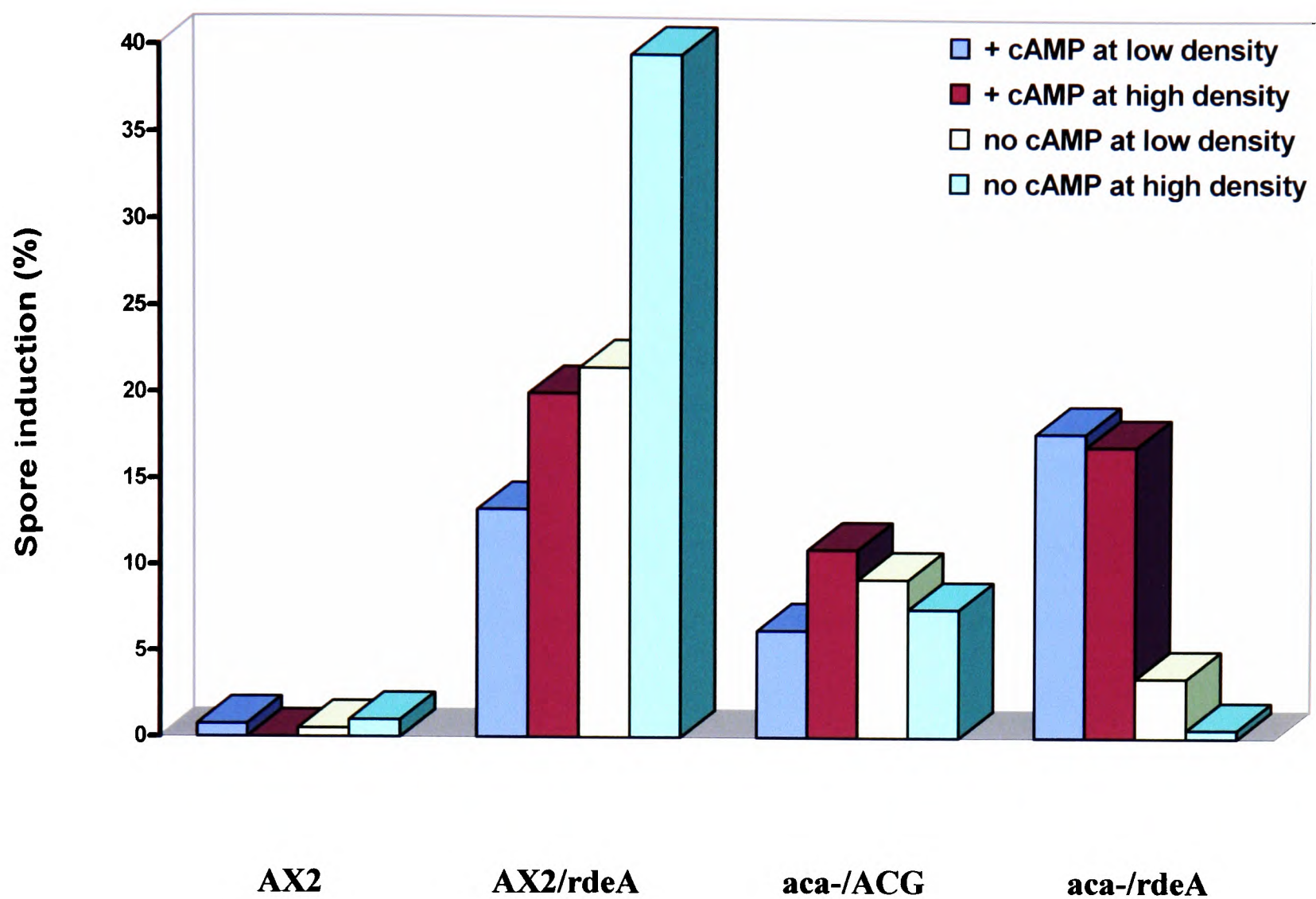


Figure 3.15 Sporogenous assays of rdeA mutants
5mM cAMP was added to the samples shown as + cAMP.
Low density stands for 5×10^5 cells / ml ; high density stands for 1×10^6 cells / ml.

Samples were incubated in 50mm tissue culture plates at 22°C for 48 hours. Spores were counted using a phase contrast microscope. All data represent means of duplicated experiments and are expressed as percentage out of total 200 cells.

was combined with 2 ml of spore induction buffer in a 50 mm tissue culture dish so that the final cell density was 5×10^5 cells / ml unless otherwise stated.

As expected, since they are not sporogenous, wild-type AX2 cells produced very few spores either in the presence or absence of 5 mM cAMP regardless of cell density.

The AX2/rdeA mutant produced many spores and the yield was not dependent on the presence of 5 mM cAMP but was greater at the higher cell density.

The aca-/ACG strain also displayed a sporogenous phenotype; in this strain the yield of spores was independent both of the presence of 5 mM cAMP and of cell density.

Most interestingly the aca-/rdeA mutant was also able to produce spores in the presence of 5 mM cAMP and spore formation was significantly reduced in the absence of 5 mM cAMP but seemingly unaffected by cell density. Thus aca/rdeA cells would appear to be able to activate PKA sufficiently to form mature spores despite lacking ACA activity.

3.3 Discussion

3.31 Does high PKA activity cause rapid development?

Sporogenous mutants, i.e. rdeA, rdeC and regA mutants show rapid development and it is thought that rapid development is due to abnormally high PKA activity, a view based mainly on the fact that the rdeC gene product is the PKA regulatory subunit.

Unfortunately it has not yet been possible to measure *in vivo* PKA activity in any of the mutants and we do not have any firm evidence of how high PKA activity arises in the rdeA and regA mutants. However, as discussed in the General Introduction

(chapter 1), the rdeA and regA mutants are both expected to lack REGA cAMP-phosphodiesterase activity and the new adenylyl cyclase was detected in just these mutants (Figure 3.6 to 3.9).

Hence it is reasonable to suppose that the high cAMP levels in vegetative rdeA and regA cells are due to accumulation of cAMP by the novel AC under conditions where destruction of the cAMP does not occur because REGA cAMP-phosphodiesterase activity is absent.

3.32 Is the mechanism of rapid development of the rdeA mutant solved?

The report of Abe and Yanagisawa that rdeA mutants generated by a chemical mutagenesis had elevated intracellular cAMP levels during vegetative growth and development gave a very simple and clear picture of how they could have high PKA activity and hence display rapid development. The fact that the new adenylyl cyclase was detected in the REMI-induced AX2/rdeA mutant might be taken as implying further support for this idea.

However my measurements in AX2/rdeA did not show elevated cAMP levels during development contrary to the related report of Abe and Yanagisawa (Figure 3.1).

Although there was no distinct peak of cAMP during development, overall the cAMP levels could not be taken as abnormally high. I will discuss how the rapid development of rdeA mutants may be explained after additional experimental data have been reported.

3.33 How are rapidly developing mutants able to produce spores in submerged monolayers?

I have shown that *aca-/rdeA* cells are sporogenous (Figure 3.15) yet they cannot aggregate under normal culture conditions. It is interesting to consider how *aca-/rdeA* cells could have high PKA activity in submerged monolayers despite the absence of ACA. One possibility is that the RDEA protein might function as a kind of glue to hold the PKA regulatory subunit and the PKA catalytic subunit together so that deletion of the glue, the RDEA protein, would result in high PKA activity. A more likely possibility in view of the current view of the roles of RDEA and REGA would be RDEA activates the REGA cAMP-PDE and this then hydrolyses the cAMP produced by the new adenylyl cyclase in wild-type cells. In *aca-/rdeA* cells on the other hand the cAMP made by the new adenylyl cyclase would be protected by the absence of the REGA cAMP-PDE and would enable the cells to have high PKA activity in submerged monolayers.

3.34 Does the novel adenylyl cyclase provide intracellular cAMP to activate PKA?

Figure 3.15 showed that *aca-/rdeA* cells required extracellular cAMP in order to produce spores in submerged monolayers whereas the *rdeA* single mutant did not. If we make the assumption that extracellular cAMP turns on a PKA-dependent pathway in submerged monolayers cells and that an adenylyl cyclase is responsible for PKA activation in the *aca-/rdeA* cells, it should follow that the adenylyl cyclase activity

should somehow be activated by extracellular cAMP. We now know that cAMP accumulation in *aca-/rdeA* cells is due to the novel adenylyl cyclase (chapter 3; Kim et al., 1998) and preliminary experiments that I have carried out gave no evidence that this enzyme is activated by extracellular cAMP. If that is the case the assumption that extracellular cAMP turns on a PKA-dependent pathway needs re-examining. It may well be instead that extracellular cAMP turns on second messenger pathways that are independent of PKA. In that case the sporogenous phenotype may require high PKA activity and in addition activation of other second messenger pathway by extracellular cAMP. The PKA independent pathway could be imagined as follows though this is pure speculation: extracellular cAMP may open Ca^{2+} channels on the plasma membrane and/or on the membrane of the ER and the elevated intracellular Ca^{2+} levels may activate Ca^{2+} / Calmodulin dependent Protein Kinase or PKC. This action would then contribute to the sporogenous phenotype.

Figure 3.15 also shows that whereas *aca-/rdeA* cells needed extracellular cAMP to produce spores in submerged monolayers, *AX2/rdeA* cells did not. Yet the novel adenylyl cyclase was detected in both strains (Figure 3.6 and 3.9). This finding could be interpreted by supposing that the new AC provides the intracellular cAMP to induce high PKA activity in both strains but that the *AX2/rdeA* mutant secretes enough cAMP into the medium to activate the other putative second messenger pathway because it has two active adenylyl cyclases, ACA and the new AC. The *aca-/rdeA* cells on the other hand would not produce enough cAMP for auto-activation of these second messenger pathway and would require supplementation of the medium with cAMP for spore formation.

This idea may gain some support from three additional findings. First the yield of spores in AX2/rdeA cells doubled when cell density was increased, as might be expected if they release cAMP into the medium and this has to reach some threshold concentration for auto-activation of the putative second messenger pathways. Second, rdeC mutants also require addition of extracellular cAMP for spore maturation in monolayers (Kay, 1989) and have been shown to possess very low levels of cAMP (Abe and Yanagisawa, 1983 ; Simon et al., 1992); they would not, therefore, be expected to release enough cAMP for auto-activation. Third the aca-/ACG strain also produced spores in submerged monolayers regardless of the presence or absence of extracellular cAMP (Figure 3.15). It would seem therefore that the high level of cAMP production by ACG in this strain satisfies the two requirements proposed above for spore maturation: high PKA activity and accumulation of extracellular cAMP to activate additional second messenger pathway. Thus in this instance at least, cAMP accumulation is a sufficient explanation for sporogeny and therefore there is no reason to invoke some direct alteration of PKA holoenzyme structure (such as the absence of an RDEA “glue”) to account for the sporogeny of rdeA cells.

3.35 Another question may be answered by the novel adenylyl cyclase.

Pitt et al (1993) have been able to induce development of aca- cells by exposing them to pulses of cAMP followed by high continuous cAMP . We still do not understand how these stimuli work inside the cells and whether they involved a signalling pathway affecting an adenylyl cyclase. It was always a possibility that the aca- cells possessed another adenylyl cyclase activity even though it could not be detected and

that they therefore retained the potential to develop provided certain conditions were fulfilled to obtain adequate adenylyl cyclase activity to induce sufficient PKA activity. The discovery of the new adenylyl cyclase may certainly have a bearing on this question.

Chapter 4

Chapter 4

Biochemical characterisation of the novel Adenylyl Cyclase and evidence for the RDEA - REGA phosphorelay system in *Dictyostelium*

4.1 Introduction

As described in the previous chapters, study of physiological aspects of the *rdeA* and *regA* mutants have led to the discovery of a novel adenylyl cyclase (chapter 3). This discovery raised questions about the nature of the new adenylyl cyclase and how it differed from the two known adenylyl cyclases, ACA and ACG. An even more intriguing question is why the new adenylyl cyclase activity could only be detected in *rdeA* and *regA* mutants and not in wild type cells (Figure 3.6-3.9). In other words how are the two proteins, the RDEA and the REGA, involved in controlling the new adenylyl cyclase activity?

4.11 How is the multi-step phosphorelay related to the new adenylyl cyclase?

As already pointed out the *rdeA* gene product displays some homology to H2-type phosphotransferases in multi-component signalling system (Chang et al, 1998). In addition, the C-terminus of the *regA* gene product encodes a cyclic AMP phosphodiesterase, while the N-terminal region is homologous to the well-characterised response regulators of bacterial and eukaryotic two-component

signalling systems (Shaulsky et al, 1996; Loomis et al, 1997; Thomason et al, 1998). It has therefore been proposed that the RDEA protein activates the REGA phosphodiesterase in a multi step phosphorelay (Chang et al, 1998; Thomason et al, 1998. See also Chapter 1 and Figure 1.1 - 1.4 for more details). When the idea was proposed, it was mostly based on sequence homology and the similarity of phenotype of *rdeA* and *regA* mutants. At that time there was little direct experimental evidence to support the hypothesis, convincing though it was to many people. Hence it was very tempting to test further the hypothesis experimentally. If the RDEA protein phosphorylates the REGA cAMP-phosphodiesterase (PDE) to induce the active state of the enzyme, *rdeA* mutants should have very low REGA PDE activity. The common feature of the *rdeA* and *regA* mutants could therefore be negligible REGA PDE activity. I shall discuss later in the current chapter how this could help to understand the reason for my detecting the new adenylyl cyclase.

4.12 Biochemical characteristics of the two known adenylyl cyclases, ACA and ACG

Apart from the possible relationship between a multi-step phosphorelay and the new adenylyl cyclase, the new enzyme itself deserved to be studied with great interest. Of the two structurally distinct forms of adenylyl cyclase previously identified in *Dictyostelium*, one, ACA, is stimulated by extracellular cAMP via a G-protein-dependent pathway and rapidly adapts (Parent and Devreotes, 1996). Its catalytic activity is increased by binding to divalent cations, for example, Mg^{2+} and Mn^{2+} (Pitt et al., 1992). It is sensitive to caffeine (Brenner and Thom, 1984) like ACG (Schaap, personal communication) and both adenylyl cyclases have optimal pH at 8.0.

Although transcripts of ACG are normally detected only during spore germination, the enzyme can be readily assayed in vegetative cells when expressed under the control of the *actin15* promoter (Conchita et al., 1995). As mentioned in chapter 3, ACG is strongly stimulated by high osmolarity and is believed to be a component of an osmosensor (van Es et al, 1996). It is known not to be activated via plasma membrane cAMP receptors and is not coupled to G proteins (Pitt et al., 1992). Its catalytic activity is also stimulated, like ACA, by binding to the divalent cations, Mg^{2+} and Mn^{2+} , showing greater sensitivity to the latter (Pitt et al., 1992). I will describe in the next section how the new adenylyl cyclase is distinctive in terms of biochemical characteristics.

4.2 Results

4.21 Biochemical characterisation of the novel adenylyl cyclase

4.211 Naming the new adenylyl cyclase

The new adenylyl cyclase activity was detected in vegetative *aca-/rdeA* cells (Figure 3.9 B) but was not evident in 6 hour starved *aca-/rdeA* cells (Figure 3.5 B). This gave the impression that the new activity might have declined after starvation in *aca-/rdeA* cells. Since there was virtually no activity detected in 6 hour starved cells I decided to see how the activity evolved over the first 6 hours of starvation. Washed *aca-/rdeA* cells were either used directly from growth medium (T0) or were first plated onto PB agar plates and incubated for 1, 2, 3, 4, 5, or 6 hours at 22°C. Subsequently they were resuspended in PB to 10^8 cells/ml, exposed to 5mM DTT for 0, 2, 5, or 10 minutes

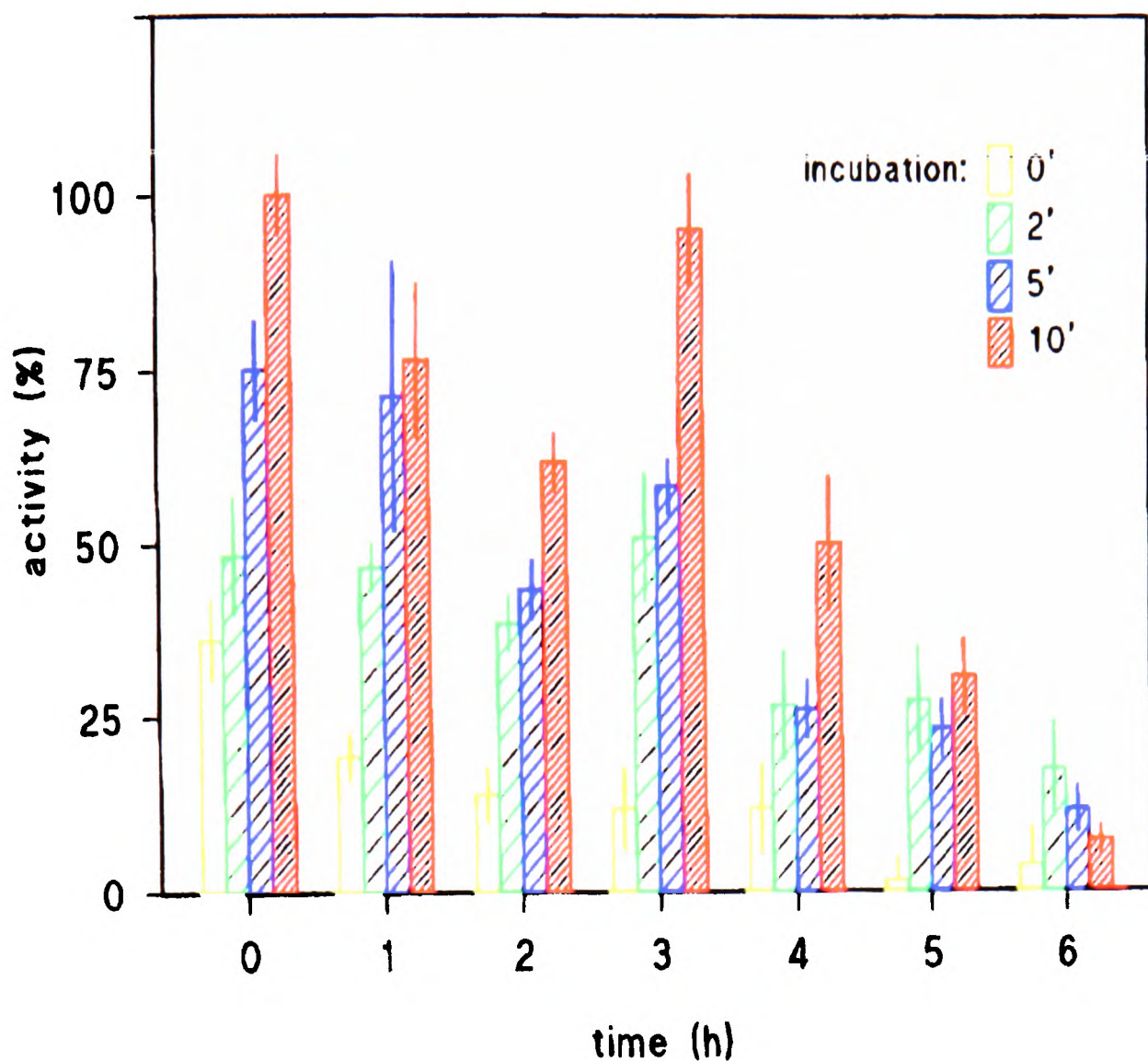


Figure 4.1 *In vivo* adenylyl cyclase activity during the starvation of *aca-/rdeA* cells.

aca-/rdeA cells were used directly from growth medium (T_0) or were plated on PB agar plates and incubated for 1, 2, 3, 4, 5, or 6 hours at 22°C. Subsequently cells were harvested and resuspended in PB at 10^8 cells/ml. The cell suspension was exposed to 5 mM DTT for 0 (in yellow), 2 (in light green), 5 (in blue), or 10 (in red) minutes and assayed for total cAMP levels. Data were standardised on the protein levels of the cell suspensions and expressed as percentage of cAMP accumulated after 10 minutes in cells at time 0 hour. Data represent means and s.e.m. of three experiments performed in triplicate.

and total cAMP measured in aliquots of the suspensions. Figure 4.1 shows maximal activity at the vegetative stage and a decrease of activity upon starvation. After 4 hours of starvation hardly any adenylyl cyclase activity was detected in the *aca-/rdeA* cells. This result coincided well with previous results (Figure 3.5 and 3.9) indicating a possible decrease of the new activity after starvation. It seemed to be reasonable to name the new enzyme as ACV, (Adenylyl Cyclase whose activity is maximal at the Vegetative stage) by analogy with ACA for Aggregation, and ACG for Germination. It was however not a very satisfactory name because we did not have any idea of the developmental regulation of the new enzyme since *aca-/rdeA* cells basically do not develop at all. Consequently the statement that the new activity was maximally expressed at the vegetative stage was only known to apply to *aca-/rdeA* cells. Dr. Pauline Schaap in Leiden University recently measured the new enzyme activity in wild-type cells (personal communication). In contrast to the profile of the activity in *aca-/rdeA* cells (Figure 4.1) the new adenylyl cyclase activity was maximal at the culmination stage in wild type development. In the light of this finding it has been agreed by workers in the field that the new adenylyl cyclase should be called ACB as a neutral term. I will refer to the new enzyme as ACB in the rest of my thesis.

4.212 Testing for G protein dependence of ACB

In order to be able to characterise ACB biochemically it was necessary to detect ACB activity *in vitro*. I therefore attempted to measure it with a similar *in vitro* assay to that developed for ACA. ACA can be measured in cell lysates, provided that ATP and GTP γ S or 2H'-cAMP are present during lysis. Since ACA is coupled to cAMP receptors in the plasma membrane, 2H'-cAMP, a non-permeable cAMP analogue, is

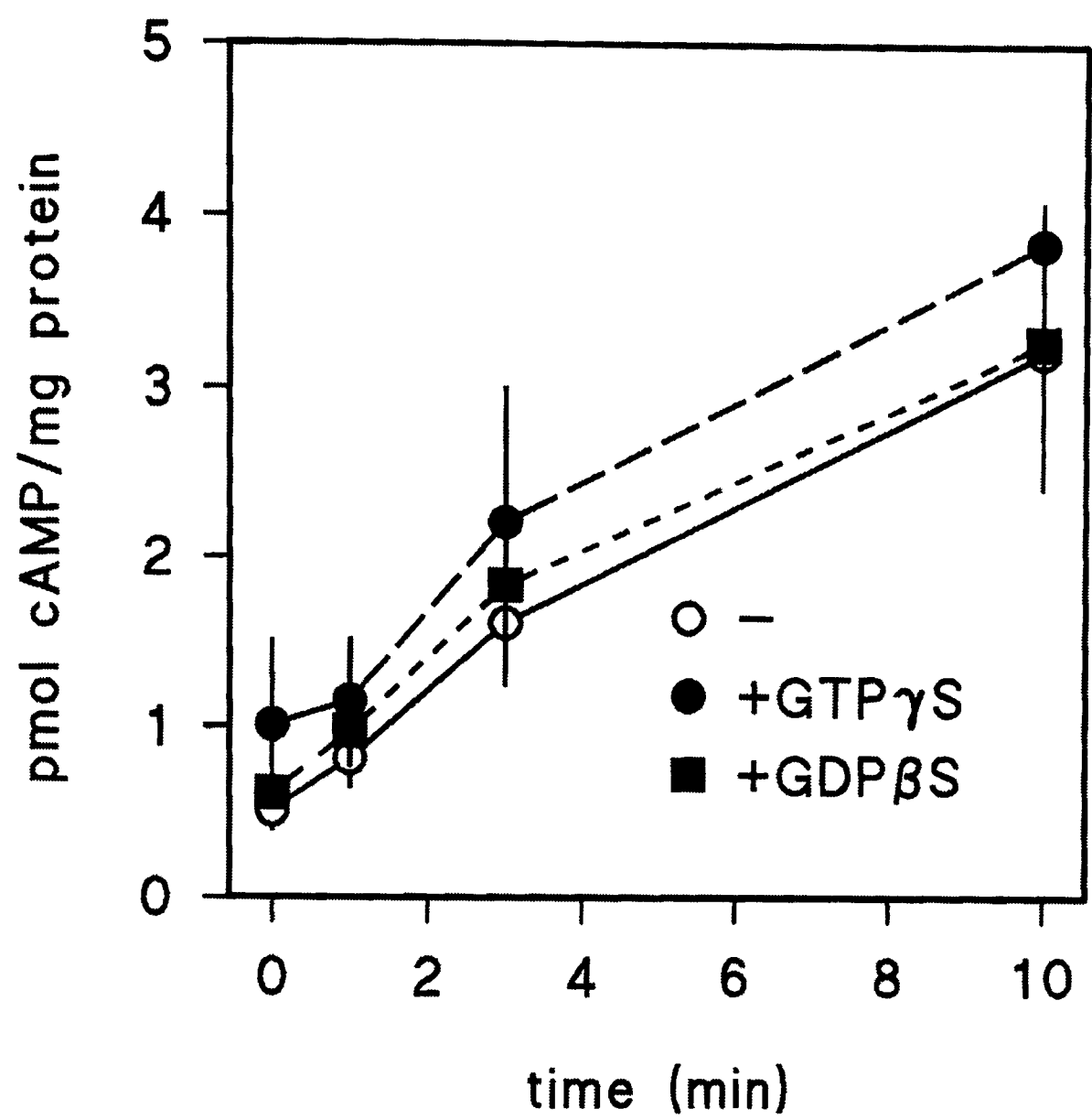
added before lysis to stimulate ACA activity. Alternatively GTP γ S can be added during lysis to activate G proteins associated with ACA. Lysis is achieved by passing the cells through a nuclepore filter (pore size; 3 μ m) by physical force. 10 mM DTT was always added from the beginning of the enzyme assay to inhibit the extracellular cAMP phosphodiesterase of *Dictyostelium* (Henderson, 1975). 2 mM MgCl₂ in the assay mixture helps to dissociate G $\beta\gamma$ subunit from G α subunit in a similar fashion to GTP γ S. The assay was stopped by adding EDTA (as a chelator) to remove Mg²⁺ because adenylyl cyclase can not use ATP as substrate in the absence of Mg²⁺. Use of vegetative *aca-/rdeA* cells was thought to be best to eliminate the possibility that the activity detected might be ACA. It would have been better to use *aca-/acg-/rdeA* triple mutant cells but these were not available. Figure 4.2 shows that an activity could be measured in the lysate of vegetative *aca-/rdeA* cells, which presumably corresponded to the activity observed in intact cells. It will also be referred to ACB.

It was of interest to ask whether ACB was coupled to G proteins like ACA. If ACB activity is dependent on a G protein, it is very likely to be a membrane bound and that could be useful information for its purification. *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer. The suspension was then lysed through nuclepore filters in the presence and absence of 30 μ M GTP γ S or 30 μ M GDP β S.

Figure 4.2 shows firstly that an adenylyl cyclase activity can be detected in lysates of vegetative *aca-/rdeA* cells (open circle), secondly that the activity is not decreased by being incubated with GDP β S (solid squares), and thirdly that it is not stimulated by

Figure 4.2 G protein dependence of ACB

aca-/rdeA cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer. The cell suspension was lysed in the presence and absence of 30 μ M GTP γ S or 30 μ M GDP β S. Open circles represent adenylyl cyclase activity in the absence of either GTP γ S or GDP β S. Solid circles represent AC activity in the presence of GTP γ S and solid squares represent AC activity in the presence of GDP β S. Data represent means and s.e.m. of two independent experiments performed in triplicate. When s.e.m. is smaller than the diameter of circles or the length of squares, it is not shown.



GTP γ S (solid circles) in the dramatic way that ACA is (Theibert and Devreotes, 1986). It could be that the activity was very slightly higher with GTP γ S but that should not be interpreted as G protein dependence. G protein dependent adenylyl cyclases like ACA show a dramatic stimulation of activity in the presence of GTP γ S.

4.213 Mg²⁺ and Mn²⁺ dependence of ACB

The catalytic activity of most adenylyl cyclases is stimulated by the binding of divalent cations to a presumed allosteric site, and the relative efficacy of Mg²⁺ and Mn²⁺ differs widely amongst different enzymes (Yang and Epstein, 1983 ; Kawabe et al., 1996 ; Pieroni et al., 1995). The responses of ACA and ACG to Mg²⁺ and Mn²⁺ have been reported (Pitts et al., 1992) and I tested that of ACB hoping to see a difference. *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer. The cell suspension was lysed, and aliquots of lysate were incubated with various concentrations of either Mg²⁺ or Mn²⁺ at 22°C for 20 minutes. The response of ACG to the divalent cations was tested in order to compare it with ACB under identical conditions (Figure 4.3 B and 4.4 B). The results (Figure 4.3 B and 4.4 B) were added to my original data in a published paper (Kim et al., 1998) and they are presented here with the permission of Dr. Pauline Schaap (personal communication).

The *aca-/ACG* strain harbours an expression vector with ACG under *actin 15* promoter control in an *aca-* background. Therefore the activity detected in lysates of vegetative *aca-/ACG* cells can be considered to be due to ACG (Conchita et al., 1995).

Responses to Mg^{2+}

Figure 4.3 shows that ACB, i.e. the activity in the lysate of vegetative *aca-/rdeA* cells, increased in response to Mg^{2+} and was highest at 10 mM Mg^{2+} (A). Data are expressed as percentage of the activity at 10 mM Mg^{2+} and were originally measured in pmol cAMP /mg protein. ACG activity also increased gradually and reached a maximum at 10 mM Mg^{2+} . While ACG seemed to maintain a plateau (B), ACB appeared to be inhibited at higher Mg^{2+} concentration (A). Thus with regard to its response to Mg^{2+} , ACB did not seem significantly different from ACG.

Responses to Mn^{2+}

Figure 4.4 shows that ACB activity was stimulated by Mn^{2+} with a maximum at 10 mM Mn^{2+} (A). Data are again expressed as percentages of the activity at 10 mM Mg^{2+} because the experiments in Figure 4.3 A and 4.4 A were done at the same time and treated in the same way. ACG activity was also quite sensitive to Mn^{2+} and reached a maximum at 1 mM Mn^{2+} . It was clearly inhibited beyond 1 mM Mn^{2+} (B). Thus with regard to the response to Mn^{2+} , ACB was clearly distinguishable from ACG, being relatively insensitive to Mn^{2+} .

ACB Vs ACA and ACG

In summary, ACB is much more efficiently stimulated by Mg^{2+} than by Mn^{2+} (see Figure 4.3 A and 4.4 A) and in this respect is reminiscent of the soluble adenylyl cyclase of Sf9 insect ovary cells (Kawabe et al, 1996) as well as of the adenylyl cyclase of *E.coli* (Yang and Epstein, 1983). In contrast, the activity of ACG (Figure

Figure 4.3 Dose response to Mg²⁺ of adenylyl cyclase activities.

A. *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer without MgCl₂. The cell suspension was lysed and lysates were incubated with 0.5 mM ATP and 10 mM DTT for 20 minutes in the presence of indicated concentrations of Mg²⁺. Data are expressed as percentage of activity obtained at 10 mM Mg²⁺ and represent means and s.e.m. of two independent experiments performed in triplicate. When s.e.m. is smaller than the diameter of circles, it is not shown. B. The data are retrieved from Kim et al. (1998).

