Characterization of Two Polypeptides Encoded by a Single Gene in *Dictyostelium discoideum*

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Abstract

The cAMP binding protein CABP1 isolated from Dictyostelium discoideum consists of two subunits, CABP1A and CABP1B, which are produced from a single gene by an unusual splicing mechanism. To characterize this mechanism, an actin-CABP1 fusion gene was constructed and introduced into D. discoideum cells by DNA-mediated transformation. Analysis of these cells demonstrated that the transcripts derived from the fusion gene were properly processed to generate both CABP1A and CABP1B. However, when the 5' splice site in the gene was mutated to conform to the D. discoideum consensus, only CABP1B was produced. These results suggest that the splicing event is regulated by the 5' splice site. By screening a cDNA library under conditions of reduced stringency with a sequence encoding CABP1, clones which code for two closely related molecules were isolated. Hybrid selection experiments indicated that these cDNAs encode polypeptides with molecular weights of 34,000 (p34) and 31,000 (p31), both of which are recognized by anti-CABP1 antibodies. Similar to the two subunits of CABP1, these two molecules appear to be encoded by a single gene and are probably generated by the same splicing mechanism described above. The CABP1 and p34/31 genes probably arose by duplication. Disruption of the gene which encodes p34 and p31 demonstrated that these two polypeptides appear to play a role in both growth and development of D. discoideum.

Résumé

La protéine CABP1, isolée de Dictyostelium discoideum, a la capacité de lier l'AMP cyclique Elle consiste en deux sous-unites, CABP1A et CABP1B, qui sont dérivées d'un gène unique par un mecanisme d'épissage inhabituel. Afin de caracteriser ce mecanisme, un gene hybride a eté obtenu par la fusion des gènes actine et CABP1 et a ete introduit dans les cellules de D. discoideum par transformation L'analyse de ces cellules a montré que les ARNm provenant de la fusion étaient épissés correctement et produisaient les deux sous-unités CABP1A et CABP1B. Cependant, lorsque le site 5' d'epissage du gene CABP1 est modifié afin d'être identique au consensus chez D discoideum, seule la sous-unité CABP1B est produite Ces resultats suggèrent que l'épissage est regulé par le site 5'. Le criblage d'une banque d'ADNc à l'aide d'une sequence codante de CABP1, dans des conditions de stringence réduite, a permis d'isoler des clones codant pour deux molécules apparentees à CABP1 La technique de sélection par hybridation indique que ces ADNc produisent des polypeptides, p34 et p31, dont le poids moléculaire est 34,000 et 31,000 respectivement Ces deux polypeptides sont reconnus par des anticorps anti-CABP1. De même que les deux sous-unites de CABP1, ces deux molécules semblent être le produit d'un gène unique et sont vraisemblablement générées par le mécanisme d'épissage decrit ci-dessus. Les gènes CABP1 et p34/31 résultent probablement d'une duplication. L'interruption du gène p34/31 montre que ces deux polypeptides semblent jouer un rôle dans la croissance et le développement de D. discoideum

Contributions to Original Knowledge

1. I have demonstrated that the two subunits of the D. discoideum cAMP binding protein CABP1 are generated from a single gene by an unusual splicing mechanism which is regulated by a deviation in the 5' splice site from the consensus sequence.

2. I have demonstrated that two polypeptides which crossreact with anti-CABP1 antibodies, p34 and p31, are in fact very closely related to CABP1

3. I have demonstrated that p34 and p31, like the two subunits of CABP1, are generated from a single gene. The extensive similarity between these four polypeptides suggests that the corresponding genes arose by duplication followed by sequence divergence. Therefore, CABP1 belongs to at least a small gene family.

4. I have demonstrated that p34 and p31 play important roles in both growth and development. Mutants in which the p34/31 gene has been disrupted grow slowly when feeding on bacteria and also show a slight delay in completing development.

5. I have demonstrated that p34 and p31, like CABP1, share significant homology with two polypeptides encoded by a bacterial plasmid which confers resistance to tellurium anions.

Preface and Acknowledgements

This thesis is assembled in accordance with the regulations of the Faculty of Graduate Studies and Research It consists of an Abstract, Résumé, Introduction (Chapter 1), Materials and Methods (Chapter 2), Results (Chapter 3), Discussion (Chapter 4), and Literature Cited.

A preliminary description of some of the data presented here can be found in the following publication:

Tsang, A , Grant, C., Kay, C Bain, G., Greenwood, M , Noce, T and Tasaka, M. (1988) Characterization of an unusual cAMP receptor and its related polypeptides in Dictyostelium discoideum Dev Genet. 9:237-245.

Three additional manuscripts have been prepared and submitted for publication:

Bain, G., Grant, C. and Tsang, A. CABP1-related polypeptides in Dictyostelium discoideum: Isolation and characterization of cDNA clones.

Bain, G. and Tsang, A. Functional analysis of two CABP1-related polypeptides in Dictyostelium discoideum by gene disruption

Grant, C., Bain, G. and Tsang, A. The molecular basis for alternative splicing of the CABP1 transcripts in Dictyostelium discoideum.

All of the data presented in this thesis is the work of the author with the following exceptions:

1 The plasmid cDNA library was constructed by Caroline Grant. She also screened it and identified the resulting clone, pC5D9, by hybrid selection.

2 The immunofluorescence microscopy was performed by Adrian Tsang.

I am indebted to my supervisor, Dr. Adrian Tsang, for his advice and encouragement during the course of this work. I would also like to thank the people who served on my supervisory committee, Drs B Brandhorst, J Monsen, E Kafer, and H Bussey In addition, I gratefully acknowledge the generosity of Dr. P. Devreotes who provided the λgt11 cDNA library, Dr. J. Williams who supplied the pB10act15BKH expression vector, and W. Witke who provided the pDneoII transformation vector. Finally, special thanks go to all the members of our lab for making it a truly enjoyable place to work: Caroline Grant, Bruce Williams, Michael Greenwood, Claire Bonfils (who also prepared the Résumé), Gillian Kent, and Carolyn Kay.

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Abbreviations

bp	base pairs
cAMP	cyclic AMP
cDNA	complementary DNA
cGMP	cyclic GMP
Ci	Curie
cpm	counts per minute
DAG	diacylglycerol
Denhardt's	1X=0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone
DIF	differentiation inducing factor
EDTA	ethylene diamine tetraacetic acid
h	hour
HPLC	high performance liquid chromatography
IP3	inositol trisphosphate
kb	kilobases
M	molar
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger RNA
PBS	phosphate buffered saline (50mM Na_2HPO_4/KH_2PO_4 pH 7.5, 150mM NaCl)
PCR	polymerase chain reaction
PKA	protein kinase A

PKC	protein kinase C
polyA+	polyadenylated
PSF	prestarvation factor
PstO	prestalk 0
PstA	prestalk A
PstB	prestalk B
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	standard saline citrate (1X=0.15M NaCl, 0 015M sodium citrate)
tRNA	transfer RNA
μCi	microcurie
μg	microgram
μl	microliter

Chapter 1: Introduction

The cellular slime mould Dictyostelium discoideum grows as single-celled amoebae which feed on bacteria. Starvation triggers a process of development and differentiation which leads ultimately to the formation of a multicellular fruiting body composed of spores supported by a column of dead stalk cells. The spores remain dormant until suitable environmental conditions trigger germination, after which asocial growth can resume.

A number of features make this organism an ideal system in which to study development and differentiation. First, since only two distinct cell types are formed, it is relatively simple. Second, it grows well in the laboratory with a short generation time. Third, the developmental cycle is complete in 24 hours and occurs very synchronously. Large amounts of cells can be isolated at any given developmental stage, thereby facilitating biochemical and molecular analyses. Fourth, growth and development are completely separate. This fact alone makes this organism extremely useful for the study of developmental processes. Finally, D discoideum normally exists in the haploid state, allowing mutants to be isolated relatively easily. Unfortunately, although this organism possesses a sexual cycle, technical difficulties prevent its use for genetic analysis. However, parasexual genetics can be used for simple complementation and linkage studies. Together, these features make this organism a powerful experimental system with which to study eukaryotic development and differentiation.

1.1: The Growth of D. discoideum

In nature, D. discoideum amoebae grow using bacteria as a food source. Folic acid released by the bacteria acts as a chemoattractant which allows the amoebae to locate them (Pan et al. 1972, 1975). The bacteria are then engulfed by phagocytosis. In the laboratory, large numbers of amoebae can be grown in petri dishes on bacterial lawns. Under these conditions, the cells divide by binary fission with a generation time of approximately 3 hours. Alternatively, axenic mutants have been isolated which can be grown in suspension in a simple liquid broth (Sussman and Sussman 1967; Watts and Ashworth 1970; Loomis 1971). Axenic cells grow considerably slower than those which feed upon bacteria, doubling every 8-10 hours.

Analysis of growing cells has demonstrated that the cell cycle is somewhat unusual in this organism. The G1 phase is either extremely short or else is completely absent (Weijer et al 1984a). Thus, there appears to be no lag between the end of mitosis and the beginning of DNA synthesis. Since DNA synthesis is completed within 15-30 minutes, the cells spend most of their time in G2 (Weijer et al. 1984a). Some other lower eukaryotes, such as Physarum and Hydra, also possess similar unusual cell cycles (Mohberg and Rusch 1971; David and Campbell 1972; Campbell and David 1974).

Growing cells secrete chemorepellents which presumably function to prevent premature starvation by dispersing the cells (Keating and Bonner 1977). Another secreted molecule released by

growing amoebae, known as prestarvation factor or PSF, apparently allows the cells to monitor their density and compare it to the concentration of the bacterial food supply (Clarke et al. 1987, 1988) When PSF reaches a relatively high level compared to the amount of bacteria present, the synthesis of specific proteins is induced (Clarke et al. 1987). Since this situation has been observed to occur approximately 3 generations before the food supply becomes limiting for growth (Clarke et al. 1987), this response may play a key role in preparing the cells for the switch from growth to development.

While the precise nature of this switch is unknown, a number of experiments suggest that amino acid starvation is the specific stimulus which initiates development (Marin 1976; Darmon and Klein 1978). When amoebae are incubated in a simple buffer supplemented with L-amino acids, development is inhibited In contrast, cells incubated in buffer alone, or in buffer containing other nutrients, develop normally. Thus, amino acid starvation, perhaps coupled to the PSF response described above, appears to be responsible for the transition from growth to development.

1.2: The Development of D. discoideum

When the food supply becomes depleted, D discoideum cells enter a developmental phase which, over a period of 24 hours, generates a multicellular fruiting body composed of two distinct cell types. The first overt sign of development occurs about 6 hours after the onset of starvation when the amoebae begin to aggregate to form multicellular mounds containing approximately 100,000 cells. The appearance of a single tip on each mound, which occurs after about 12 hours of development, signifies the beginning of morphogenesis. The tipped aggregates gradually elongate to form the so-called first finger or standing slug stage. The slugs can slowly fall over and migrate in response to certain environmental cues such as heat and light. By this time in development, precursors of the two mature cell types known as prespore and prestalk cells can be detected by immunological and biochemical means These precursors are present in the slug in a very specific pattern. Prestalk cells are localized in the anterior 20% of the slug while prespore cells make up the posterior. This localization is not absolute; a small proportion of the cells in the posterior of the slug possess the characteristics of prestalk cells (Sternfeld and David 1981, 1982, Devine and Loomis 1985) and are therefore called "anterior-like" In addition, a small group of prestalk cells is located at the extreme posterior of the slug These rearguard cells eventually form the basal disc which supports the mature fruiting body

During slug migration, if an adequate food supply is

encountered, the slug can dissociate into individual cells and asocial growth can resume following a period of programmed dedifferentiation (Finney et al. 1979). In fact, this process of dedifferentiation can occur at all but the latest stages of development, indicating that the developmental process is not irreversible until it is almost complete If the appropriate environmental cues do not trigger dedifferentiation, culmination ensues In this process, the slug rears onto its posterior end and backward migration of the prestalk cells through the prespore zone pulls the spore mass to the top Terminal differentiation of the two cell types occurs during this stage. The entire developmental cycle is complete in about 24 hours (for a review of this process, see Loomis 1982).

1.2.1: Aggregation

The formation of multicellular aggregates early in the development of D. discoideum is the result of a chemotactic process. The attractant molecule has been identified as cAMP (Konijn et al. 1967). A cAMP receptor which mediates chemotaxis is present on the surface of developing cells (Malchow and Gerisch 1974). This receptor has been identified by labelling intact cells with 8-N_3-[^{32}P]cAMP (Juliani and Klein 1981; Theibert et al. 1984) It has subsequently been purified (Klein et al. 1987a) and anti-receptor antibodies have been prepared (Klein et al. 1987b). These antibodies have been used to isolate cDNA clones which encode the receptor (Klein et al. 1988).

After a few hours of starvation, some cells spontaneously

begin to emit pulses of cAMP (Gerisch and Wick 1975). These pulses travel through the cell monolayer by diffusion Other cells detect the extracellular cAMP through the cell-surface cAMP receptor. After the binding of cAMP to this receptor, the cells elongate and move up the cAMP gradient They continue to move until the peak of the cAMP wave has passed. Biochemical analysis has demonstrated that both guanylate cyclase (Mato et al. 1977; Wurster et al. 1977; Mato and Malchow 1978) and adenylate cyclase (Roos and Gerisch 1976; Klein et al 1977) are transiently activated within the cell after cAMP binds to the cell-surface receptor.

The activation of adenylate cyclase leads to an increase in the intracellular concentration of cAMP, which is then secreted This response is amplified by the binding of the secreted cAMP to the cell-surface receptor, generating a positive feedback loop between the receptor and adenylate cyclase. Because the activation of adenylate cyclase is transient, intracellular cAMP levels peak in 2 to 3 minutes and then decline (Janssens and Van Haastert 1987). The activity of extracellular and membrane-bound cAMP-phosphodiesterases then reduces the concentration of extracellular cAMP (Malchow et al. 1972; Pannbacker and Bravard 1972). This signal relay process leads to the amplification and propagation of the original cAMP signal throughout the cell population.

The transient activation of guanylate cyclase appears to play an important role in chemotaxis A mutant has been isolated which

exhibits very long periods of chemotactic movement during aggregation (Ross and Newell 1981). Analysis of this mutant has revealed that this phenotype is due to prolonged intervals of high intracellular cGMP concentration caused by a great reduction in the level of intracellular cGMP-phosphodiesterase (Van Haastert et al. 1982). Other factors that may play an important role in the chemotactic response include actin polymerization (McRobbie and Newell 1984) and myosin phosphorylation (Berlot et al 1985).