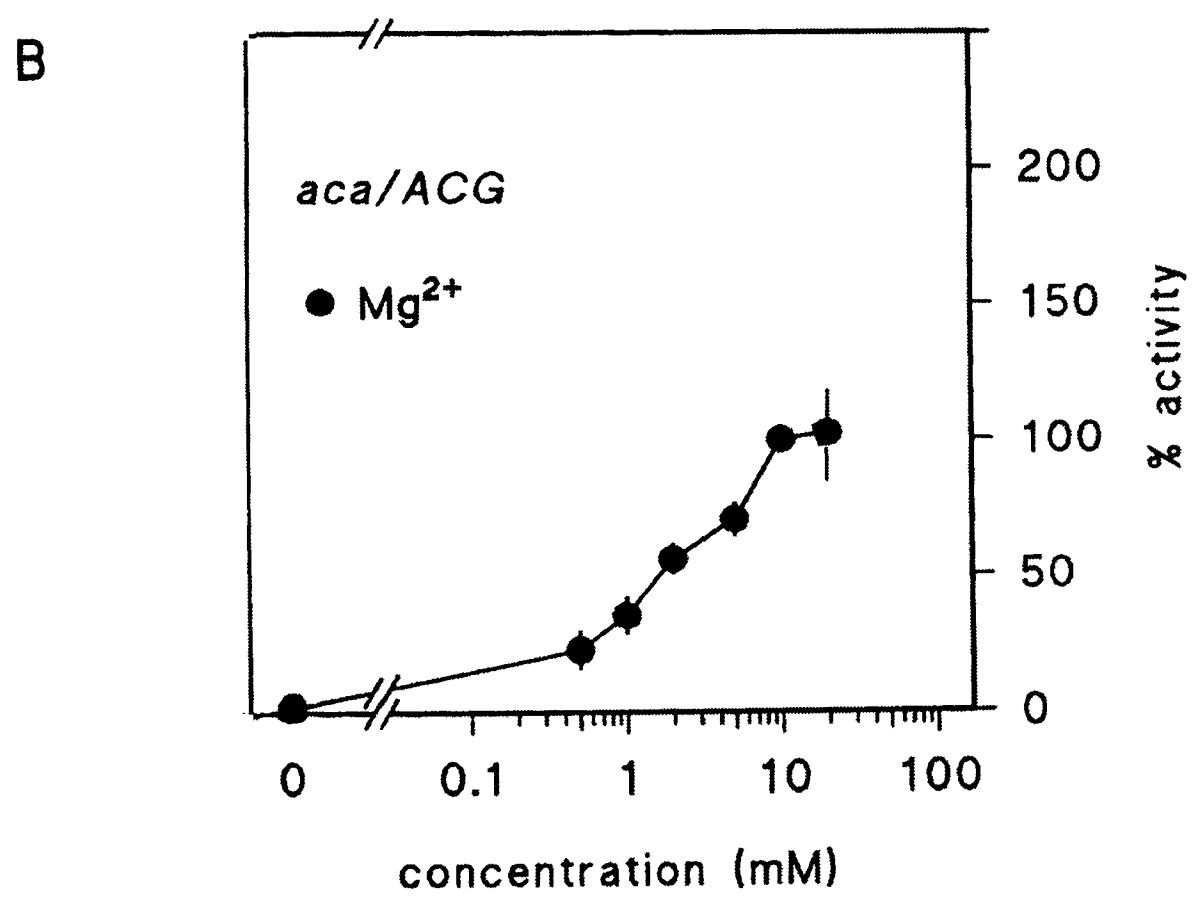
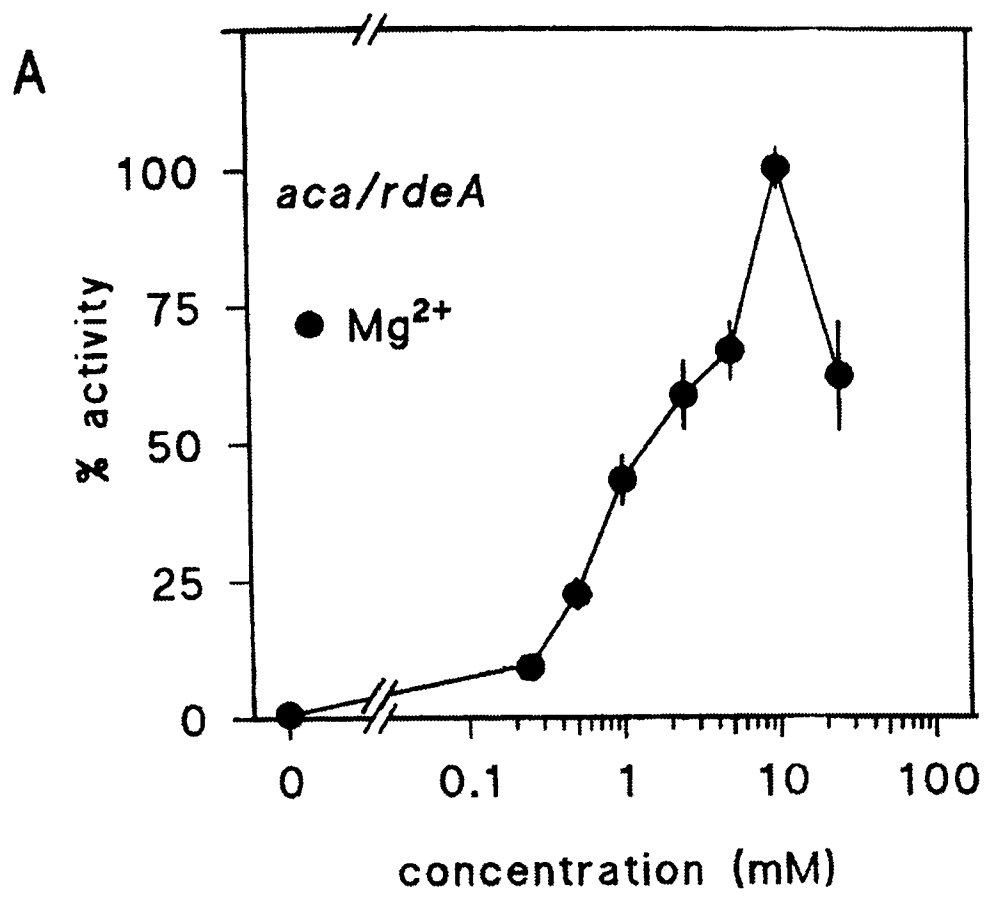
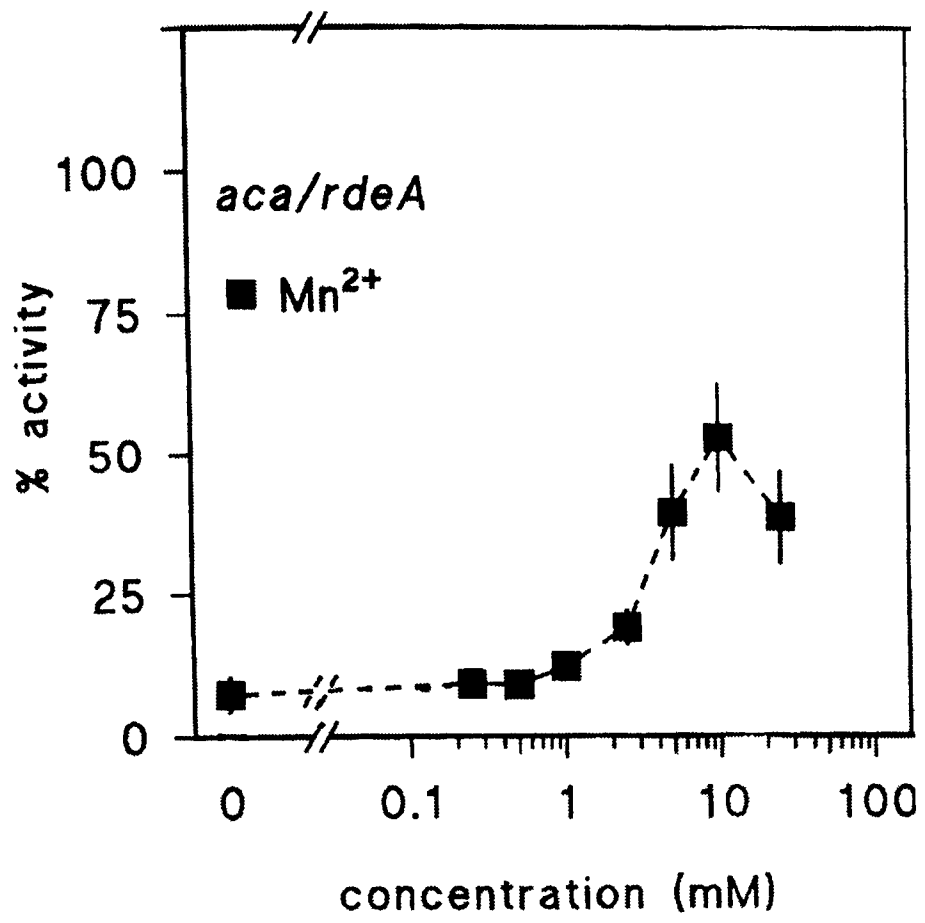


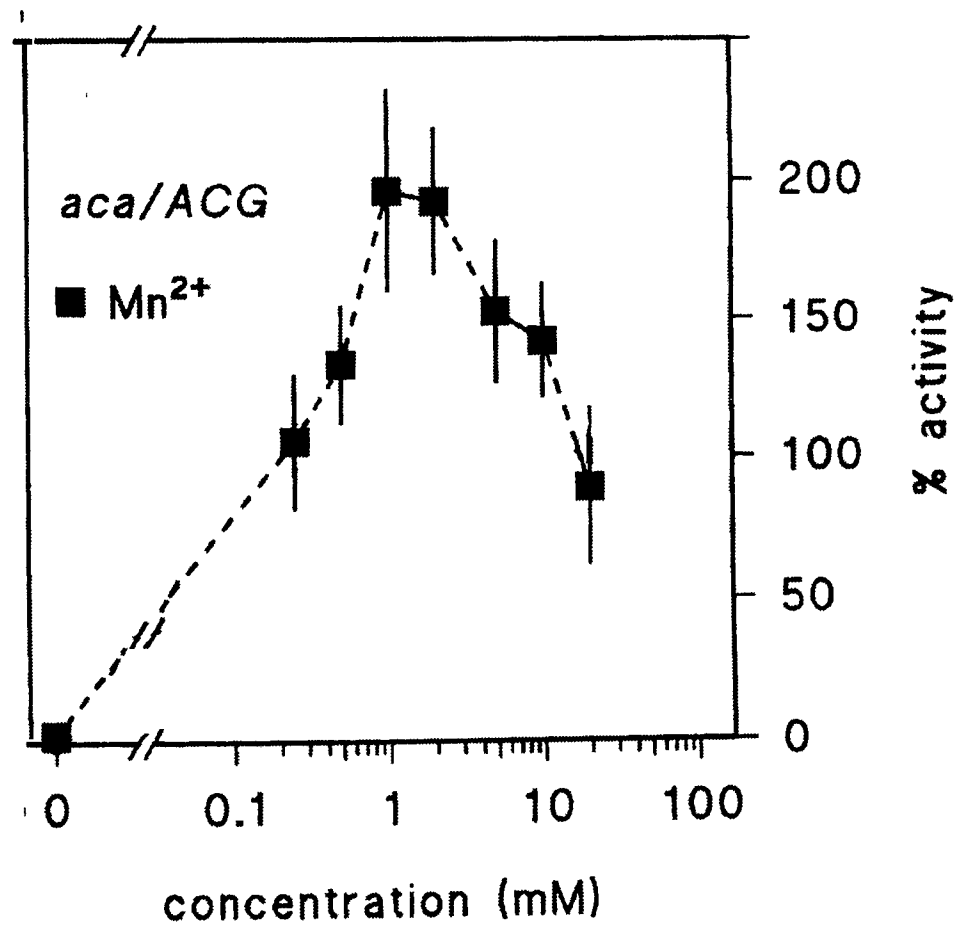
Figure 4.4 Dose response to Mn²⁺ of adenylyl cyclase activities.

A. *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer without MgCl₂. The cell suspension was lysed through nuclepore filter and lysates were incubated with 0.5 mM ATP and 10 mM DTT for 20 minutes in the presence of indicated concentrations of Mn²⁺. Data are expressed as percentage of activity obtained at 10 mM Mg²⁺ at Figure 4.2. Both experiments (Fig 4.2 and Fig 4.3) were done and calculated at the same time. Data also represent the means and s.e.m. of two independent experiments performed in triplicate. When s.e.m. is smaller than the length of squares, it is not shown. B. The data are retrieved from Kim et al. (1998).

A



B



4.3 B and 4.4 B) and of ACA (Pitt et al., 1992), is more responsive to Mn^{2+} than to Mg^{2+} , like standard mammalian forms of adenylyl cyclase (Pieroni et al., 1995) as well as the soluble adenylate cyclase in rat testis (Braun and Dods, 1975), the adenylyl cyclase in particulate preparations from epimastigote forms of *Trypanosoma cruzi* (Da Silveira et al., 1977) and the adenylyl cyclase of *Saccharomyces cerevisiae* (Heideman et al., 1987).

4.214 ACB is very sensitive to caffeine.

How are ACA and ACG inhibited by caffeine?

ACA is known to be inhibited by caffeine (Brenner and Thom, 1984) as is ACG (Dr. Pauline Schaap, personal communication). When extracellular cAMP binds to the cAMP receptor 1, cAR1, in the plasma membrane, the $G\alpha_2$ protein is activated transiently and leads the $G\beta\gamma$ subunit to be dissociated resulting somehow in the creation of a binding site for CRAC (cytosolic regulator of ACA) on the plasma membrane (Theibert and Devreotes, 1986 ; Lilly and Devreotes, 1994 and 1995 ; Insall et al., 1994). The activated $G\beta\gamma$ subunit is thought to bind to a PH (pleckstrin homology) domain of CRAC and the complex then causes CRAC to be translocated to the plasma membrane to activate ACA (Lilly and Deverotes, 1995). The inhibitory effect of caffeine on ACA was thought to involve blocking translocation of CRAC to the plasma membrane (Schaap, personal communication).

However this explanation is not applicable to ACG because ACG requires an entirely different pathway to be activated, without involvement of cAMP receptors, G protein and CRAC. Caffeine has been reported to release Ca^{2+} into the cytoplasm from the

endoplasmic reticulum (ER) (Islam et al., 1998) and some adenylyl cyclases are known to be controlled by Ca^{2+} , for example Ca^{2+} inhibitable adenylyl cyclase of the rat striatum (Chern et al., 1996) and Ca^{2+} /CaM stimulated adenylyl cyclase activity in a neuroblastoma (Kluxen et al., 1996). However such an explanation probably does not apply to ACG activity because it is not regulated by Ca^{2+} at physiological concentrations (Schaap et al., 1995).

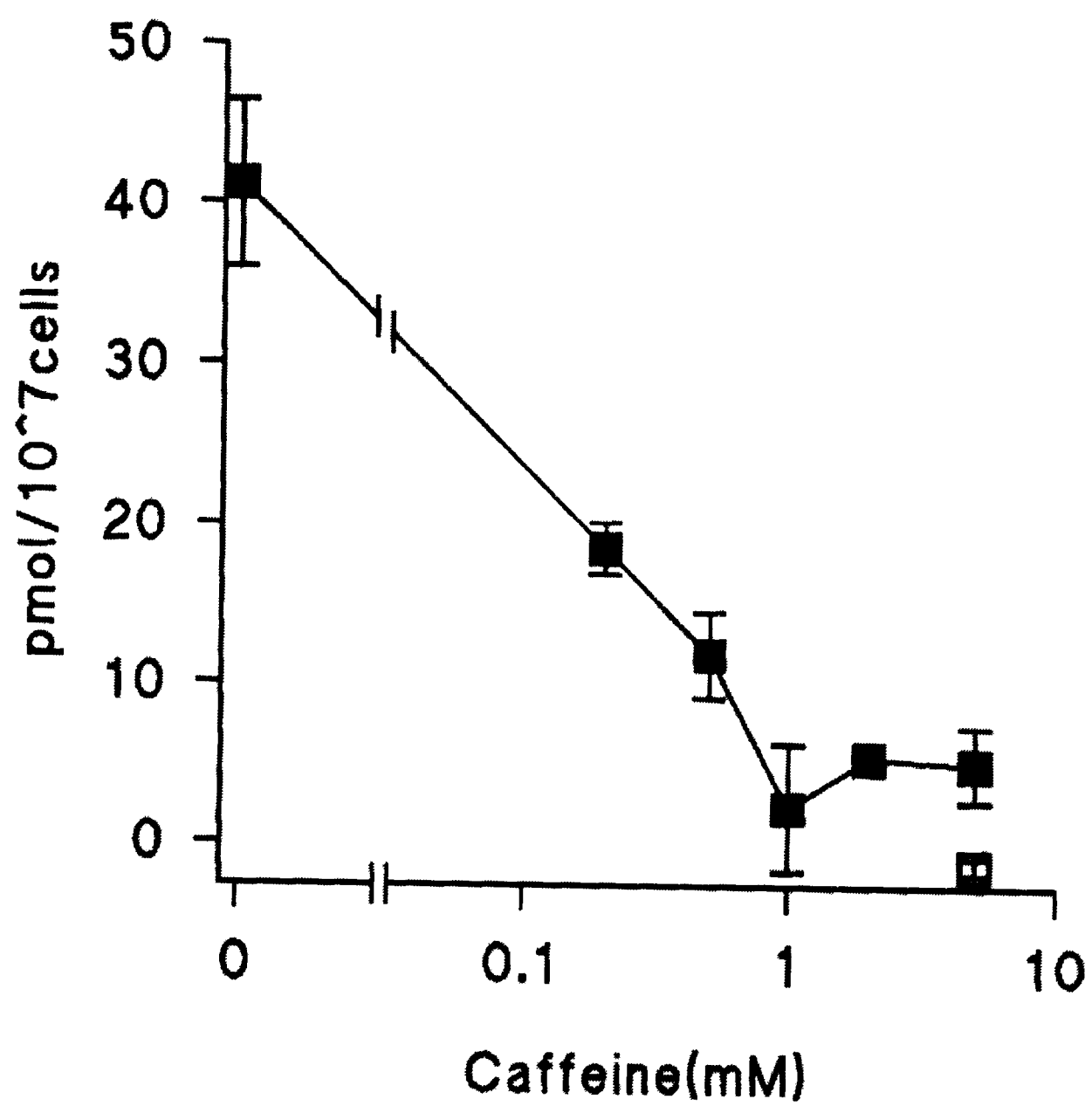
Response of ACB to caffeine

Dose dependence of ACB in response to caffeine was tested as follows. *aca-/rdeA-* cells were harvested from growth medium, washed once and resuspended directly in PB at 10^8 cells/ml. Aliquots of cell suspension were incubated with various concentrations of caffeine at 22°C for 20 minutes and cAMP levels determined by isotope dilution assay (van Haastert, 1984). Figure 4.5 shows that ACB activity was very effectively inhibited by caffeine considering that activity was virtually abolished at 1 mM caffeine. How does caffeine inhibit activation of ACB? Since ACB, like ACG, is not coupled to a G protein (Figure 4.2), caffeine can not inhibit its activity by blocking translocation of the CRAC- $\text{G}\beta\gamma$ complex to the plasma membrane as was thought to occur for ACA.

We do not yet know if ACB activity is influenced by Ca^{2+} . I have tested the effects of Ca^{2+} , Calmodulin (Ca^{2+} binding protein to activate PKC), Calmidazolium (an inhibitor of calmodulin) on ACB activity. The results however were not reproducible and further experiment should be done in future. If we assume that ACB is inhibited by Ca^{2+} /Calmodulin we may explain the inhibition of ACB by caffeine as follows.

Figure 4.5 ACB is very sensitive to caffeine.

aca-/rdeA- cells were harvested from growth medium, washed once and then resuspended in PB. The cell suspension was incubated for 20 minutes with 5 mM DTT in the presence of indicated concentrations of caffeine. Solid squares represent cAMP accumulated by 10^7 cells after incubation and open squares represent cAMP accumulated by 10^7 cells at time 0 min. Data represent means and s.e.m. of two independent experiments performed in triplicate. Data are presented in semi-logarithmic scale and standard error means are given as bar. When s.e.m. is smaller than the length of squares, it is not shown.



Caffeine might cause Ca^{2+} to be released into cytoplasm and the increased intracellular Ca^{2+} concentration might inhibit ACB activity. However there may well be other explanations.

4.215 pH dependence of ACB activity

ACA and ACG are known to have distinct peaks in their pH profiles, at pH 8.0 and pH 7.8 respectively (Schaap et al., 1995). In *Dictyostelium*, ammonia has been given much attention as a molecule influencing culmination (Hopper et al., 1993). Much study has been carried out by many researchers in an attempt to understand how depletion of ammonia might increase PKA activity and allow *Dictyostelium* to culminate (Davies et al., 1993; Gross, 1994; Gee et al., 1994). No effect of intracellular pH or Ca^{2+} on either ACA or ACG could be found to account for a possible control of PKA activity by ammonia (Schaap et al., 1995). The issue has remained as an exciting conundrum in *Dictyostelium* and the rather radical suggestions were made that ammonia could influence a PKA independent pathway or inhibit an as yet undiscovered adenylyl cyclase (Schaap et al., 1995). Therefore it was very exciting to test the pH dependence of ACB and the experiment was done as follows in brief. *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer. The cell suspension was lysed and aliquots of lysate were incubated at each indicated pH, from pH 6.0 to pH 9.0 at interval of 0.2 unit at 22°C for 10 minutes. The data for wild-type ACA and *aca-/ACG* are reproduced from Schaap et al., 1995 as presented in Kim et al., (1998) with Dr. Schaap's permission. My own data are in blue to distinguish them from Dr Schaap's in black (Figure 4.6). ACB activity has a fairly broad pH optimum or a plateau from

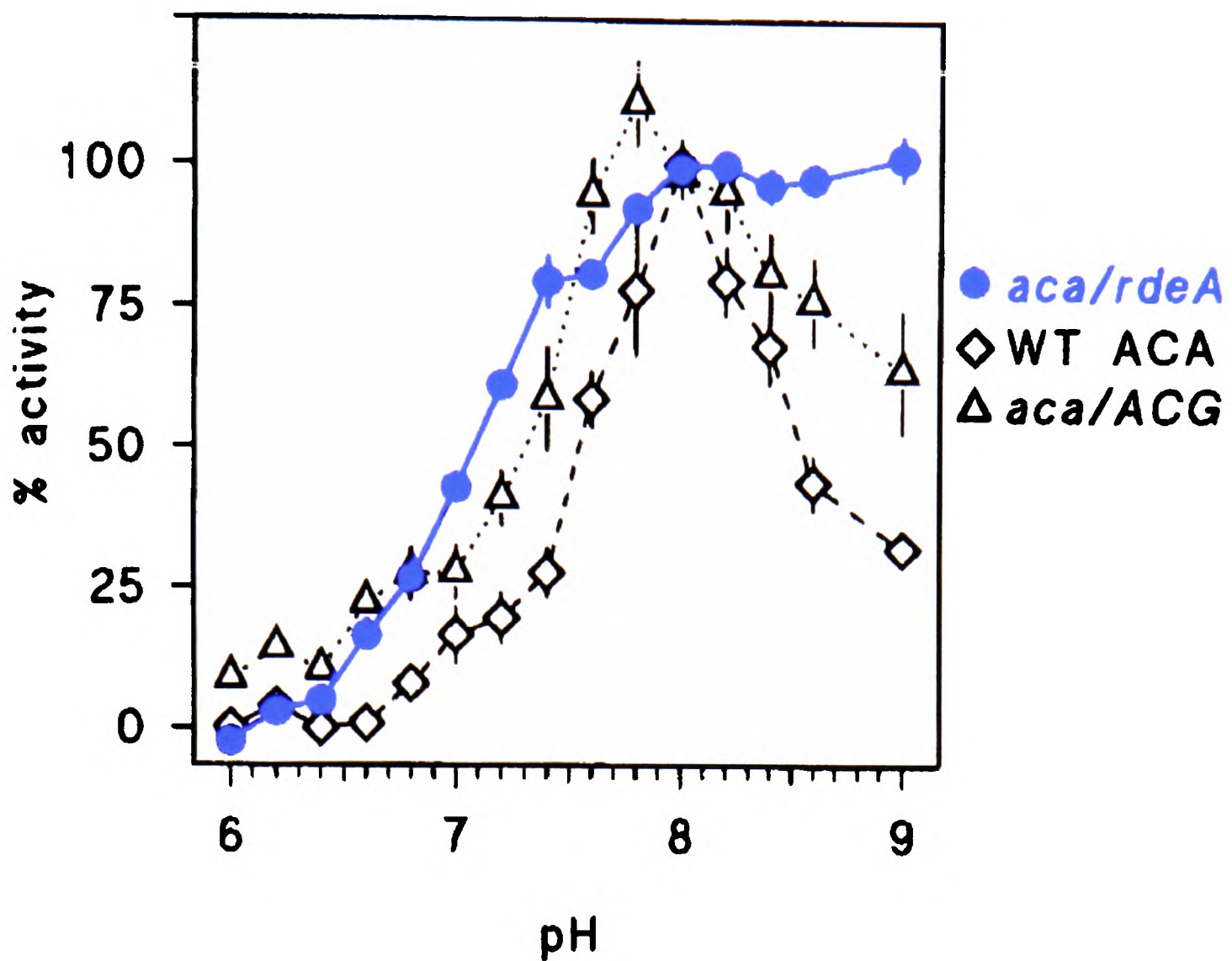


Figure 4.6 Effect of pH on adenylyl cyclase activities.

aca-rdeA cells were harvested from growth medium, washed once with PB and resuspended in modified lysis buffer (2 mM MgCl_2 and 250 mM sucrose in 2 mM Tris, pH 7.5) at 10^8 cells/ml. Lysates were incubated with 0.5 mM ATP and 10 mM DTT for 10 minutes along with 0.5 M Tris-maleate of indicated pH values. Reactions were terminated by adding 10 μl of 0.2 M EDTA, pH 8.0 followed by boiling for 1 min. The data for *aca-/ACG* and ACA in starved wild type cells stimulated by $\text{GTP}\gamma\text{S}$ are retrieved from Schaap et al. (1995) in the same way as done in Kim et al. (1998). My original results are clearly distinguished in blue from the retrieved. Data represent means and s.e.m. of two independent experiments performed in triplicate and they are expressed as percentage of the adenylyl cyclase activity at pH 8.0 of *aca-rdeA* lysate. When s.e.m. is smaller than the length of symbols, it is not shown.

pH 7.8 to pH 9.0 while ACA and ACG have somewhat distinct optima at pH 8.0.

ACB is thus slightly different from ACA and ACG in pH profile but the new adenylyl cyclase does not seem to provide a solution to the ammonia effect on the basis of intracellular pH. This will be discussed more later in this chapter.

4.22 ACB in relation to the multi-step phosphorelay in *Dictyostelium*

If the novel adenylyl cyclase activity can be detected in *rdeA* and *regA* cells because they lack a cAMP phosphodiesterase activity, it should be possible to detect the same activity in lysates of *aca-* cells by inhibiting the cAMP phosphodiesterase by pharmacological means. In an attempt to do this IBMX (isobutyl methylxanthine) was employed, a well-known inhibitor of mammalian cAMP phosphodiesterases (Barber and Butcher, 1980) which has also been shown to inhibit the REGA phosphodiesterase (Thomason et al, 1998). *aca-* and *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in lysis buffer. The cell suspensions were lysed and aliquots of cell lysates were incubated in the presence or absence of 0.1 mM IBMX at 22°C. The reaction was terminated by adding 10 µl of 0.2M EDTA, pH 8.0 at the indicated time periods and cAMP levels were determined. Figure 4.7 shows that no cAMP synthesis could be detected in lysates of vegetative *aca-* cells in the absence of IBMX. However, in its presence *aca-* lysates accumulated as much cAMP as *aca-/rdeA-* lysates. There appeared to be a slight stimulation of activity when the *aca-/rdeA-* lysate was supplemented with IBMX. This could be due to the presence of an additional IBMX-sensitive cAMP phosphodiesterase or to residual REGA phosphodiesterase activity in *rdeA* cells.

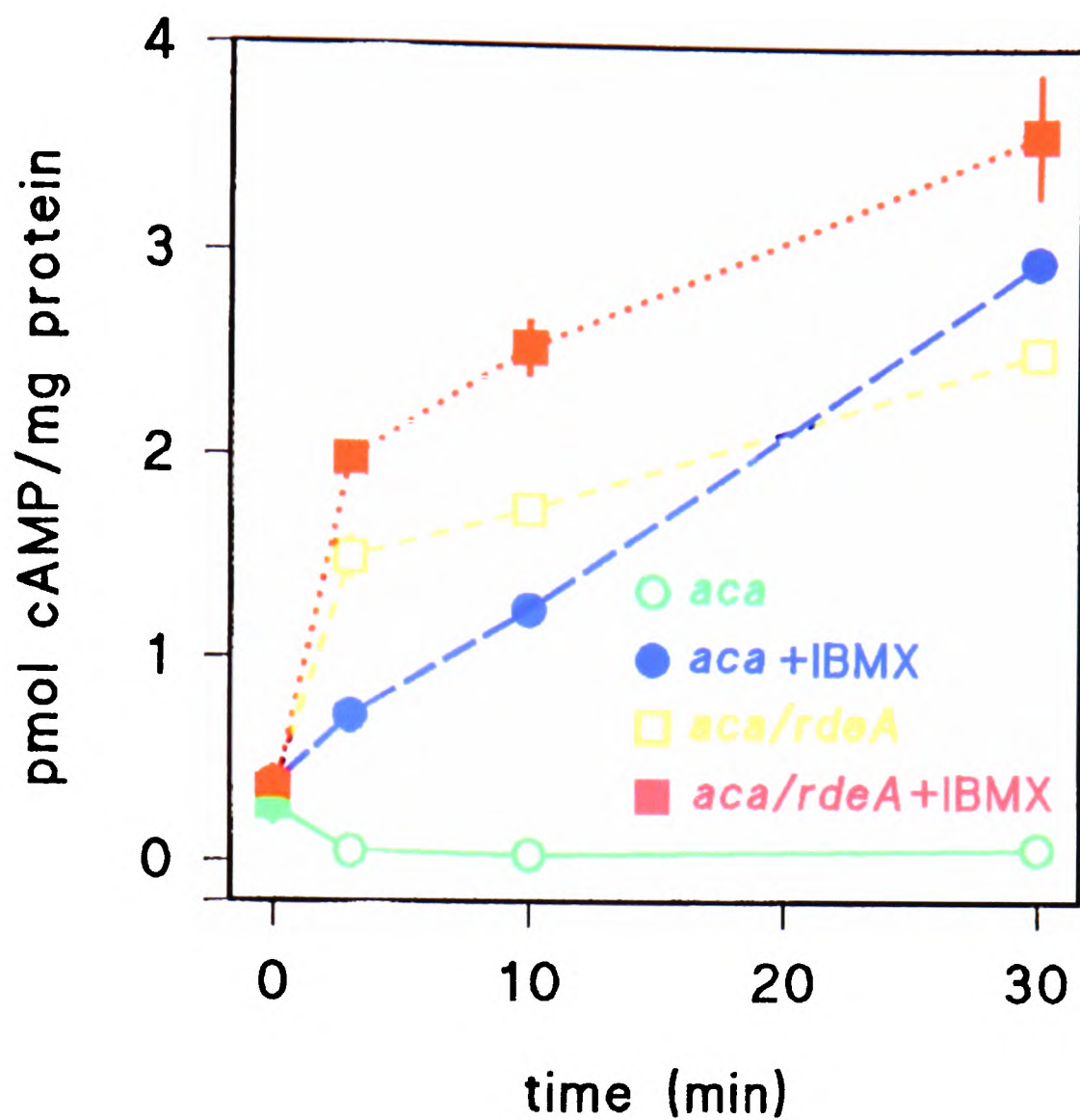


Figure 4.7 ACB revealed by IBMX, an inhibitor of cAMP-phosphodiesterases. *aca*- and *aca/rdeA* cells were harvested from growth medium, washed once and resuspended in PB at 10^8 cells/ml. Aliquots of lysates were incubated with or without 0.1 mM IBMX. Vegetative *aca*- lysates did not accumulate cAMP (yellow green) without IBMX but the same lysates with IBMX (blue) accumulated cAMP similarly to vegetative *aca/rdeA* lysates (yellow). cAMP accumulation in vegetative *aca/rdeA* lysates was slightly stimulated in the presence of IBMX (red). Data were standardised at protein concentration and represent two independent experiments performed in triplicate. Standard error means are given as bars and they are not shown when s.e.m. is smaller than the diameter of circles or the length of squares.

4.3 Discussion

4.31 cAMP produced by ACB is down regulated by an RDEA-REGA phosphorelay.

As noted in the Introduction, it has recently been proposed that the *rdeA* gene encodes a histidine phosphotransferase that relays a phosphoryl group from an unknown histidine kinase to the response regulator of REGA and in so doing activates the REGA-cAMP phosphodiesterase (Chang et al., 1998; Thomason et al, 1998). In support of this, it has been shown experimentally that it is the phosphorylated form of the REGA cAMP-phosphodiesterase that is enzymatically active (Thomason et al, 1998). According to such a scheme *rdeA* mutants should possess the unphosphorylated form of REGA and should therefore lack the REGA cAMP-PDE activity. The *regA* cells should have no REGA cAMP-PDE activity regardless of the state of phosphorylation of the response regulator if the knock-out insertion is in the cAMP-PDE domain. I have indeed proven that *regA* cells do not have any REGA cAMP-PDE activity at any time from the vegetative stage throughout development (see chapter 6).

The idea of a phosphorelay that activates REGA is further supported by the finding that the mammalian cAMP-phosphodiesterase inhibitor IBMX, shown to inhibit the REGA cAMP-phosphodiesterase (Thomason et al., 1998), permits detection of a similar activity in lysates of vegetative *aca-* cells. It should be noted that the novel adenylyl cyclase activity was also detected in an *aca-/acg-* double mutant by adding IBMX (Kim et al., 1998). The fact that the novel AC activity was detected either by disruption of the *rdeA* gene or by inhibiting the REGA cAMP-phosphodiesterase supports the idea that the RDEA protein activates the REGA cAMP-

phosphodiesterase in a multi-step phosphorelay.

4.32 ACB may provide intracellular cAMP to activate PKA.

In early development

ACB was shown to be active at the vegetative stage at which neither ACA nor ACG is expressed in *aca-/rdeA* cells (Figure 3.9 and 4.1). Since the regulatory subunit of PKA has very high affinity for cAMP ($K_d = 3$ nM; de Gunzburg, 1984) minute amount of cAMP produced by ACB in the absence of ACA and ACG may be enough to activate PKA. Hence it is possible to attribute PKA dependent gene expression early in development to the novel adenylyl cyclase. The untranslatable, RNA polymerase II-dependent gene (*dutA*) of *Dictyostelium* is expressed in early development and its expression is completely dependent on PKA activity (Kumimoto et al., 1996). Expression of Discoidin I, one of the preaggregative genes, is also absolutely dependent on cAMP-dependent protein kinase activity (Schulkes and Schaap, 1995). Despite this the expression of these two genes is normal in *aca-* cells (Kumimoto et al., 1996). A possible explanation is that PKA is activated by the cAMP produced by ACB (in the absence of ACA) in early development. Other explanations have also been proposed for the presence of PKA catalytic activity in the absence of ACA early in development. For instance PKA regulatory subunits may be expressed a little later than PKA catalytic subunits so that free PKA catalytic subunits are present regardless of cAMP availability.

In culmination

The maximal activity of ACB at the onset of starvation in *aca-/rdeA* cells (Figure 4.1)

does not provide any idea of the developmental regulation of ACB during normal development, as mentioned earlier. According to a recent report, ACB activity goes up substantially at culmination (Dr. Pauline Schaap, personal communication). This encourages the idea that ACB could provide the intracellular cAMP to activate PKA during the whole of development. The *rdeA* gene was shown to be expressed at the mRNA level from the vegetative stage until 16-18 hours after onset of development (Chang et al., 1998) while *regA* was shown to be expressed throughout development (Shaulsky et al., 1996). It can be speculated that accumulation of cAMP resulting from ACB activity remains low in the wild type cells until just before culmination due to active REGA phosphodiesterase phosphorylated by RDEA. Around culmination the REGA phosphodiesterase may become inactive because of some kind of inhibition of the RDEA phosphotransferase. Large amount of cAMP would then be produced by ACB and could induce high enough PKA activity for *Dictyostelium* to make fruiting bodies.

4.33 What if ACB mediates ammonia depletion leading to culmination?

This question was briefly addressed in section 4.215 “pH dependence of ACB activity” where it was concluded that the pH profile of ACB (Figure 4.6) did not help one to understand how ammonia depletion could increase PKA activity if the mechanism involved an effect of intracellular pH on ACB. However there might be another way to understand this. It has been reported that Ca^{2+} is sequestered in a subpopulation of acidic vesicles driven by a proton gradient such that alkalisation would dissipate the gradient and increase cytosolic Ca^{2+} levels (Gross et al., 1988 ;

Rooney and Gross, 1992). If ACB activity is inhibited by cytosolic Ca^{2+} , this might explain how PKA is activated upon ammonia depletion: when ammonia is present, cytosolic Ca^{2+} levels are elevated and ACB activity is accordingly inhibited and PKA activity is not high enough to induce culmination because of insufficient cAMP. Once ammonia is depleted, cytosolic Ca^{2+} levels drop, ACB becomes active and culmination occurs due to high PKA activity induced by active ACB. It should be very interesting to test this idea in future.

Chapter 5

Chapter 5

Testing possible interaction between the RDEA and the REGA proteins using the yeast two-hybrid system

5.1 Introduction

5.11 Why should the phosphorelay model be tested by the yeast two-hybrid system?

If RDEA is the upstream activator of REGA in a multi-step phosphorelay system it is possible that the RDEA and the REGA proteins form a reasonably stable complex while a phosphate is transferred between them. In order to get further evidence to support the phosphorelay model, it was decided to test for a potential protein interaction between RDEA and REGA using the yeast two-hybrid system. Why was the yeast two-hybrid system thought to be a sensible way to test for a protein interaction that is probably only maintained during phosphotransfer? RDEA shows good homology with YPD1 around the site of histidine phosphorylation in Figure 1.2 B, and yeast YPD1 was shown to provide the biological function of RDEA in *Dictyostelium* when the YPD1 was expressed in an *rdeA* knock-out strain (Chang et al., 1998). The response regulator domain of REGA shows good homology with that of yeast SSK1 in Figure 1.3 B. Even though it is not certain yet if the histidine kinase responsible for phosphorylating RDEA has been found, the putative multi-step phosphorelay in *Dictyostelium* appeared to be similar to that of *S.cerevisiae* (See Figure 1.4). The latter has been proven by *in vitro* phosphate transfer and by *in vivo* demonstration of interaction between SLN1 and YPD1 and between YPD1 and SSK1

using the yeast two hybrid system (Posas et al., 1996). Given this successful outcome of the yeast two-hybrid test, it seemed worthwhile to use it to test a potential interaction between RDEA and REGA.

5.12 The yeast two hybrid system

Protein-protein interactions are involved in many important biological functions such as transcriptional regulation, receptor mediated signal transduction and antigen-antibody recognition. They have been studied by biochemical methods for instance, co-immunoprecipitation, crosslinking or co-fractionation by chromatography (Paterson et al., 1997 ; Kolosha and Martin, 1997 ; Ray and Prefontaine, 1994 ; Sollner et al., 1992). A genetical technique was developed based on the *S. cerevisiae* GAL4 transcriptional activator (Fields and Song, 1989). GAL4 is one of the proteins taking part in galactose metabolism in *S. cerevisiae* and it has a DNA binding domain (1-147) and two transcriptional activation domains (148-196 and 768-881) as shown in Figure 5.1 B.2. In wild-type yeast cells GAL 80, a negative regulator of GAL4, is bound to GAL4 preventing it from binding to the UAS (upstream activation sequence) in the absence of galactose as carbon source. When galactose is available, GAL 80 is detached from GAL4 and the DNA binding domain of GAL4 binds to the UAS. The transcriptional activation domain of GAL4 allows GAL1 to be transcribed and galactose can then be metabolised to glucose to be used for ATP production (Darnell et al ; see also Figure 5.1 A). The yeast two-hybrid system utilises the independent functioning of the DNA binding and the transcriptional activation domains of GAL4 i.e. each domain retains its function if expressed separately. Only if the transcriptional activation domain can be brought physically near to the DNA

binding domain can the reconstituted GAL4 function as a transcription activator.

Figure 5.1 B.1 shows how the properties of GAL4 can be used to test a possible interaction between two known proteins. (Both proteins do not necessarily need to be known, as will be discussed later.) Two proteins expected to interact can be fused, one (X) to the DNA binding domain and the other (Y) to the transcriptional activation domain. When those two fusion proteins are expressed in the Y190 strain (see Figure 5.1 legend for its genotype) especially made for two-hybrid screening (Harper et al., 1993 ; Flick and Johnston, 1990), the GAL4 DNA binding domain can be brought close to the GAL4 transcriptional activation domain if the two proteins interact in *S. cerevisiae*. The outcome can be monitored by seeing whether “reporters” such as His⁺ and lacZ⁺ are expressed depending on the presence or absence of GAL4 transcriptional activation activity (Fields and Song, 1989 ; Chien et al., 1991 ; Fields and Sternglanz, 1994).

The two-hybrid system is not only used to test a possible interaction between two specific proteins but also to screen protein candidates potentially interacting with an interesting protein. Since many transcriptional factors or signalling molecules work in complexes, the two-hybrid screen has played an important role in revealing new components interacting with proteins of interest. For instance p21 Cdk-Interacting Protein Cip1 was found to interact with the cyclin-dependent kinase Cdk2 (Harper et al., 1993) and the protein phosphatase type 1 catalytic subunit PP-1 α 2 to interact with the retinoblastoma protein p110^{RB} (Durfee et al., 1993). In my experiment the two-hybrid system was used to test a possible interaction between two known proteins, RDEA and REGA.

Figure 5.1 Diagrams describing the yeast two-hybrid system.

A Galactose metabolism in wild type yeast cells.

When galactose is available, Gal80, a negative regulator of Gal4 becomes dissociated from Gal4 transcriptional regulator. Then Gal4 regains its function as a transcriptional activator and makes the *gal1* gene transcribed. This gene product is involved in metabolising galactose to glucose.

UAS; Upstream Activation Sequence

B.1 A schematic diagram for the two-hybrid system.

One protein, X is expressed in frame fusion to Gal4 DNA binding domain (vertical stripes in red). The other protein, Y is expressed in frame fusion to Gal4 transcriptional activation domain (horizontal stripes in blue with a black star at centre). When the two proteins, X and Y interact with each other, the Gal4 transcriptional activation domain can approach very close to the Gal4 DNA binding domain. Under this condition the reconstituted form of Gal4 transcriptional activator can induce transcription of the reporter genes such as His and LacZ.

UAS; Upstream Activation Sequence

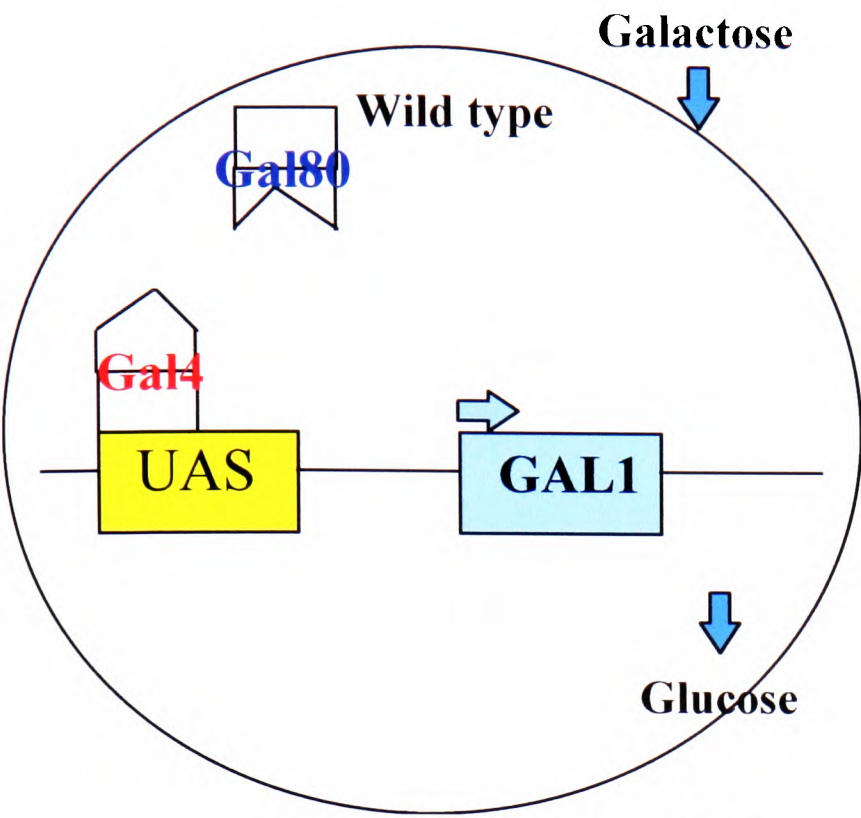
Genotype of the Y190 strain;

MATa, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3, 112*, *gal4Δ*, *gal80Δ*, *cyh^r2*, *LYS2::GAL1_{UAS}-HIS3_{TATA}-HIS3*, *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*
(Harper et al., 1993)

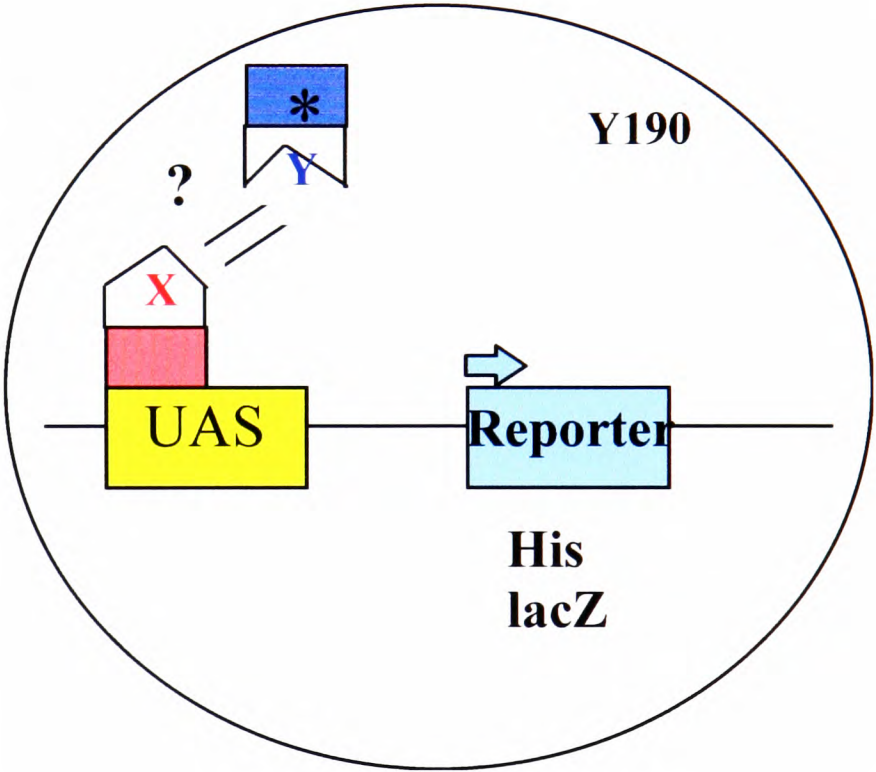
B.2 A primary structure of native Gal4 protein

The intact gene encodes a DNA binding domain (vertical stripes in red) and two transcriptional activation domains (horizontal stripes in blue). The second transcriptional activation domain has higher activity than the first one and the second part (marked with a black star) is used in the two-hybrid system.

A

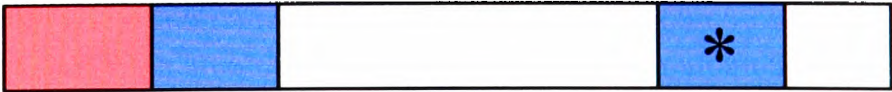


B.1



B.2

GAL4 ;



5.2 Results

5.21 Molecular cloning in order to express 4 fusion proteins.

If the RDEA and the REGA protein interact, the interaction may be very transient, occurring only during phosphotransfer, and the region involved would presumably be confined to the N terminal half of RDEA (1-130, Figure 1.2 A) and the response regulator region of REGA (155-432, Figure 1.3 A). It might have been a good idea to use these restricted regions of the two proteins to test for interaction but it was decided that full length proteins should be expressed as a first step. If the first test were positive, then the specific regions involved could be defined thereafter. There was a worry that the natural folding of the proteins might be distorted by deletion, though this seemed rather unlikely.

The pAS2 and pACT2 vectors and the Y190 yeast strain were kindly given by Adam Kuspa (Baylor College of Medicine, Houston). The pAS2 plasmid (Harper et al., 1993) was developed from the pAS1 plasmid (Durfee et al., 1993) with an inserted CYH2 (Figure 5.2) and pAS2 is equivalent to pAS1-CYH2.

pAS2-rdeA was generated by ligating the RDEA coding region as a NcoR1-BamH1 fragment into the multiple cloning site immediately after GAL4 (1-147) in pAS2. The ligated plasmid was transformed into competent *E.coli* cells, amplified and concentrated to 1 µg/µl as described in section 2.421: “The construction of rdeA-pAS2”. pAS2-regA was generated by ligating the REGA coding region as an EcoR1-Sal1 (equivalent to Xho1) fragment into the multiple cloning site immediately after

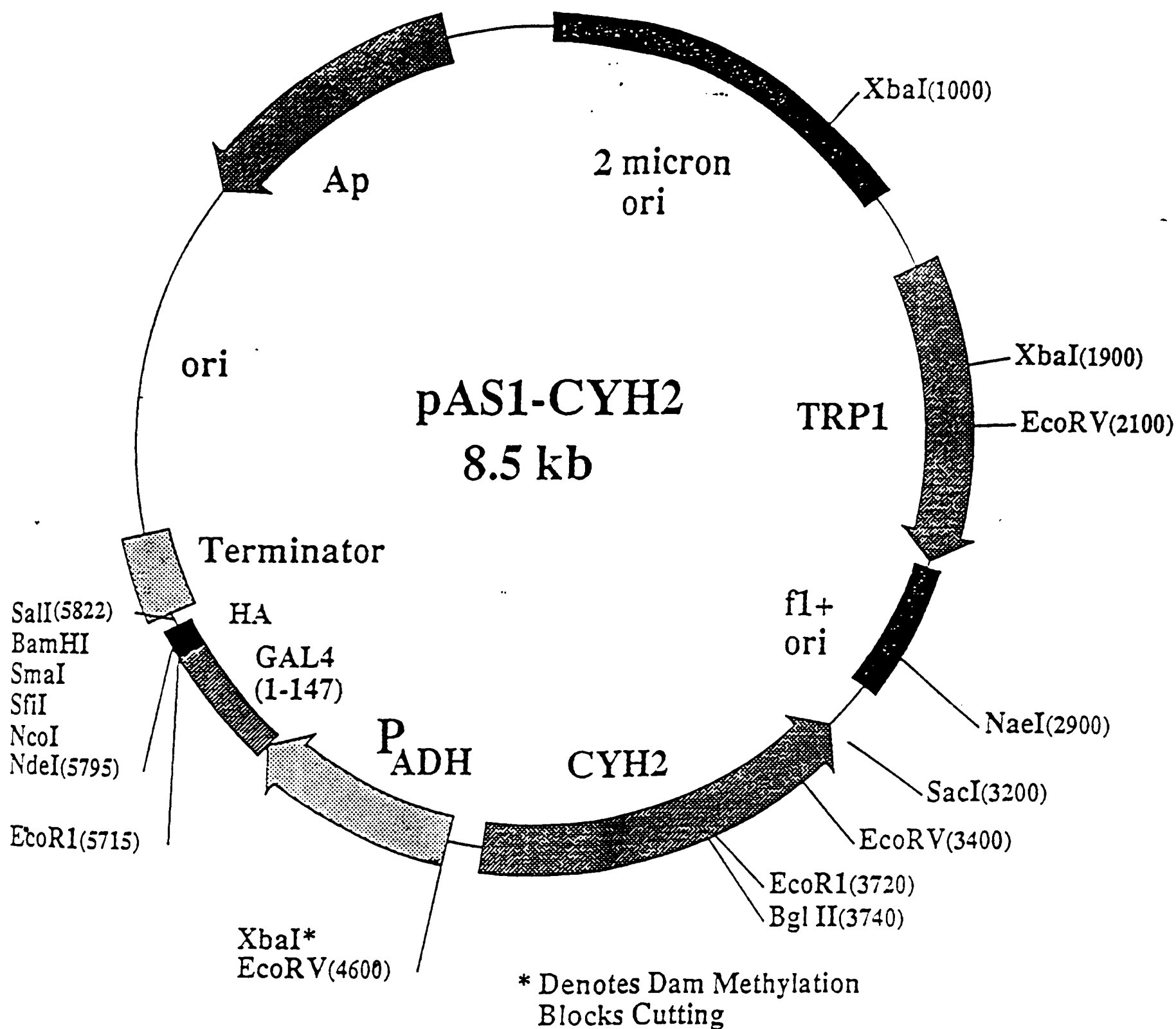
GAL4 (1-147) in pAS2. The ligated plasmid was transformed into competent *E.coli* cells, amplified and concentrated to 1 µg/µl as described in section 2.424: “The construction of regA- pAS2”.

pACT2 (GeneBank accession number U29899) has a GAL4 activation domain connected to a multiple cloning site as shown in Figure 5.3. pACT2-regA was generated by ligating the coding region for REGA as an EcoR1-Xho1 fragment into the multiple cloning site. The ligated plasmid was transformed into competent *E.coli* cells, amplified and concentrated to 1 µg/µl as described in 2.422: “The construction of regA- pACT2”. Similarly pACT2-rdeA was generated by ligating the RDEA coding region as a NcoR1-BamH1 fragment into pACT2. The ligated plasmid was transformed into competent *E.coli* cells, amplified and concentrated to 1 µg/µl as described in 2.423: “The construction of rdeA- pACT2”.

Why should the 2-hybrid analysis be performed as two independent tests?

Altogether 4 plasmids were constructed for two test sets. Test set 1 was supposed to detect an interaction between the RDEA protein fused to the GAL4 DNA binding domain (RDEA:DNA BD) and the REGA protein fused to the GAL4 Transcription Activation Domain (REGA:Tx AD). Test set 2 was to detect an interaction between the RDEA protein fused to the GAL4 Transcription Activation Domain (RDEA:Tx AD) and the REGA protein fused to the GAL4 DNA Binding Domain (REGA:DNA BD).

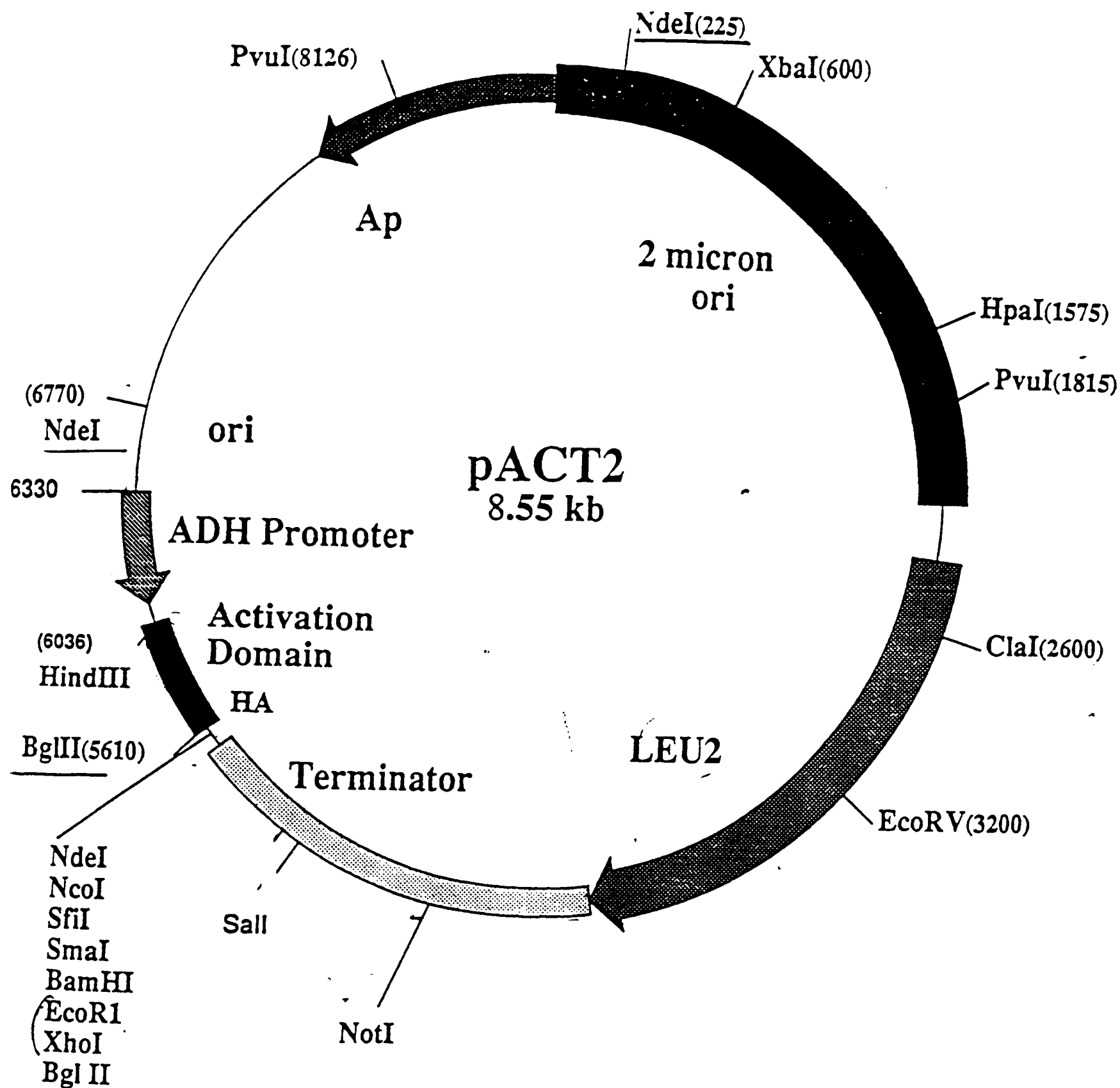
It is possible that when the fusion proteins are expressed in Y190, the RDEA and REGA proteins could fold incorrectly because of having been fused to other



CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA C
NdeI NcoI SmaI BamHI SalI
Sfi I

Figure 5.2 A plasmid map of pAS1-CYH2 (=pAS2)

The above map is quoted from Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805-816



CAT ATG GCC ATG GAG GCC CCG GGG ATC CGA ATT CGA GCT CGA GAG ATC T
 NdeI NcoI SfiI SmaI BamHI EcoRI XhoI BglII

Figure 5.3 A plasmid map of pACT2

GeneBank accession number U29899. A previous version, pACT was published in Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes and Dev.* 7:555-569

fragments such as DNA BD and Tx AD. DNA BD is 147 amino acid long and Tx AD is 113 amino acid long. The two domain themselves might well have different structures and each domain could well change the folding of the RDEA and the REGA proteins differently. If the folding process went wrong, there would be no way to see this unless the structure of each protein was examined by x-ray crystallography or by NMR. As this is impractical, it is evident that performing the reciprocal tests should provide more chance of obtaining correctly folded fusion proteins than doing only one test. This experimental tactic reflects one aspect of the many reasons there can be for a negative outcome.

Critical steps in yeast transformation.

For successful yeast transformation, freshly prepared competent cells were made for transformations (as described in section 2.431 “Preparation of competent yeast cells”) and all plasmids were transformed at 1 µg/µl to get generous yields of transformants and reproducible results.

5.22 Test set 1

5.221 Colony-lift filter assays for Test set 1

Principles of test set 1

Test set 1 was performed to see if RDEA:DNA BD could interact with REGA:Tx AD (Figure 5.4). Numerous control experiments were included to be certain that blue transformants could be interpreted as indicators of a real interaction between RDEA and REGA (Table 5.1). The pAS2-rdeA plasmid has TRP1 as a selective marker and

the pACT2-regA plasmid has LEU as a selective marker. The host strain Y190 carries mutations trp1-901, leu2-3 and his3-200 as shown in the legend to Figure 5.1.

Histidine was always added to allow colonies to be formed. The his deletion was present to permit double selection to reduce the number of false positive colonies in the two-hybrid screening. When both plasmids shown in Figure 5.4 A were transformed into Y190, the transformants could grow on SD plate supplemented with all amino acids listed in “10X dropout of 2.41 Media for yeast growth” except for Trp and Leu. If RDEA interacts with REGA, the GAL4 transcriptional activation domain can be brought near to the GAL4 DNA binding domain. In doing so the GAL4 transcriptional activator will regain its activity and allow His and lacZ to be transcribed (Figure 5.4 B).

Interpretation of the results of the test set 1

Table 5.1 shows the results of the Test set 1 and clearly indicates that no interaction between RDEA and REGA was detected. When the pAS2 plasmid alone was transformed into Y190, transformants had to be plated on SD (Synthetic Dropout; minimal medium without any amino acid) supplemented with all amino acids except Trp (described as -Trp in Table 5.1) for selection. When the pACT2 plasmid alone was transformed into Y190, transformants had to be plated onto SD supplemented with all amino acids except Leu (described as -Leu in Table 5.1) for selection.

As a most basic control, nothing but carrier DNA was added and no colonies formed on any of the three dishes, -Leu, -Trp, -Leu and -Trp. Carrier DNA (single stranded DNA) only helps plasmid DNA to enter yeast cells. pCL1 carrying the native GAL4 coding sequence was transformed in as a positive control and the good sized (2-3mm)

colonies formed all turned blue after colony-lift filter assay (see section 2.45 “Colony-lift filter assay”).

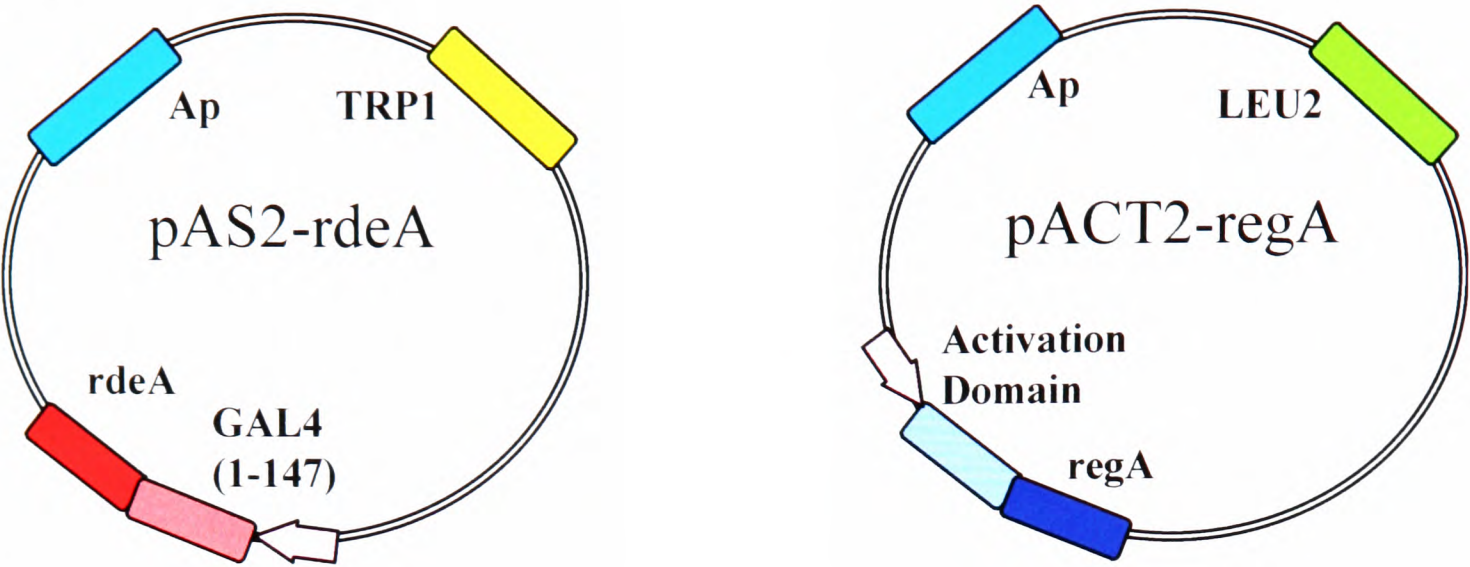
To check whether any endogenous transcriptional factor interacted with the GAL4 DNA binding domain or if the GAL4 transcriptional activation domain somehow managed to bind to UAS, pAS2 vector alone, pACT2 vector alone and the combination of the two vectors were transformed into Y190. All colonies formed turned white as shown between the 5th line and 7th line of Table 5.1.

The fusion proteins had also to be checked for self-induced GAL4 activity. Whether the RDEA domain of RDEA:DNA BD interacted with any endogenous transcription factor or if the REGA domain of REGA:Tx AD bound to the UAS was also checked by transforming the pAS2/rdeA plasmid alone and the pACT2/regA plasmid alone into Y 190 . All colonies turned white as shown in the 8th and 9th lines of Table 5.1.

Another level of self-induced GAL4 activity was tested to deal with the following possibility. If the RDEA domain of RDEA:DNA BD interacted with the GAL4 transcriptional activation domain or the REGA domain of REGA:Tx AD interacted with the GAL4 DNA binding domain, the system could not be used to test an interaction between RDEA and REGA. The 11th and 12th lines of Table 5.1 shows that there was no interaction to prevent the system being used.

All control experiments proved that if transformants expressing RDEA:DNA BD and REGA:TxAD had turned blue, the result could be accepted as evidence for direct interaction between RDEA and REGA. The truth however was that all the colonies were white even when the experiment was repeated. Test set 1 appears to show that an interaction can not be proven by means of the yeast two-hybrid system.