The transient nature of the activation of the adenylate and guanylate cyclases is the result of an adaptation process (Devreotes and Steck 1979; Van Haastert and Van der Heijden 1985). The cells respond only to increases in the fractional occupancy of the cell-surface cAMP receptors while the degree of receptor occupancy controls the magnitude of the response. The cellular response declines rapidly when receptor occupancy remains constant. When the cAMP stimulus is removed, deadaptation occurs which causes the receptors to regain their cAMP responsiveness.

The precise molecular basis of the adaptation process remains unknown Comparison of the kinetics of excitation, adaptation, and deadaptation for the adenylate and guanylate cyclases suggests that separate adaptation processes are involved for the two enzymes (Snaar-Jagalska et al. 1988b). In the case of adenylate cyclase, cAMP-induced phosphorylation of the cell-surface receptors has been implicated in the adaptation process (Vaughan and Devreotes 1989) although other factors appear to be involved as well (Snaar-Jagalska and Van Haastert 1990). Intriguingly, the

Dictyostelium homolog of the ras oncogene appears to play a role in the adaptation of guanylate cyclase (Van Haastert et al. 1987)

Thus, aggregation is the result of two separate processes coordinated by cell-surface cAMP receptors The first of these, signal relay, involves the transient activation of adenylate cyclase and serves to propagate the cAMP signal throughout the cell population. Then, the chemotactic response associated with guanylate cyclase activation causes the cells to move toward the aggregation centers from which the cAMP pulses originate As the cells move closer together, they elongate and become attached end-to-end, forming streams. After approximately 30 to 50 waves of cAMP, mounds containing about 100,000 cells have formed.

Analysis of the kinetics of binding of cAMP to the cell has demonstrated the existence of two classes of cAMP receptors (Van Haastert and De Wit 1984). These two classes differ in the rate of cAMP dissociation. The fast-dissociating receptors are more abundant and appear to be coupled to adenylate cyclase and the signal relay pathway while the slow-dissociating receptors are linked to guanylate cyclase and the chemotaxis pathway (Van Haastert 1985). It is not yet known if these two populations of receptors are encoded by different genes or if they represent different kinetic forms of the same molecule.

Several cDNA clones encoding a cell surface cAMP receptor which is expressed during aggregation have recently been isolated (Klein et al. 1988). Analysis of its deduced primary structure suggests that it is an integral membrane protein containing 7

membrane-spanning domains. Unsurprisingly, elimination of the receptor by antisense RNA mutagenesis completely blocks aggregation and subsequent development (Klein et al. 1988; Sun et al. 1990) It has recently been reported that Dictyostelium contains a family of genes which are closely related to these receptor cDNAs and that these genes are differentially regulated during development (cited in Devreotes 1989). Thus, different cellular responses to extracellular cAMP such as the transient activation of adenylate and guanylate cyclases may be mediated by receptor subclasses encoded by different genes

The signal transduction pathways which couple the cell-surface cAMP receptors to the signal relay and chemotactic responses have been intensively investigated. Biochemical analysis has demonstrated that guanine nucleotides reduce the affinity of the cell-surface receptors for cAMP and that GTP stimulates both guanylate and adenylate cyclase activities in isolated membranes (Van Haastert 1984; Thiebert and Devreotes 1986; Van Haastert et al. 1986). Finally, cAMP stimulates GTPase activity in membrane preparations (Snaar-Jagalska et al. 1988a). These data strongly suggest that the cell-surface cAMP receptors interact with G proteins. This class of proteins, which bind GTP, are heterotrimeric and possess an intrinsic GTPase activity They have been found to be associated with many eukaryotic transmembrane receptors (reviewed in Gilman 1989). Binding of the ligand to the receptor leads to the exchange of bound GDP for GTP by the $G\alpha$ subunit, which causes the release of the $G\beta$-$G\gamma$ subunits

from the complex. The activated subunits are then able to trigger various intracellular responses. Meanwhile, the GTPase activity of the Gα subunit hydrolyses the bound GTP, which allows the G protein complex to reassociate, thereby terminating the response

Genetic experiments also support the hypothesis that the cell-surface cAMP receptors in Dictyostelium are coupled to G proteins. These experiments have involved the analysis of mutants that are deficient in either signal relay or chemotaxis The Frigid A mutants (Coukell et al. 1983) cannot aggregate due to their inability to produce a chemotactic response to cAMP. Although these mutants contain cell-surface cAMP receptors which appear to be normal (Kesbeke et al. 1988), extracellular cAMP cannot activate either of the adenylate or guanylate cyclases in vivo. In vitro, a GTP-stimulated adenylate cyclase activity can be detected In addition, guanine nucleotides have no effect on the affinity of the receptor for cAMP in these mutants, and cAMP has no effect on GTPase and GTP binding activity in membranes isolated from Frigid A mutants (Kesbeke et al 1988) These results demonstrate that a defective G protein appears to be present in Frigid A mutants and that this G protein is involved in the chemotactic response.

Another class of aggregation-deficient mutants has also been analyzed. In these synag mutants, the signal relay system is completely inactive. Extracellular cAMP cannot activate adenylate cyclase. However, this developmental defect can be overcome if cAMP pulses are supplied either by wildtype cells or by an

exogenous source of cAMP (Thiebert and Devreotes 1986). The chemotaxis pathway is intact, and there do not appear to be any defects in the cell-surface cAMP receptors or the adenylate cyclase enzyme itself. A more detailed analysis of one mutant, synag 1, has demonstrated that GTP cannot stimulate adenylate cyclase activity in vitro. This defect can be complemented by a protein present in wildtype cells (Thiebert and Devreotes 1986). Therefore, the signal relay pathway triggered by the binding of cAMP to the cell-surface receptors requires at least one extra factor in addition to the receptors, the adenylate cyclase enzyme and the G protein involved in the chemotactic response.

Direct evidence proving that G proteins are present in D. discoideum has been provided by the isolation of cDNA clones which encode Gα and Gβ subunits. Two separate Gα subunits, Gα1 and Gα2, have been cloned (Pupillo et al. 1989). These molecules show high homology to mammalian Gα subunits. In addition, a cDNA clone encoding a Gβ subunit has also been isolated and demonstrates similar high homology to Gβ subunits isolated from mammals (Pupillo et al. 1988).

A clear link between the cell-surface cAMP receptors and one of the cloned Gα subunits has recently been demonstrated. All 4 members of the Frigid A complementation group have been shown to possess reduced Gα2 mRNA and protein levels compared to wildtype strains (Kumagai et al. 1989). Moreover, the mutant with the strongest phenotype possesses a deletion which removes most of the Gα2 gene. In addition, it has recently been reported that binding

of cAMP to the cell surface receptors induces the phosphorylation of Gα2 (Gunderson and Devreotes 1990). Taken together, the biochemical, molecular and genetic data clearly indicate that the Gα2 polypeptide is associated with the cell-surface cAMP receptors and that this molecule plays a direct role in transducing the extracellular cAMP signal to the chemotactic pathway.

The precise function of the Gα1 subunit is not known Overexpression of this polypeptide causes severe growth and developmental defects (Kumagai et al. 1989). Vegetative cells which express large amounts of Gα1 are larger than control cells and contain many nuclei. When starved, most of the cells cannot aggregate and those that do form abnormal fruiting bodies. Interestingly, when the cells are grown in shaking culture, they are indistinguishable from wildtype cells. Therefore, Gα1 appears to perform functions in both growth and development.

In higher eukaryotes, membrane receptors which transduce an extracellular signal with the aid of G proteins often employ a phosphatidyl inositol signalling pathway (reviewed in Berridge 1989). In this pathway, receptor-induced activation of the G protein stimulates phospholipase C, which leads to the hydrolysis of phosphatidyl inositol bisphosphate to yield inositol trisphosphate (IP3) plus diacylglycerol (DAG). IP3 triggers the release of calcium ions into the cytoplasm from intracellular stores while DAG activates protein kinase C (PKC) Phosphorylation of specific target proteins by PKC and the activation of various Ca++-dependent events then produces a

specific cellular response.

There is a large body of evidence which suggests that a similar phosphatidyl inositol pathway operates in the chemotactic response to extracellular cAMP (reviewed in Newell et al.1988). First, an inositol cycle almost identical to that present in mammalian cells has been detected in Dictyostelium (Van Lookeren Campagne et al. 1988; Van Haastert et al. 1989). Second, treatment of permeabilized cells with IP3 triggers calcium release (Europe-Finner and Newell 1986b) Third, many of the cellular responses involved in chemotaxis such as the activation of guanylate cyclase and actin polymerization can be induced by treating permeabilized cells with IP3 or calcium (Europe-Finner and Newell 1985, 1986a; Small et al 1986). Fourth, stimulating cells with extracellular cAMP leads to the rapid formation of IP3 (Europe-Finner and Newell 1987a; Van Haastert et al. 1989). Fifth, treatment of permeabilized cells with GTP or nonhydrolyzable GTP analogues also stimulates IP3 formation (Europe-Finner and Newell 1987b; Van Haastert et al. 1989). Finally, two PKC-like enzyme activities have recently been detected in Dictyostelium (Ludérus et al. 1989; Jiminez et al. 1989). Experiments with a specific activator of PKC, phorbol 12-myristate 13-acetate, suggest that the activation of this enzyme does in fact play an important role in the chemotactic pathway (Thiery et al. 1988)

Thus, aggregation is the result of two separate pathways which are both activated by the binding of extracellular cAMP to

the cell-surface receptors. Comparatively little is known about the signal relay pathway which amplifies and propagates the original cAMP stimulus throughout the cell population The chemotactic pathway, however, has been analyzed in detail. The binding of cAMP to the surface receptors appears to trigger chemotaxis through the action of a G protein-linked signal transduction pathway which involves phosphatidyl inositol. The analysis of this pathway may lead to a better understanding of similar transmembrane signalling pathways in higher eukaryotes

1.2.2: Post-Aggregative Development

The early stages of development lead to the formation of a multicellular aggregate. The remainder of the developmental cycle involves both morphogenesis and cellular differentiation to produce the mature fruiting body.

Shortly after aggregation, a tip forms on each multicellular mound. This tip plays an important regulatory role in post-aggregative development, presumably due to its ability to generate oscillatory cAMP signals (reviewed in Schaap 1986). Under its influence, the mound slowly elongates to form the standing slug, which can then fall over and migrate in response to specific environmental cues.

At this time, prestalk and prespore cells can be detected These two classes of cells can be separated by centrifugation through percoll gradients (Tsang and Bradbury 1981; Ratner and Borth 1983), allowing biochemical and molecular analysis. As

described in section 1.2, the prestalk cells are located in the anterior 20% of the slug while the prespore cells make up the rest.

Analysis of these precursor cells by two dimensional SDS polyacrylamide gel electrophoresis has demonstrated that prespore cells express a number of polypeptides that are not present at other stages of development (Ratner and Borth 1983; Morrisey et al 1984). Several of these polypeptides are also expressed in mature spore cells. In contrast, the majority of polypeptides specific to stalk cell differentiation do not appear until later in development.

The ability to obtain relatively pure populations of prespore and prestalk cells has led to the isolation of cDNA clones which are complementary to mRNAs expressed preferentially in one or the other cell type (Mehdy et al. 1983; Barklis and Lodish 1983; Ozaki et al. 1988) While it has been relatively easy to obtain prespore-specific cDNAs, many of the original prestalk cDNAs have been found to be only marginally specific to that cell type. Recently, however, two clones designated pDd56 and pDd63 have been obtained which appear to be expressed only in prestalk cells (Jermyn et al 1987). Based on a comparison of the expression of these cDNAs with some of the earlier prestalk clones, it has been suggested that there are two classes of prestalk-specific mRNAs. The first class, which appears to be the most abundant, consists of sequences which are initially expressed in all cells during aggregation and then are selectively

lost from prespore cells. The second class, typified by pDd56 and pDd63, are expressed only in prestalk cells (Jermyn et al. 1987) The protein products of pDd56 and pDd63 have recently been identified and consist of polypeptides which form part of the extracellular sheath which surrounds each slug (McRobbie et al. 1988a,b).

The mechanism which specifies cell identity is unknown. It has been suggested that cell fate is determined by its stage in the cell cycle when development is initiated (Weijer et al. 1984b; Gomer and Firtel 1987). Alternatively, differences in the metabolic state of cells at the time of starvation may specify cell fate. It has been proposed that cells with low energy reserves may become prestalk cells (Wang et al. 1988a). Another model suggests that differences in intracellular pH determine cell fate (Gross et al. 1983). Finally, gradients of specific morphogens have been invoked to explain both cell fate and the specific prestalk/prespore pattern present in the slug (reviewed in Williams 1988; Williams et al. 1989b).

The isolation of cDNAs (and their corresponding genes) which are expressed specifically in either prespore or prestalk cells has allowed a detailed analysis of differentiation and pattern formation to be performed. The anatomy of the prestalk zone in the slug has been analyzed by using the promoters of the prestalk-specific genes pDd56 and pDd63 to specifically tag cells in which these genes are expressed (Jermyn et al. 1989). This was accomplished by fusing these promoters to reporter genes and then

introducing the resulting constructs separately in D. discoideum cells by DNA-mediated transformation

Careful analysis of the two groups of transformants has revealed the existence of at least 3 classes of prestalk cells in the slug (Jermyn et al. 1989). The first class, designated prestalk A (PstA), is restricted primarily to the front 10% of the slug These cells express the pDd63 reporter gene. A few of the cells in the prespore region also express this marker. Presumably, these consist of the anterior-like cells described in section 1.2. A second class of prestalk cells express the pDd56 reporter gene. These prestalk B (PstB) cells are located in the central core which occupies the anterior 10-20% of the length of the slug. It does not usually extend to the extreme tip. In addition, the anterior-like cells of the prespore zone also appear to express this marker. Finally, a third class of cells designated prestalk 0 (Pst0) is found in the region between the anterior 10% of the slug and the beginning of the prespore zone. These cells do not express either of the reporter genes. The rearguard cells at the extreme posterior of the slug also appear to belong to this class.

Thus, at least three distinct classes of prestalk cells can be detected (Jermyn et al. 1989). PstA cells express the pDd63 gene and are found in the anterior 10% of the slug. PstB cells are localized to a central core of the prestalk zone and express the pDd56 gene. Due to technical difficulties, it is not clear whether these cells also express the pDd63 gene. Finally, the

posterior half of the prestalk zone contains PstO cells which do not express either gene. These results demonstrate that the prestalk zone of D. discoideum is considerably more complex than originally believed. As more truly prestalk-specific genes are isolated, it is possible that additional classes of prestalk cells will be identified.