A



B

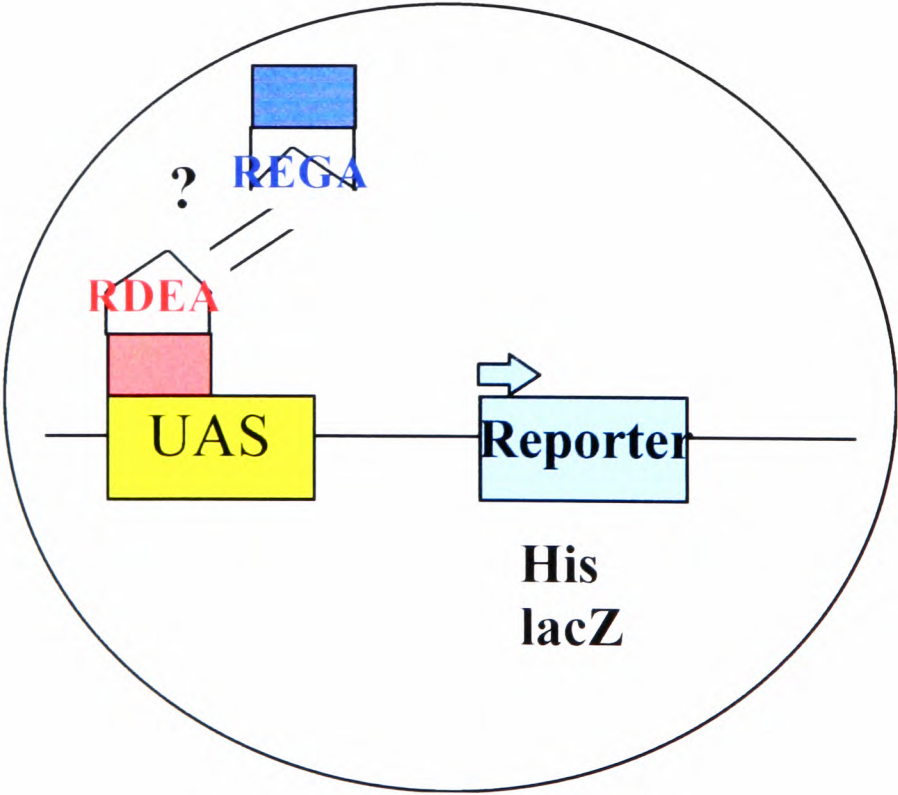


Figure 5.4 A basic scheme of test set I

A. The two plasmids to be transformed into the Y190. pAS2-rdeA plasmid has an rdeA gene immediately under the GAL4 DNA Binding Domain (GAL4 DB) and pACT2-regA plasmid has an regA gene immediately under the GAL4 transcription Activation Domain (GAL4 AD). They contain TRP1 and LEU2 as a selective marker respectively and ampicillin resistance gene for DNA manipulation. Pink arrow represents a promoter region for the fusion proteins.

B. A simple diagram of test set I. Testing possible interaction between RDEA-GAL4 DB and REGA-GAL4 AD.

Plasmids transformed	Proteins expressed in Y190	Plates used for selection	Results of colony lift assay
1.None	Nothing but carrier DNA	- Leu	No colony
2.None	Nothing but carrier DNA	- Trp	No colony
3.None	Nothing but carrier DNA	- Leu - Trp	No colony
4.pCL1	native gal	- Leu	Blue
5.pAS2	DNA BD	- Trp	White
6.pACT2	Tx AD	- Leu	White
7.pAS2 + pACT2	DNA BD & Tx AD	- Leu - Trp	White
8.pAS2/rdeA	RDEA:DNA BD	- Trp	White
9.pACT2/regA	REGA:Tx AD	- Leu	White
10. pACT2/regA-PDE	REGA PDE:Tx AD	- Leu	White
11.pAS2/rdeA +pACT2	RDEA:DNA BD & Tx AD	- Leu - Trp	White
12.pAS2 + pACT2/regA	DNA BD & REGA:Tx AD	- Leu - Trp	White
13.pAS2 + pACT2/regA-PDE	DNA BD REGA & PDE:Tx AD	- Leu - Trp	White
14.pAS2/rdeA + pACT2/regA	RDEA:DNA BD & REGA:Tx AD	- Leu - Trp	White
15.pAS2/rdeA + pACT2/regA-PDE	RDEA:DNA BD & REGA PDE:Tx AD	- Leu - Trp	White

Table 5.1 Results of colony-lift filter assays for test set I.
Abbreviations:
DNA BD; the GAL4 DNA Binding Domain,
Tx AD; the GAL4 Transcriptional Activation Domain.
Test set I was repeated twice.

Apart from the main test the possibility of an interaction between RDEA and the cAMP-phosphodiesterase domain of REGA (REGA PDE) was also tested. There turned out to be no interaction between them (See 10th, 13th and 15th lines of Table 5.1). This negative result would have supported the phosphorelay model if an interaction between RDEA and REGA had been demonstrated, as the two proteins did not interact when the response regulator of REGA was absent. But this control was no longer necessary.

5.222 Western Blotting analysis of the Test set 1

Before coming to a firm conclusion about the absence of an interaction between RDEA and REGA, it was necessary to check that the fusion proteins (RDEA:DNA BD and REGA:Tx AD) were fully expressed. Therefore Western blotting was carried out with appropriate controls. Transformants carrying the pACT2 vector alone, the pACT2-regA plasmid alone and pACT2-regA as well as pAS2-rdeA were inoculated into SD-Leu, SD-Leu and SD-Leu-Trp media respectively. Similarly transformants with pAS2 vector alone, pAS2-rdeA plasmid alone and pAS2-rdeA as well as pACT2-regA were inoculated into SD-Trp, SD-Trp and SD-Leu-Trp media respectively. Protein extracts were prepared as described in section 2.463 “Procedures” and loaded on SDS PAGE gels after being boiled.

The in-frame fusion of REGA to the GAL4 transcriptional activation domain

Figure 5.6 A (upper panel) tests if the fusion protein, REGA:Tx AD had been expressed in the double transformant containing both pAS2/rdeA and pACT2/regA

plasmids. Anti-monoclonal GAL4 transcriptional activation domain antibody raised in rabbit was used as a primary antibody. Anti-polyclonal rabbit antibody was used as a secondary antibody. The REGA protein is 793 amino acid long and the GAL4 transcriptional activation domain is 113 amino acid long. The expected molecular weight of the REGA: TX AD was 99,660 Da (average molecular weight of an amino acid is about 110 Da.) and that of the GAL4 transcriptional activation domain itself was 12,430 Da.

A band of about 100 kDa was detected in the second lane as well as the third lane, which meant that REGA:Tx AD was expressed both in the single transformant with pACT2/regA alone and in the double transformant with pAS2/rdeA and pACT2/regA. However a band of roughly 12 kDa could not be detected in the first lane where protein extract from the transformant with pACT2 alone was loaded. It would have been better to use a higher percentage of SDS gel especially for this small band. However 10 % SDS gel had to be used for detecting the expected band of 100 kDa of interest.

The in-frame fusion of RDEA to the GAL4 DNA binding domain

Figure 5.6 B (lower panel) tested if the fusion protein, RDEA: DNA BD had been expressed in the double transformant containing pAS2/rdeA and pACT2/regA plasmids. Anti-monoclonal GAL4 DNA binding domain antibody raised in rabbit was used as primary antibody and anti-polyclonal rabbit antibody was used as secondary antibody. The RDEA protein is 254 amino acid long and the GAL4 DNA binding domain is 147 amino acid long. The expected molecular weight of the RDEA: DNA BD was 44,110 Da and that of the GAL4 DNA binding domain itself was 16,170 Da.

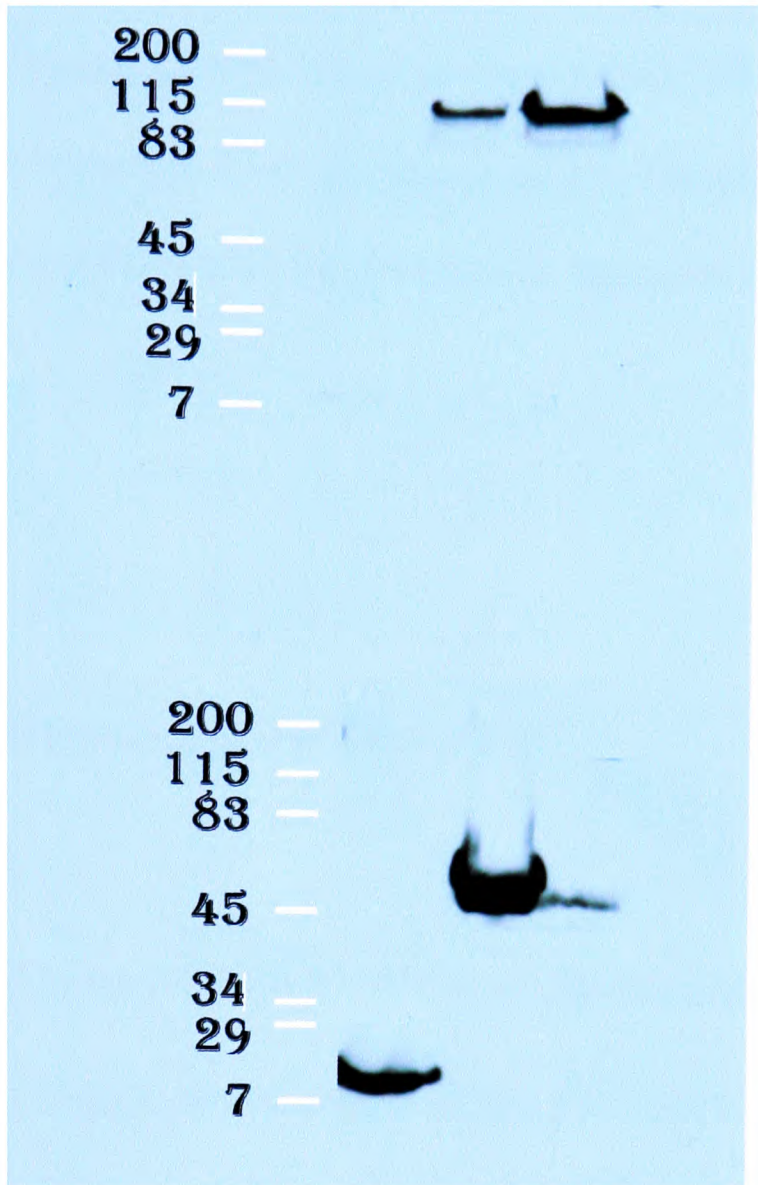
Figure 5.6 Western Blotting of Test set I

A. Protein extracts from transformants with the pACT2 vector alone (1st lane), the pACT2/regA plasmid alone (2nd lane) and both pACT2/regA and pAS2/rdeA plasmids (3rd lane) were loaded and run on a 10 % SDS gel. Anti Gal4 transcriptional activation domain monoclonal antibody was used as primary Ab.

B. Protein extracts from transformants with the pAS2 vector alone (1st lane), pAS2/rdeA plasmid alone (2nd lane) and both pAS2/rdeA and pACT2/regA plasmids (3rd lane) were loaded and run on a 10% SDS gel. Anti Gal4 DNA binding domain monoclonal antibody was used as primary Ab.

A

**Marker pACT2 pACT2 pACT2/regA &
/regA pAS2/rdeA**



B

**Marker pAS2 pAS2/ pAS2/rdeA &
rdeA pACT2/regA**

A band of approximately 16 kDa was detected in the first lane where protein extract from the transformant with pAS2 vector alone was loaded. A 45 kDa band was detected in the second as well as the third lanes, which meant that RDEA: DNA BD was expressed both in the single transformant with pAS2/rdeA alone and in the double transformant with pAS2/rdeA and pACT2/regA. Thus according to the result of Western blotting in Figure 5.6 both fusion proteins, RDEA: DNA BD and REGA:Tx AD were expressed in the double transformant. Therefore the failure to detect interaction between RDEA and REGA proteins was not due to the absence of the fusion proteins.

5.23 Test set 2

5.231 Colony-lift filter assays for Test set 2

Principles of test set 2

Test set 2 was designed to see if RDEA:Tx AD could interact with REGA:DNA BD (Figure 5.5). Numerous control experiments were also included as in Test set 1 to be certain that blue transformants could be interpreted as indicating real interaction between RDEA and REGA (Table 5.2). Both plasmids shown in Figure 5.5 A were transformed into Y190, and transformants harbouring the two plasmids, pAS2/regA and pACT2/rdeA were selected. If REGA interacts with RDEA the GAL4 transcriptional activator will regain its activity and allow reporter genes, His and lacZ to be transcribed (Figure 5.5 B).

Interpretation of the results of the test set 2

Table 5.2 shows the results of Test set 2 and again clearly indicates that no interaction between RDEA and REGA was detected. The basic negative and positive controls all worked as before and the check whether any endogenous transcriptional factor interacted with the GAL4 DNA binding domain or if the GAL4 transcriptional activation domain somehow managed to bind to UAS also gave satisfactory results. (5th and 7th lines of Table 5.2.)

The fusion proteins were also checked for self-induced GAL4 activity as well as whether the REGA domain of REGA: DNA BD interacted with any endogenous transcription factor or the RDEA domain of RDEA:Tx AD bound to the UAS. All colonies turned white as shown in the 8th and 9th lines of Table 5.2.

Another level of self-induced GAL4 activity was tested to deal with the following possibility. If the REGA domain of REGA: DNA BD interacted with the GAL4 transcriptional activation domain or the RDEA domain of RDEA:Tx AD interacted with the GAL4 DNA binding domain, the system could not be used to test an interaction between RDEA and REGA. The 10th and 11th lines of Table 5.2 shows that there was no interaction to prevent the system from being used.

All control experiments thus showed that if transformants expressing REGA: DNA BD and RDEA:TxAD turned blue, the result could be accepted as evidence for direct interaction between RDEA and REGA. Unfortunately all the colonies were white and test set 2 also appears to show that an interaction cannot be detected by the yeast two-hybrid system.

5.232 Western Blotting analysis of the Test set 2

Before coming to a firm conclusion about no interaction between RDEA and REGA,

it had to be determined whether the fusion proteins (REGA: DNA BD and RDEA:Tx AD) were fully expressed. Therefore Western blotting was carried out with appropriate controls. Transformants carrying the pACT2 vector alone, the pACT2-rdeA plasmid alone and pACT2-rdeA as well as pAS2-regA were inoculated into SD-Leu, SD-Leu and SD-Leu-Trp media respectively. Transformants with pAS2 vector alone, pAS2-regA plasmid alone and pAS2-regA as well as pACT2-rdeA were inoculated into SD-Trp, SD-Trp and SD-Leu-Trp media respectively. Protein extracts were prepared as described in section 2.463 “Procedures” and loaded on SDS PAGE gel after being boiled.

The in-frame fusion of RDEA to the GAL4 transcriptional activation domain

Figure 5.7 A (upper panel) tested if the fusion protein RDEA:Tx AD had been expressed in the double transformant containing both pAS2/regA and pACT2/rdeA plasmids. Anti-monoclonal GAL4 transcriptional activation domain antibody raised in rabbit was used as a primary antibody. Anti-polyclonal rabbit antibody was used as a secondary antibody. The expected molecular weight of the RDEA:Tx AD was 40,307 Da and that of the GAL4 transcriptional activation domain itself was 12,430 Da.

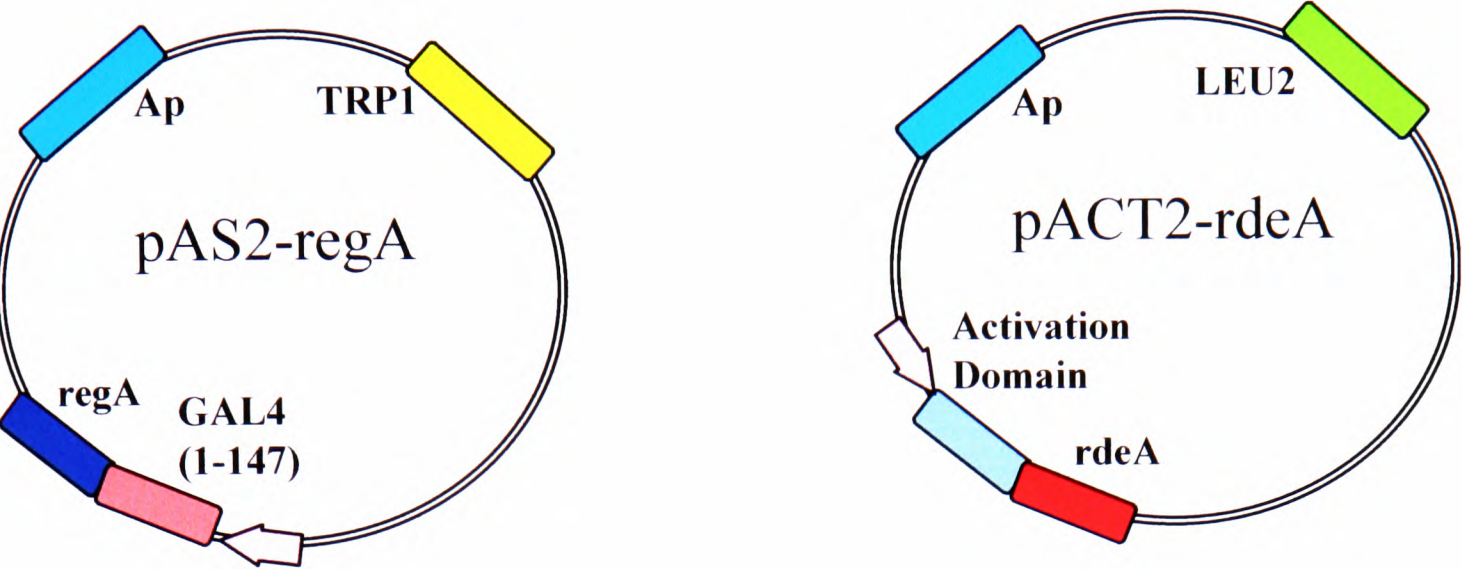
A band of about 40 kDa was detected in the second lane as well as the third lane, which meant that RDEA:Tx AD was expressed both in the single transformant with pACT2/rdeA alone and in the double transformant with pAS2/regA and pACT2/rdeA. However a band of roughly 12 kDa could not be detected in the first lane where protein extract from the transformant with pACT2 alone was loaded. As in the previous case the band of 12 kDa might have run out of 10 % SDS protein gel.

Figure 5.5 A basic scheme of test set II

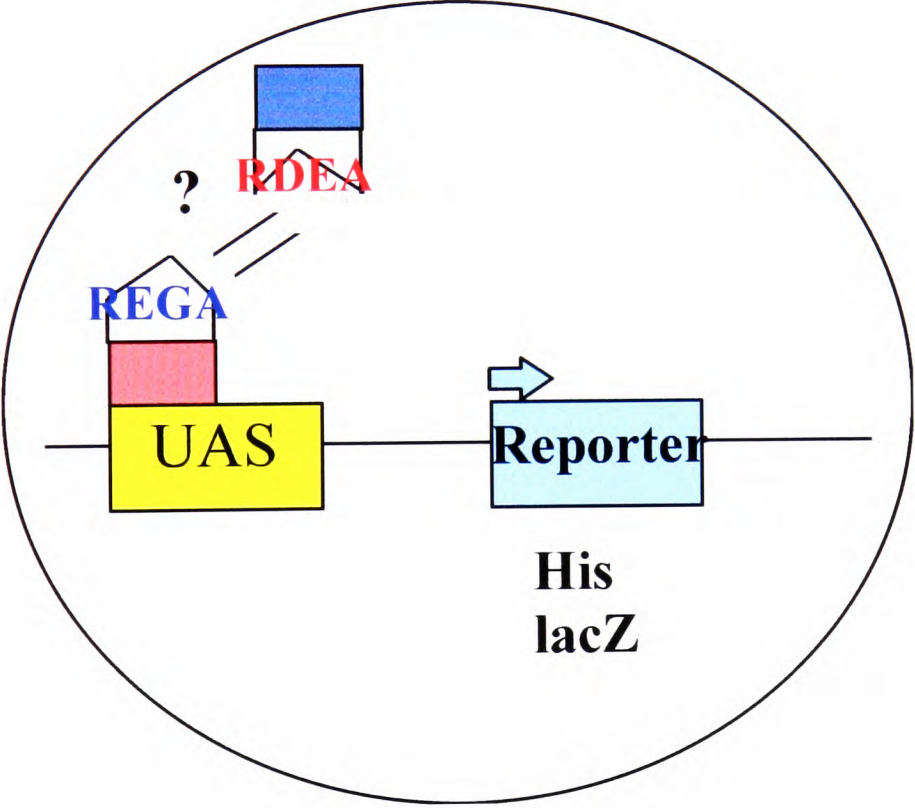
A. The two plasmids to be transformed into the Y190. pAS2-regA plasmid has an regA gene immediately under the GAL4 DNA Binding Domain (GAL4 DB) and pACT2-rdeA plasmid has an rdeA gene immediately under the GAL4 transcriptional Activation Domain (GAL4 AD). They contain TRP1 and LEU2 as a selective marker respectively and ampicillin resistance gene for DNA manipulation. Pink arrow represents a promoter region for the fusion proteins.

B. A simple diagram of test set II. Testing possible interaction between REGA-GAL4 DB and RDEA-GAL4 AD.

A



B



Plasmids transformed	Proteins expressed in Y190	Plates used for selection	Results of colony lift assay
1.None	Nothing but carrier DNA	- Leu	No colony
2. None	Nothing but carrier DNA	- Trp	No colony
3.None	Nothing but carrier DNA	- Leu - Trp	No colony
4.pCL1	native gal	- Leu	Blue
5.pAS2	DNA BD	- Trp	White
6.pACT2	Tx AD	- Leu	White
7.pAS2 + pACT2	DNA BD & Tx AD	- Leu - Trp	White
8.pAS2/regA	REGA: DNA BD	- Trp	White
9.pACT2/rdeA	RDEA: Tx AD	- Leu	White
10.pAS2/regA + pACT2	REGA: DNA BD & Tx AD	- Leu - Trp	White
11.pAS2 + pACT2/rdeA	DNA BD & RDEA: Tx AD	- Leu - Trp	White
12.pAS2/regA + pACT2/rdeA	REGA: DNA BD & RDEA: Tx AD	- Leu - Trp	White

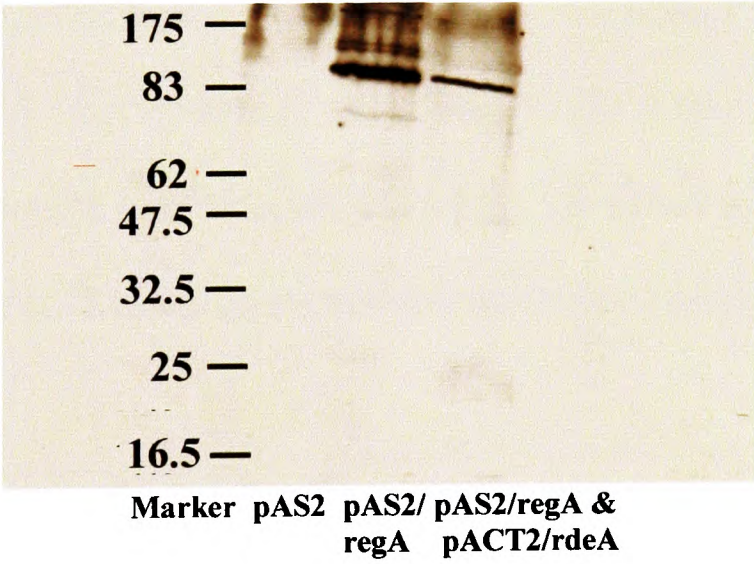
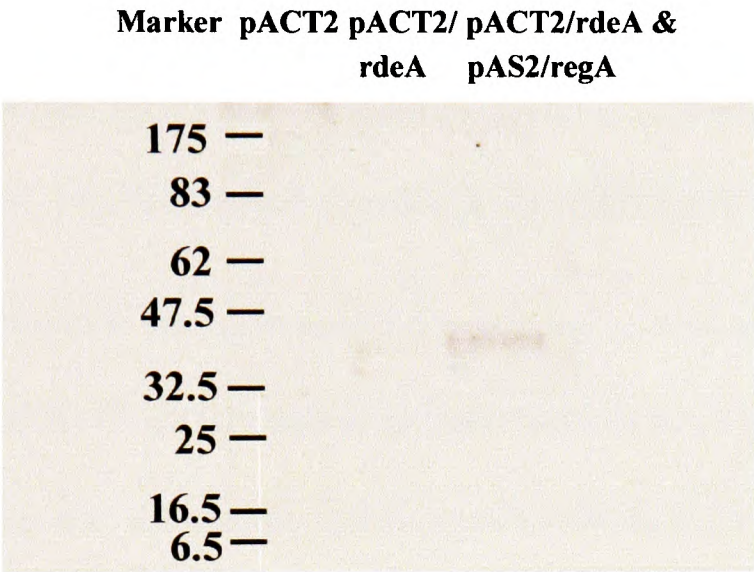
Table 5.2 Results of colony-lift filter assays for test set II.
Abbreviations:
DNA BD; the GAL4 DNA Binding Domain,
Tx AD; the GAL4 Transcriptional Activation Domain

Figure 5.7 Western Blotting of Test set II

A. Protein extracts from transformants with the pACT2 vector alone (1st lane), the pACT2/rdeA plasmid alone (2nd lane) and both pACT2/rdeA and pAS2/regA plasmids (3rd lane) were loaded and run on a 10 % SDS gel. Anti Gal4 transcriptional activation domain monoclonal antibody was used as primary Ab.

B. Protein extracts from transformants with the pAS2 vector alone (1st lane), the pAS2/regA plasmid alone (2nd lane) and both pAS2/regA and pACT2/rdeA plasmids (3rd lane) were loaded and run on a 10 % SDS gel. Anti Gal4 DNA binding domain monoclonal antibody was used as primary Ab.

A



B

The in-frame fusion of REGA to the GAL4 DNA binding domain

Figure 5.7 B (lower panel) tested if the fusion protein, REGA: DNA BD had been expressed in the double transformant containing both pAS2/regA and pACT2/rdeA plasmids. Anti-monoclonal GAL4 DNA binding domain antibody raised in rabbit was used as primary antibody and anti-polyclonal rabbit antibody was used as secondary antibody. The expected molecular weight of the REGA: DNA BD was 103,400 Da and that of the GAL4 DNA binding domain itself was 16,170 Da.

A band of about 100 kDa was detected in the second as well as the third lanes, which meant that the REGA: DNA BD was expressed both in the single transformant with pAS2/regA alone and in the double transformant with pAS2/regA and pACT2/rdeA. A band of 16 kDa could not be detected in the first lane where protein extract from the transformant with the pAS2 alone was loaded. The same reason in the previous section could be applied to this occasion as well. According to the result of Western blotting in Figure 5.7 both fusion proteins, REGA: DNA BD and RDEA:Tx AD were expressed in the double transformant. Therefore, the failure to detect interaction between RDEA and REGA proteins was not due to the absence of the fusion proteins.

5.3 Discussion

5.31 The interaction between the RDEA and the REGA proteins is probably too transient

The osmosensory system of *S. cerevisiae* is quite similar to the one proposed in *Dictyostelium*, as was mentioned at the end of section 5.12 “A possible multi-step phosphorelay system in *Dictyostelium*”. Complementation by the yeast YPD1 protein

of the *rdeA* null mutant phenotype strongly implies that the function of the RDEA protein is specifically devoted to phosphate transfer. The intact RDEA protein (254 amino acids) is twice as long as the YPD1 protein (167 amino acid) (Chang et al., 1998 ; Posas et al., 1996). Secondary structure prediction for RDEA indicates that a 5 or 6 α helix-bundle structure at the N-terminus is connected to a long loop possessing neither α helix nor β sheet. This helix bundle structure is very well conserved in the family of histidine phosphatetransferases (HPt) within the HPt domain. Members of the HPt family may well have different tertiary structures in other parts of the protein and carry other functional groups in addition to the HPt domain. It has been reported that the imidazol group of the histidine residue is usually exposed to solvent (Kato et al., 1997) which makes sense in understanding how phosphates might be transferred between a histidine residue of one protein and an aspartate residue of another protein. The secondary structure of RDEA predicted by PHD, DSC programs also indicated the strong possibility that the histidine residue is accessible from the solvent although the residue itself might be located in a little loop between the α helices, unlike in ArcB. When phosphotransfer occurs between RDEA and REGA, the process could be extremely transient and the small exposed area around the histidine (his 65) might be the only part involved in protein interaction.

Positive aspects of the absence of an interaction proven by the yeast two-hybrid system

The above idea can also explain how yeast YPD1 was able to complement the *rdeA* null mutant. If the interaction between RDEA and REGA had involved a tight and specific complex involving several regions of each protein, it is very unlikely that

YPD1 of *S. cerevisiae*, only about half the size of RDEA, could replace the function of RDEA. The interaction may well be too transient to be detected in the yeast two-hybrid system but sufficient to allow YPD1 to function in the entirely different *Dictyostelium* system. New evidence in favour of the phosphorelay model has been provided in the form of a demonstration of *in vitro* phosphate transfer between RDEA and REGA (P. Thomason, personal communication). The existence of this possible multi-step phosphorelay system in *Dictyostelium* adds weight to the speculation that systems of this kind are utilised in many eukaryotic organisms.

5.32 Upstream regulators of the RDEA phosphotransferase

Candidates for phosphorylation of the RDEA protein

Apart from testing for a possible interaction between the RDEA and the REGA proteins, the yeast two-hybrid system should also be very useful for finding upstream regulators of RDEA. There are a few candidates for the role of histidine kinase in the proposed model, such as dhkA (Wang et al., 1996 ; Loomis et al., 1997) and dokA (Schuster et al., 1996). However neither dhkA null mutants nor dokA null mutants displayed rapid development, unlike rdeA or regA null mutants. In addition neither dhkA- nor dokA- mutants are sporogenous (Wang et al., 1996 ; Schuster et al., 1996). Therefore DHKA and DOKA are unlikely to be upstream regulators of RDEA. Another hybrid histidine kinase, dhkC, was recently identified. Null mutants develop rapidly but form roughly normal fruiting bodies (Singleton et al., 1998). We cannot yet be confident that DHKC is the sensor kinase that would complete the proposed model.

How to identify the real histidine kinase

The basic difficulty in excluding or confirming possible candidates for the histidine kinase in the model comes from having no idea of how many upstream regulators of RDEA could be present. It is possible that there is only one histidine kinase feeding RDEA, as in the phosphorelay system of *S. cerevisiae*, SLN1-YPD1-SSK1. Given that circumstance a kinase knockout mutant should be rapid developing as well as sporogenous. In that case it is likely that the upstream regulator of RDEA protein has not been found yet. If however several histidine kinases exist that phosphorylate RDEA as in the sporulation system of *B. subtilis* (Burbulys et al., 1991) and they can somehow compensate for each other's absence, it is not sufficient to eliminate candidates simply by comparing phenotypes of knock-out mutants. At this point the yeast two-hybrid screening would appear to be a good way of helping to solve the problem.

How can the yeast two-hybrid system be useful to target histidine kinases?

Once the *rdeA* gene had been cloned in a bait vector, pAS2 in fusion with the GAL4 DNA binding domain, the cloned plasmid could be transformed into strain Y190 that had already been transformed with a *Dictyostelium* expression library. After colony-lift filter assays had been performed, plasmids would be rescued from blue colonies. Assuming that upstream regulators of the RDEA should interact the RDEA, we would expect that all possible upstream histidine kinase should be picked up by the two-hybrid screen provided they were represented in the library. It would be very exciting if DHKA, DOKA and DHKC were all detected, perhaps along with some new histidine kinases. But success would not be guaranteed in view of the fact that I

could not detect an interaction between RDEA and REGA with this system. It is possible that the interaction between histidine kinases and RDEA is also transient in which case it would be almost impossible to detect upstream regulators by this approach. The answer should be obtained in the near future.

Chapter 6

Chapter 6

Further insight into the rdeA-regA multi-step phosphorelay

6.1 Introduction

6.11 A current view of how ACB was detected

The new adenylyl cyclase, ACB was detected in vegetative rdeA and regA mutant cells. The same activity was also detected in lysate of vegetative aca- cells in the presence of IBMX, an inhibitor of the REGA cAMP-phosphodiesterase (Chapter 4, Kim et al., 1998). This result was interpreted as follows. The rdeA mutant could not phosphorylate the response regulator of the REGA protein because of the absence of the RDEA protein. The phosphorylated state of the REGA cAMP-PDE was shown to be the enzymatically active state (Thomason et al., 1998) hence the rdeA mutant should contain the inactive form of the REGA cAMP-PDE. If cAMP produced by ACB was hydrolysed by the REGA-PDE in wild-type cells, the cAMP made by ACB should be protected in the rdeA and regA mutants due to the absence of the REGA-PDE activity.

6.12 An assumption underlies the current understanding.

Basically, it was assumed that vegetative wild-type cells should have high enough REGA-PDE activity to destroy essentially all cAMP produced by ACB. And vegetative cells of the rdeA and regA mutants should have sufficiently low REGA-PDE activity to allow the cAMP produced by ACB to be detected. This explanation

also assumes that the REGA-PDE is the only intracellular cAMP-phosphodiesterase or at least the major intracellular phosphodiesterase in *Dictyostelium*. There might be other intracellular phosphodiesterases that are also IBMX sensitive. If the REGA-PDE activity were less than half of the total intracellular PDE activity and any cAMP produced was randomly destroyed by the REGA-PDE or by other intracellular PDEs, it would be incorrect to say that the cAMP produced by ACB was hydrolysed specifically by the REGA-PDE. It appeared to be exciting to open the black box to see if intracellular cAMP-phosphodiesterase activities would turn out to be as expected or not.

6.13 Testing the assumption

I tried to measure total intracellular PDE activity under the condition in which the new adenylyl cyclase activity was detected. Since cAMP accumulation in vegetative *rdeA* lysates was measured in the presence of 10 mM DTT, I measured total PDE activities in lysates prepared in the same way. As I will describe in the next section, considerable intracellular PDE activity was detected in *rdeA* and *regA* cells and the amount of activity was similar to wild-type cells. This result gave a hint of the possible presence of other intracellular PDEs aside from the REGA-PDE. The unexpected intracellular PDE activity made us realise that more attention should be paid to focusing on the specific REGA-PDE activity and immuno-precipitation by anti-*regA* antibody was employed in order to measure this activity. This gave us more convincing evidence for the presence of other intracellular PDEs and pointed to a possible complexity of the proposed *rdeA-regA* multi-step phosphorelay.

The data to be presented in the current chapter could well be used to argue against the proposed model. However if we believe the model on the basis of the many other supporting data, the negative-looking data may provide new insight into the regulation of PKA activity in the form of a possible complex of ACB and REGA.

6.2 Result

6.21 Measurement of total intracellular cAMP-PDE activity

The first step was to see if the *rdeA* mutants have reduced intracellular cAMP-PDE activity. Since ACB was detected at the vegetative stage of AX2/*rdeA* and *aca-/rdeA* cells I measured PDE activity at the same stage. Cells were harvested from log phase, washed once with PB buffer and resuspended in Lysis buffer 1 (for total intracellular PDE assay, Henderson, 1975; 50 mM Tris-HCl, 12.5 mM MgSO₄, 250 mM Sucrose, pH to 7.5) at 10⁸ cells / ml. The cell suspension was lysed either through a nuclepore filter (pore size; 3 µm) or by being frozen in dry ice followed by thawing on crushed ice. The extracellular cAMP-phosphodiesterase of *Dictyostelium* is known to be almost 99 % inhibited by 10 mM DTT (dithiothreitol) (Henderson, 1975). Since I wanted to measure total intracellular PDE activity, the cell lysate was always assayed in the presence of 10 mM DTT with 0.3 µM ³H-cAMP as substrate and alkaline phosphatase. The principle of the PDE assay I used is as follows; PDE converts cAMP to 5' AMP and the phosphate group is then removed by excess alkaline phosphatase. In the assay solution there would be a mixture of cAMP, the substrate, that is negatively charged, and adenosine, the product, which is uncharged. At the end of the assay, dowex, a strong basic anion exchanger is added as an ethanolic slurry. This

stops the reaction and adsorbs unhydrolysed cAMP, quenching its radioactivity. The scintillation counter then detects the radioactivity of the adenosine that remains in solution.

Intracellular cAMP-PDE activities in the *rdeA* mutants

Figure 6.1 shows a comparison of total intracellular PDE activities at the vegetative stage of *aca*- and *aca*-/*rdeA* cells. Because ACB activity was not detected in *aca*- cells in the absence of IBMX, but was detected in *aca*-/*rdeA* cells (Figure 4.7), this was interpreted as indicating that the *aca*- cells have much higher intracellular PDE activity than the *aca*-/*rdeA* cells. Since the REGA-PDE was the only known intracellular cAMP-phosphodiesterase, it was tempting to think that the active form of REGA-PDE was responsible for the high intracellular PDE activity in the *aca*- cells and that the inactive form of REGA-PDE was responsible for the low intracellular PDE activity in the *aca*-/*rdeA* cells. However I obtained quite unexpected results. The *aca*-/*rdeA* cells had, if anything, higher intracellular PDE activity than the *aca*- cells (Figure 6.1) and the AX2/*rdeA* cells had higher activity than AX2 cells (Figure 6.2).

At this point the question inevitably arises of how cAMP produced by ACB may be protected in *rdeA* mutants but not in wild type, in spite of such similar levels of intracellular PDE activities. The data raised the question of whether it was due to the REGA-PDE or another factor. One way to answer these questions may be to immunoprecipitate REGA-PDE with anti-regA antibody and measure specifically REGA-PDE activity. Then total intracellular PDE activity could be compared to REGA-PDE activity to see if there is any significant activity due to other intracellular PDEs. The

Figure 6.1 Comparison of total intracellular cAMP-PDE activity levels between vegetative *aca-* cells and *aca-/rdeA* cells.

aca- and *aca-/rdeA* cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated with 0.3mM ^3H -cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 30°C for 10, 20 or 30 minutes. 3-5 independent experiments were performed in duplicate. Means and error bars are shown.

Total intracellular cAMP-PDE activity detected in vegetative cells.

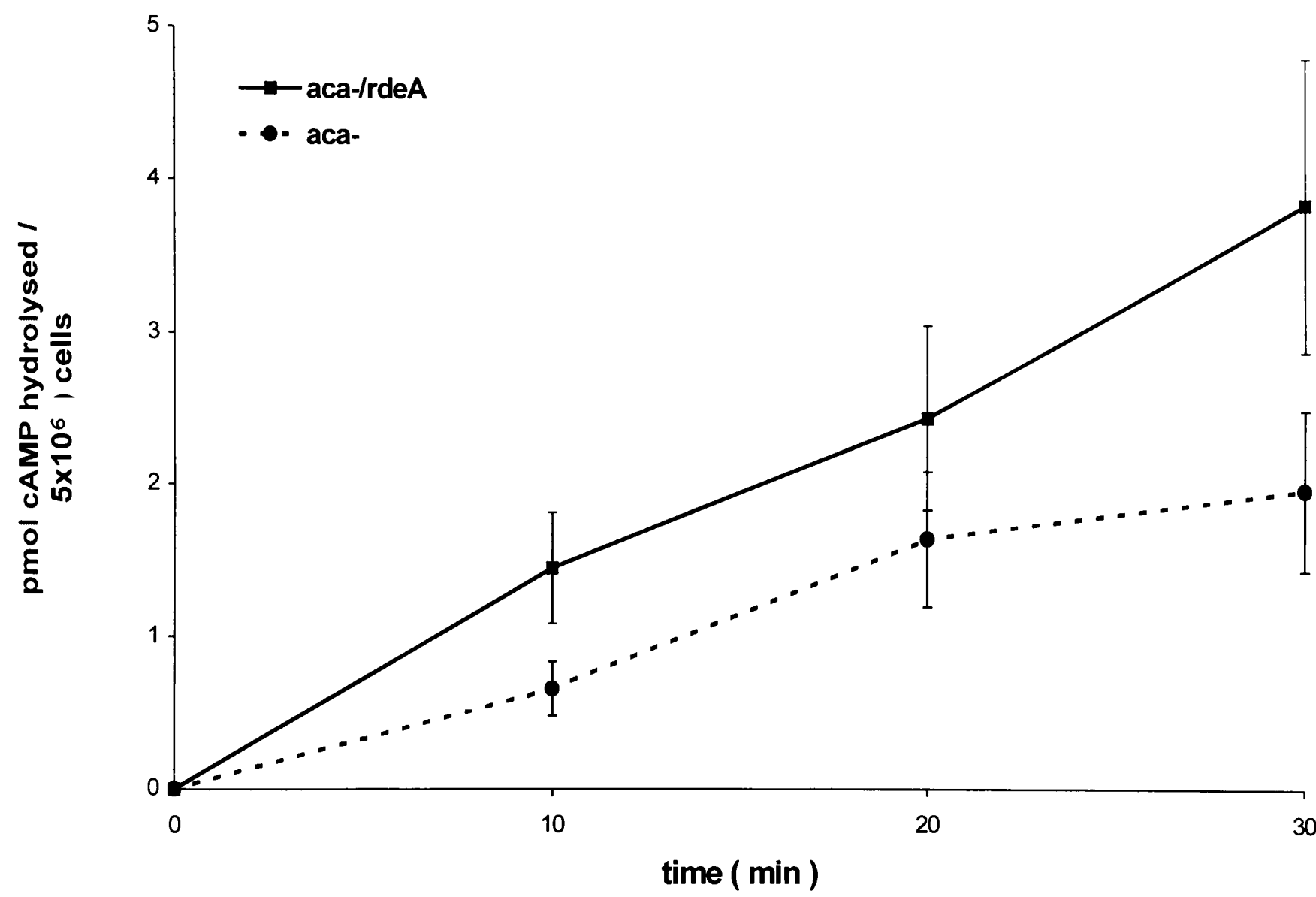
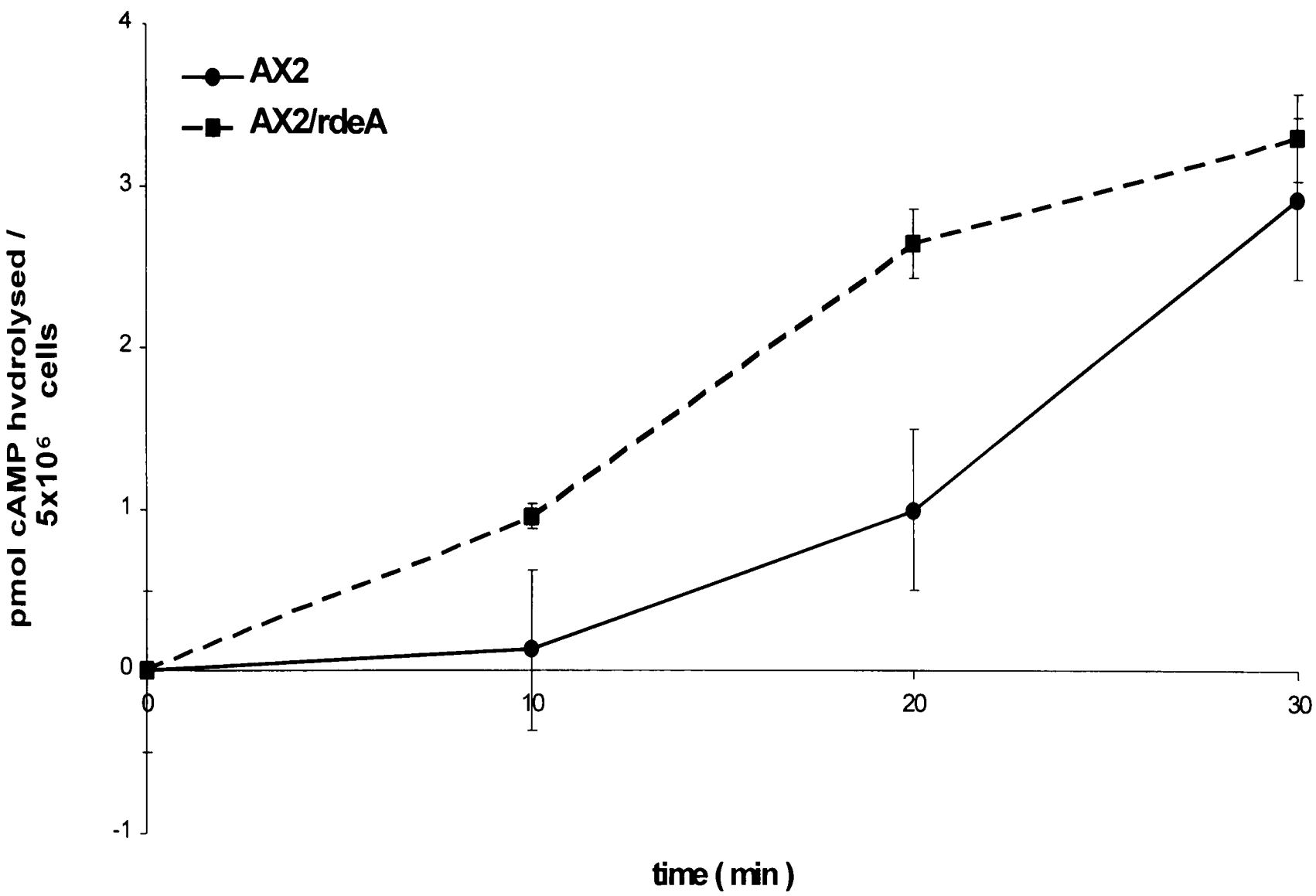


Figure 6.2 Comparison of total intracellular cAMP-PDE activity levels between vegetative AX2 cells and AX2/rdeA cells.

AX2 and AX2/rdeA cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated with 0.3mM ^3H -cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 30°C for 10, 20 or 30 minutes. 2 independent experiments were performed in duplicate. Means and error bars are shown.

Total intracellular cAMP-PDE activity detected
in vegetative cells.



experiments were carried out and the results are presented in the later part of this chapter.

6.22 Effect of IBMX and Rolipram on intracellular cAMP-PDEs

REGA-PDE is known to be inhibited by IBMX, a non-specific cAMP-phosphodiesterase inhibitor and not by Rolipram, a type IV cAMP-phosphodiesterase inhibitor (Thomason et al., 1998). If *Dictyostelium* has other intracellular cAMP-PDEs, one cannot know which types they belong to and how many there are unless one purifies them all. Even though there are some disadvantages in relying on inhibition studies to infer anything about the identities of the PDE, it still looked interesting to try such studies.

Effect of IBMX

Figure 6.3 A shows that 100 mM IBMX inhibits the intracellular PDE activity in the vegetative lysate of *aca-* cells by 50 %. The purified REGA-PDE was previously shown to be also about 50 % inhibited by the same IBMX concentration (Thomason et al., 1998). Intracellular PDE activity in the *aca-/rdeA* cells was inhibited by 100 mM IBMX in a similar manner to the *aca-* lysate (Figure 6.3 B). It seemed encouraging that PDE in wild-type cells (*aca-*) responded to IBMX in the same way as the REGA-PDE. However the data did not tell us anything about whether the PDE activity (*aca-/rdeA*) was the same as in the wild-type (*aca-*).

The possibility of the presence of another intracellular PDEs was addressed in Figure 6.3 C. A significant amount of total intracellular PDE activity was detected in a

vegetative lysate of AX2/regA cells and the activity appeared not to be inhibited by IBMX. This activity is not due to the REGA-PDE since the regA gene is disrupted in the AX2/regA cells nor can it be due to extracellular PDE because the assay was done at 10 mM DTT, a concentration that inhibits almost 99 % of the extracellular PDE (Henderson, 1975). This is therefore first direct evidence for the presence of another intracellular PDE in *Dictyostelium*.

Effect of Rolipram

Figure 6.4 A shows that 2 μ M Rolipram does not inhibit the intracellular PDE activity of AX2 cells. The REGA-PDE was shown not to be inhibited by Rolipram (Thomason et al., 1998). REGA-PDE is expected to be active in the wild-type, AX2 or aca-, cells and the intracellular PDE activity detected in lysates of wild-type cells (Figure 6.3 A and 6.4 A) behaved in this respect like REGA-PDE.

The intracellular PDE activity of AX2/rdeA cells was also not inhibited by 2 mM Rolipram (Figure 6.4 B). The presence of intracellular PDE activity in AX2/rdeA cells was confirmed in a further experiment and the PDE activity in that lysate was again not inhibited by Rolipram (Figure 6.4 C). Since Rolipram is known to inhibit type IV cAMP-phosphodiesterase (Beavo, 1995), none of PDEs detected in the AX2, AX2/rdeA and AX2/regA is likely to be similar to type IV.

6.23 Immuno-precipitation of the REGA cAMP-PDE

6.231 Measurement of REGA-PDE activity at the vegetative stage

Figure 6.3 A Effect of IBMX on intracellular cAMP-PDE activity detected in vegetative aca- cells.

aca- cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated in the presence of 0.1 mM IBMX with 0.3mM ^3H -cAMP, excess alkaline phosphatase and 10 mM DTT at 30°C for 10, 20 or 30 minutes. 3 independent experiments were performed in duplicate. Means and error bars are shown.

IBMX inhibits the intracellular PDE in aca- cells.

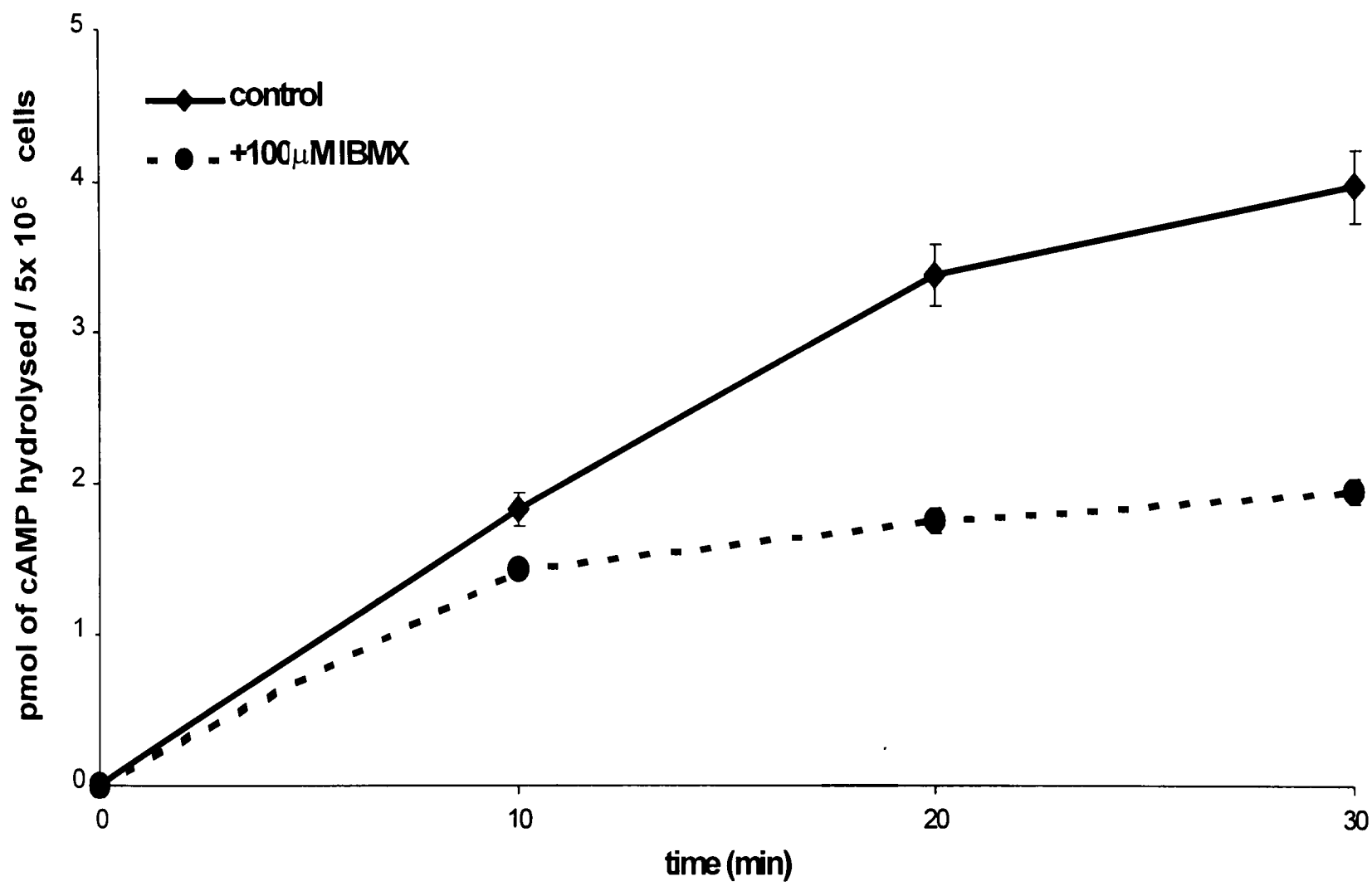


Figure 6.3 B Effect of IBMX on intracellular cAMP-PDE activity detected in vegetative *aca-/rdeA* cells.

aca-/rdeA cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated in the presence of 0.1 mM IBMX with 0.3mM ^3H -cAMP, excess alkaline phosphatase and 10 mM DTT at 30°C for 10, 20 or 30 minutes. 3 independent experiments were performed in duplicate. Means and error bars are shown.

IBMX inhibits the intracellular PDE in *aca-/rdeA* cells.

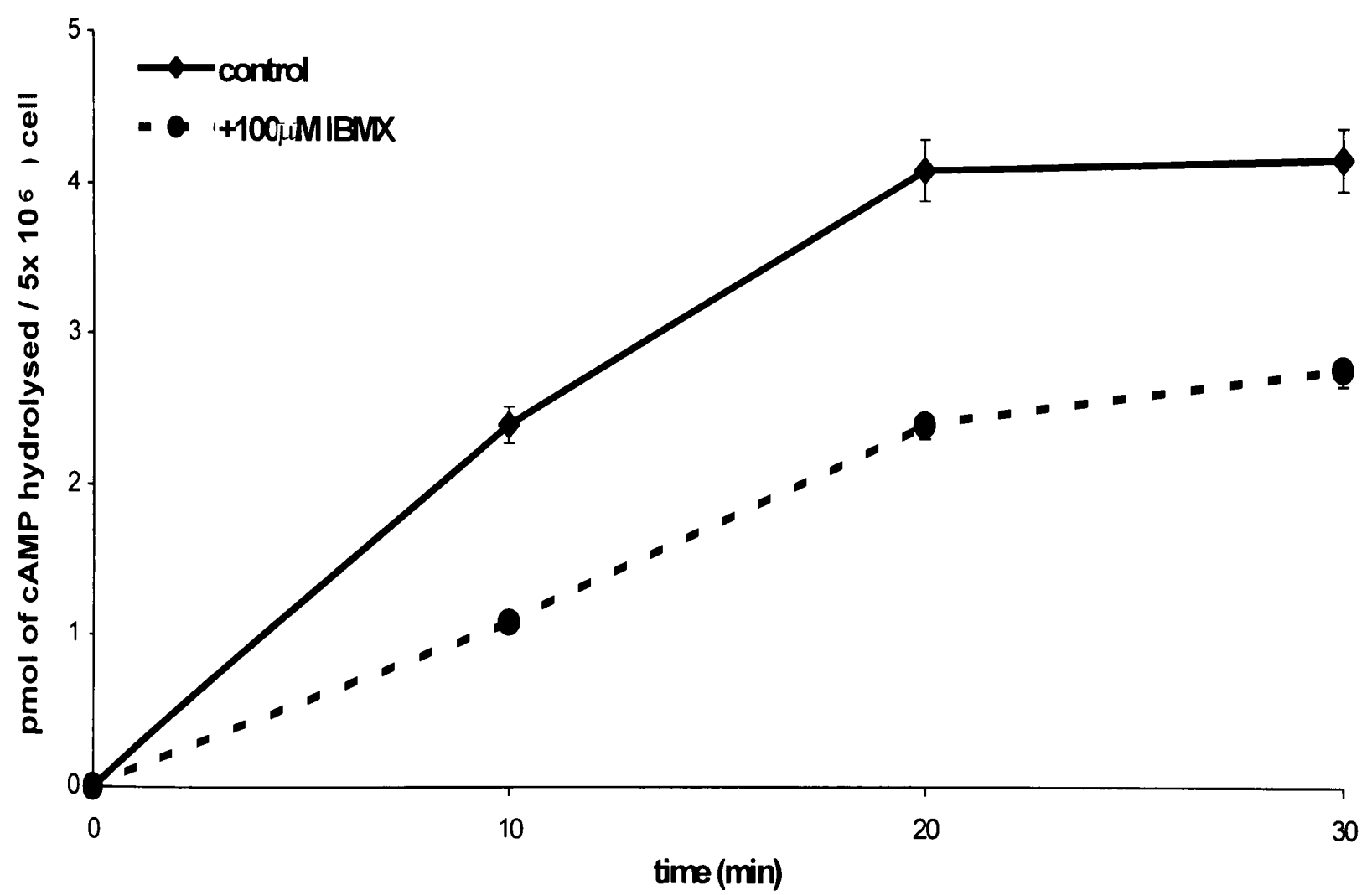


Figure 6.3 C Effect of IBMX on intracellular PDE activity detected in vegetative AX2/regA cells.

AX2/regA cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated in the presence of 0.1 mM IBMX with 0.3mM ^3H -cAMP, excess alkaline phosphatase and 10 mM DTT at 30°C for 10, 20 or 30 minutes. 2 independent experiments were performed in duplicate. Means and error bars are shown.

The intracellular PDE activity detected in AX2/reg cells was not inhibited by IBMX.

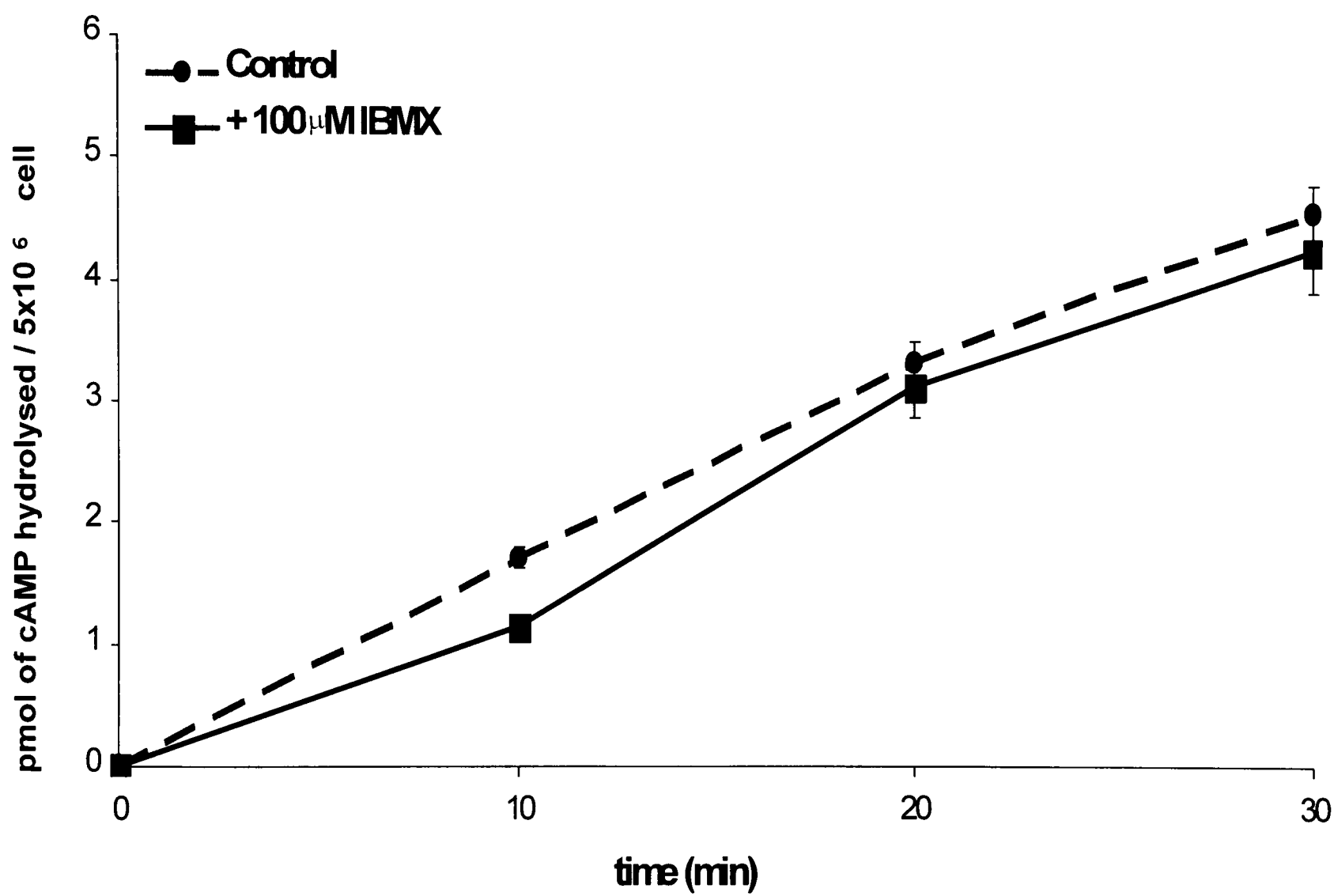


Figure 6.4 A Effect of Rolipram on intracellular cAMP-PDE activity detected in vegetative AX2 cells.

AX2 cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated in the presence of 2 μ M rolipram with 0.3mM 3 H-cAMP, excess alkaline phosphatase and 10 mM DTT at 30°C for 10, 20 or 30 minutes. 2 independent experiments were performed in duplicate. Means and error bars are shown.

The intracellular PDE activity detected in AX2 cells was not inhibited by Rolipram.

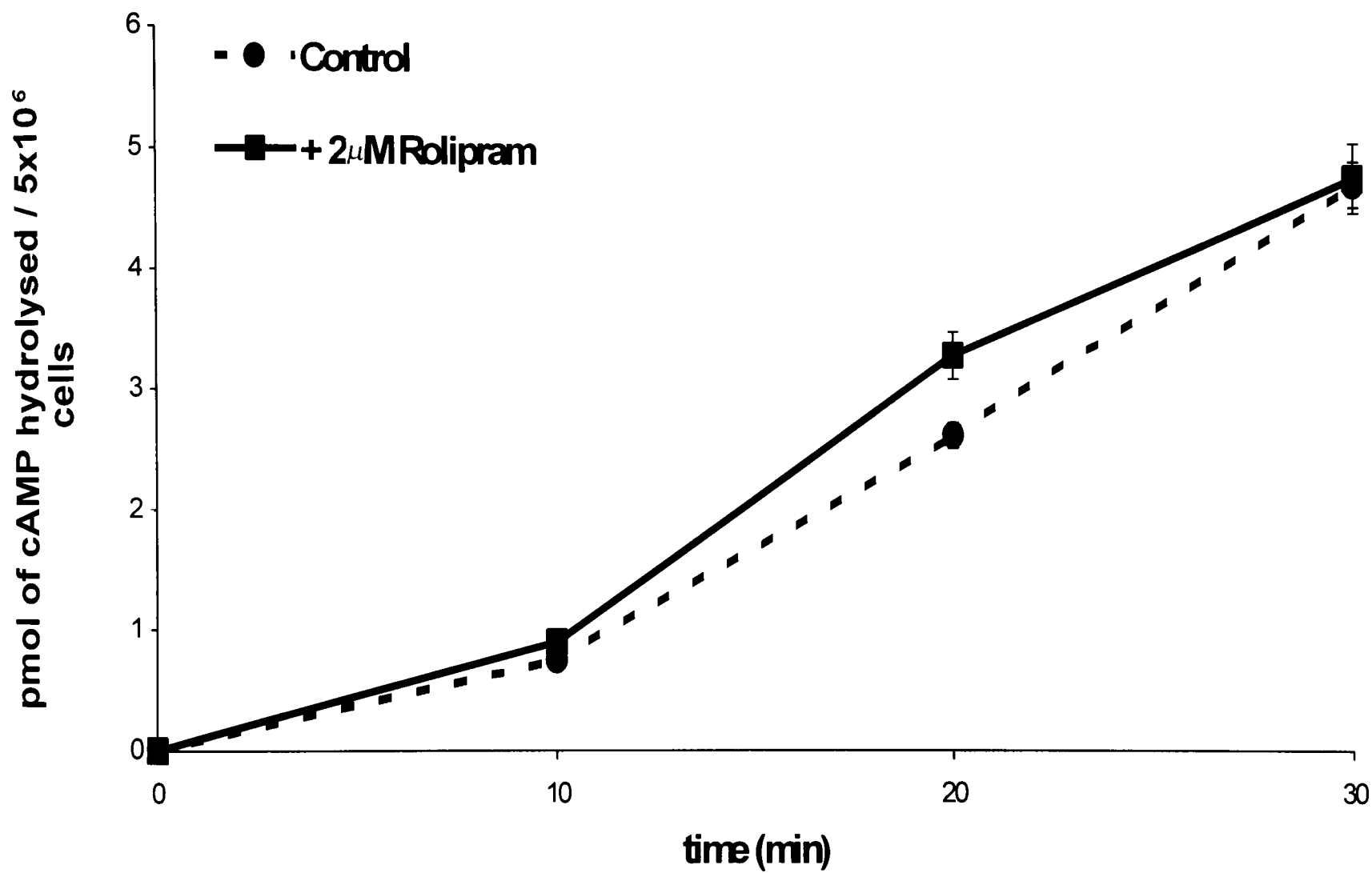


Figure 6.4 B Effect of Rolipram on intracellular PDE activity detected in vegetative AX2/rdeA cells.

AX2/rdeA cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated in the presence of 2 μ M rolipram with 0.3mM 3 H-cAMP, excess alkaline phosphatase and 10 mM DTT at 30°C for 10, 20 or 30 minutes. 2 independent experiments were performed in duplicate. Means and error bars are shown.

The intracellular PDE activity detected in *AX2/rdeA* cell
was not inhibited by Rolipram.