A similar approach has been taken to trace the origins of prespore, PstA and PstB cells during slug formation and to examine the mechanisms involved in establishing the prestalk-prespore pattern during development (Williams et al. 1989a). Transformants carrying a prespore-specific reporter gene were analyzed in conjunction with those possessing the pDd56 and pDd63 marker constructs described above to determine when the different cell types can first be detected. In addition, the localization of these cells within the aggregate at various stages of development was examined. The results obtained from these experiments indicate that both prestalk and prespore cells appear in the aggregate several hours before the tip is formed. Prespore cells occupy most of the aggregate but are absent from a thin layer at the base as well as from the tip. Both PstA and PstB cells appear to originate at the base of the aggregate. The PstA cells then rapidly migrate to the tip, while a second population of PstB cells differentiates in the central core of the anterior of the standing slug. Thus, both cell sorting and positionally localized differentiation contribute to Dictyostelium morphogenesis.

After the establishment of the prestalk/prespore pattern, the mature fruiting body is produced by terminal differentiation and morphogenesis as described in section 1.2. By studying the processes which occur during the post-aggregative development of D discoideum, it may be possible to learn more about the mechanisms involved in cellular differentiation and pattern formation in more complex organisms.

1.3: Factors Which Affect the Development of D. discoideum

A number of small diffusible molecules have been shown to play important roles in regulating the development of D. discoideum. These include cAMP, adenosine, ammonia, and differentiation inducing factor (DIF). Other potentially important regulatory substances have also been detected but have yet to be characterized (Kay 1982; Mehdy and Firtel 1985, Wilkinson et al. 1985; Kumagai and Okamoto 1986).

In addition to its role as a chemoattractant during aggregation, cAMP promotes spore differentiation and also regulates much of the gene expression which occurs during development. The regulatory effects of cAMP on spore cells have been shown by a number of experiments. When intact slugs are allowed to migrate on agar containing 1mM cAMP, the proportion of prespore cells increases by 5% (Schaap and Wang 1986). In addition, when gradient-purified prespore cells are agitated in a simple salt solution, they lose prespore markers. This effect can be eliminated by the addition of cAMP while cAMP-phosphodiesterase or caffeine (which represses adenylate cyclase activity) accelerates it (Weijer and Durston 1985) Similarly, the proportion of prespore cells in intact slugs can be reduced by immersing them in a buffer containing cAMP-phosphodiesterase; this effect is blocked by the addition of cAMP (Wang et al. 1988b) Finally, so-called sporogenous mutants have been isolated Individual amoebae of these mutants will differentiate to form spore cells with high efficiency in the presence of cAMP (Town et

al 1976; Kay 1982) Taken together, these results demonstrate that extracellular cAMP stimulates spore cell differentiation.

Intracellular cAMP also appears to be important for the differentiation of spore cells The ability of the sporogenous mutants to differentiate into spore cells can be blocked by treatments which inhibit intracellular cAMP accumulation (Riley and Barclay 1986). In addition, treatment of isolated wildtype cells with the membrane-permeable cAMP analogue 8-Bromo-cAMP induces spore cell differentiation at a high frequency (Kay 1989) Finally, drug treatments or mutations that reduce intracellular cAMP levels inhibit spore differentiation while conditions that increase intracellular cAMP concentration promote it (Riley et al. 1989).

At least part of the regulatory effects of cAMP on spore cell differentiation can presumably be explained by its effects on developmental gene activity. Prior to aggregation, genes regulated by cAMP can be divided into a number of classes (reviewed in Firtel et al. 1989). The so-called pulse-induced genes are not expressed in growing cells but become active after 2-3 hours of starvation. They are maximally expressed in aggregating cells The expression of these genes is stimulated by nanomolar pulses of cAMP and repressed by constant amounts of cAMP In contrast, the pulse-repressed genes are repressed by pulses, but not by constant levels, of cAMP. The expression of another class of genes which are active only during early development is repressed by cAMP. These genes require a constant

cAMP stimulus for repression (Singleton et al 1988) Finally, the genes encoding cAMP phosphodiesterase and an inhibitor of this enzyme are both regulated by cAMP. Phosphodiesterase expression is induced by both pulses and continuous levels of cAMP while the inhibitor shows the opposite regulation

Post-aggregative gene expression is also known to be regulated by cAMP. When slugs are disaggregated and the cells then shaken rapidly in suspension, the transcription rate of many genes is reduced, as is the stability of the corresponding mRNAs. Most of these genes are expressed preferentially in prespore cells. The addition of cAMP to the cells prevents both these effects (Chung et al. 1981; Mangiarotti et al 1983, 1989, Landfear et al. 1982; Barklis and Lodish 1983, Mehdy et al 1983)

A number of experiments have demonstrated that almost all of the effects of cAMP on gene expression are mediated through the cell-surface cAMP receptors involved in aggregation Experiments using cAMP analogues demonstrate that the ability of such analogues to regulate gene expression closely parallels their affinity for the cell-surface cAMP receptors (Haribabu and Dottin 1986; Oyama and Blumberg 1986a; Gomer et al 1986). Moreover, mutants which lack this receptor demonstrate aberrant expression of at least some of these genes (Sun et al 1990) In addition, analysis of aggregation mutants has shown that the expression of some of these genes is essentially normal in Synag strains but blocked in FrigidA strains (Mann et al 1988) Furthermore, the expression of most of these genes is normal under conditions which

inhibit the accumulation of intracellular cAMP (Oyama and Blumberg 1986a; Gomer et al. 1986) Finally, the expression of at least some of these genes can be induced by treating permeabilized cells with IP3 and DAG analogues (Ginsberg and Kimmel 1989). Taken together, these data imply that only extracellular cAMP is involved in regulating the expression of most of these genes and that this regulation is mediated by the cell-surface cAMP receptors and the chemotaxis pathway described in section 1.2.1.

There is one exception to the above data. One of the pulse-repressed genes, M4-1, requires the activation of adenylate cyclase and the resulting increase in intracellular cAMP levels for repression (Kimmel and Carlisle 1986; Kimmel 1987). Thus, intracellular cAMP appears to regulate at least one gene during development.

The precise mechanism by which cAMP regulates gene expression is unknown. However, the analysis of a number of cAMP-responsive promoters has found that discrete cis acting sequences appear to be involved (Pears and Williams 1987; Datta and Firtel 1987, 1988; Pavlovic et al. 1989· May et al. 1989; Haberstroh and Firtel 1990). A developmentally regulated and cAMP-inducible trans-acting factor which specifically binds to at least some of these sequences has recently been identified and partially characterized (Hjorth et al. 1989, 1990)

Adenosine has also been found to regulate the development of D. discoideum. This molecule is generated as a consequence of cAMP degradation. It appears to function as an antagonist of

cAMP. The addition of adenosine to cells early in development reduces the number of aggregation centers (Newell and Ross 1982a) During later development, adenosine inhibits many cAMP-mediated responses such as signal relay, the cGMP response, and chemotaxis by blocking the binding of cAMP to the cell-surface cAMP receptors (Van Haastert 1983: Thiebert and Devreotes 1984, Van Lookeren Campagne et al. 1986). Furthermore, adenosine inhibits the stabilization of prespore markers by cAMP in gradient-purified prespore cells (Weijer and Durston 1985) or in intact slugs treated with cAMP phosphodiesterase (Wang et al. 1988b) Conversely, the reduction of adenosine levels in intact slugs by treatment with adenosine deaminase leads to the appearance of prespore cells in the prestalk zone (Schaap and Wang 1986). Finally, adenosine inhibits the accumulation of prespore-specific mRNAs and stimulates that of prestalk-specific mRNAs (Spek et al. 1988). Thus, in general, cAMP and adenosine appear to be mutually antagonistic.

During development, large amounts of protein are degraded which leads to the formation of a substantial quantity of ammonia. This molecule inhibits stalk cell differentiation. Enzymatic depletion of ammonia induces stalk cell differentiation in intact slugs (Wang and Schaap 1989). In addition, a mutant that is hypersensitive to ammonia possesses a reduced prestalk zone (Newell and Ross 1982b) Ammonia also appears to promote spore differentiation In submerged cultures, it is required for spore cell formation (Sternfeld and David 1979). Also, in shaking

cultures where cAMP relay is blocked, ammonia is necessary for optimal expression of prespore genes (Oyama and Blumberg 1986b). Finally, when sporogenous mutants are incubated in submerged culture at high cell density, both stalk and spore cells form. The proportion of cells which differentiate into spores can be increased by ammonium salts (Gross et al 1983).

One other molecule which affects the development of D. discoideum has been characterized. Differentiation Inducing Factor, or DIF, was detected by its ability to induce isolated cells incubated at low density to form stalk cells (Town et al. 1976) When analyzed by HPLC, five peaks of DIF activity can be detected (Kay et al 1983). The majority of the activity is present in one of the peaks. The molecule responsible for this peak, DIF1, has been purified and its structure has been determined (Morris et al. 1987). At least two of the other peaks consist of related molecules of unknown function (Morris et al. 1988). They have been named DIF2 and DIF3.

Several lines of evidence suggest that DIF plays a crucial role in stalk cell differentiation. First, when slugs or aggregates are placed on agar containing DIF, the prestalk zone becomes enlarged (Kay et al. 1983). Second, its accumulation is developmentally regulated. A small amount of DIF is produced early in development but then its level rises substantially between the loose mound and tipped aggregate stages (Brookman et al. 1982). Third, mutants have been isolated which produce only very small amounts of DIF (Kopachik et al. 1983). These mutants,

unlike wildtype strains, will not form stalk cells when incubated at high density in the presence of cAMP but will do so when purified DIF is added. In addition, they cannot complete development. They become arrested at the loose mound stage Moreover, they express prespore but not prestalk markers. Fourth, when prespore cells isolated from slugs are treated with DIF, they redifferentiate into stalk cells (Kay and Jermyn 1983). Finally, DIF represses the transcription of a prespore gene (Early and Williams 1988) and induces the transcription of the prestalk genes pDd56 and pDd63 (Williams et al. 1987) These results suggest that DIF plays an essential role in stalk cell differentiation

The induction of stalk cell differentiation by DIF appears to be a two-stage process (Sobelewski et al. 1983). In the first step, cAMP is required to bring the cells to a DIF-responsive state. In the second step, which appears to be inhibited by cAMP (Berks and Kay 1988), DIF induces prestalk and stalk cell differentiation. These results suggest that cAMP is a general inducer of development since it is required for both spore and stalk cell differentiation, while DIF specifically promotes stalk cell formation.

Thus, four different molecules which appear to regulate differentiation have been characterized in D. discoideum. A model explaining both cellular differentiation and pattern formation in this organism has recently been described (Williams 1988; Williams et al. 1989b). This model suggests that morphogenetic gradients of these 4 regulatory molecules control development.

In addition to its role as a chemoattractant, during the formation of the aggregate cAMP will also presumably act to promote cell differentiation. According to data described previously, this will lead to the formation of prespore cells unless DIF is present. Since DIF does not accumulate to appreciable levels until after loose mounds have formed (Brookman et al 1982), it is likely that most cells in early development will initially be directed down a single differentiation pathway. However, by the time tight aggregates have formed, DIF will be present and should therefore trigger prestalk cell differentiation. Based on the pattern of expression in tight aggregates of two genes which are induced by DIF (pDd56 and pDd63), it has been suggested that a high concentration of DIF may be found at the base of the aggregate, causing PstA and PstB cells to initially differentiate there (Williams et al. 1989b). The DIF concentration is proposed to be regulated by the antagonistic effects of ammonia (Wang and Schaap 1989), which is generated by protein degradation. Perhaps ammonia is lost preferentially from the base of the aggregate and thus generates a higher effective concentration of DIF in this region. In addition, the higher concentration of ammonia in the rest of the aggregate may promote prespore differentiation as described earlier, thereby reinforcing the emerging prespore/prestalk pattern. Then, the observed migration of PstA cells to the tip may occur in response to the cAMP signals which are emitted by this region of the aggregate (Schaap 1986). The initial PstB

population, on the other hand, appears to be chemotactically inactive and so will remain at the base to be lost in the slime trail as the slug migrates away from its site of origin.

As the slug forms, the tip continues to generate cAMP signals which are relayed throughout the slug (Schaap 1986). In the prestalk zone, however, the effects of cAMP could be repressed by adenosine as described earlier. Since the enzymes which generate adenosine are present at a higher concentration in the prestalk zone (Armant and Rutherford 1979; Armant and Stetler 1980; Brown and Rutherford 1980) this hypothesis seems reasonable. The posterior of the slug would maintain a relatively high effective concentration of cAMP which would promote and maintain prespore differentiation.

The precise anatomy of the prestalk zone can also be explained by these morphogenetic gradients. It appears that stalk cell differentiation occurs as a progression from PstO to PstA to PstB and finally to fully differentiated stalk cells (Williams et al. 1989b). The transition from one stage to the next is apparently triggered by increasing concentrations of DIF. It has been suggested that cAMP may block the PstA-PstB conversion, and that terminal stalk cell differentiation is inhibited by ammonia (Williams et al. 1989b). Thus, the PstO zone of the slug could consist of cells prevented from expressing prespore markers by the high concentration of adenosine in this region as described above while they cannot progress into PstA cells because of a relatively low DIF concentration. At the tip, however, a relatively high DIF

concentration could trigger PstA and PstB cell differentiation. Unfortunately for this hypothesis, direct measurements of DIF in sections of migrating slugs suggest that the DIF concentration is in fact highest at the posterior (Brookman et al. 1987). However, since only about a 2-fold difference was detected, and the assay measured total DIF and not the actual fraction that is active, it is possible that these measurements do not truly reflect the situation in vivo Since 2 DIF-dependent genes are expressed almost exclusively in the tip of migrating slugs (Williams et al. 1989a), it appears that the tip does contain a relatively high effective concentration of DIF.