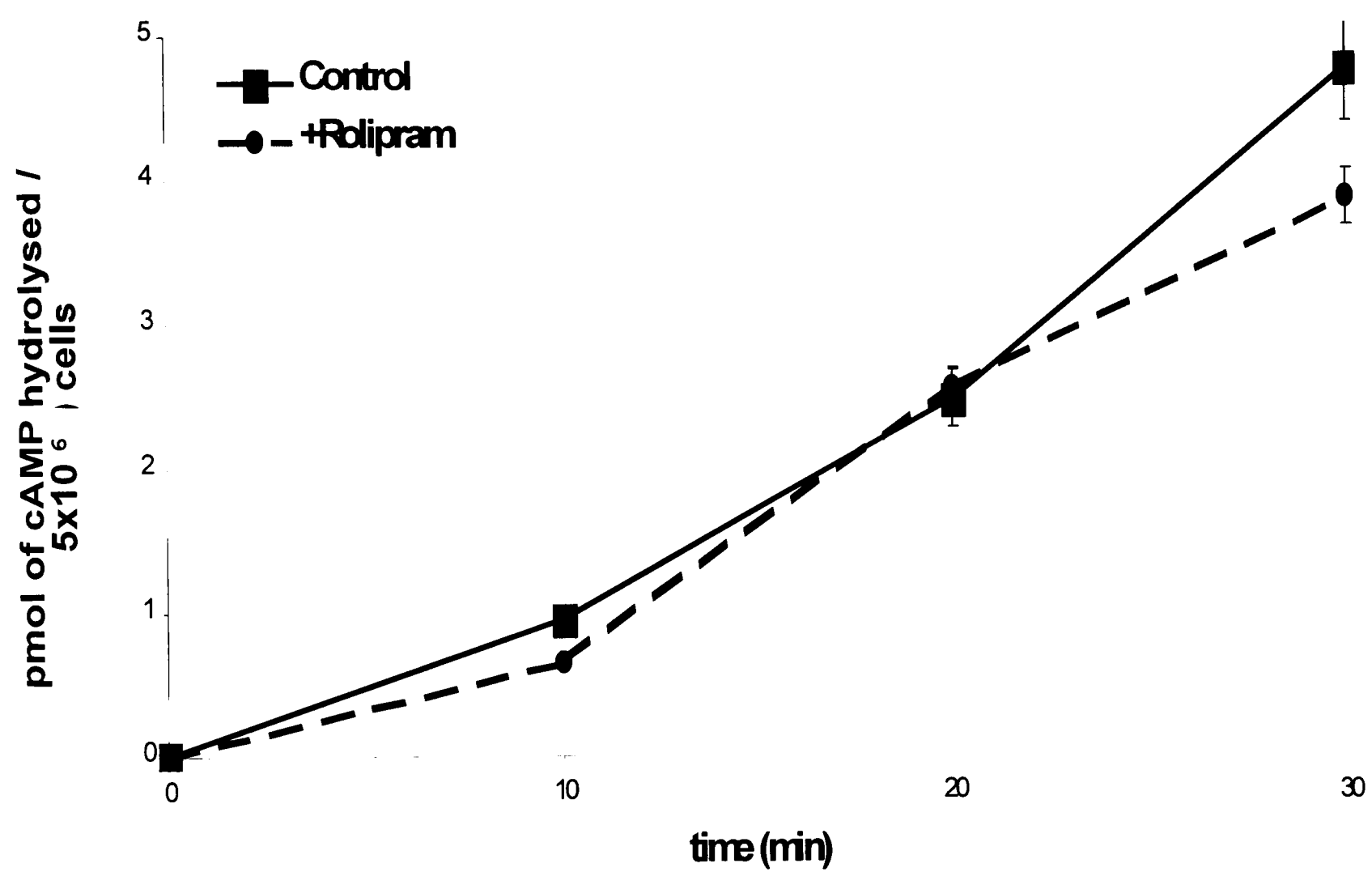
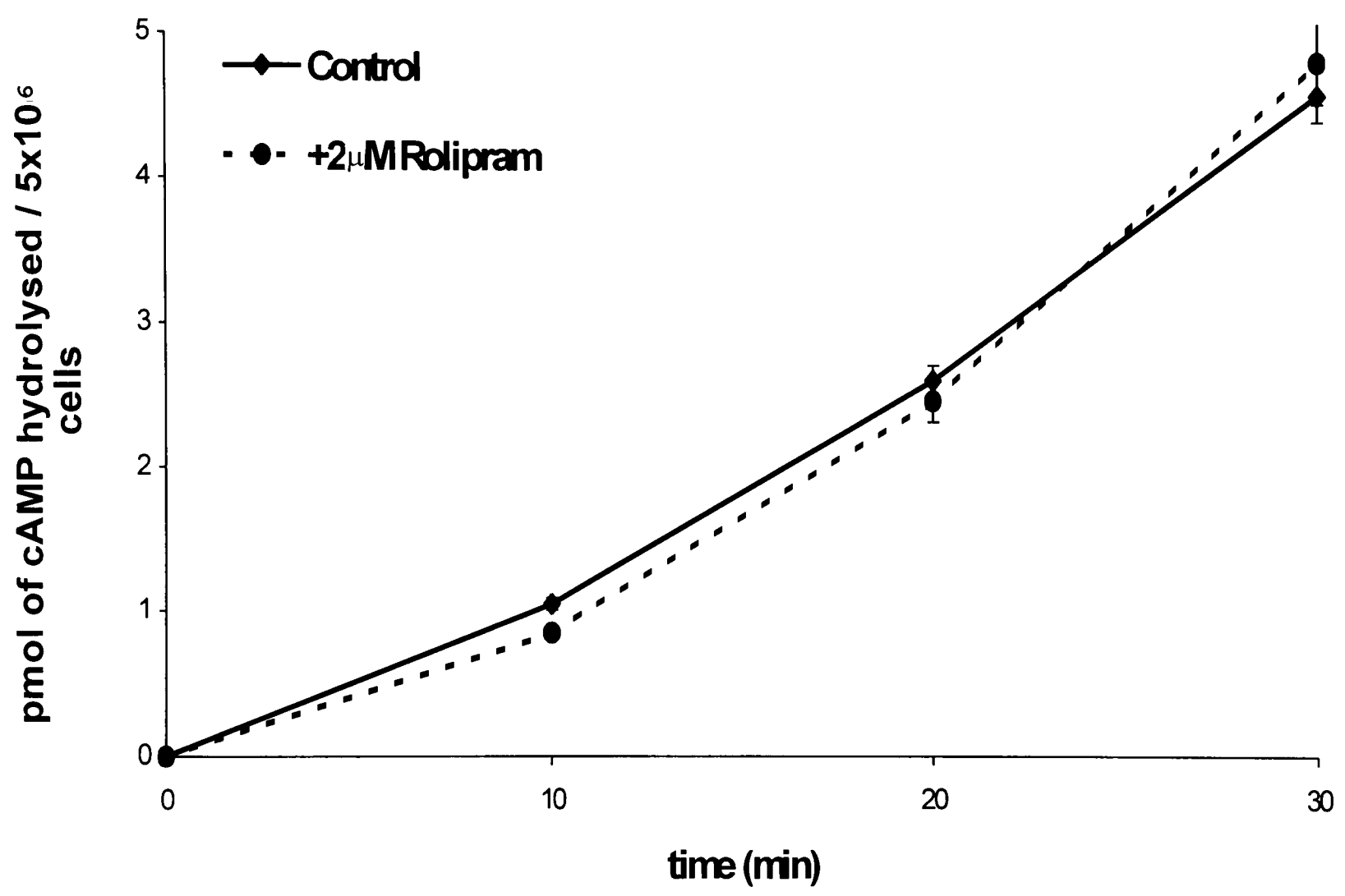


Figure 6.4 C Effect of Rolipram on intracellular cAMP-PDE activity detected in vegetative AX2/regA cells.

AX2/regA cells were harvested from growth medium, washed once with PB and resuspended in Lysis buffer 1 (Henderson, 1975). The cell suspension was incubated in the presence of 2 μ M rolipram with 0.3mM 3 H-cAMP, excess alkaline phosphatase and 10 mM DTT at 30°C for 10, 20 or 30 minutes. 2 independent experiments were performed in duplicate. Means and error bars are shown.

The intracellular PDE activity detected in *AX2/regA* cell
was not inhibited by Rolipram.



As discussed in the later part of 6.21 "Measurement of total intracellular cAMP-PDE activity", immuno-precipitation was carried out to see whether the intracellular PDE activity in vegetative lysates of AX2/rdeA cells was due to the REGA-PDE. Figure 6.5 B illustrates how the activity due specifically to the REGA-PDE was measured. Cells were harvested from log phase, washed once with PB buffer and resuspended in Immuno-Precipitation buffer (50 mM K_1/K_2PO_4 pH 7.3, 1 mM $MgCl_2$, 1 % BSA and 10 % glycerol) containing protease inhibitors (tablets, diluted 1:50) at 10^8 cells / ml. Cell suspension was frozen in dry-ice as aliquots of 200 μ l. The frozen pellets were lysed on ice in IP buffer and precleared by centrifugation. The supernatant was incubated with protein A Sepharose and the anti-RegA antibody, R1/2F for 1 hour at 4°C. The Sepharose beads were pelleted, and the pellet washed 6 times in 8 bed volumes (or 6 x 0.9 ml) of IP buffer (minus the BSA). The beads were then resuspended in Lysis buffer 2 (for REGA-PDE assay, Thomason et al., 1998; 50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 10 % glycerol, pH 8.0). Finally the immuno-precipitate was assayed for PDE activity at 25 °C in the presence of 10 mM DTT with 0.3 μ M 3H -cAMP as substrate and excess alkaline phosphatase. The levels of cAMP hydrolysed were determined in the same way as described in the earlier part of 6.21 "Measurement of total intracellular cAMP-PDE activity".

Figure 6.5 A shows that the experiment itself worked and that only negligible REGA-PDE activity was detected in vegetative AX2/regA cells. REGA-PDE activity was present in the immuno-precipitate of AX2/rdeA cells as in AX2 cells. From these data it cannot be concluded that the rdeA mutant has any major reduction in REGA-PDE activity because of a block in the putative rdeA-regA phosphorelay. However the

result in Figure 6.5 A is far from answering many questions, such as “Is all the intracellular PDE activity detected in AX2/rdeA due to the REGA-PDE?” “Is the proposed multi-step phosphorelay wrong?” and “How was it possible that ACB was detected in the rdeA mutant but not in the wild-type?” As a next step, I wanted to measure REGA-PDE activity and total intracellular PDE activity throughout development in all the strains. It seemed especially important to know whether REGA-PDE activity perished in AX2/rdeA cells after the onset of the development.. These experiments were also aimed to confirming the presence of another intracellular cAMP-PDE in AX2/regA cells and secondly at determining the proportion of the PDE activity in AX2 and AX2/rdeA that is due to REGA-PDE.

6.232 Comparison between REGA-PDE activity and total intracellular PDE activity during development

Cells in log phase (3×10^6 cells / ml to 7×10^6 cells /ml) were washed three times with LPS (Lower Pad Solution). Washed pellets were resuspended at 10^8 cells / ml in LPS and 400 μ l, equivalent to 4×10^7 cells was plated evenly onto the whole of a Millipore filter (dark grey, diameter; 46 mm). The plated cells were developed in dark and well-moisturised condition at 22°C. Developing cells were harvested from the filters by scrubbing them with a spatula. When they form multi-cellular structures more care is required when they are collected into 400 μ l of Immuno-Precipitation buffer in an Eppendorf tube. The suspensions were frozen as aliquots of 200 μ l in dry ice and lysed on ice. Lysates from the later stages of development were pipetted up and down several times after being thawed on ice. Immuno-precipitation followed by

PDE assay was performed as described in the earlier part of this section. To measure total intracellular PDE activity developing cells were collected into Lysis buffer 1 (Henderson E.J., 1975; 50 mM Tris-HCl, 12.5 mM MgSO₄, 250 mM Sucrose, pH to 7.5) instead of IP buffer. Assays were done as described in 6.21 "Measurement of total intracellular cAMP-PDE activity".

AX2

Figure 6.6 shows that REGA-PDE is not the only intracellular phosphodiesterase in the wild-type. REGA-PDE activity was detected from the vegetative stage onwards and my results were quite similar to the report of Thomason et al., (1998). Total intracellular PDE activity was greater than REGA-PDE activity alone throughout the development of AX2 cells and increased as the cells developed. Thus there again seemed to be one or more other intracellular PDE in *Dictyostelium*. It will be interesting to know whether it or they, is/are related to the rdeA-regA pathway.

AX2/rdeA

Figure 6.7 shows firstly that REGA-PDE activity is present during the development of AX2/rdeA cells and secondly that other intracellular PDE activities are present. Total intracellular PDE activity in the AX2/rdeA cells appeared to decrease after 18-20 hours but this may not be meaningful since the mutant has already completed development by that time. It was not possible to identify if the other intracellular PDE activities (total activity subtracted by the REGA-PDE activity) detected in AX2 and AX2/rdeA were due to the same enzymes. However it is possible that the enzyme in

the wild type is different from that in the *rdeA* mutant based on preliminary experiments to determine their K_m values. I found it very difficult to get good hyperbolic curves (activity vs conc. of substrate) and so it was almost impossible to obtain reliable and consistent values for K_m and V_{max} . I admit that if more than two PDEs were present as I suspected, my approach was like trying to figure out a few pairs of parameters with only one equation. The experiment would give better results if repeated after purification of the intracellular PDEs.

AX2/*regA*

Figure 6.8 shows that no REGA-PDE activity is detectable during development of AX2/*regA* cells but that nevertheless intracellular PDE activity is present. In terms of total intracellular PDE activities the AX2/*rdeA* and AX2/*regA* strains do not appear to be very different from the wild type. Since all the strains have similar levels of total intracellular PDE activities during development it cannot be concluded in any straightforward way that ACB was detected in the *rdeA* and *regA* mutants due to the absence of intracellular cAMP-PDE activities. A possible explanation of this paradox will be discussed in the following section.

6.233 Comparison of the REGA-PDE activity during development of *rdeA* and wild-type cells

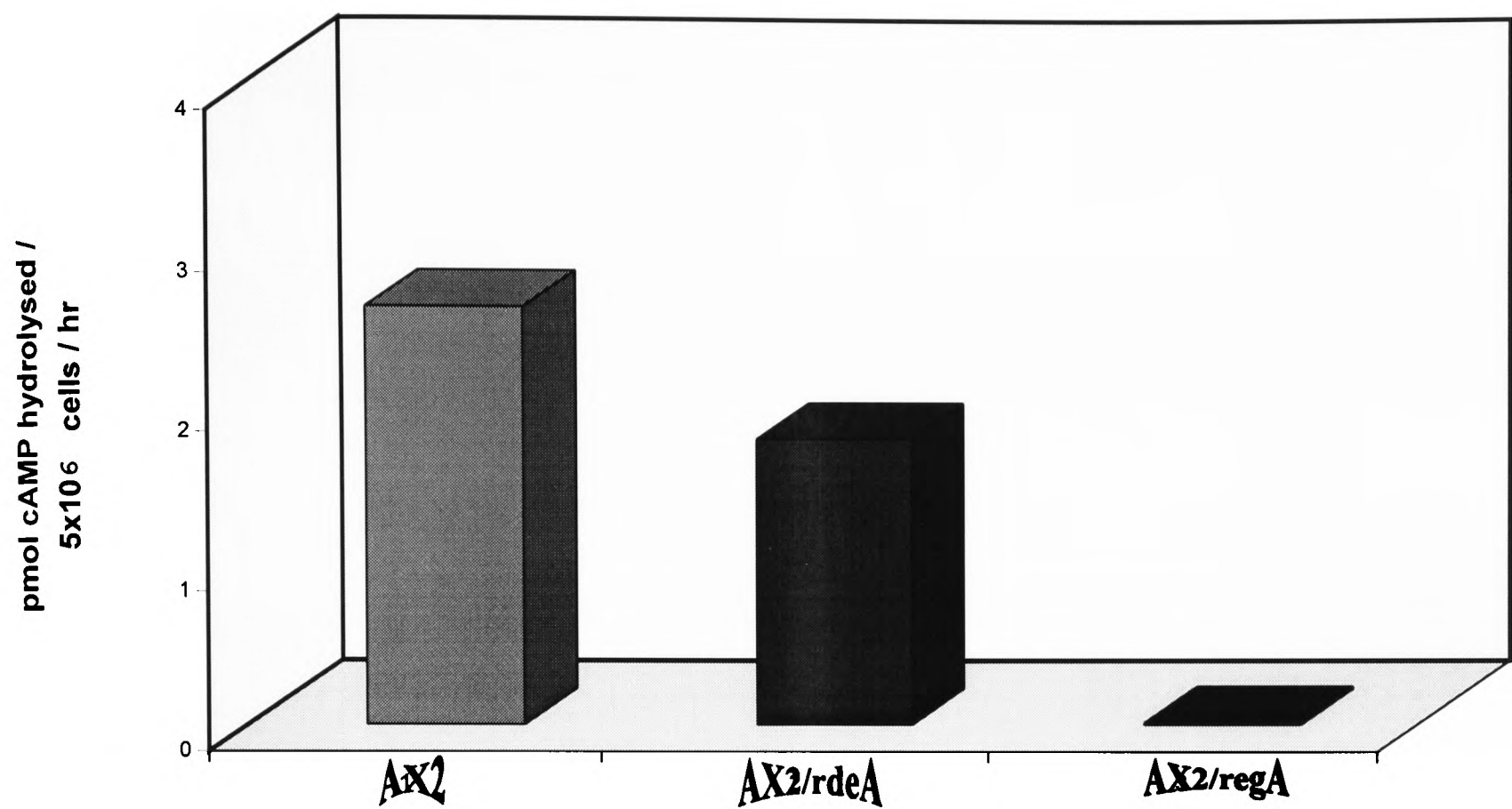
Figure 6.9 shows that AX2/*rdeA* has a level of REGA-PDE activity similar to wild-type. However unlike AX2/*rdeA*, no significant REGA-PDE activity is detectable in AX2/*regA*. It was expected that REGA-PDE activity in AX2/*rdeA* should be closer to

Figure 6.5 REGA cAMP-PDE activity detected in vegetative cells.

- A. Similar levels of REGA-PDE activity were detected in AX2 and AX2/rdeA cells.
The activity was absent in AX2/regA cells.
- B. A diagram describing a method to detect specific REGA-PDE activity.

A

The REGA PDE activity detected in vegetative cells.



B

The REGA-PDE activity measurement

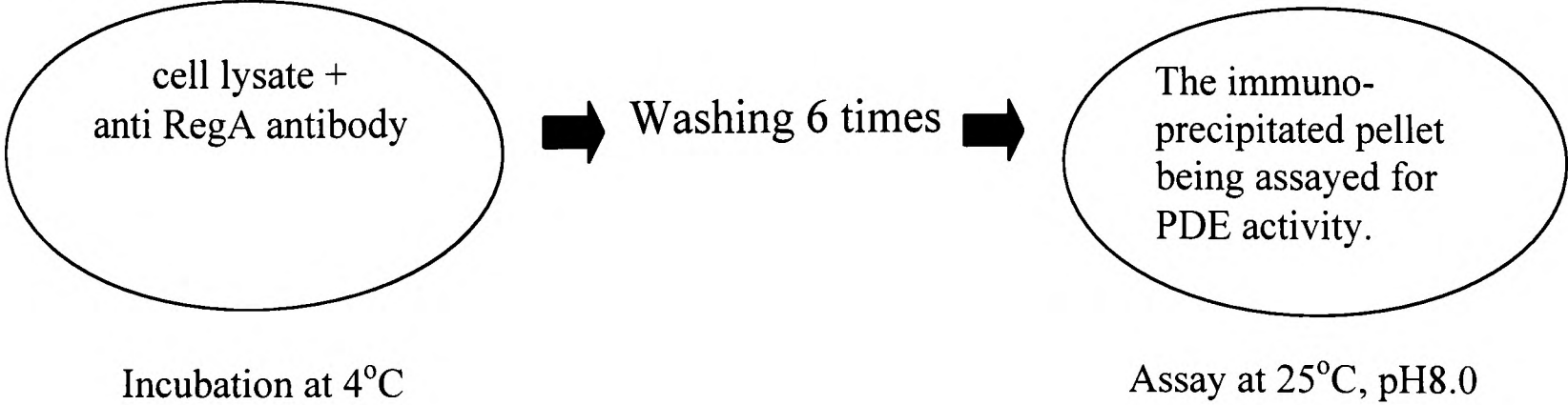


Figure 6.6 Total intracellular cAMP-PDE activity vs REGA cAMP-PDE activity during the development of AX2 cells.

AX2 cells were developed on Millipore filters at 22°C for indicated time periods.

For measurement of total intracellular cAMP-PDE activity, developing cells were harvested in Lysis buffer 1 (Henderson, 1975) and lysed. Lysates were incubated with 0.3mM ^3H -cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr.

For measurement of REGA cAMP-PDE activity, developing cells were harvested in Immuno-precipitation buffer and the immuno-precipitate was resuspended in Lysis buffer 2 (Thomason et al., 1998). The suspension was incubated with 0.3mM ^3H -cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr. Data are means of a duplicated experiment.

Intracellular PDE activities during development of AX2 cells.

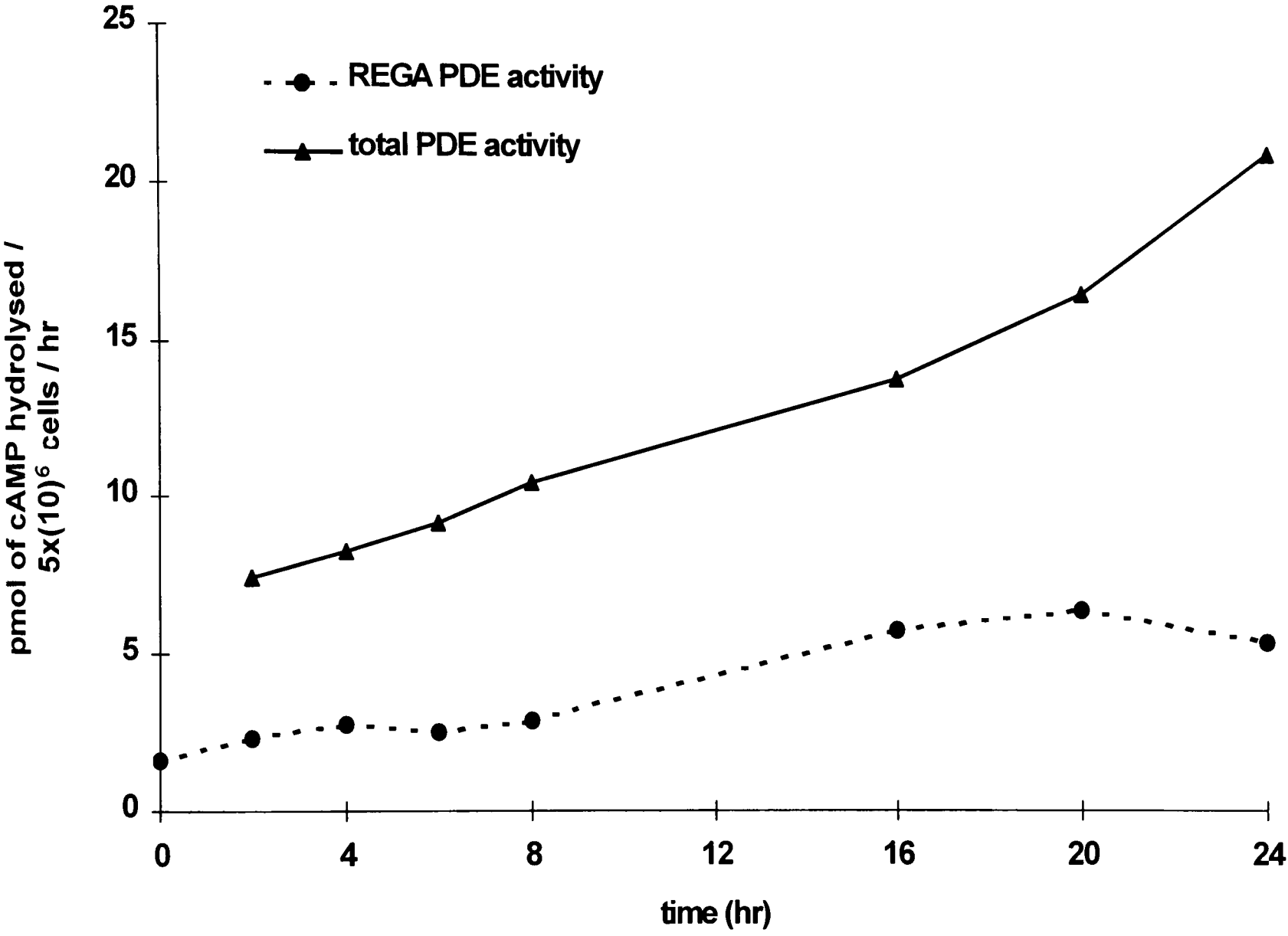


Figure 6.7 Total intracellular cAMP-PDE activity vs REGA cAMP-PDE activity during the development of AX2/rdeA cells.

AX2/rdeA cells were developed on Millipore filters at 22°C for indicated time periods. For measurement of total intracellular cAMP-PDE activity, developing cells were harvested in Lysis buffer 1 (Henderson, 1975) and lysed. Lysates were incubated with 0.3mM ³H-cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr.

For measurement of REGA cAMP-PDE activity, developing cells were harvested in Immuno-precipitation buffer and the immuno-precipitate was resuspended in Lysis buffer 2 (Thomason et al., 1998). The suspension was incubated with 0.3mM ³H-cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr. Data are means of a duplicated experiment.

**Intracellular PDE activities during development of
AX2/rdeA cells.**

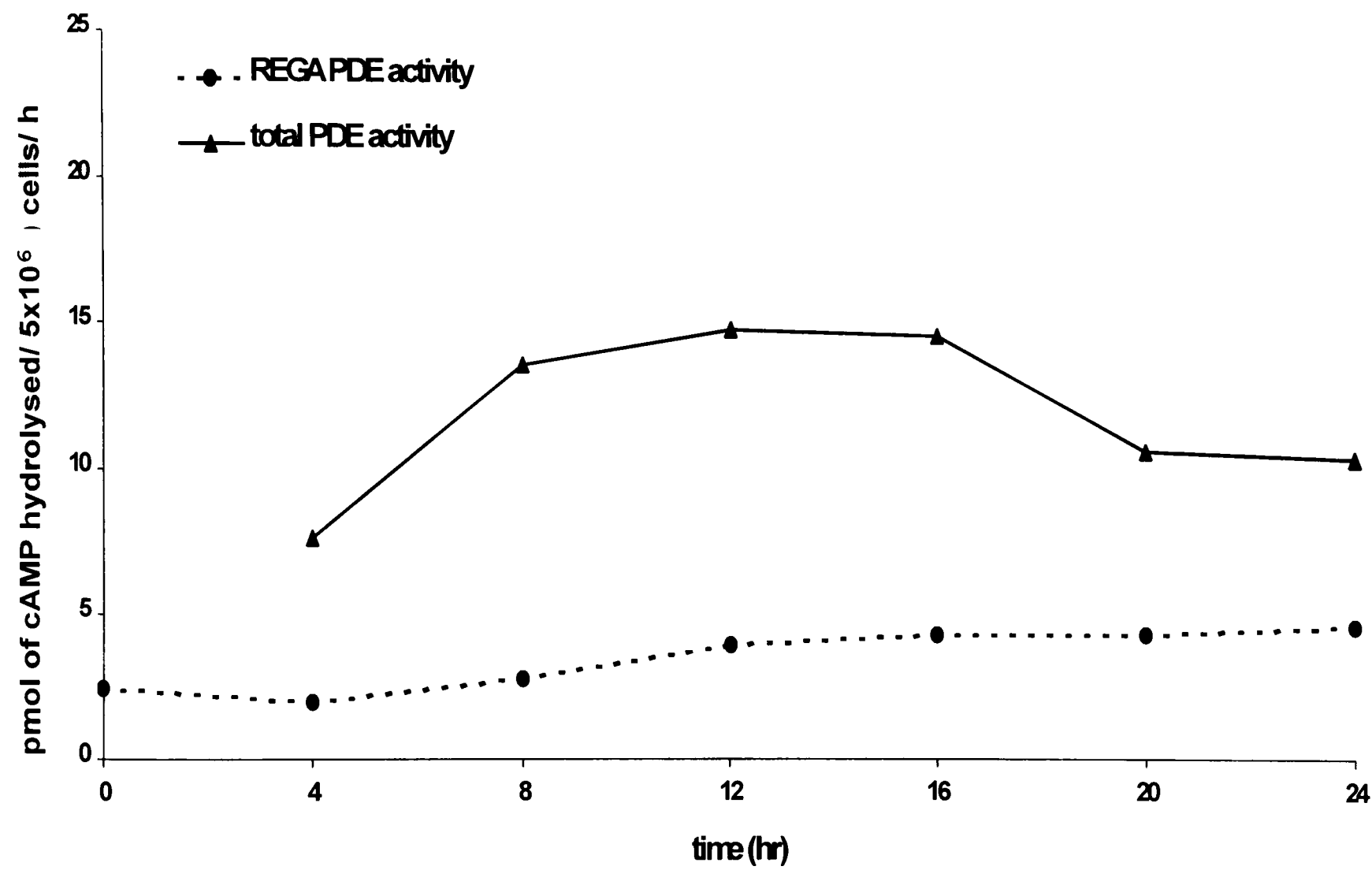


Figure 6.8 Total intracellular cAMP-PDE activity vs REGA cAMP-PDE activity during the development of AX2/regA cells.

AX2/regA cells were developed on Millipore filters at 22°C for indicated time periods. For measurement of total intracellular cAMP-PDE activity, developing cells were harvested in Lysis buffer 1 (Henderson, 1975) and lysed. Lysates were incubated with 0.3mM ^3H -cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr.

For measurement of REGA cAMP-PDE activity, developing cells were harvested in Immuno-precipitation buffer and the immuno-precipitate was resuspended in Lysis buffer 2 (Thomason et al., 1998). The suspension was incubated with 0.3mM ^3H -cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr. Data are means of a duplicated experiment.

Intracellular PDE activities during development of AX2/regA cells.

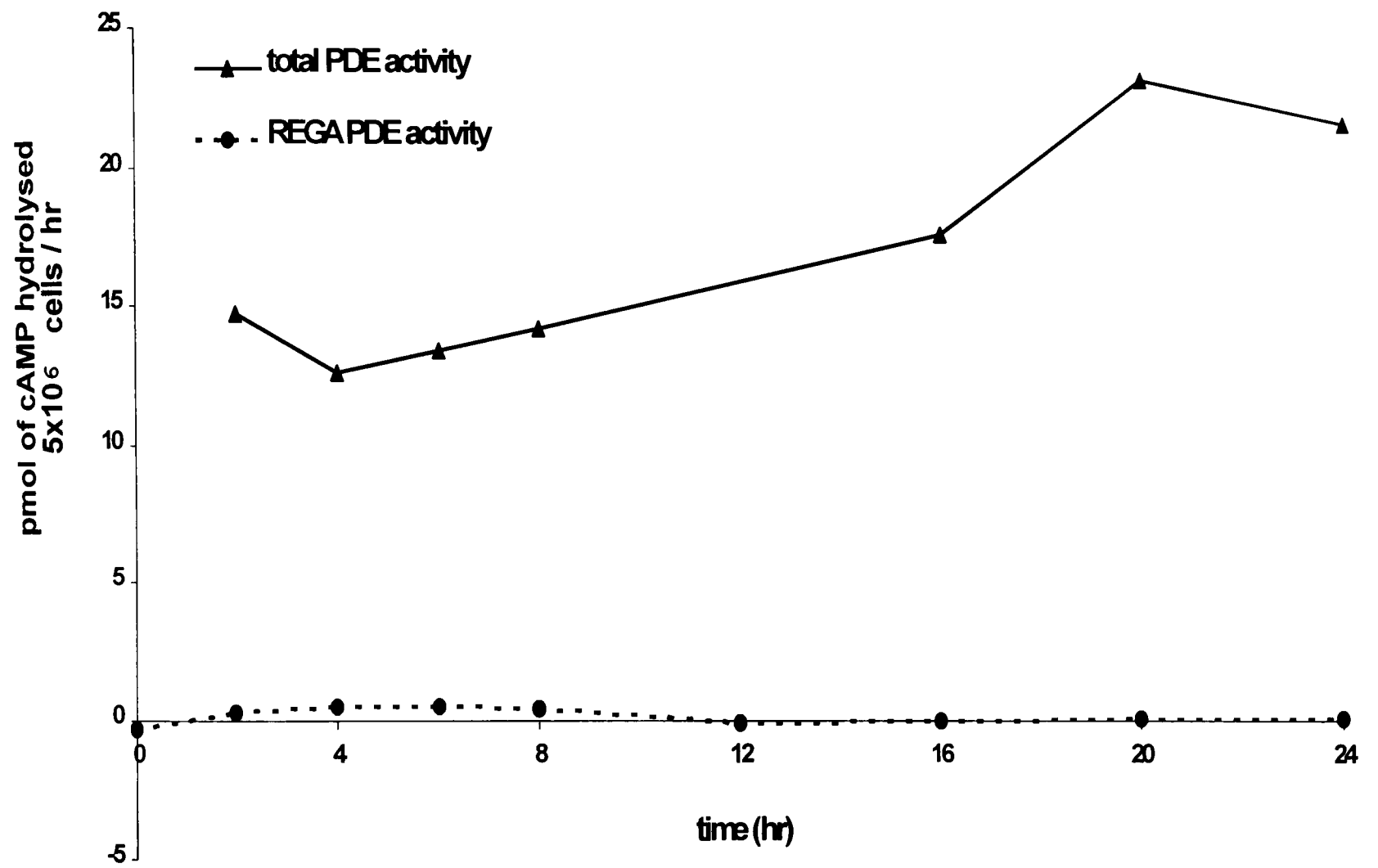
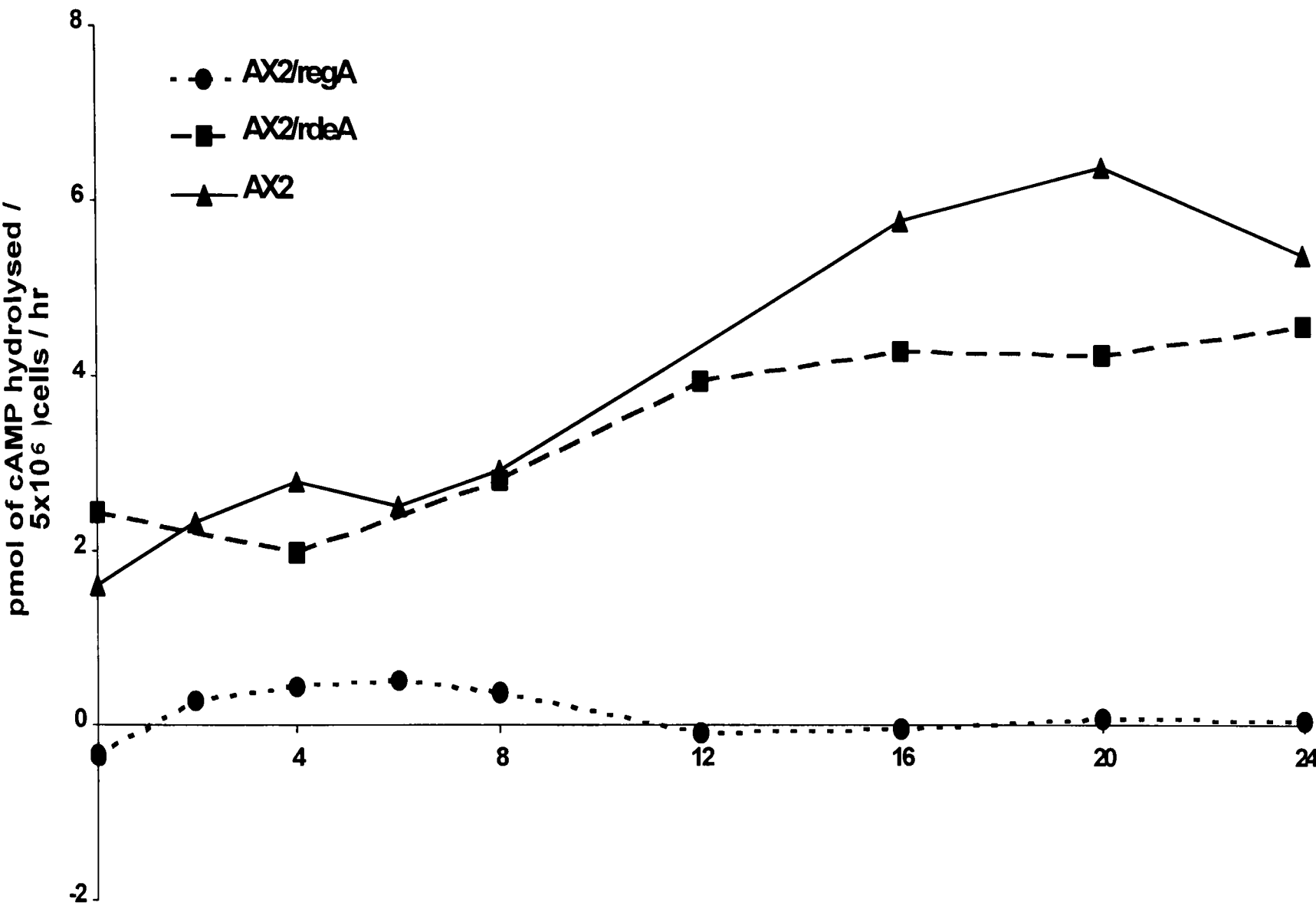


Figure 6.9 Comparison of REGA cAMP-PDE activity in AX2, AX2/rdeA and AX2/regA.

Cells were developed on Millipore filters at 22°C for indicated time periods.

Developing cells were harvested in Immuno-precipitation buffer and the immuno-precipitate was resuspended in Lysis buffer 2 (Thomason et al., 1998). The suspension was incubated with 0.3mM ³H-cAMP as substrate and excess alkaline phosphatase in the presence of 10 mM DTT at 25°C for 1 hr. Data are means of a duplicated experiment.

The REGA cAMP-PDE activities during development



AX2/regA than to AX2. Therefore do AX2/rdeA cells possess the active form of REGA-PDE? If so, this could imply that the hypothesis that RDEA activates REGA-PDE by phosphorylation might be wrong. Indeed the data might have been considered a clear disproof of the model if there had not been other convincing data supporting it, such as reverse and forward phosphotransfer between RDEA and REGA demonstrated by Dr. Peter Thomason (personal communication).

The problem in interpreting these data may derive either from the experimental approach used to make inferences about the presence of phosphorylated forms of REGA-PDE or from complications in the way the response regulator of REGA is phosphorylated. Basically the problem is that we cannot be sure that the REGA-PDE activity measured in the wild-type corresponds to its phosphorylated state even though we are sure that the activity is due to the REGA-PDE. There are three reasons why this may not be the case.

Explanation I

According to Dr. Peter Thomason, the phosphorylated form of REGA is very sensitive to temperature and he always makes sure that all *in vitro* phosphotransfer experiments are carried out at 4°C. During immuno-precipitation anti-regA antibody was incubated with cell lysate at 4°C and the acyl bond was likely to be stable. However in order to assay PDE activities, the immuno-precipitated pellet had to be brought to 25°C, usually for 1 hour, and it is possible that by the start of the assays all of the phosphorylated REGA-PDE has been dephosphorylated. In that case what I measured could be the basal activity of the unphosphorylated REGA-PDE.

With respect to the stability of the phosphorylated state of REGA, it was initially reported that the REGA RR domain had low intrinsic phosphatase activity (Thomason et al., 1998) unlike CheY, whose phosphorylated form is very unstable due to high intrinsic phosphatase activity (Lukat et al., 1992). However Drs. Thomason and Kay now think that this is incorrect and it is actually quite unstable (personal communication) and we are therefore inclined to believe that phospho-REGA becomes dephosphorylated during the PDE assay at 25°C.

Explanation II

Another possibility could be related to in the immuno-precipitation procedure itself. When anti-regA antibody binds to REGA, the structure of the Ab-Ag complex might interrupt the interface between the response regulator of REGA and the REGA cAMP-PDE domain. Djordjevic et al., 1998 have defined the structural basis of how phosphorylation of the response regulator of CheB causes the methylesterase, CheB, to be activated. Che A and Che B proteins are part of a complex two-component signalling system controlling bacterial chemotaxis. RDEA displays homology to CheA, an unorthodox histidine kinase (Chapter 1, general introduction) and Che B possesses a response regulator region associated with a methylesterase.

The x-ray crystal structure of CheB (Djordjevic et al., 1998) revealed that the N-terminal response regulator is closely opposed to the C-terminal methylesterase

domain at the interdomain interface. The interdomain interface includes the Asp (56) of the N-terminal domain that receives phosphate from CheA or low molecular weight phospho-donors, and Ser (164), the active site of the C-terminal methylesterase. When the N-terminal domain is unphosphorylated in the intact CheB, the interdomain interface allows very little solvent accessibility. Substrate cannot access to the active site, Ser (164) and therefore CheB remains inactive. When the N-terminal domain is phosphorylated, it causes propagation of conformational changes to the interdomain interface. The C-terminal domain becomes separated from the N-terminal domain and there is free access to the active site. In brief the unphosphorylated response regulator domain obstructs access to the active site of the CheB methylesterase.

It is possible that this CheA-CheB study might apply to the RdeA-RegA case. The anti-regA antibody, R1/2F that I used recognises the cAMP-phosphodiesterase domain. The antibody may bind to the region, a putative interdomain interface between the response regulator and the cAMP-PDE domain. Hence the active site of the PDE domain may be obstructed regardless of the state of phosphorylation of the response regulator. Or the antibody may block access of the PDE domain to the substrate, cAMP while it does not interfere with the interdomain interface. In either case it is possible that one can only measure the basal activity of the unphosphorylated REGA-PDE as long as immuno-precipitation is performed.

Explanation III

Apart from auto-phosphatase activity of REGA, the proposed rdeA-regA system might include protein aspartate phosphatases controlling the levels of the phosphorylated form of REGA. In *B. subtilis* the products of rapA and rapB are reported to be protein-aspartate phosphatases that dephosphorylate the response regulator, Spo0F (Perego et al., 1994 ; Perego and Hoch, 1996 a,b). Therefore it is possible that the phosphorylated form of REGA is very unstable not only due to auto-phosphatase activity but also to the presence of protein aspartate phosphatases.

In summary

As discussed above, it is incorrect to conclude that the data in Figure 6.9 disprove the proposed model. At the same time it is disappointing to find that there may be no way to measure the extent of *in vivo* phosphorylation of REGA. If I had been able to measure a difference between phosphorylated REGA-PDE activity and unphosphorylated activity, the approach taken in Figure 6.9 might have given clear support for the model. However if the REGA RR domain can in fact be phosphorylated in the absence of RDEA, we do not know how to account for detection of ACB in rdeA and regA mutants. One possibility will be discussed next.

6.234 A special relationship between ACB and REGA?

Leaving aside the problem of AX2/rdeA for a moment, my results show clearly that REGA-PDE activity is present in the wild-type but not in the regA mutant. Moreover

in the absence of IBMX the novel adenylyl cyclase was detected only in the *rdeA* and *regA* mutants not in the wild type (Figure 3.6 to 3.9; Kim et al., 1998). Despite this another way to account for the finding that intracellular PDE levels are similar in AX2, AX2/*rdeA* and AX2/*regA* (Figure 6.6 to 6.8) is to assume that ACB and REGA form a multi-molecular complex. If that is the case the cAMP made by ACB might be preferentially hydrolysed by the REGA cAMP-PDE and this activity might not need to be a major component of total intracellular PDE. If the other intracellular cAMP-PDE(s) destroyed the cAMP made by ACB inefficiently either because there is little of them or they have low affinities for cAMP, ACB would be detected only if the REGA-PDE were inactive. This would explain (1) why cAMP produced by ACB did not seem to be affected by the presence of the additional intracellular cAMP-PDEs in the wild type and the *rdeA* and *regA* mutants and (2) why REGA cAMP-PDE seems to be a negligible fraction of total intracellular cAMP-PDE activity. How can the idea of an ACB-REGA complex be tested?

Co-immunoprecipitation of ACB by anti-regA antibody

If the proposed ACB-REGA complex were rather stable, ACB might be pulled down by co-immuno-precipitation with anti-regA antibody. How could we recognise a specific band among many non-specific bands when the immuno-precipitate is stained with Commassie Blue on an SDS gel? Possible candidates should be bands present only in the immuno-precipitate from AX2 cells but not from AX2/*regA* cells. I would not expect that any band of about 28 kDa that might be detected would correspond to RDEA; the yeast two-hybrid system is known to be more sensitive for detecting

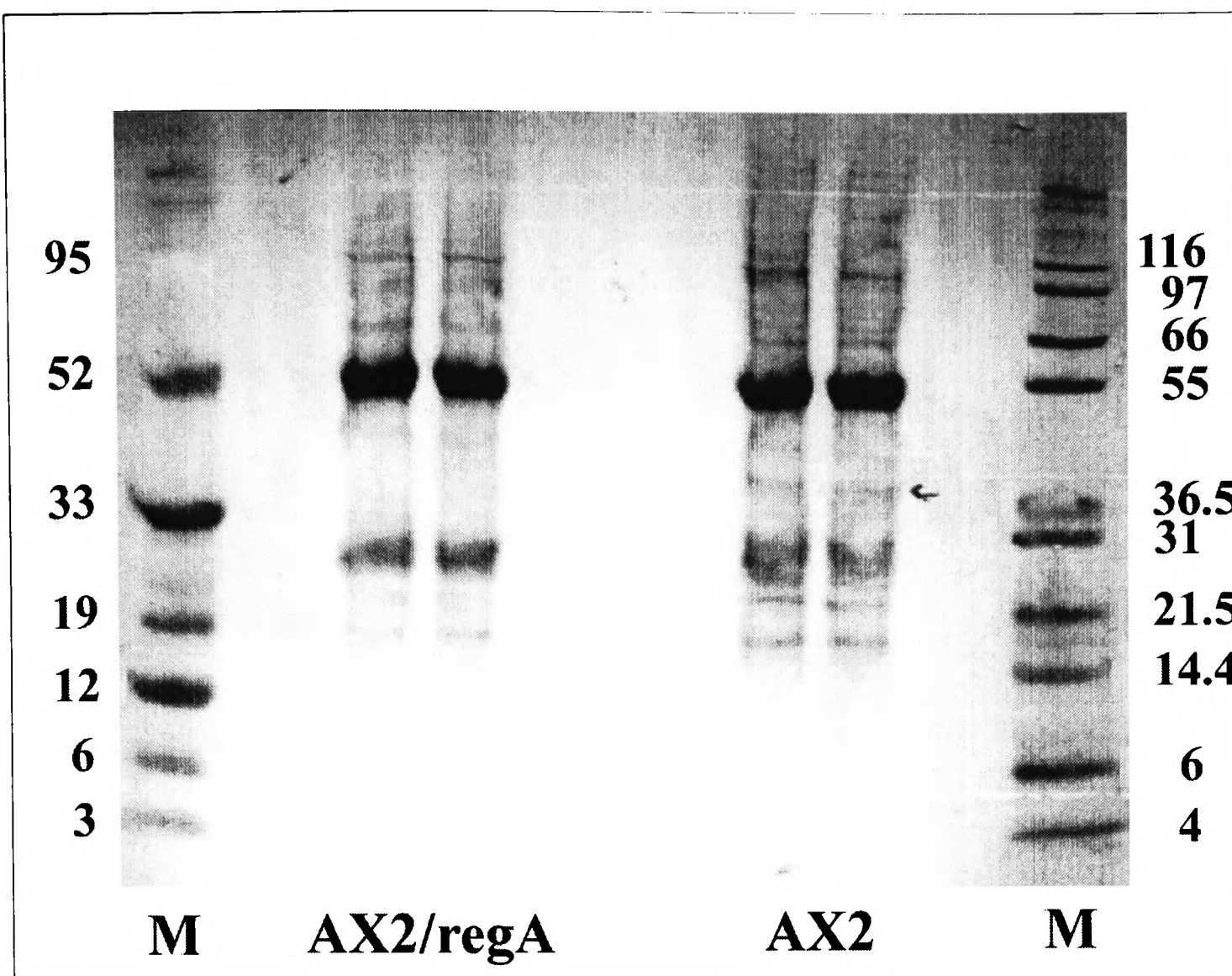


Figure 6.10 Co-immunoprecipitation with anti-regA antibody in AX2 and AX2/regA

Lysates of AX2 and AX2/regA were incubated with anti-regA antibody at 4°C. After being washed 6 times, the immuno-precipitate was resuspended in SDS gel loading buffer. Protein samples were loaded on a SDS gel with two different markers in either side. Most of the bands present in AX2 sample (in duplicate) were detected in AX2/regA sample (in duplicate) except for a single band of 37.5 kDa.

protein-protein interactions than immuno-precipitation and yet interaction between RDEA and REGA could not be demonstrated by the yeast two-hybrid system (Chapter 5).

To examine this possibility vegetative cells of AX2 and AX2/regA were harvested, lysed and immuno-precipitated with anti-regA antibody. The immuno-precipitates were prepared in SDS gel-loading buffer and boiled for 1-2 minutes before electrophoresis. The SDS-gel was stained with Commassie blue for 3 hours at room temperature and de-stained overnight. Figure 6.10 shows that there is a single band of 37.5 kDa detected in AX2 and absent in AX2/regA. It was very exciting to see that the idea seemed to work. The next step was to have the band micro-sequenced to check if the protein could be an adenylyl cyclase. Micro sequencing of the band did not produce any good answer because of N-terminal blocking (A. Willis, Immunochemistry , Oxford). If I can continue to work on it in future I will try again, taking into account the lessons I have learnt about how to overcome N-terminal blocking.

6.3 Discussion

6.31 The phosphorelay model could be far more complex than what we propose.

The work presented in this chapter was mainly aimed at getting direct evidence in

favour of the idea that both *rdeA* and *regA* mutants lack the REGA-PDE activity responsible for hydrolysis of the cAMP produced by ACB. Many workers have attempted to detect direct *in vivo* consequences of defects in upstream components of multi-step phosphorelay systems. It has usually turned out to be very difficult to achieve this since systems tend to be interconnected within an organism and the function of one protein is frequently compensated for by another.

Two-component systems are quite often the initial components of complex transcriptional processes or enzyme cascades (Posas et al., 1996; Burbulys et al., 1991; Uhl and Miller, 1996). Such systems may take inputs from various sources apart from direct cognate phosphotransferases, such as low-molecular weight donors to monitor the state of metabolisms, or from intermediate components of independent systems. The two-component signalling system of *Escherichia coli* controlling chemotaxis is a good example of the rather complex processes that may be involved in phosphorylation of response regulators. For example Lukat et al. (1992) found that in the absence of CheA, the histidine phosphotransferase, the downstream response regulators, CheB and CheY can be phosphorylated *in vitro* by intermediary metabolites such as acetyl phosphate and carbamoyl phosphate.

In the chemotaxis phosphorelay of *Escherichia coli*, CheA, the direct cognate phosphotransferase, as well as possible low molecular weight phospho-donors functioning as part of a mechanism monitoring the state of metabolism, may therefore phosphorylate CheB and CheY. CheB, once phosphorylated by CheA becomes an active methylesterase and removes a methyl group from the receptor. Since methylated receptors stimulate autophosphorylation of CheA, phospho-CheB

functions as a negative feedback of the system (Swanson et al., 1993 ; Djordjevic et al., 1998)

Thus the proposed multi-step phosphorelay of *Dictyostelium* may turn out to be rather more complicated than what we suppose at the moment. As far as the histidine kinases are concerned, a possible complexity has already been discussed (chapter 5). There might also be other response regulators and other intermediate components dephosphorylating REGA or blocking phospho-flow from RDEA to REGA. The proposed RDEA-REGA phosphorelay might also require a coupling protein between these two proteins such that phosphotransfer from RDEA to REGA can only occur when the two proteins are pulled closely to each other via the coupling protein.

Such a coupling protein might resemble CheW, a small coupling protein acting between CheA and chemotaxis receptors that contributes to controlling phospho-flow from CheA to CheY (Swanson et al., 1993). In addition the response regulator (RR) of REGA might be phosphorylated by low molecular weight phospho-donors *in vivo*. It has been shown that the RR domain of REGA can be phosphorylated by acetyl-phosphate *in vitro* (Thomason et al., 1998). It seems possible that the proposed RDEA-REGA relay may sense the state of cellular metabolites and that the RR domain of REGA can be phosphorylated in the absence of the RDEA phosphotransferase. However if the phosphorylated form of REGA exists in *rdeA* mutant, we would not be able to reconcile this possibility with the fact that the phenotype of *rdeA* mutant is more or less the same as *regA* mutant. Furthermore if REGA cAMP-PDE is active in the *rdeA* mutant unlike the *regA* mutant, we could not explain the fact that ACB was detected in both mutants.

6.32 A possible complex of ACB and REGA

Membrane-bound forms of adenylyl cyclase have molecular weight; of 100-120 kDa and many bacterial adenylyl cyclases are 80-200 kDa (Yang and Epstein, 1983; Danchin, 1993). Soluble adenylyl cyclases are much smaller; the soluble AC in *Spodoptera frugiperda* (Sf9) cells is 25-40 kDa and the soluble AC in mammalian testis is 50-52 kDa (Kawabe et al., 1996; Braun, 1991). Since the band of interest in relation to a possible ACB-REGA complex is about 37,5 kDa, it might be the catalytic subunit of a multimeric enzyme, or ACB might be similar to a soluble adenylyl cyclase. It was found that ACB activity seemed to be present on the plasma membrane as well as in the cytoplasm (Kim et al., 1998). This result was interpreted as showing that ACB might resemble yeast adenylyl cyclase, a loose membrane-bound type of enzyme. When membrane fractions are separated from soluble fractions, the catalytic subunit of the yeast AC is detached from the membrane domain. It has been reported that there is a knock-out mutant of a novel adenylyl cyclase (Dr. W. Loomis, personal communication). He suggested that his adenylyl cyclase is similar to bacterial AC and that it might turn out to be ACB. There seems to be a good possibility that ACB is a membrane-bound AC and it can be imagined that the catalytic region of ACB interacts with REGA in the cytoplasm. All these ideas are speculative at this stage and experiments should certainly be carried out to test them.

6.33 What if PKA is regulated by an ACB-REGA complex?

It has been proposed that REGA-PDE is stimulated by binding to the PKA regulatory

subunit (Shaulsky et al., 1998). Therefore one could imagine that the ACB-REGA complex might include PKA as well. It seems an interesting possibility that the cAMP produced by ACB is dedicated to the activation of PKA and that the high PKA activity required for culmination is mainly dependent on ACB. As mentioned in chapter 4, it has been reported that ACB is present at high levels during culmination in wild type cells (P. Schaap, personal communication). It is possible that the RDEA-REGA phosphorelay system is active from the vegetative to the slug stage and then is somehow inhibited at culmination. For instance, ammonia depletion may turn off the phosphorelay, or RDEA, the intermediate component, may become inactive or unavailable. Possibly also phosphatases may be identified that shut down the relay. While the phosphorelay is active, the cAMP produced by ACB may be almost completely hydrolysed by the REGA-PDE and the PKA regulatory subunit may not have a chance to “see” this cAMP. When the phosphorelay becomes inactive around culmination, the cAMP produced by ACB would be protected by the absence of REGA-PDE activity. If PKA were situated close to ACB in a complex, all the cAMP made by ACB could bind to the PKA regulatory subunit. Then PKA activity could be greatly stimulated without a dramatic increase of total intracellular cAMP level.

According to this scheme in the rapidly developing mutants, the RDEA-REGA phosphorelay would be turned off throughout development. The cAMP produced by ACB would be protected even at the vegetative stage (Figure 3.6 to 3.9 ; Kim et al., 1998) and all the cAMP produced by ACB would be available to interact with the PKA R subunit. Hence the *rdeA* and *regA* mutants would have high PKA activity throughout their development without there being a markedly elevated level of total intracellular cAMP. If this idea is right, it would be possible to generalise that high

PKA activity is the main factor inducing rapid development in *rdeA*, *rdeC* and *regA* mutants. However the *rdeA* and *regA* mutants would not need to contain elevated intracellular cAMP levels and it will be remembered that I in fact did not detect elevated cAMP levels in *rdeA* (Chapter 3).

6.34 The presence of another intracellular cAMP-PDE

I have shown that there is probably at least one other intracellular PDE present in *Dictyostelium* (Figure 6.8). Purification of the protein(s) and study of their characteristics should be useful. When their genes are identified, it would be interesting if any of them turned out to be another response regulator-associated PDE.

Chapter 7

Chapter 7

General Discussion

7.1 Summary

Disruption of either the *rdeA* or *regA* gene leads to rapid development in *Dictyostelium*. The *rdeA* gene product displays homology to H2-type phosphotransferases in two component signalling systems while *regA* encodes a cAMP-phosphodiesterase with an associated response regulator (Chang et al., 1998 ; Shaulsky et al, 1996). It has therefore been proposed that RDEA activates the REGA-phosphodiesterase in a multi-step phosphorelay (Chang et al., 1998 ; Thomason et al., 1998). I have examined cAMP accumulation in *rdeA* and *regA* mutants and a novel adenylyl cyclase, ACB, was discovered during the course of that work (Kim et al., 1998). ACB is not regulated by G-proteins and is relatively insensitive to stimulation by Mn^{2+} ions. Addition of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) permitted detection of ACB in lysates of *aca*- cells. The fact that disruption of *rdeA* as well as inhibition of the REGA-phosphodiesterase by IBMX permitted detection of ACB strongly supports the idea that RDEA activates the REGA phosphodiesterase in a multi-step phosphorelay.

There has been growing evidence for phosphotransfer between RDEA and the REGA *in vitro* (Dr. Peter Thomason, personal communication) yet the yeast two-hybrid system could not demonstrate interaction between them. This implies that the interaction is very transient like a hit-and-run process.

Intracellular cAMP-phosphodiesterase activity is present during development of both *rdeA* and *regA* cells as well as wild type while REGA cAMP-PDE activity is present only in the wild type and *rdeA* but not in the *regA* mutant. ACB was detected in the *regA* and *rdeA* cells but not in the wild type in the absence of IBMX. These results may mean that cAMP made by ACB is destroyed exclusively by the REGA cAMP-PDE and therefore that ACB may exist in a complex with REGA.

7.2 Possible roles of ACB

It is possible to attribute PKA dependent gene expression early in development to the novel adenylyl cyclase. Discoidin I and the untranslatable, RNA polymerase II-dependent gene (*dutA*) are expressed in early development and their expression is completely dependent on the PKA catalytic activity (Kumimoto et al., 1996; Schulkes and Schaap, 1995). According to a recent report, ACB activity goes up before culmination and stays at a high level (Dr. P. Schaap, personal communication). This suggests that ACB could provide the intracellular cAMP to activate PKA throughout development or at least at culmination. Around the culmination stage the REGA phosphodiesterase may remain in the unphosphorylated form because of some kind of inhibition of the RDEA phosphotransferase. High levels of cAMP would then be produced by ACB and would induce high enough PKA activity for *Dictyostelium* to initiate fruiting body formation.

Ammonia depletion triggers culmination and this is thought to be due to elevation of PKA activity (Hopper et al., 1993; Gross, 1994). If ACB activity is inhibited by

cytosolic Ca^{2+} , this might explain how PKA is activated upon ammonia depletion: when ammonia is present, cytosolic Ca^{2+} levels are elevated and ACB activity is accordingly inhibited; PKA activity is not high enough to induce culmination because of insufficient cAMP provided by ACB. Once ammonia is depleted, cytosolic Ca^{2+} levels drop, ACB becomes active and culmination occurs due to high PKA activity induced by active ACB.

Pitt et al., 1993 have demonstrated that *aca-* cells are able to develop in response to pulses of cAMP followed by high continuous cAMP or 2H'-cAMP. It seems very well possible that the *aca-* cells possess ACB activity under these conditions even though it is undetectable in standard assays. The stimulus regime described above might somehow provide cellular conditions to activate ACB in the *aca-* cells so that high enough PKA activity was available for culmination. Alternatively ACB may be "constitutively " active and the extracellular cAMP stimuli satisfy other requirements for gene expression.

7.3 PKA regulation by an ACB-REGA complex

The results presented in chapter 6 suggested the idea that ACB may exist in complex with REGA. Furthermore REGA-PDE has been shown to be activated by binding to the PKA regulatory subunit (Shaulsky et al., 1998). Consequently the PKA catalytic subunit might be under control of an ACB-REGA-PKAR complex and so the cAMP produced by ACB could be dedicated to activating PKA. If the *rdeA-regA* phosphorelay becomes inactive at culmination as discussed in chapter 6, the cAMP

produced by ACB would be protected by the absence of the REGA-PDE activity and would be produced close to the PKA regulatory subunit. Therefore PKA activity would increase enough to trigger culmination without there needing to be a dramatic increase in intracellular cAMP levels.

The above idea suggests a novel mode of PKA regulation; PKA activity is down-regulated by a multi-step phosphorelay. As a downstream target of the crucial vertebrate hedgehog morphogens, PKA plays important roles in cell-type specification during the entire course of morphogenesis (Epstein et al., 1996 ; Hammerschmidt et al., 1996 ; Ohlmeyer and Kalderon, 1997). It will be interesting to search for similar pathways in higher eukaryotes in the fields of mammalian development, hormone action and signal transduction.

7.4 Mechanism of rapid development

In *rdeA* and *regA* mutants, the RDEA-REGA phosphorelay would be turned off throughout development. The cAMP produced by ACB would be protected by the absence of the REGA-PDE activity from the vegetative stage onwards (Figure 3.6 to 3.9; Kim et al., 1998) and all the cAMP due to ACB might be used to activate the PKA R subunit. If PKA, ACB and REGA were in a complex together, PKA would be efficiently activated by the cAMP made by ACB when REGA-PDE activity absent. Hence the *rdeA* and *regA* mutants could have high PKA activity throughout their development, but ACB might not produce a general elevation of intracellular cAMP levels. *rdeC* mutants lack a functional PKA regulatory subunit so that their PKA

activity is constitutive (Simon et al., 1992). One might therefore make a generalisation that high PKA activity is the main factor inducing rapid development in all three classes of mutant. cAMP-dependent protein kinase (PKA) is known to play an important role in many aspects of eukaryotic development (Blair, 1995) as well as in learning and memory (Kandel and Abel, 1995) and in *Dictyostelium*, genetic manipulation has demonstrated that it is essential for gene expression throughout development (Mann et al, 1997). Thus the level of PKA activity is likely to have a profound influence on the rate of development in many organisms.

Human somatic cells have a limited life span in culture and interestingly the same is true of the budding yeast *Saccharomyces cerevisiae*. Although there is as yet little evidence that PKA activity level controls the rate of development of the life span of most other eukaryotic organisms it does seem to influence life span in *S. cerevisiae* (Sun et al., 1994). Replicative life-span in this yeast was found to decrease on disruption of BCY1, which encodes the PKA regulatory subunit. This could perhaps be comparable to the rapid development of *rdeC* mutant of *Dictyostelium* which also lack a functional PKA R subunit. The molecular or cellular mechanism of ageing have been studied in *Caenorhabditis elegans*, *S. cerevisiae* and *Drosophila melanogaster* as well as in mice and men. (Hekimi et al., 1998 ; Proft et al., 1995 ; Gray et al., 1997). Two main views of ageing seem to have emerged: on the one hand animals with faster metabolic rates appear to have shorter life-span implying that damage resulting from metabolism and a counteracting response that can repair the damage may interact to determine life-span at the cellular level. On the other hand there is ample evidence

that life-span is genetically determined and that genetic factors can predominate over metabolic status (Guarente, 1997). It has been suggested that the genetics of ageing should be approached with genetically tractable organisms such as *C. elegans* and *Drosophila* (Martin, 1997). I believe that *Dictyostelium* is also a suitable organism for elucidating the molecular mechanisms of developmental rate and ageing.

7.5 A proposed multi-step phosphorelay

In addition to the phosphorelay systems of *Dictyostelium* discussed in this thesis multi-step phosphorelays have been reported in several other eukaryotic organisms such as *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Neurospora crassa* in addition to *Dictyostelium discoideum* (Posas et al., 1996 ; Chang et al., 1993 ; Alex et al., 1996). Multi-step phosphorelays may therefore be found to represent as important a conserved signalling pathway in eukaryotes as they are already known to be in many prokaryotes (Burbulys et al., 1991 ; Parkinson, 1993; Uhl and Miller, 1996 ; Bourret et al, 1996). The RDEA-REGA pathway of *Dictyostelium* is incomplete at this stage and a few candidates are available for the role of histidine kinase in the model such as dhkA, dokA and dhkC. (Wang et al., 1996 ; Loomis et al., 1997 ; Schuster et al., 1996 ; Singleton et al., 1998) However we are not sure about whether any of them is the upstream regulator of the RDEA and it will be of great interest to know how the RDEA-REGA pathway is controlled, by what histidine kinases, and what environmental stimuli.

Multi-step phosphorelay systems tend to include complex additional inputs. Thus when a response regulator is phosphorylated in response to environmental changes, specific phosphatases can reverse its state of phosphorylation or the phosphorylated response regulator can itself have auto-phosphatase activity (Lukat et al., 1992 ; Perego et al., 1994 ; Perego and Hoch, 1996 a,b). No phosphatase taking part in the RDEA-REGA pathway has yet been identified but it is likely that this phosphorelay will have additional components. Candidates may include a phosphatase dephosphorylating RDEA and/or REGA, a coupling protein to the RDEA contributing to the interaction between RDEA and REGA and possibly another intracellular cAMP-phosphodiesterase regulated by a response regulator. I have presented evidence for the presence of another intracellular cAMP-PDE in Chapter 6 and it interests me whether and how its presence is related to the phosphorelay. According to Dr. William Loomis the new adenylyl cyclase he has identified in a REMI mutant has an associated response regulator (personal communication). If that enzyme proves to be ACB, then the RDEA phosphorelay or a separate phosphorelay system may control ACB activity directly, as well as by controlling the life time of its product.