A complex set of morphogen interactions has been invoked to generate the relative distribution of PstA and PstB cells in the prestalk zone of the migrating slug (Williams et al. 1989b). The presence of high levels of DIF in the tip as described above should activate general prestalk cell differentiation. However, it is known that cAMP is initially required to bring cells to a DIF-responsive state (Sobelewski et al. 1983) while the continued presence of cAMP then acts to repress accumulation of the pDd56 protein (Berks and Kay 1988). Since the presence of this protein defines a cell as being PstB (Jermyn et al. 1989), the existence of a low concentration of cAMP in the core of the tip would allow this cell type to differentiate at this location. In contrast, a high concentration of cAMP in the outer layers of the tip would prevent the PstA cells from becoming PstB cells. The generation of regions of high and low cAMP concentration in the tip may be

the result of the ability of ammonia to inhibit cAMP synthesis and/or secretion (Schindler and Sussman 1977). The preferential loss of ammonia from the periphery of the slug could generate a radial gradient of cAMP with the lowest concentration being in the core. Thus, the precise PstA-PstB pattern in the slug could be generated by interactions between DIF, ammonia and cAMP.

Although the model proposed above can adequately explain cellular differentiation and pattern formation in D discoideum, it is highly speculative. Its validity can only be assessed by accurately measuring the effective concentrations in the aggregate and slug of all the morphogenetic molecules involved. Such analyses should lead to a better understanding of these complex processes in this organism.

1.4 Intracellular cAMP Binding Proteins

As described in preceding sections, both extracellular and intracellular cAMP play important roles in regulating the development of D. discoideum. While all the effects of extracellular cAMP appear to be mediated chrough the cell-surface cAMP receptors and various G protein linked signal transduction pathways, very little is known about the mechanisms which mediate the effects of intracellular cAMP. To address this problem, intracellular cAMP binding proteins have been investigated.

The most intensively studied intracellular cAMP binding protein in Dictyostelium consists of the regulatory subunit of a cAMP-dependent protein kinase or PKA (Sampson 1977; Leichtling et al. 1982; De Gunzberg and Veron 1982; Part et al. 1985). In other eukaryotes, all cAMP-dependent processes appear to be mediated by such enzymes (reviewed in Krebs 1989), which consist of regulatory (R) and catalytic (C) subunits. In mammals, these enzymes possess a tetrameric subunit structure of R_2C_2. The Dictyostelium PKA, in contrast, is a simple RC dimer (De Gunzberg et al. 1984). The binding of cAMP to the R subunits causes the holoenzyme to dissociate. The freed catalytic subunits are then able to phosphorylate specific target proteins.

To investigate the role of this enzyme in Dictyostelium development, cDNAs encoding the regulatory subunit have been isolated (Mutzel et al. 1987). The predicted amino acid sequence of this polypeptide is very similar to those of the regulatory subunits isolated from yeast and mammals. The regulatory subunit

is expressed only at very low levels during growth and then increases during early development until just after aggregation, when it is maximally expressed (De Gunzberg et al 1986).

Overexpression of the regulatory subunit in Dictyostelium cells, which should constitutively repress the catalytic subunit, blocks aggregation (Simon et al 1989). However, since large amounts of the polypeptide were produced, this defect may be the result of a significant decrease in the levels of free cytoplasmic cAMP rather than the absence of the kinase activity. A more careful analysis of the function of PKA during development has been reported (Firtel and Chapman 1990). In these experiments, mutated forms of a mouse PKA regulatory subunit unable to bind cAMP were overexpressed in Dictyostelium cells. Since it is known that the mammalian R subunit can associate with the Dictyostelium C subunit in vitro (Chevalier et al. 1986), the kinase activity in these cells should be strongly supressed. In addition, there should be no major decrease in the concentration of intracellular cAMP. These cells cannot aggregate. Thus, PKA appears to play an important role in at least the early stages of development.

One other intracellular cAMP binding protein from Dictyostelium, designated CABP1, has recently been characterized (Tsang and Tasaka 1986; Kay et al. 1987; Tsang et al. 1987; Tsang et al. 1988). This molecule is composed of two subunits, CABP1A and CABP1B which have molecular weights of 43,000 and 38,000 respectively. Monoclonal antibodies have been raised independently against each subunit Interestingly, all such

antibodies crossreact with both subunits along with several additional polypeptides. Three of the immunologically related polypeptides, which have molecular weights of 62,000 (p62), 34,000 (p34), and 31,000 (p31), also copurify with CABP1.

CABP1 is present at relatively low levels in the cytoplasm of growing cells and then gradually accumulates during development. Furthermore, there is a shift in the subcellular localization of CABP1 during development such that a significant amount of the protein is found in the nucleus. Thus, this molecule may play an important regulatory role.

Two of the polypeptides which crossreact with anti-CABP1 antibodies, p34 and p31, show a somewhat different pattern of expression and localization. These two molecules cannot be detected by Western blotting in growing cells but appear soon after development is initiated. Their levels then remain constant throughout the rest of the developmental cycle. They appear to be localized almost exclusively in the nucleus. Most of the other immunologically related polypeptides are also developmentally regulated.

cDNA clones encoding both subunits of CABP1 have recently been isolated (Grant and Tsang, manuscript submitted). Analysis of these clones has revealed that the two polypeptides are identical except for the presence of an additional 37 amino acids near the amino terminus of CABP1A. A single gene encodes both polypeptides through an alternative splicing mechanism (Grant et al., manuscript submitted).

Southern blotting using CABP1 cDNA probes has shown that several related genes appear to be present in the genome of Dictyostelium. This result agrees well with the observation that several polypeptides are immunologically related to CABP1. Taken together, the data suggest that this protein may belong to a family of related molecules.

This thesis describes the characterization of the alternative splicing mechanism which generates the two subunits of CABP1 from a single gene In addition, the isolation and characterization of cDNA clones which encode p24 and p31, two of the polypeptides which crossreact with anti-CABP1 antibodies and also copurify with CABP1, are presented. Finally, the effects of disrupting the gene which encodes these two molecules are described.

Chapter 2: Materials and Methods

2.1: Growth and development of D. discoideum. The wild-type strain NC4 and its axenic derivative AX2 were used for these experiments. Both strains were grown in two-membered cultures with Enterobacter aerogenes on SM agar (Sussman 1966) at 22°C. Strain AX2 was sometimes grown axenically in HL5 medium (Watts and Ashworth 1970) at 22°C. Amoebae grown on bacteria were harvested just before the bacterial lawns began to clear and plated for development on non-nutrient agar (Sussman 1966). Alternatively, they were resuspended to a density of $5x10^7$ cells/ml in KK2 (20mM potassium phosphate pH 6.2) One ml of this suspension was then spread over the surface of a 47mm-diameter polycarbonate membrane filter (Nuclepore) supported on two layers of filter paper (Whatman No 1) saturated with KK2. The filter was then incubated at 22°C and development was monitored periodically using a dissecting microscope

2.2: Growth rate measurements A lawn of E. aerogenes was grown on SM agar (Sussman 1966) and then washed off the plate into a large volume of KK2. The bacteria were pelleted by centrifugation at 5000xg, washed twice, resuspended in 10ml of the same buffer, and then 2 5ml portions of the suspension were placed into 25ml flasks Each flask was inoculated with $2.5x10^6$ cells of the appropriate Dictyostelium strain and then agitated at 22°C. At 4h intervals, small aliquots were removed and cell density was determined with a hemocytometer. Alternatively, Dictyostelium cells were grown axenically in HL5 medium at 22°C. Cell density

was determined every 8h using a hemocytometer.

2.3: Isolation of RNA. Vegetative or developing cells were collected by centrifugation at 450xg, resuspended in sterile HMK buffer (Cocucci and Sussman 1970) at a concentration of 10^8 cells/ml and lysed by the addition of SDS to 0.5%. Total cellular RNA was purified by phenol/chloroform extraction and precipitated with ethanol. Cytoplasmic RNA was obtained using a slight modification of the above procedure. After resuspending the cells in HMK buffer, cell membranes were disrupted by the addition of NP40 to 2%. The nuclei were removed from the lysate by centrifugation at 10,000xg and the supernatant was recovered and extracted with phenol and chloroform. The purified cytoplasmic RNA was then ethanol precipitated. Affinity chromatography over oligo(dT) cellulose columns (Pharmacia) was employed to isolate polyA$^+$ RNA.

2.4: Isolation of DNA. Genomic DNA was isolated by a miniprep procedure Approximately 10^8 cells were collected by centrifugation at 450xg and resuspended in 1ml of 50mM Hepes pH 7.5, 5mM magnesium acetate and 10% (w/v) sucrose They were lysed by the addition of NP40 to 2%. Nuclei were collected by a brief centrifugation in a microfuge and suspended in 200μl of 10mM tris-HCl pH 7.5, 5mM EDTA. After the addition of proteinase K (Boehringer Mannheim) to 100μg/ml and SDS to 2%, the lysates were incubated at 65°C for 4h and then extracted with phenol and chloroform. The purified nucleic acids were collected by precipitation with ethanol and resuspended in 50μl of sterile

distilled water. Alternatively, to prepare large amounts of genomic DNA, nuclei purified as described (Kay et al. 1987) were resuspended in 0.2M EDTA at a concentration of 10^9 nuclei/ml and lysed by the addition of SDS to 2%. DNA was then purified by centrifugation through CsCl density gradients (Firtel and Bonner 1972)

2.5: Construction and screening of cDNA library. A cDNA library was constructed from a mixture of $polyA^+$ RNAs prepared from NC4 cells that had developed for 12 and 20h. Blunt-ended double-stranded cDNA was synthesized and homopolymer dC tails were added as described (Maniatis et al. 1982) to allow annealing to PstI-cut, dG-tailed pBR322 (Bethesda Research Laboratories). The annealed DNA was transformed into Escherichia coli DH1 cells as described (Hanahan 1985).

To screen the library, recombinant clones were grown in microtitre dishes at 10 clones/well and then transferred to nitrocellulose (Schleicher and Schuell) (Maniatis et al. 1982). The filters were prehybridized at 32°C for two hours in 5X SSC, 1X Denhardt's solution, 1% SDS, 20mM sodium phosphate pH 6.5, 100μg/ml denatured salmon sperm DNA, 30μg/ml polyA and 50% formamide. Hybridization was performed in the same solution using a nick-translated cDNA clone encoding a portion of CABP1 at 32°C for 16h. Filters were washed briefly in 2X SSC, 0.1% SDS at room temperature, then for two 30-min periods in the same solution at 60°C and finally in 0.1X SSC, 0.1% SDS at room temperature for 30 min. Positive clones were detected by

exposing the filters to Kodak X-omat film at -70°C with intensifying screens.

2.6 Isolation of full length cDNA clones. To isolate full length cDNAs, a λgt11 cDNA library, kindly provided by Dr Peter Devreotes (Klein et al. 1988), was screened Recombinant phage were transferred to nitrocellulose filters as described (Maniatis et al. 1982). The filters were prehybridized at 42°C for 2h in 6X SSC, 10X Denhardt's solution, 1% SDS, and 50μg/ml denatured salmon sperm DNA. The filters were then probed with a nick-translated cDNA isolated from the plasmid library described in section 2.5 in a hybridization solution composed of 50% formamide, 6X SSC, 1% SDS, and 50μg/ml denatured salmon sperm DNA at 42°C for 16h The filters were rinsed briefly in 2X SSC, 0.1% SDS at room temperature followed by two 30-min washes in 0 1X SSC, 0 1% SDS at 68°C and then exposed to Kodak X-omat film at -70°C with intensifying screens. Positive plaques were purified to homogeneity by rescreening under the same conditions.

2.7: Hybrid selection and cell-free translation. Hybrid selection was performed essentially as described (Maniatis et al 1982) A 20μg sample of each cDNA clone was denatured and then immobilized on a 3mm square of nitrocellulose (BA85; Schleicher and Schuell) The filters were placed in a solution composed of 50% formamide, 20mM Pipes pH 6.5, 0.2% SDS, 0.4M NaCl, 100μg/ml yeast tRNA and 500μg/ml polyA+ RNA isolated from HL-60 cells that had been developed for 17h. After a 3h incubation at 50°C, the filters were washed thoroughly at 65°C in 10mM tris-HCl pH 8.0, 0.15M

NaCl, 1mM EDTA, and 0 5% SDS. A final wash was performed in the same buffer lacking SDS. The mRNAs bound to the filters were then eluted by boiling in 300μl of water containing 30μg yeast tRNA. After concentration by ethanol precipitation, the resulting mRNAs were translated in vitro using a rabbit reticulocyte lysate (Bethesda Research Laboratories) in the presence of [^{35}S]methionine (Amersham). The translation products were then immunoprecipitated as described in section 2.16.

2 8· DNA sequencing. cDNAs were subcloned into the plasmid vector Bluescript (Stratagene) and single stranded DNA was prepared (Vieira and Messing 1987). The nucleotide sequences were determined by the chain termination method (Sanger et al. 1977) using [^{35}S]dATP (Amersham) as the radiolabel Double-stranded DNA sequencing in the vector pBR322 was accomplished using a synthetic oligonucleotide primer (Hattori and Sakaki 1986). DNA sequencing reactions were analyzed by electrophoresis through 6% polyacrylamide gels containing 8M urea

2.9. DNA blot hybridization. Ten microliters of genomic DNA prepared by the miniprep procedure described in section 2 4 or 1μg of CsCl purified material was digested with various restriction endonucleases according to the recommendations of the enzyme manufacturer (Bethesda Research Laboratories), fractionated by electrophoresis through 1% agarose gels, and transferred to Nytran membranes (Schleicher and Schuell) as described (Maniatis et al. 1982). The filters were prehybridized and hybridized using the same conditions employed to screen the λgt11 library as

described in section 2.6 at either 37°C (moderate stringency) or 42°C (high stringency). The blots were probed with cDNA fragments labeled to a specific activity of $1x10^9$ cpm/μg by the random primers technique (Feinberg and Vogelstein 1983). The blots were washed briefly in 2X SSC 0.1% SDS at room temperature and then twice for 30 min in 2X SSC, 0.1% SDS at 65°C (moderate stringency) or 0.1X SSC, 0.1% SDS at 68°C (high stringency) and exposed to Kodak X-Omat film at -70°C with intensifying screens.

2.10: RNA blot hybridization. Five micrograms of polyA$^+$ RNA isolated from cells at various times of development was fractionated on 1.5% agarose formaldehyde gels and transferred to Genescreen membrane (New England Nuclear) (Maniatis et al. 1982) The membrane was prehybridized and hybridized using the high stringency conditions employed for DNA blot analysis as described in section 2.9. The blot was probed with a cDNA fragment labelled by the random primers technique (Feinberg and Vogelstein 1983) to a specific activity of $1x10^9$ cpm/μg. The filter was washed briefly in 2X SSC, 0.1% SDS at room temperature and then twice in 0.1X SSC, 0.1% SDS at 70°C for 30 min each. The blot was exposed to Kodak X-Omat film at -70°C with an intensifying screen.