The structure of RDEA itself is of great interest. Secondary structure prediction analysis that I have carried out confirms that RDEA belongs to the superfamily of histidine phosphotransferases whose structure is composed of 5-6 α helices. ArcB, the cytoplasmic domain of a histidine phosphotransferase of *E.coli* has been crystallised (Kato et al., 1997) and it was shown that the critical histidine was located within one of the α helices and that the residue was exposed to solvent. Hence phosphates are accessible from the outside to this particular histidine residue packed in the α helice

bundle. Tertiary structure analysis gave a clear idea of how ArcB may function as a phosphotransferase. The results I obtained by secondary structure prediction indicated that the histidine residue in RDEA may have rather a unique position: Although the protein does have 5-6 α helices, the histidine (65) itself may be located in a loop between the 2nd and 3rd α helices, unlike in ArcB. The tertiary structure of RDEA obtained by NMR or crystallography should reveal how RDEA can accommodate such a difference and function efficiently as a histidine phosphotransferase.

References

References

- Abe, K., Saga, Y., Okada, H., and Yanagisawa, K. (1981) Differentiation of *Dictyostelium discoideum* mutant cells in a shaken suspension culture and the effect of cyclic AMP. *J. Cell. Sci.* 51: 131-142
- Abe, K., and Yanagisawa, K. (1983) A new class of rapidly developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. *Dev. Biol.* 95:200-210
- Aelst, L.V., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993) Complex formation between RAS and RAF and other protein kinases. *Proc. Natl. Acad. Sci. USA* 90:6213-6217
- Allen, J.B., Walberg, M.W., Edwards, M.C. and Elledge, S.J. (1995) Finding prospective partners in the library: the two-hybrid system and phage display find a match. *Trends in Bioch. Sciences* 20:511-516
- Ambros, V. and Moss, E.G. (1994) Heterochronic genes and the temporal control of *C. elegans* development. *Trends in Genet.* 10: 123-127
- Anjard, C., Pinaud, S., Kay, R. and Reymond, C.D. (1992) Overexpression of DdPK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*. *Development* 115:785-790
- Appleby, J.L., Parkinson, J.S. and Bourret, R.B. (1996) Signal transduction via the multi-step phosphorelay: not necessarily a road less travelled. *Cell* 86:845-848
- Barber, R. and Butcher, R.W. (1980) The turnover of cyclic AMP in cultured fibroblasts. *J. Cyclic. Nucleotide Res.* 6:3-14
- Beavo, J.A. (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiological Reviews* 75:725-748
- Bhat, N.R. (1995) Signal transduction mechanisms in glial cells. *Dev. Neurosci.* 17: 267-284
- Biondi, R. M., Schneider, B., Passeron, E., and Passeron, S. (1998). Role of Mg²⁺ in

- nucleoside diphosphate kinase autophosphorylation. Arch. Biochem. Biophys. 353: 85-92
- Bominaar, A. A., Kesbeke, F. and Van Haastert, P. J. (1994) Phospholipase C in *Dictyostelium discoideum*. Cyclic AMP surface receptor and G-protein-regulated activity in vitro. Biochem. J. 297: 181-187
- Bonner, J.T. (1947) Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. J. Exp. Zoo. 106: 1-26
- Braun, T. (1991) Purification of soluble form of adenylyl cyclase from testes. Methods. Enzymol. 195:130-136
- Braun, T. and Dods, R.F. (1975) Development of a Mn-2+-sensitive, "soluble" adenylyl cyclase in rat testis. Proc. Natl. Acad. Sci. USA 72:1097-1101
- Bremer, H. and Dennis, P. P. (1987) Modulation of chemical composition and other parameters of the cell by growth rate. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, pp. 1527-1542. Edited by F. C. Neidhardt and others. Washington DC. American Society for Microbiology.
- Brenner, M. (1978). Cyclic AMP levels and turnover during development of the cellular slime mold *Dictyostelium discoideum*. Dev. Biol. 64: 210-223
- Brenner, M. and Thoms, S. D. (1984) Caffeine blocks activation of cyclic AMP synthesis in *Dictyostelium discoideum*. Dev. Biol. 101: 136-146
- Brookman, J. J., Jermyn, K. A. and Kay, R. R. (1987) Nature and distribution of the morphogen DIF in the *Dictyostelium* slug. Development 100: 119-124
- Brookman, J.J., Town, C.D., Jermyn, K.A. and Kay, R.R. (1982) Developmental regulation of stalk cell differentiation-inducing factor in *Dictyostelium discoideum*. Dev. Biol. 91: 191-196
- Chang, W. T., Thomason, P. A., Gross, J. D. and Newell, P. C. (1998) Evidence that the RdeA protein is a component of a multistep phosphorelay modulating rate of development in *Dictyostelium*. EMBO J. 17: 2809-2816

Chen, M.-Y., Insall, R. and Devreotes., P. (1996) Signalling through chemoattractant receptors in *Dictyostelium*. Trends in Genet.12:52-57

Chern, Y., Lee, E.H., Lai, H.L., Wang, H.L., Lee, Y.C. and Ching, Y.H. (1996) Circadian rhythm in the Ca(2+)-inhibitable adenylyl activity of the rat striatum. FEBS Lett. 385: 205-208

Chien, C.T., Bartel, P.L., Sternglans, R and Fields, S. (1991) The two-hybrid system; A method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA 88:9578-9582

Clarke, M., Kayman, S.C. and Riley, K. (1987) Density-dependent induction of discoidin-I synthesis in exponentially growing cells of *Dictyostelium discoideum*. Differentiation 34:79-87

Conchita, C.G.M., Schulkes, Irene Verkerke-van Wijk and Schaap, P. (1995) Transformation with vectors harbouring the NEO^R selection marker induces germination-specific adenylyl cyclase activity in *Dictyostelium* cells. Exp. Cell Res. 220:505-508

Coukell, M. B., Cameron, A. M., and Adames, N. R. (1992). Involvement of intracellular calcium in protein secretion in *Dictyostelium discoideum*. J. Cell. Sci. 103: 371-380

Coukell, M. B. and Chan, F. K. (1980) The precocious appearance and activation of an adenylate cyclase in a rapid developing mutant of *Dictyostelium discoideum*. FEBS Lett. 110: 39-42

Danchin, A. (1993) Phylogeny of adenylyl cyclases. Adv. Second Messenger Phosphoprotein Res. 27:109-162

Darmon, M. and Klein, S.C. (1978) Effects of amino acids and glucose on adenyl cyclase and cell differentiation of *Dictyostelium discoideum*. Dev. Biol. 63:377-389

Darnell, J., Lodish, H. and Baltimore, D. Chapter 11: Gene control and the molecular genetics of development in eukaryotes in Molecular Cell Biology. Scientific American Books. pp 411-413, 2nd edition (1990)

Da Silveira, J.F., Zingales, B. and Colli, W. (1977) Characterisation of an adenylyl cyclase activity in particulate preparations from epimastigote forms of *Trypanosoma*

cruzi. Biochim. Biophys. Acta. 481:722-733

Datta, S. Gomer, R.H., and Firtel, R.A. (1986) Spatial and temporal regulation of a foreign gene by a prestalk-specific promoter in transformed *Dictyostelium discoideum*. Mol. Cell. Biol. 6: 811-821

Davies, L., Satre, M., Martin, J. B., and Gross, J. D. (1993). The target of ammonia action in *Dictyostelium*. Cell 75: 321-327

de Gunzburg, J., Franke, J., Kessin, R.H., and Veron, M. (1986) Detection and developmental regulation of the mRNA for the regulatory subunit of the cAMP-dependent protein kinase of *D. discoideum* by cell-free translation. EMBO J. 5: 363-367

de Gunzburg, Part, D., Guiso, N. and Veron, M. (1984) An unusual adenosine 3',5'-phosphate dependent protein kinase from *Dictyostelium discoideum*. Biochemistry 23: 3805-3812

Devreotes, P. N. (1983). The effect of folic acid on cAMP-elicited cAMP production in *Dictyostelium discoideum*. Dev. Biol. 95: 154-162

Devreotes, P. N. (1994). G protein-linked signaling pathways control the developmental program of *Dictyostelium*. Neuron 12: 235-241

Djordjevic, S., Goudreau, P.N., Xu, Q., Stock, A.M. and West, A.H. (1998) Structural basis for methylesterase CheB regulation by a phosphorylation-activation domain. Proc. Natl. Acad. Sci. USA 95:1381-1386

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes and Dev. 7:555-569

Early, V.E., and Williams J.G. (1988) A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro. Development 103: 519-523

Epstein, D.J., Marti, E., Scott, M.P. and McMahon, A.P. (1996) Antagonising cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signalling pathway. Development 122:2885-2894

Fields, S. and Sternglanz, R. (1994) The two-hybrid system: an assay for protein-protein interactions. *Trends in Genetics* 10:286-292

Firtel, R. A. (1995). Integration of signalling information in controlling cell-fate decisions in *Dictyostelium*. *Genes Dev* 9: 1427-1444

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

Flick, J.S. and Johnston, M. (1990) Two systems of glucose repression of the GAL1 promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10:4757-4769

Franke, J., and Kessin, R. (1977) A defined minimal medium for axenic strains of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* 74:2157-2161

Franke, J., and Kessin, R. (1992) The cyclic nucleotide phosphodiesterases of *Dictyostelium discoideum*: Molecular genetics and biochemistry. *Cell. Signal.* 4: 471-478

Gee, K., Russell, F., and Gross, J. (1994). Ammonia hypersensitivity of slugger mutants of *D. discoideum*. *J Cell Sci* 107: 701-708

Gray, M.D., Shen, J.C., Kamath-Loeb, A.S., Blank, A, Sopher, B.L., Martin, G.M., Oshima, J. and Loeb, L.A. (1997) The Werner syndrom protein is a DNA helicase. *Nature Genetics* 17:100-103

Gross, J. D. (1994) Developmental decisions in *Dictyostelium discoideum*. *Microbiol. Rev.* 58:330-351

Gross, J.D., Bradbury, J., Kay, R.R., and Peacey, M.J. (1983) Intracellular pH and the control of cell differentiation in *Dictyostelium discoideum*. *Nature* 303: 244-245

Gross, J.D., Peacey, M.J. and von Strandmann, R.P. (1988) Plasma membrane proton pump inhibition and stalk cell differentiation in *Dictyostelium discoideum*. *Differentiation* 38: 91-98

Guarente, L. (1997) What makes us tick? *Science* 275:943-944

- Hadas, H., Einav, M., Fishov, I. And Zaritsky, A. (1997) Bacteriophage T4 development depends on the physiology of its host *Escherichia coli*. *Microbiology*. 143: 179-185
- Hammerschmidt, M., Bitgood, M.J. and McMahon, A.P. (1996) Protein kinase A is a common negative regulator of Hedgehog signalling in the vertebrate embryo. *Genes and Dev*. 10:647-658
- Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805-816
- Harwood, A.J., Early, A., and Williams, J.G. (1993) A repressor controls the timing and spatial localisation of stalk cell-specific gene expression in *Dictyostelium*. *Development* 118: 1041-1048
- Harwood, A.J., Hopper, N.A., Simon, M.N., Bouzid. S., Veron. M., and Williams, J.G. (1992a) Multiple roles for cAMP-dependent protein kinases during *Dictyostelium* development. *Dev. Biol.* 149:90-99
- Harwood, A.J., Hopper, N.A., Simon, M.N., Driscoll, D.M., Veron, M., and Williams, J.G. (1992b) Culmination in *Dictyostelium* is regulated by cAMP-dependent protein kinase. *Cell* 69: 615-624
- Harwood, A., Plyte, S. Woodgett, J. Strutt, H. and Kay, R. (1995) Glycogen synthase kinase 3 regulates cell fate in *Dictyostelium*. *Cell* 80:139-148
- Heideman, W., Casperson, G.F. and Bourne, H.R. (1987) Adenylyl cyclase in yeast. Hydrodynamic properties and activation by trypsin. *J. Biol. Chem.* 262:07087-7091
- Hekimi, S., Lakowski, B., Barnes, T.M. and Ewbank, J.J. (1998) Molecular genetics of life span in *C. elegans*: how much does it teach us? *Trends in Genet.* 14:14-19
- Henderson, E. J. (1975) The cyclic adenosine 3':5'-monophosphate receptor of *Dictyostelium discoideum*. Binding characteristics of aggregation-competent cells and variation of binding levels during the life cycle. *J. Biol. Chem.* 250: 4730-4736
- Hereld, D., and Devreotes, P. N. (1992). The cAMP receptor family of *Dictyostelium*. *Int. Rev. Cytol.* 35-47.

- Hicks, K.A., Millar, A.J., Carre, I.A., Somers, D.E., Straume, M., Meeks, Wagner, D.R. and Kay, S.A. (1996) Conditional circadian dysfunction of the *Arabidopsis* early-flowering 3 mutant. *Science* 274:790-792
- Hoch, J.A. (1993) Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annu. Rev. Microbiol.* 47:441-465
- Hopper, N.A., Anjard, C. Reymond, C.D., and Williams, J.G. (1993a) Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinases and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. *Development* 119: 147-154
- Hopper, N.A., Harwood, A.J., Bouzid. S., Veron. M., and Williams, J.G. (1993b) Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP-dependent protein kinases and evidence for its upstream regulation by ammonia. *EMBO J.* 12: 2459-2466
- Insall, R., Kuspa, A., Lilly, P.J., Shaulsky, G., Levin, L.R., Loomis, W.F., and Devreotes, P. (1994) CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. *J. Cell Biol.* 126:1537-1545
- Ishige, K., Nagasawa, S., Tokishita, S. and Muzuno, T. (1994) A novel device of bacterial signal transducers. *EMBO J.* 13:5195-5202
- Islam, M.S., Leibiger, I., Leibiger, B., Rossi, D., Sorrentino, V., Ekstrom, T.J., Westerblad, H., Andrade, F.H. and Berggren, P.O. (1998) In situ activation of the type 2 ryanodine receptor in pancreatic beta cells requires cAMP-dependent phosphorylation. *Proc. Natl. Acad. Sci. USA* 95:6145-6150
- Jacobshagen, S., Kindle, K.L. and Johnson, C.H. (1996) Transcription of CABII is regulated by the biological clock in *Chlamydomonas reinhardtii*. *Plant. Mol. Biol.* 31:1173-1184
- Jermyn, K.A., Berks, M., Kay, R.R., and Williams, J.G. (1987) Two distinct classes of prestalk-enriched mRNA sequence in *Dictyostelium discoideum*. *Development* 100:745-755

Jermyn, K.A., Duffy, K.T.I., and Williams, J.G. (1989) A new anatomy of the prestalk zone in *Dictyostelium*. *Nature* 340:144-146

Jermyn, K.A., and Williams, J.G. (1991) An analysis of culmination in *Dictyostelium* using prestalk and stalk-specific cell autonomous markers. *Development* 111:779-787

Johnson, R. L., Saxe, C. L. d., Gollop, R., Kimmel, A. R. and Devreotes, P. N. (1993) Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. *Genes Dev.* 7: 273-282

Johnson, R.L., Van Haastert, P.J.M., Kimmel, A.R., Saxe, C.L., Jastorff, B., and Devreotes, P.N. (1992) The cyclic nucleotide specificity of three cAMP receptors in *Dictyostelium*. *J. Biol. Chem.* 267:4600-4607

Jonassen, T., Proft, M., Randez-Gil, F., Schultz, J. R., Marbois, B. N., Entian, K.Dd. and Clarke, C. F. (1998). Yeast Clk-1 homologue (Coq7/Cat5) is a mitochondrial protein in coenzyme Q synthesis. *J. Biol. Chem.* 273: 3351-3357

Kale, S.P. and Jazwinski, S.M. (1996) Differential response to UV stress and DNA damage during the yeast replicative life span. *Dev. Genet.* 18:154-160

Kandel, E. and Abel, T. (1995) Neuropeptides, adenylyl cyclase, and memory storage *Science* 268: 825-826

Kato, M., Mizuno, T., Shimizu, T and Hakoshima, T. (1997) Insights into multistep phosphorelay from the crystal structure of the C-terminal HPt domain of ArcB. *Cell* 88:717-723

Kawabe, J., Toya, Y., Schwencke, C., Oka, N, Ebina, T. and Ishikawa, Y. (1996) Soluble adenylyl cyclase from *Spodoptera frugiperda* (Sf9) cells. Purification and biochemical characterisation. *J. Biol. Chem.* 271:20132-20137

Kay, R. R. (1987) Cell differentiation in monolayers and the investigation of slime mold morphogens. *Methods Cell Biol.* 28: 433-448

Kay, R.R. (1989) Evidence that elevated intracellular cyclic AMP triggers spore maturation in *Dictyostelium*. *Development* 105:753-759

Kay, R. R., Dhokia, B., and Jermyn, K. A. (1983) Purification of stalk-cell-inducing morphogens from *Dictyostelium discoideum*. Eur. J. Biochem. 136: 51-56

Kay, R.R. and Jermyn, K.A. (1983) A possible morphogen controlling differentiation in *Dictyostelium*. Nature 303: 242-244

Kennaway, D. J. (1998). Generation and entrainment of biological rhythms. Clin. Exp. Pharmacol. Physiol. 25: 862-865

Kim, H.J., Chang, W.T., Meima, M., Gross, J.D. and Schaap, P. (1998) A novel adenylyl cyclase detected in rapidly developing mutants of *Dictyostelium*. J. Biol. Chem. 273:30859-30862

Kluxen, F.W., Hulster, A., Droste, M. and Pfeuffer, T. (1996) The expression of a novel Ca²⁺/CaM stimulated adenylyl cyclase activity in the neuroblastoma cell line Lan-1 is regulated by cell density. Brain. Res. 740:169-174

Knecht, D.A., Cohen, S.M., Loomis, W.F., and Lodish, H.F. (1986) Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low-copy and high-copy transformation vectors. Mol. Cell. Biol. 6: 3973-3983

Kondo, T., Mori, T., Lebedeva, N.V., Aoki, S., Ishiura, M. and Golden, S.S. (1997) Circadian rhythms in rapidly dividing cyanobacteria. Science 275:224-227

Kopachik, W., Oohata, A., Dhokia, B., Brookman, J. J., and Kay, R. R. (1983) *Dictyostelium* mutants lacking DIF, a putative morphogen. Cell 33: 397-403

Kumimoto, H., Yoshida, H., and Okamoto, K. (1996) Expression of *Dictyostelium* early gene, dutA, is independent of cAMP pulses but dependent on protein kinase A. FEMS Microbiol. Lett. 140: 121-124

Kyriacou, C. P., Oldroyd, M., Wood, J., Sharp, M. and Hill, M. (1990) Clock mutations alter developmental timing in *Drosophila*. Heredity Edinburgh 64: 395-401

Lacombe, M-L., Podgorski, G.J., Franke, J., and Kessin, R.H. (1986) Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum*. J. Biol. Chem. 261:16811-16817

Lakowski, B. and Hekimi, S. (1996). Determination of life span in *C. elegans* by four clock genes. *Science* 272: 1010-1013

Lilly, P. J., and Devreotes, P. N. (1995) Chemoattractant and GTP gamma S-mediated stimulation of adenylyl cyclase in *Dictyostelium* requires translocation of CRAC to membranes. *J. Cell Biol.* 129: 1659-1665

Liu, G., Kuwayama, H., Ishida, S., and Newell, P. C. (1993). The role of cyclic GMP in regulating myosin during chemotaxis of *Dictyostelium*: evidence from a mutant lacking the normal cyclic GMP response to cyclic AMP. *J. Cell Sci.* 106: 591-595

Liu, G., and Newell, P. C. (1991) Evidence of cyclic GMP may regulate the association of myosin II heavy chain with the cytoskeleton by inhibiting its phosphorylation. *J. Cell Sci.* 98: 483-490

Liu, G., and Newell, P. C. (1994) Regulation of myosin regulatory light chain phosphorylation via cyclic GMP during chemotaxis of *Dictyostelium*. *J. Cell Sci.* 107 1737-1743

Loomis, W.F., Shaulsky, G., Wang, N. (1997) Histidine kinases in signal transduction pathways of eukaryotes. *J. Cell Sci.* 110:1141-1145

Louis, J., Ginsburg, G., and Kimmel, A. (1994) A cAMP receptor regulates axial patterning and cellular differentiation during late development of *Dictyostelium*. *Genes Dev.* 8: 2052-2061

Lukat, G.S., McCleary, W.R., Stock, A.M. and Stock, J.B. (1992) Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. *Proc. Natl. Acad. Sci. USA* 89:718-722

Maeda, Y., Ohmori, T., Abe, T., Abe, F., and Amagai, A. (1989) Transition of starving *Dictyostelium* cells to differentiation phase at a particular position of the cell cycle. *Differentiation* 41:169-175

Mann, S. K., Brown, J. M., Briscoe, C., Parent, C., Pitt, G., Devreotes, P. N., and Firtel, R. A. (1997). Role of cAMP-dependent protein kinase in controlling aggregation and postaggregative development in *Dictyostelium*. *Dev. Biol.* 183: 208-221

Mann, S.K.O., Firtel, R.A. (1991) A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech. Dev.* 35:89-101

Mann, S.K.O., Firtel, R.A. (1993) cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during *Dictyostelium* development. *Development* 119: 135-146

Mann, S., Richardson, D., Lee, S., Kimmel, A., and Firtel, R.A. (1994) Expression of cAMP-dependent protein kinase in prespore cells is sufficient to induce spore cell differentiation in *Dictyostelium*. *Proc. Natl. Acad. Sci. USA* 91: 10561-10565

Martin, G. M. (1978). Genetic syndromes in man with potential relevance to the pathobiology of ageing. *Birth Defects* 14: 5-39

Martin, G.M. (1997) Genetics and the pathology of ageing. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 352:1773-1780

Newell, P. C., Malchow, D., and Gross, J. D. (1995) The role of calcium in aggregation and development of *Dictyostelium*. *Experientia* 51: 1155-1165

Newell, P. C., Telser, A., and Sussman, M. (1969) Alternative developmental pathways determined by environmental conditions in the cellular slime mold *Dictyostelium discoideum*. *J. Bacteriol.* 100: 763-768

Ohlmeyer, J.T. and Kalderon, D. (1997) Dual pathways for induction of wingless expression by protein kinase A and Hedgehog in *Drosophila* embryos. *Genes Dev.* 11: 2250-2258

Parent, C. A., and Devreotes, P. N. (1996) Molecular genetics of signal transduction in *Dictyostelium*. *Annu. Rev. Biochem.* 65: 411-440

Paterson, R.G., Johnson, M.L. and Lamb, R.A. (1997) Paramyxovirus fusion (F) protein and hemagglutinin-neuraminidase (HN) protein interactions: intracellular retention of F and HN does not affect transport of the homotypic HN or F protein. *Virology* 237:1-9

Perego, M., Hanstein, C., Welsh, K.M., Djavakhishvii, T., Glaser, P. and Hoch, J.A. (1994) Multiple protein-aspartate phosphatases provide a mechanism for the integration

of diverse signals in the control of development in *B. subtilis*. *Cell* 70:1047-1055

Perego, M. and Hoch, J.A. (1996a) Protein aspartate phosphatases control the output of two-component signal transduction systems. *Trends in Genet.* 12:97-101

Perego, M. and Hoch, J.A. (1996b) Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* 93:1549-1533

Pieroni, J.P., Harry, A., Chen, J., Jacobowitz, O., Magnusson, R.P. and Iyengar, R. (1995) Distinct characteristics of the basal activities of adenylyl cyclases 2 and 6. *J. Biol. Chem.* 270:21368-21373

Pitt, G.S., Milona, N., Borleis, J., Lin, K.C., Reed, R.R., and Devreotes, P.N. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell* 69: 305-315

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

Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C. and Saito, H. (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 “two-component” osmosensor. *Cell* 86:865-875

Raper, K.B. (1940) Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. Elisha. Mitchell. Sci. Soc.* 56: 241-282

Ray, A. and Prefontaine, K.E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA* 91:752-756

Rooney, E. K., and Gross, J. D. (1992). ATP-driven Ca²⁺/H⁺ antiport in acid vesicles from *Dictyostelium*. *Proc Natl Acad Sci USA* 89: 8025-8029

Ross, F. M., and Newell, P. C. (1979). Genetics of aggregation pattern mutations in the

cellular slime mould *Dictyostelium discoideum*. J. Gen. Microbiol. 115: 289-300

Ross, F. M., and Newell, P. C. (1981). Streamers: chemotactic mutants of *Dictyostelium discoideum* with altered cyclic GMP metabolism. J. Gen. Microbiol. 127: 339-350

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

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

Schaap, P., Brandt, R., and van Es, S. (1995). Regulation of *Dictyostelium* adenylylcyclases by morphogen-induced modulation of cytosolic pH or Ca²⁺ levels. Dev. Biol. 168: 179-188

Schindler, J., and Sussman, M. (1977). Ammonia determines the choice of morphogenetic pathways in *Dictyostelium discoideum*. J. Mol. Biol. 116: 161-169

Schulkes, C., and Schaap, P. (1995). cAMP-dependent protein kinase activity is essential for preaggregative gene expression in *Dictyostelium*. FEBS Lett. 368: 381-384

Schuster, S. S., Noegel, A. A., Oehme, F., Gerisch, G., and Simon, M. I. (1996). The hybrid histidine kinase DokA is part of the osmotic response system of *Dictyostelium*. EMBO J. 15: 3880-3889

Shaulsky, G., Escalante, R., and Loomis, W. F. (1996). Developmental signal transduction pathways uncovered by genetic suppressors. Proc. Natl. Acad. Sci. USA 93: 15260-15265

Shaulsky, G., Fuller, D., and Loomis, W. F. (1998). A cAMP-phosphodiesterase controls PKA-dependent differentiation. Development 125: 691-699

Siegert, F., and Weijer, C.J. (1992) Three-dimensional scroll waves organize *Dictyostelium* slugs. Proc. Natl. Acad. Sci. USA 89:6433-6437

- Simon, M. N., Driscoll, D., Mutzel, R., Part, D., Williams, J., and Veron, M. (1989). Overproduction of the regulatory subunit of the cAMP-dependent protein kinase blocks the differentiation of *Dictyostelium discoideum*. EMBO J. 8: 2039-2943
- Simon, M. N., Pelegri, O., Veron, M., and Kay, R. R. (1992). Mutation of protein kinase A causes heterochronic development of *Dictyostelium*. Nature 356: 171-172
- Singleton, C.K., Zinda, M.J., Mykytka, B. and Yang, P. (1998) The histidine kinase dhkC regulates the choice between migrating slugs and terminal differentiation in *Dictyostelium discoideum*. Dev. Biol. 203:345-357
- Sobolewski, A., Kwong, L., and Weeks, G. (1988) Stalk cell formation in monolayers of *Dictyostelium discoideum* V12-M2. Dev. Genet. 9: 597-605
- Sollner, T., Rassow, J., Wiedmann, M., Schlossmann, J., Keil, P., Neupert. W. and Pfanner, N. (1992) Mapping of the protein import machinery in the mitochondrial outer membrane by crosslinking of translocation intermediates. Nature 355:84-87
- Somers, D.E., Webb, A.A., Pearson, M. and Kay, S.A. (1998) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. Development 125:485-494
- Sun, T.J., and Devreotes, P.N. (1991) Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. Genes Dev. 5: 572-582
- Sun, J., Kale, S.P., Childress, A.M., Pinswasdi, C. and Jazwinski, S.M. (1994) Divergent roles of RAS1 and RAS2 in yeast longevity. J. Biol. Chem. 269:18638-18645
- Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. Methods Cell Biol. 28: 9-29
- Sutoh, K. (1993). A transformation vector for *Dictyostelium discoideum* with a new selectable marker bsr. Plasmid 30: 150-154
- Suzuki, N., Shimamoto, A., Imamura, O., Kuromitsu, J., Kitao, S., Goto, M. and Furuichi, Y. (1997). DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system Nucleic Acids Res. 25: 2973-2978

Swanson, R.V., Schuster, S.C. and Simon, M.I. (1993) Expression of CheA fragments which define domains encoding kinase, phosphotransfer, and CheY binding activities. *Biochemistry* 32:7623-7629

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

Thomasson, P. A., Traynor, D., Cavet, G., Chang, W. T., Harwood, A. J., and Kay, R. R. (1998) An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*. *EMBO J.* 17: 2838-2845

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680

Touhara, K., Inglese, J., Pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994) Binding of G protein $\beta\gamma$ -subunits to pleckstrin homology domains. *J. Biol. Chem.* 269:10217-10220

Town, C.D., Gross, J.D., Kay, R.R. (1976) Cell differentiation without morphogenesis in *Dictyostelium discoideum*. *Nature* 262: 717-719

van Es, S., Viridy, K. J., Pitt, G. S., Meima, M., Sands, T. W., Devreotes, P. N., Cotter, D. A., and Schaap, P. (1996). Adenylyl cyclase G, an osmosensor controlling germination of *Dictyostelium* spores. *J. Biol. Chem.* 271: 23623-23625

van Haastert, P.J.M. (1984) A method for studying cAMP-relay in *Dictyostelium discoideum*: the effect of temperature on cAMP-relay. *J. General Micro.* 130:2559-2564

Wang, M., Aerts R.J., Spek, W., and Schaap, P. (1988) Cell cycle phase in *Dictyostelium discoideum* is correlated with the expression of cyclic AMP production, detection, and degradation. Involvement of cyclic AMP signalling in cell sorting. *Dev. Biol.* 125: 410-416

Wang, B., and Kuspa, A. (1997). *Dictyostelium development* in the absence of cAMP

Science 277: 251-254

Wang, M.M. and Reed, R.R. (1993) Molecular cloning of the olfactory neuronal transcriptional factor Olf-1 by genetic selection in yeast. *Nature* 364:121-126

Wang, M., and Schaap, P. (1985) Correlation between tip dominance, prestalk/prespore pattern, and cAMP relay efficiency in slugs of *Dictyostelium discoideum*. *Differentiation* 30: 7-14

Wang, N., Shaulsky, G., Escalante, R., and Loomis, W.F. (1996) A two-component histidine kinase gene that functions in *Dictyostelium* development. *EMBO J.* 15:3890-3898

Wang, Z.Y. and Tobin, E.M. (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93:1207-1217

Ward, M.P., Gimeno, C.J., Fink, G.R. and Garrett, S. (1995) SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. *Mol. Cell Biol.* 15: 6854-6863

Watts, D. J., and Ashworth, J. M. (1970). Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* 119: 171-174

Weijer, C. J., Duschl, G., and David, C. N. (1984). A revision of the *Dictyostelium discoideum* cell cycle. *J. Cell Sci.* 70: 111-131

Williams, J. (1997) Prestalk and stalk cell heterogeneity in *Dictyostelium*. In Maeda, Y., Inouye, K., and Takeuchi, I., (ed.), *Dictyostelium-A model system for cell and developmental biology*. Universal Academy Press, Tokyo, pp. 293-304

Williams, J., Hopper, N., Early, A., Traynor, D., Harwood, A., Abe, T., Simon, M., and Veron, M.(1993) Interacting signalling pathways regulating prestalk cell differentiation and movement during the morphogenesis of *Dictyostelium*. *Development (Suppl)*: 1-7

Williams, J.G., Ceccarelli, A., McRobbie, S., Mahbubani H., Kay, R.R., Early, A., Berks, M. and Jermyn, K.A. (1987) Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphagen. *Cell* 49:185-192

Williams, J.G., Duffy, K.T., Lane, D.P., McRobbie, S.J., Harwood, A.J., Traynor, D., Kay, R.R., and Jermyn, K.A. (1989) Origins of the prestalk-prespore pattern in *Dictyostelium*. Development. Cell 59:1157-1163

Wu, L. and Franke, J. (1990) A developmentally regulated and cAMP-repressible gene of *Dictyostelium* discoideum: cloning and expression of the gene encoding cyclic nucleotide phosphodiesterase inhibitor. Gene 91:51-56

Wu, L., Hansen, D., Franke, J., Kessin, R.H., and Podgorski, G.J. (1995) Regulation of *Dictyostelium* early development genes in signal transduction mutants. Dev. Biol. 171:149-158

Yang, J. K. and Epstein, W. (1983) Purification and characterisation of adenylate cyclase from *Escherichia coli* K12. J. Biol. Chem. 258:3750-3758

Yang, X., Hubbard, J.A. and Carlson, M. (1992) A protein kinase substrate identified by the two-hybrid system. Science 257:680-682

Yonemura, I., Motoyama, T., Hasekura, H. and Boettcher, B. (1991). Relationship between genotypes of longevity genes and developmental speed in *Drosophila melanogaster*. Heredity Edinburgh. 66: 143-149

Publications

Publications

Kim, H.J., Chang, W.T., Meima, M., Gross, J.D. and Schaap, P. (1998) A novel adenylyl cyclase detected in rapidly developing mutants of *Dictyostelium*. J. Biol. Chem. 273:30859-30862