2.11: Construction of actin 15-CABP1 fusions. To express CABP1 under the control of the actin 15 promoter in Dictyostelium cells, a cDNA clone encoding CABP1A (Grant and Tsang, manuscript submitted) was digested with SmaI and EcoRV to release a fragment containing the entire open reading frame present in the cDNA plus a small amount of sequence derived from the polylinker of the

plasmid vector. After the addition of HindIII linkers, this fragment was inserted into the HindIII site of the expression vector pB10Act15BKH (obtained from Dr. J.G. Williams). Constructs containing the insert in the correct orientation for expression were identified by restriction analysis.

To construct a plasmid expressing a mutated CABP1 under the control of the actin 15 promoter, oligonucleotide-directed mutagenesis (Zoller and Smith 1987) was employed to alter the 5' splice site present in the CABP1A cDNA from GTAATA to GTAAGT. The presence of the desired mutation was confirmed by DNA sequence analysis. The mutated cDNA was isolated by digestion with SmaI and EcoRV, ligated to HindIII linkers, and inserted into the HindIII site of pB10Act15BKH This construct is identical to the plasmid expressing the wildtype CABP1A cDNA except for the two-nucleotide change introduced by mutagenesis

2.12 Construction of actin 15-pDdM34 fusion. To express a full length p34 cDNA in Dictyostelium cells under the control of the actin 15 promoter, oligonucleotide-directed mutagenesis (Kunkel 1985) was used to introduce a HindIII site at the translation initiation codon of the clone pDdM34. The presence of the desired mutation was confirmed by DNA sequence analysis. Because of an additional HindIII site in the polylinker of this plasmid, digestion with HindIII released a fragment containing the entire open reading frame of the cDNA plus 3' untranslated sequences. This fragment was inserted into the HindIII site of the expression vector pB10Act15BKH and plasmids containing the insert

in the correct orientation for expression were identified by restriction analysis. The resulting construct expresses the open reading frame present in pDdM34 under the control of the actin 15 promoter.

2.13: Construction of gene disruption vector To construct a vector designed to disrupt the p34/31 gene by homologous recombination, the cDNA clone pDdM115 was digested completely with EcoRI and then partially with Sau3A. The longest EcoRI-Sau3A fragment was inserted into the transformation vector pDneoII (Witke et al. 1987) (obtained from W. Witke). The resulting construct contains a truncated cDNA fragment which lacks approximately 100 nucleotides of coding sequence from both the 5' and 3' ends.

2.14: Transformation of Dictyostelium. Plasmid DNA was introduced into strain AX2 by the calcium phosphate coprecipitation technique as described (Early and Williams 1987) Stable transformants were selected in HL5 medium (Watts and Ashworth 1970) containing the antibiotic G418 (Gibco) at a concentration of 20μg/ml. For transformants carrying expression constructs, the resulting colonies were pooled and grown in HL5 containing G418 or else on bacterial lawns as described in section 2.1. For gene disruption experiments, individual colonies were isolated and grown separately in HL5 containing G418 or else on bacterial lawns.

2.15: Metabolic labelling of Dictyostelium cells with [^{35}S]methionine. To accomplish metabolic labelling, bacterially-grown cells were harvested, washed 3 times with KK2, and then plated for development on polycarbonate membrane filters as described in section 2.1. Each filter contained $5x10^7$ cells. Following a 3h incubation at 22°C, the filters were transferred to a clean, dry petri dish and then 20μCi of [^{35}S]methionine (Amersham) were added directly to them. After a 3h labelling period, the cells were washed off the filters by vortexing in 10ml of KK2 and collected by centrifugation at 450xg. They were lysed in 1ml of PBS containing 1% NP40, 0.1% Tween 20, 0.1% TritonX100, and 0.1% SDS. Insolubic material was removed by a brief centrifugation in a microfuge.

2.16: Immunoprecipitation. Cell lysates or in vitro translation reactions containing [^{35}S]methionine-labelled proteins were immunoprecipitated using the anti CABP1 monoclonal antibody 9B (Tsang and Tasaka 1986). After the addition of antibody, the extracts were incubated at 4°C for 16h. Antibody-antigen complexes were precipitated with protein A-Sepharose (Pharmacia) and suspended in SDS sample buffer (Laemmli 1970). The resulting polypeptides were analyzed by SDS-PAGE and detected by fluorography (Skinner and Griswold 1983).

2.17 Immunoblot Analysis. Protein samples were prepared by lysing vegetative or developing cells in SDS sample buffer (Laemmli 1970). The protein concentration in each extract was determined as described (Esen 1978). Ten micrograms of each

sample were resolved by SDS-PAGE and then transferred to Immobilon membranes (Millipore) according to the manufacturer's specifications. The following manipulations were all carried out at room temperature unless otherwise indicated. The blots were incubated in buffer A (10mM tris-HCl pH 8.0, 150 mM NaCl containing 10% (w/v) milk powder) for 1h and then probed with monoclonal antibody 9B (Tsang and Taseka 1986) in buffer A + 0.3% Tween 20 at 4°C for 16h. Nonspecifically bound antibody was removed by 3 brief washes in buffer B (10mM tris-HCl pH 8.0, 150mM NaCl + 0.3% Tween 20). The blots were incubated with [^{125}I]labelled goat-antimouse antibodies (New England Nuclear) in buffer A + 0.3% Tween 20 for 2h, washed extensively in buffer B, and exposed to Kodak X-Omat film at -70°C with an intensifying screen.

2.18: Polymerase Chain Reaction. Cytoplasmic RNA was isolated as described in section 2.3 from cells which had developed for 2h. First strand cDNA was synthesized from 2μg of total RNA using an oligonucleotide primer (5'CGAAGAGCGCCTTGCATACC 3') corresponding to a segment near the 3' end of the CABP1 transcripts. The reaction was carried out with 5 units of AMV reverse transcriptase (Promega) at 42°C for 1h in a buffer containing 50mM KCl, 10mM tris HCl pH 8.3, 4mM $MgCl_2$, 0.01% gelatin, and all 4 deoxyribonucleotide triphosphates each at a concentration of 1mM. The reverse transcriptase was then inactivated by a 5 min incubation at 95°C. To amplify the resulting single-stranded DNA molecules, the reaction was diluted

5-fold with 50mM KCl, 10mM tris-HCl pH 8.3, 1.5mM $MgCl_2$, and 0.01% gelatin. Then a second oligonucleotide primer (5'CAAGCTTGGATCGAATTCCGGTATA 3') corresponding to the 5' end of the actin 15-CABP1 fusion transcripts was added along with 2 units of Taq DNA polymerase (Pharmacia). The reaction was first incubated at 94°C for 1 min to denature the molecules, followed by a 1 min incubation at 58°C to anneal the primers to the templates, and finally incubated for 2 min at 72°C to allow extension of the primers. A total of 35 cycles of amplification were performed. Genomic DNA samples (5ng) were amplified under the same conditions. The amplification products were analyzed by electrophoresis through 1.2% agarose gels followed by DNA blot analysis as described in section 2.9.

2.19. Immunofluorescence microscopy. Cells were developed as described in section 2.1 for 5h and then washed and resuspended in 20mM potassium phosphate buffer at a concentration of approximately $1x10^6$ cells/ml. A drop of cell suspension was placed on a coverslip, incubated at room temperature for 20 min, and then fixed by immersion in 100% methanol for 20 min at -20°C. The coverslips were incubated for 30 min at room temperature in 3% bovine serum albumin in phosphate buffered saline (PBS; 50mM Na_2HPO_4/KH_2PO_4 pH 7.5 containing 150mM NaCl) followed by an overnight incubation at 4°C with the anti-CABP1 monoclonal antibody 9B (Tsang and Tasaka 1986). They were then rinsed for 10 min in PBS and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse IgG for 1h at room

temperature. After a final rinse with PBS, the stained cells were examined under a Leitz Orthoplan fluorescence microscope

Chapter 3: Results

3.1 Analysis of the Mechanism Which Generates the Two Subunits of CABP1 From a Single Gene

3.1.1 Expression of wildtype and mutant CABP1A cDNAs under the control of the actin 15 promoter

As described in section 1.4, the two subunits of CABP1 appear to be produced from the same gene. Transcripts containing the entire open reading frame present in the CABP1 gene encode CABP1A while CABP1B mRNAs are produced by a splicing reaction which occasionally removes a small segment of coding sequence from the primary transcript. This segment is flanked precisely by GT/AG eukaryotic splice consensus signals. A more detailed comparison of the sequence surrounding the 5' splice site in CABP1 (GTAATA) to the *Dictyostelium* consensus (GTAAGT) (Grant *et al.* manuscript submitted) has revealed a 2 nucleotide difference. It has been suggested that this deviation may be important in regulating the splicing event which generates the CABP1B transcript (Grant 1990). To test this hypothesis, site-directed mutagenesis experiments have been performed.

Oligonucleotide-directed mutagenesis (Zoller and Smith 1987) was employed to make a 2-nucleotide mutation in a full-length CABP1A cDNA. This mutation converts the 5' splice site in the cDNA to the *Dictyostelium* consensus. It should be noted that this change alters 2 amino acids in the open reading frame present in the cDNA. The resulting fragment was inserted into the expression

vector pB10Act15BKH. This vector contains the complete actin 15 gene, which has been slightly modified by the insertion of a multilinker cloning site, and its promoter It has previously been used to express a mouse thymidylate synthase gene in D discoideum (Chang et al. 1989). After insertion of the mutated CABP1A cDNA fragment, the resulting construct codes for a translational fusion consisting of the first 14 codons of the modified actin 15 gene linked to the open reading frame present in the cDNA. In addition, due to the cloning strategy employed to insert the cDNA in the proper reading frame, 18 additional nucleotides derived from polylinker sequences contained in the cDNA clone are present in the expression plasmid Thus, this plasmid will express the open reading frame contained in the mutated CABP1A cDNA along with an extra 20 amino acids fused to the amino terminus. As a control, an identical expression construct was made using the wildtype CABP1A cDNA Fig 1A illustrates the details of these constructs

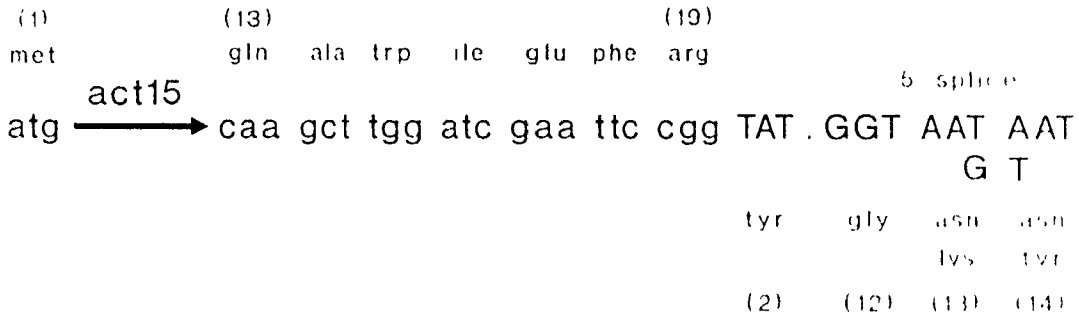
The plasmids were introduced into D. discoideum strain AX2 by the calcium phosphate coprecipitation technique (Early and Williams 1987). Approximately 150 stable transformants for each construct were obtained and pooled separately for analysis The cells were analyzed by metabolic labelling with [^{35}S]methionine followed by immunoprecipitation with the anti-CABP1 monoclonal antibody 9B (Tsang and Tasaka 1986) This antibody recognizes both subunits of CABP1 as well as several additional polypeptides The immunoprecipitated products were resolved by SDS-PAGE and

Figure 1. CABP1 polypeptides expressed in transformants carrying wildtype and mutant expression constructs.

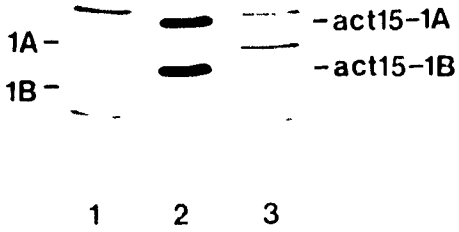
A Actin 15-CABP1 fusion constructs. The structure of the amino terminus of the fusion polypeptides is shown. The nucleotide sequences provided by the vector are shown in small letters while those present in the CABP1A cDNA are in capitals. The numbers above the sequence represent amino acid position in the fusion while those below the sequence indicate the codon position in the CABP1A cDNA. The 5' splice site is underlined. The mutated actin 15-CABP1 fusion construct is identical except for the two nucleotide change introduced to convert the 5' splice site to the Dictyostelium consensus. These modifications are indicated underneath the underlined sequence, as are the resulting amino acid changes.

B Immunoprecipitation analysis of transformants. Transformants carrying the expression constructs described above were analyzed by metabolic labelling with [^{35}S]methionine followed by immunoprecipitation with an anti-CABP1 monoclonal antibody. The resulting polypeptides were resolved by SDS-PAGE and detected by fluorography. As a control, cells transformed with the expression vector alone were treated similarly. The endogenous CABP1A and CABP1B subunits are labelled, as are the fusion polypeptides. The immunoprecipitated products obtained from cells transformed with pB10Act15BKH (Lane 1), the wildtype actin 15-CABP1 expression construct (Lane 2), and the mutated actin 15-CABP1 expression construct (Lane 3), are shown.

A


(1)
met
(13)
gln ala trp ile glu phe
(19)
arg
act15
atg
caa gct tgg atc gaa ttc cgg TAT . GGT AAT AAT
G T
tyr gly asn asn
lys tyr
(2) (12) (13) (14)

B


1A –
1B –
–act15–1A
–act15–1B
1 2 3

detected by fluorography The results are shown in Fig. 1B. Cells transformed with the expression vector alone possess only the two subunits of CABP1 as well as a few additional immunologically related polypeptides (Fig. 1B, lane 1). In contrast, cells transformed with the wildtype CABP1A expression construct contain large amounts of plasmid-encoded CABP1A and CABP1B, which can be distinguished from the endogenous molecules by virtue of their slight increase in size due to the additional 20 amino acids fused to their amino termini (Fig. 1B, lane 2). Thus, expression of a wildtype CABP1A cDNA under the control of the actin 15 promoter leads to the synthesis of both CABP1A and CABP1B, directly demonstrating that these two polypeptides are the products of a single gene. Intriguingly, the only plasmid-encoded molecule expressed in cells transformed with the mutated CABP1A cDNA corresponds to CABP1B, which is present at relatively low levels (Fig. 1B, lane 3). These results suggest that conversion of the 5' splice site in CABP1 to the Dictyostelium consensus leads to constitutive splicing of the primary transcript, thereby leading to the synthesis of only CABP1B.

It should be mentioned that cells containing the mutated CABP1A expression construct sometimes contain an additional polypeptide which is recognized by the antibody. It migrates slightly faster than the wildtype actin 15-CABP1A fusion. The presence of this molecule seems to depend upon the age of the cells. It has only been seen in cells that are relatively young. As the culture ages, it completely disappears. The precise origin

of this polypeptide is unknown (see section 4.1 for discussion)

3.1.2: Analysis of mRNAs produced by wildtype and mutant CABP1A cDNAs

To confirm the results shown in Fig. 1, the mRNAs produced by the wildtype and mutant CABP1A expression constructs have been examined using the polymerase chain reaction (PCR) (Saiki et al. 1988) This technique provides a very sensitive method to detect specific transcripts

Cytoplasmic RNA was isolated from the two transformed populations as well as from a control transformant which contains only the expression vector pB10Act15EV4 Transcripts derived from both the endogenous CABP1 gene as well as the expression constructs were converted to single-stranded cDNA using reverse transcriptase and an oligonucleotide primer located near the 3' end of the mRNAs. The plasmid-encoded transcripts were then specifically amplified using a second oligonucleotide primer corresponding to their 5' ends In addition, the same pair of primers was used to amplify the actin15-CABP1A fusion genes from genomic DNA isolated from the two populations of transformants. The amplification strategy is illustrated in Fig 2A

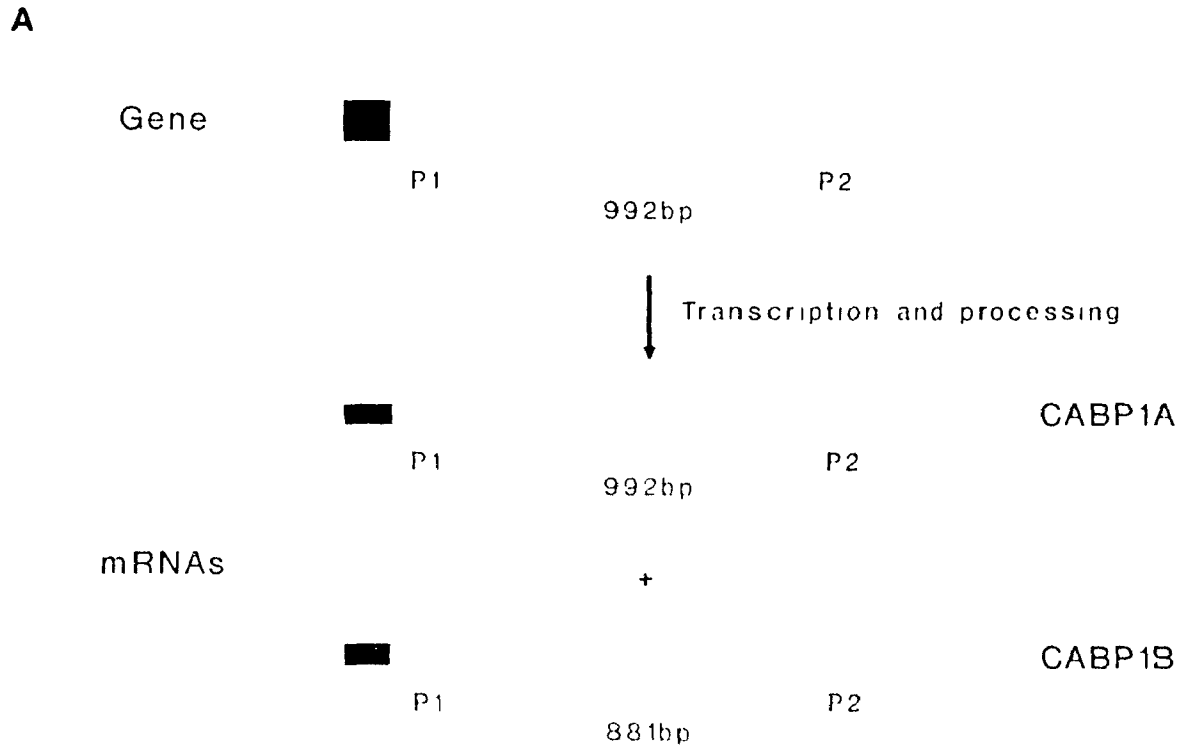
The PCR products were analyzed by agarose gel electrophoresis followed by Southern blotting. The filters were probed under conditions of high stringency with a cDNA fragment corresponding to the 3' half of the CABP1 gene, this fragment is present in both CABP1A and CABP1B transcripts The results of these experiments

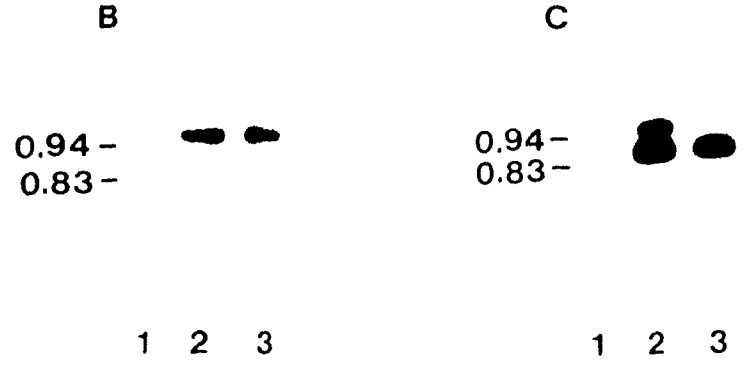
Figure 2 PCR analysis of actin-CABP1 fusion genes and transcripts derived from them.

A. Strategy for analysis by PCR. Untranslated sequences are represented by a line while coding sequence is depicted by a rectangle The sequences provided by the expression vector are represented by the solid zone. The region which is alternatively spliced to generate the CABP1B transcript is indicated by diagonal lines The locations of the two primers used for PCR, P1 and P2, are shown The predicted sizes of the PCR products are indicated

B PCR analysis of genomic DNA isolated from various transformants. PCR products were fractionated by agarose gel electrophoresis, blotted to a nylon membrane, and probed with a CABP1 cDNA fragment. The positions of the 0 94 and 0 83 kb size markers obtained by digesting λ DNA with EcoRI and HindIII are indicated The PCR products obtained from genomic DNA isolated from cells transformed with the expression vector pB10Act15BKH (Lane 1), the wildtype actin 15-CABP1 expression construct (Lane 2), and the mutated actin 15-CABP1 expression construct (Lane 3) are shown.

C PCR analysis of RNA transcripts isolated from various transformants. The PCR reactions were analyzed as above. PCR products obtained from cytoplasmic RNA isolated from cells transformed with pB10Act15BKH (Lane 1), the wildtype actin-CABP1 expression construct (Lane 2), and the mutated actin 15-CABP1 expression construct (Lane 3) are shown.


A
Gene
P1
P2
992bp
Transcription and processing
CABP1A
P1
P2
992bp
mRNAs
+
CABP1B
P1
P2
881bp


B
C
0.94 –
0.83 –
0.94 –
0.83 –
1 2 3
1 2 3

are shown in Figs 2B and 2C. As expected, a single fragment of 992bp is obtained from genomic DNA isolated from transformants carrying both expression constructs (Fig. 2B, lanes 2 and 3). This observation indicates that no gross rearrangements of the fusion genes have occurred during the transformation process. In addition, no amplification product is obtained from genomic DNA isolated from cells transformed with pB10Act15BKH (Fig. 2B, lane 1), demonstrating that the 992bp fragment is in fact specific to cells carrying the expression constructs.

Amplification of cDNA generated from cytoplasmic RNA isolated from cells transformed with the wildtype CABP1A expression construct produces 2 products of 992bp and 881bp (Fig. 2C, lane 2). The longer fragment corresponds to CABP1A transcripts while the smaller one corresponds to mRNAs encoding CABP1B. This result clearly demonstrates that the two polypeptides are produced by an RNA processing event. In contrast, amplification of cytoplasmic RNA obtained from transformants carrying the mutated CABP1A expression construct yields only the CABP1B-specific 881bp fragment (Fig 2C, lane 3) This result agrees perfectly with the immunoprecipitation data presented in Fig 1B and demonstrates clearly that conversion of the 5' splice site in CABP1 to the Dictyostelium consensus leads to constitutive splicing. It should be noted that the RNA samples were all isolated from cells that had been in culture for several weeks; therefore, the second immunoreactive polypeptide sometimes expressed in cells transformed with the mutant CABP1A expression construct (described

in section 3.1.1) was not present. Finally, as expected, amplification of cytoplasmic RNA isolated from cells transformed with the expression vector pB10Act15BKH produces no products (Fig. 2C, lane 1). In addition, examination of all the PCR products obtained in these experiments by agarose gel electrophoresis followed by staining with ethidium bromide revealed that no fragments other than those detected by Southern blotting (Fig. 2B and 2C) were generated, thereby demonstrating the specificity of the reactions.

3.2 Isolation and Characterization of cDNAs Encoding CABP1-Related Polypeptides

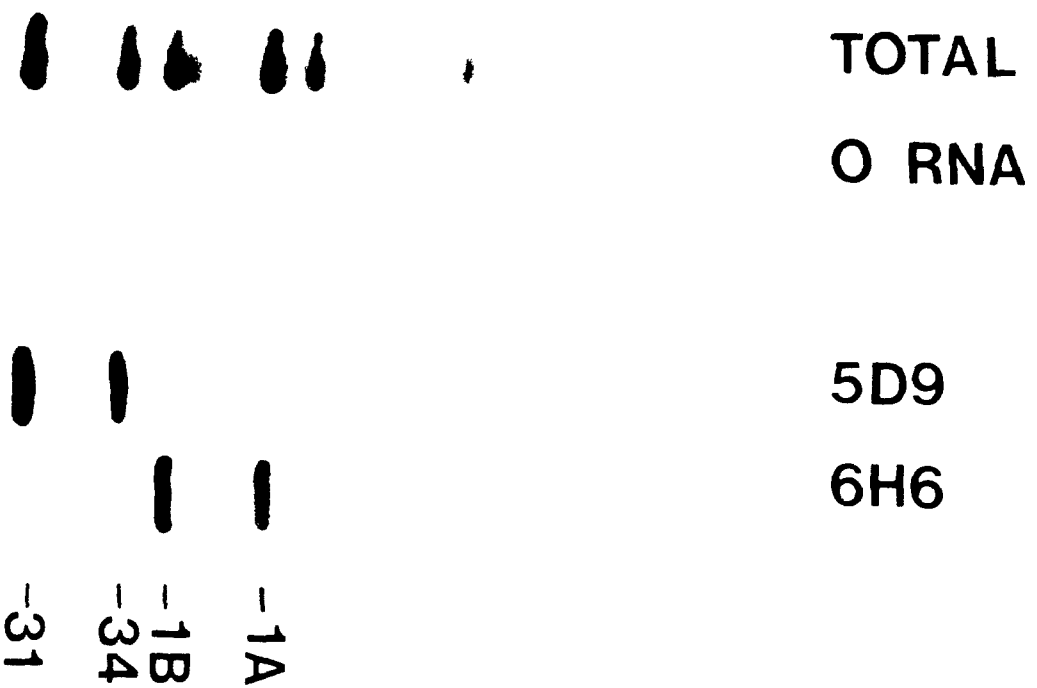
3.2.1 Isolation of cDNAs encoding p34 and p31

To isolate clones encoding related polypeptides, a cDNA library was screened with a 0.67kb cDNA containing a portion of the CABP1 coding sequence (Grant and Tsang manuscript submitted) under conditions of reduced stringency. Besides CABP1 cDNAs, this approach led to the isolation of a single related cDNA species, termed pC5D9, from the plasmid library. In order to identify this clone, hybrid selection experiments were performed. PolyA$^+$ RNA isolated from cells that had developed for 17h was hybridized under stringent conditions to denatured, filter-bound pC5D9 DNA. After washing the filters to remove nonspecifically bound material, RNA that had hybridized to pC5D9 was eluted and translated *in vitro* using a rabbit reticulocyte lysate. The translation products were analyzed by immunoprecipitation using an anti-CABP1 monoclonal antibody (Tsang and Tasaka 1986) followed by SDS polyacrylamide gel electrophoresis. As Fig. 3 shows, under stringent conditions pC5D9 selects RNA species which encode polypeptides with molecular weights of 34,000 and 31,000, both of which are recognized by the anti-CABP1 antibody. This observation suggests that pC5D9 contains sequences present in the transcripts coding for p34 and p31 and that these two transcripts share substantial homology at the nucleotide level.

The insert in pC5D9 is only about 400 nucleotides long. To

Figure 3. Identification of pC5D9 by hybrid selection.

Messenger RNAs complementary to pC5D9 were isolated by hybrid selection, translated in vitro, and analyzed by immunoprecipitation followed by SDS-PAGE. As a positive control, the CABP1 cDNA pC6H6 was also used to select RNA. Lane 1, immunoprecipitated products obtained from the translation of 1μg polyA$^+$ RNA; Lane 2, no exogenous RNA added to translation reaction; Lane 3, immunoprecipitated products obtained from the translation of RNAs complementary to pC5D9, Lane 4, immunoprecipitated products obtained from the translation of RNAs complementary to pC6H6. The two subunits of CABP1 as well as p34 and p31 are labelled.


TOTAL
O RNA
5D9
6H6
-31
-34
-1B
-1A

isolate full-length cDNAs for p34 and p31, a λgt11 cDNA library, kindly provided by Dr Peter Devreotes (Klein et al. 1988), was screened using pC5D9 as a probe Highly stringent hybridization conditions were chosen such that the probe would not hybridize to CABP1 sequences, under these conditions, pC5D9 does not hybridize to CABP1 cDNAs or to the CABP1 gene on Southern blots After screening approximately 200,000 plaques, twelve positive clones were obtained with insert sizes ranging from 500bp to 1 0kb To confirm that all of these clones corresponded to pC5D9 sequences and not to CABP1 transcripts, Southern analysis was performed None of the twelve cDNAs hybridized to CABP1 probes under conditions of high stringency Therefore, these sequences most likely represent cDNA clones of p34 and/or p31

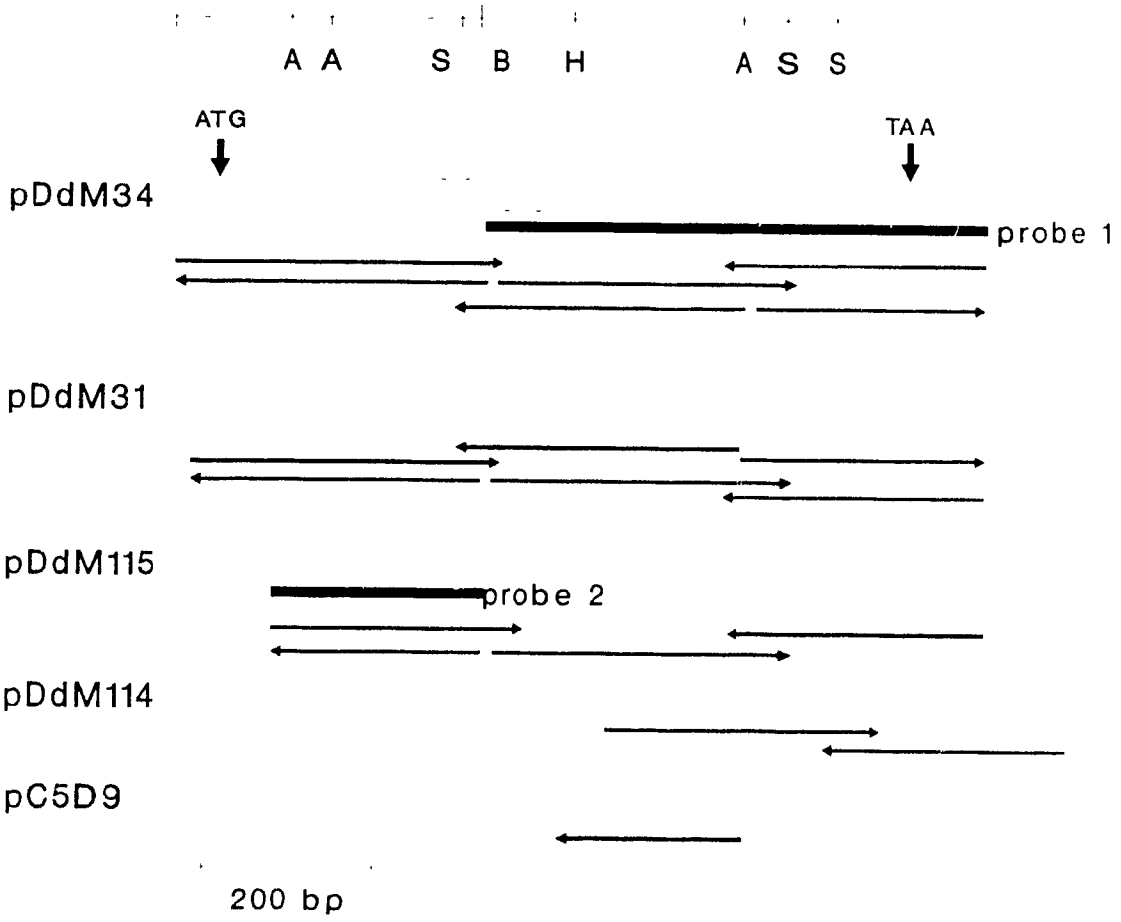
3 2.2: Sequence analysis of cDNAs

To facilitate further analysis, the longest cDNAs were subcloned into Bluescript plasmids Fig 4 describes the resulting clones and their restriction map as well as the strategy employed to determine their nucleotide sequences

The two longest cDNAs pDdM34 and pDdM31, have been sequenced completely on both strands Each clone contains only a single long open reading frame flanked by 5' and 3' untranslated sequences In each case, the first ATG appears likely to be the initiation codon since the sequences around it resemble the D discoideum consensus for translation initiation (Steel and Jacobson 1988). Furthermore, the sequence upstream of this codon in both clones is extremely A+T rich which is typical of

Figure 4 CABP$_L$-related cDNA clones.

cDNAs isolated from the phage library were subcloned into the EcoRI site of the plasmid vector Bluescript. The clone isolated from the plasmid library, pCFD9 is contained in the PstI site of pBR322. The open reading frame in each clone is represented by an open rectangle, untranslated sequences are indicated by a line. The restriction fragment used to probe the Southern blot (Fig. 6) is labelled probe 1, while the segment used to probe the Northern blot (Fig. 9) is designated probe 2. The strategy used to determine the nucleotide sequences of the cDNA clones is indicated by the arrows. The restriction map of the cDNA clones is located at the top of the figure. A, AluI, S, Sau3A; B, BamHI; H, HincII.


A A S B H A S S
ATG
TAA
pDdM34
probe 1
pDdM31
pDdM115
probe 2
pDdM114
pC5D9
200 bp

untranslated flanking regions of mRNAs in this organism. Both open reading frames end with TAA termination codons, as is the case for almost all D. discoideum genes sequenced to date.

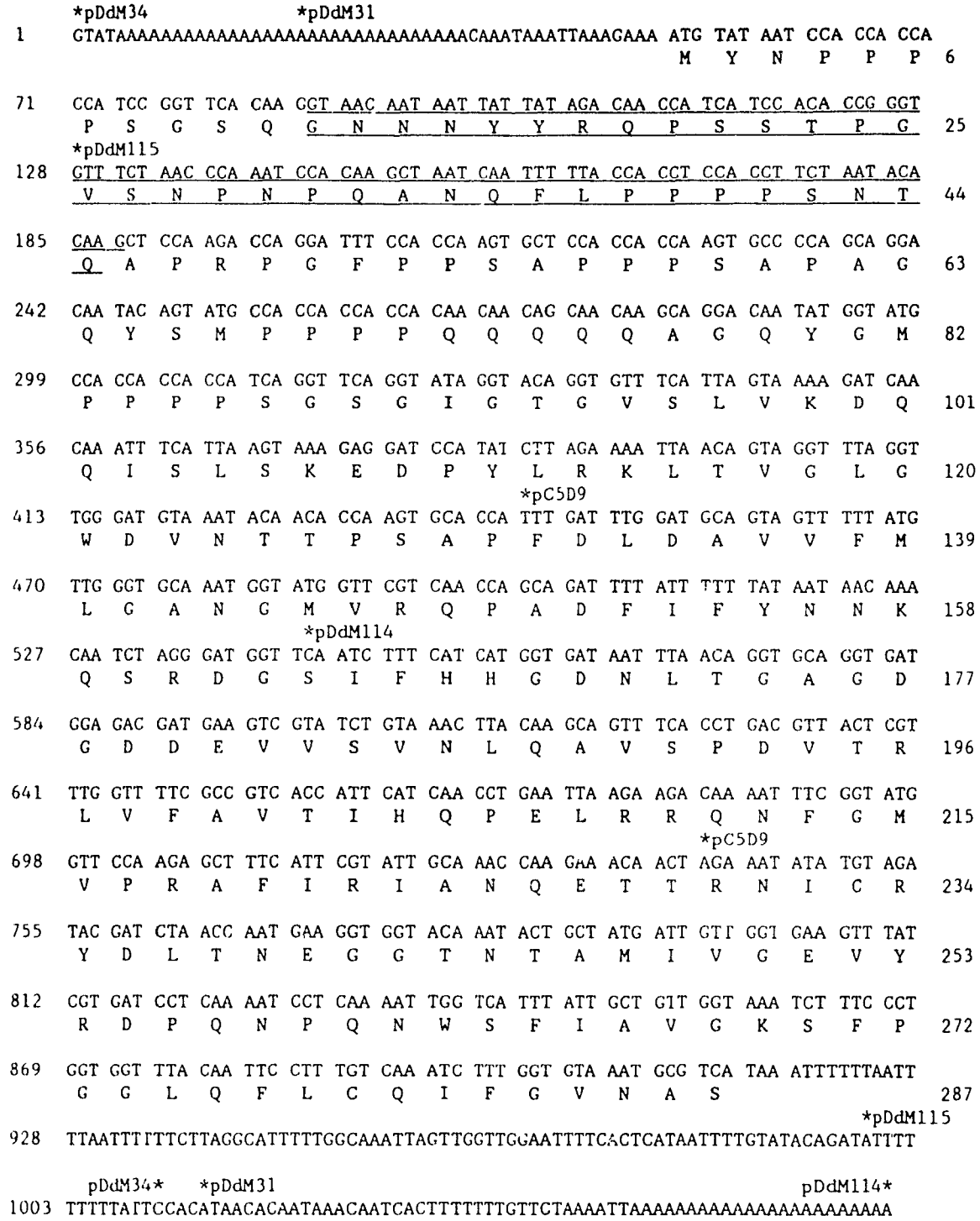
The shorter cDNAs have not been sequenced entirely on both strands However, these clones completely overlap portions of the longer cDNAs described above with the exception of pDdM114, which contains extra 3' untranslated sequence including a consensus polyadenylation signal, AATAAA, and a polyA tail.

The original cDNA identified and shown to contain portions of the transcripts which encode p34 and p31 by hybrid selection, pC5D9, was sequenced using supercoiled, double-stranded plasmid DNA as a template in conjunction with a synthetic oligonucleotide primer This technique was employed to avoid the problems associated with trying to sequence through the G/C homopolymer tracts added to each end of this clone during library construction Since the nucleotide sequence of pC5D9 perfectly matches sequences present in the other cDNAs, it is probable that the longer clones consist of more complete copies of the p34 and p31 transcripts and do not represent other CABP1-related messages. The nucleotide and deduced amino acid sequences of the cDNAs are illustrated in Fig. 5

Remarkably, pDdM34 and pDdM31 are completely identical except for the presence of a 102-nucleotide segment located at the 5' end of pDdM34 just downstream of the translation initiation codon. This element does not disrupt the open reading frame, thereby leading to the production of a protein containing an extra 34

Figure 5. Nucleotide and deduced amino acid sequences of cDNA clones.

The sequence of the two longest cDNAs, pDdM34 and pDdM31, was determined completely on both strands The remaining clones have not been sequenced entirely on both strands The 5' and 3' borders of all cDNAs are indicated The 3' end of pC5D9 was not determined The border that is marked represents the 3' end of the sequencing data for this clone The sequence which is underlined indicates the 102 nucleotide element which is absent from pDdM31 Nucleotide numbers are on the left while amino acid numbers are on the right.


*pDdM34
*pDdM31
1 GTATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAAATAAATTAAAGAAA ATG TAT AAT CCA CCA CCA
M Y N P P P 6
71 CCA TCC GGT TCA CAA GGT AAC AAT AAT TAT TAT AGA CAA CCA TCA TCC ACA CCG GGT
P S G S Q G N N N Y Y R Q P S S T P G 25
*pDdM115
128 GTT TCT AAC CCA AAT CCA CAA GCT AAT CAA TTT TTA CCA CCT CCA CCT TCT AAT ACA
V S N P N P Q A N Q F L P P P P S N T 44
185 CAA GCT CCA AGA CCA GGA TTT CCA CCA AGT GCT CCA CCA CCA AGT GCC CCA GCA GGA
Q A P R P G F P P S A P P P S A P A G 63
242 CAA TAC AGT ATG CCA CCA CCA CCA CAA CAA CAG CAA CAA GCA GGA CAA TAT GGT ATG
Q Y S M P P P P Q Q Q Q Q A G Q Y G M 82
299 CCA CCA CCA CCA TCA GGT TCA GGT ATA GGT ACA GGT GTT TCA TTA GTA AAA GAT CAA
P P P P S G S G I G T G V S L V K D Q 101
356 CAA ATT TCA TTA AGT AAA GAG GAT CCA TAT CTT AGA AAA TTA ACA GTA GGT TTA GGT
Q I S L S K E D P Y L R K L T V G L G 120
*pC5D9
413 TGG GAT GTA AAT ACA ACA CCA AGT GCA CCA TTT GAT TTG GAT GCA GTA GTT TTT ATG
W D V N T T P S A P F D L D A V V F M 139
470 TTG GGT GCA AAT GGT ATG GTT CGT CAA CCA GCA GAT TTT ATT TTT TAT AAT AAC AAA
L G A N G M V R Q P A D F I F Y N N K 158
*pDdM114
527 CAA TCT AGG GAT GGT TCA ATC TTT CAT CAT GGT GAT AAT TTA ACA GGT GCA GGT GAT
Q S R D G S I F H H G D N L T G A G D 177
584 GGA GAC GAT GAA GTC GTA TCT GTA AAC TTA CAA GCA GTT TCA CCT GAC GTT ACT CGT
G D D E V V S V N L Q A V S P D V T R 196
641 TTG GTT TTC GCC GTC ACC ATT CAT CAA CCT GAA TTA AGA AGA CAA AAT TTC GGT ATG
L V F A V T I H Q P E L R R Q N F G M 215
*pC5D9
698 GTT CCA AGA GCT TTC ATT CGT ATT GCA AAC CAA GAA ACA ACT AGA AAT ATA TGT AGA
V P R A F I R I A N Q E T T R N I C R 234
755 TAC GAT CTA ACC AAT GAA GGT GGT ACA AAT ACT GCT ATG ATT GTT GGT GAA GTT TAT
Y D L T N E G G T N T A M I V G E V Y 253
812 CGT GAT CCT CAA AAT CCT CAA AAT TGG TCA TTT ATT GCT GTT GGT AAA TCT TTC CCT
R D P Q N P Q N W S F I A V G K S F P 272
869 GGT GGT TTA CAA TTC CTT TGT CAA ATC TTT GGT GTA AAT GCG TCA TAA ATTTTTTAATT
G G L Q F L C Q I F G V N A S 287
*pDdM115
928 TTAATTTTTTCTTAGGCATTTTTGGCAAATTAGTTGGTTGGAATTTTCACTCATAATTTTGTATACAGATATTTT
pDdM34* *pDdM31 pDdM114*
1003 TTTTTATTCCACATAACACAATAAACAATCACTTTTTTTGTTCTAAAATTAAAAAAAAAAAAAAAAAAAAAAA

amino acids compared to the polypeptide encoded by pDdM31. The only other difference between these clones is that they contain slightly different amounts of untranslated flanking sequences. The simplest explanation for these results is that p34 and p31 are very closely related, and that pDdM34 and pDdM31 are complementary to the transcripts which encode these polypeptides.

3.2.3: DNA blot analysis

To determine the number of genes encoding p34 and p31, Southern analysis was performed. Two micrograms of genomic DNA was digested with various restriction enzymes, fractionated by agarose gel electrophoresis and transferred to a nylon membrane. The blot was then probed using the 3' BamHI-EcoRI fragment common to both pDdM34 and pDdM31 (probe 1 in Fig. 4). As Fig. 6A shows, only a single band hybridized to the probe under conditions of high stringency for all enzymes tested. Even if the stringency of hybridization was reduced the only additional bands recognized by this probe contain the CABP1 gene (Fig. 6B). Therefore, a single gene appears to encode both p34 and p31.

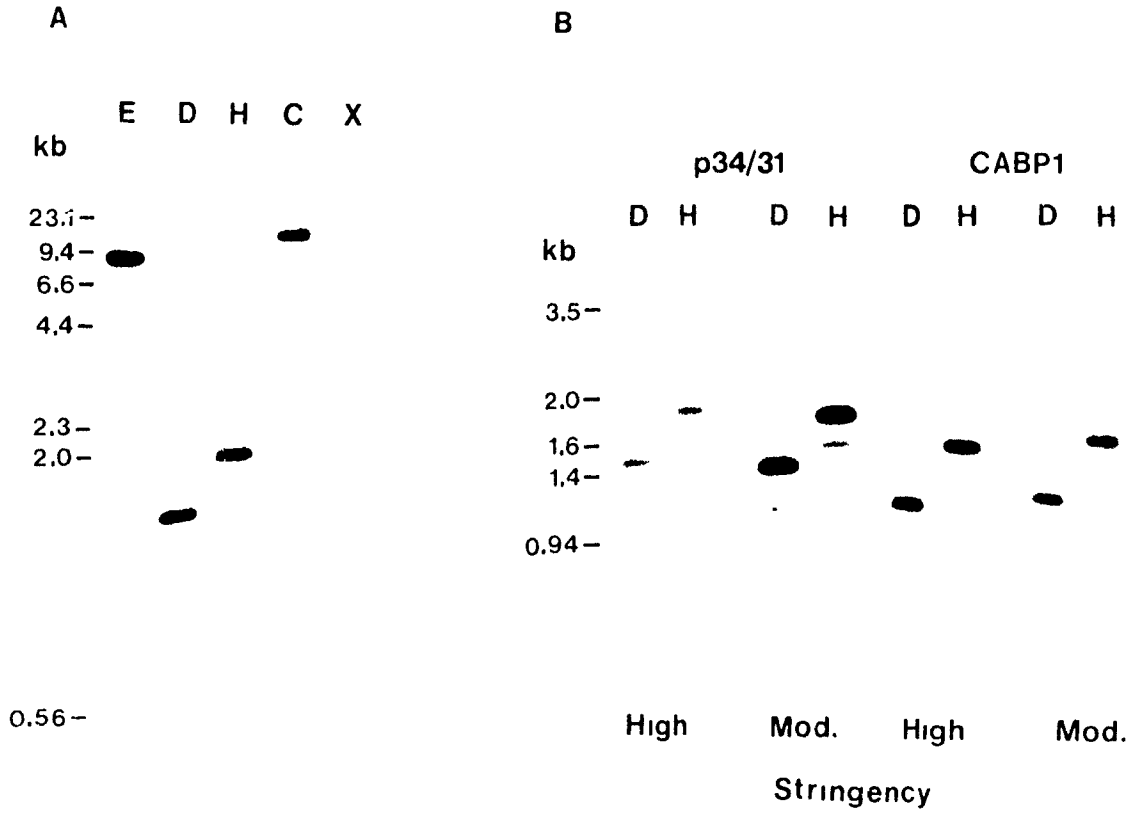
3.2.4: Expression of actin 15-pDdM34 fusion

Further evidence indicating that p34 and p31 are encoded by a single gene has been obtained by expressing pDdM34 under the control of the actin 15 promoter. This was accomplished by placing a HindIII site at the translation initiation codon of this clone and then inserting the resulting restriction fragment into the expression vector pB10Act15BKH. This construct codes for a translational fusion consisting of the first 14 codons of the

Figure 6 DNA blot analysis.

A Genomic DNA was digested with a variety of enzymes, size-fractionated on an agarose gel, transferred to a nylon membrane, and then probed under conditions of high stringency with a restriction fragment common to both pDdM34 and pDdM31 (probe 1 in Fig. 2) E, EcoRI; D, DraI, H, HinfI; C, ClaI; X, XhoII.

B Genomic DNA was digested with DraI (D) or HinfI (H) and blotted as above Identical blots were hybridized with either the probe used above or else with a restriction fragment corresponding to the 3' half of CABP1 under varying degrees of stringency.


A
E D H C X
kb
23.1–
9.4–
6.6–
4.4–
2.3–
2.0–
0.56–
B
p34/31
CABP1
D H D H D H D H
kb
3.5–
2.0–
1.6–
1.4–
0.94–
High
Mod.
High
Mod.
Stringency

modified actin 15 gene linked to the open reading frame present in pDdM34 (Fig. 7A). Stable transformants were isolated and analyzed by metabolic labelling with [^{35}S]methionine followed by immunoprecipitation with a monoclonal antibody which recognizes CABP1 as well as p34 and p31 (Tsang and Tasaka 1986). The immunoprecipitated products were resolved by SDS polyacrylamide gel electrophoresis and detected by fluorography. As Fig. 7B clearly shows, transformants carrying this construct express large amounts of plasmid-encoded p34 and p31, which can be distinguished from the endogenous molecules by virtue of their slight increase in size. This result indicates that these two polypeptides are the products of a single open reading frame. Furthermore, the information required to generate the two polypeptides is most likely present in the coding region of pDdM34. The observation that p34 and p31 can both be expressed from the same cDNA provides strong evidence that the two polypeptides are the products of one gene.

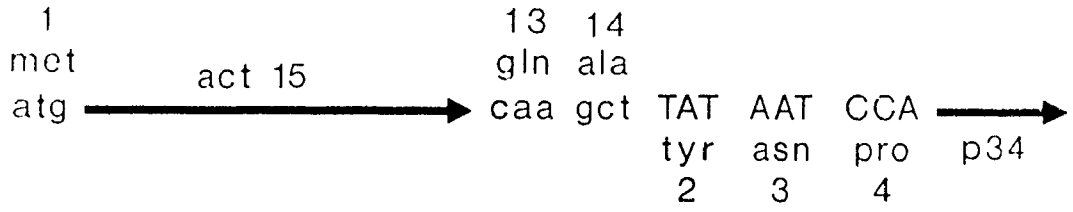
Attempts to use the overexpressing strain for a biochemical analysis of p34 and p31 have not been successful. A more detailed examination of this strain has revealed that the overexpression of these polypeptides is not very stable. In addition, immunofluorescence experiments have revealed that the overexpressed molecules appear to be forming aggregates in the cytoplasm and therefore may not be functional (Fig. 8).

Figure 7 Expression of pDdM34 Under the control of the actin 15 promoter.

A. Actin 15-pDdM34 fusion construct The nucleotide sequences provided by the expression vector pB10Act15BKH are shown in small letters, while those derived from pDdM34 are in capitals The numbers above the sequence represent amino acid position in the fusion while those below the sequence indicate the codon position in the open reading frame of pDdM34

B Immunoprecipitation analysis of transformants After introducing the expression construct into Dictyostelium cells, the transformants were analyzed by metabolic labelling with [^{35}S]methionine followed by immunoprecipitation The two subunits of CABP1 are labelled along with p34 and p31 Lane 1, immunoprecipitated products obtained from untransformed cells, Lane 2, immunoprecipitated products obtained from cells transformed with pB10Act15BKH, Lane 3, immunoprecipitated products obtained from cells transformed with the actin 15-pDdM34 fusion construct

A

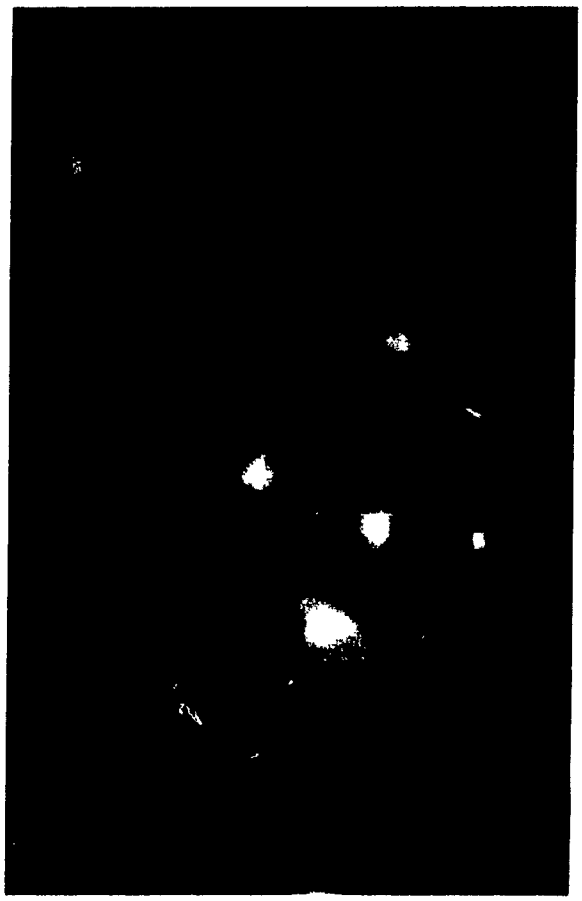

1
met
atg
act 15
13
gln
caa
14
ala
gct
TAT
tyr
2
AAT
asn
3
CCA
pro
4
p34

B

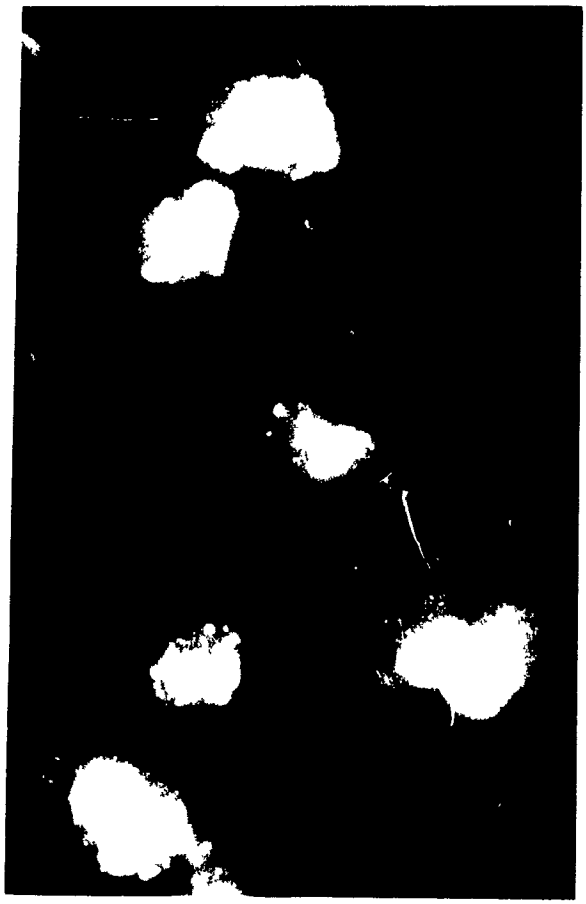

1
2
3
1A
1B
p34
p31

Figure 8 Immunofluorescence microscopy of cells overexpressing p34 and p31

Transformants carrying pB10Act15BKH (control) or the actin 15-pDdM34 fusion construct (overexpression) were stained with an anti-CABP1 monoclonal antibody and then treated with TRITC-conjugated secondary antibodies. The cells were then examined by immunofluorescence microscopy



control



overexpression

3 2 5. RNA blot analysis

To examine the pattern of expression of the transcripts complementary to pDdM34 and pDdM31 5μg of polyA$^+$ RNA isolated from cells at various times of development was fractionated by electrophoresis through denaturing agarose gels and transferred to nitrocellulose The blot was probed with the 5' EcoR1-BamHI fragment from pDdM115 (probe 2 in Fig 4) Highly stringent conditions were employed to prevent hybridization to CABP1 RNAs As is shown in Fig. 9, three transcripts with lengths of approximately 1 15kb, 1 25kb and 1 4kb were detected at all times of development The same results were obtained when 3' probes were used Sizes were determined by probing identical blots with CABP1, actin and discoidin I probes

The 100 nucleotide difference in size between the two smaller transcripts agrees well with our sequencing data and suggests that these two RNA species are complementary to pDdM34 and pDdM31 Since our cDNA clones contain 1 0kb and 1.1kb of sequence, and polyA tails on mRNAs in D discoideum are approximately 100 nucleotides long, it is probable that these clones contain most of the sequence present in their complementary transcripts The origin of the 1 4kb transcript is not known (see Dicussion)

3 2.6. Derived amino acid sequences of p34 and p31 and comparison to CABP1

The derived amino acid sequences of the polypeptides encoded by pDdM34 and pDdM31 are shown in Fig 5 These polypeptides have calculated molecular weights of 30,963 and 27,255, respectively

Figure 9 RNA blot analysis of transcripts complementary to pDdP134 and pDdP131

PolyA^+ RNA (5μg) isolated from cells which had developed for 0, 9, 12, or 16 h was fractionated by electrophoresis through an agarose formaldehyde gel and transferred to nitrocellulose. The blot was probed under stringent conditions with a restriction fragment from the cDNA clone pDdP115 (probe 2 in Fig. 7).

0 9 12 16

This is somewhat lower than their apparent molecular weights as determined by SDS polyacrylamide gel electrophoresis It is unlikely that this discrepancy is due to post-translational modifications since the polypeptides produced by <u>in vitro</u> translation followed by immunoprecipitation comigrate with the polypeptides synthesized <u>in vivo</u> (A. Tsang, personal communication) In addition, since the expression of pDdM34 in <u>Dictyostelium</u> cells leads to the production of the appropriate-sized molecules (Fig 7), the difference between the predicted and observed sizes is not the result of a cloning artifact. A more likely explanation is the amino acid composition of these polypeptides Both molecules have a very high content of proline and glutamine High proline content has been found to cause abnormal migration on SDS polyacrylamide gels (Driever and Nusslein-Volhard 1988) Furthermore, it has recently been demonstrated that the subunits of CABP1 also migrate slower than expected, presumably due to a high proline/glutamine content (Grant and Tsang, manuscript submitted).

As described earlier, p34 and p31 are identical except for the presence of a 34-amino acid segment inserted near the amino terminus of p34 Interestingly, we have found that the two subunits of CABP1 are also identical except for the presence of an additional 37 amino acids at the amino terminus of the larger polypeptide, CABP1A The results described in section 3.1 demonstrate that the mRNA encoding the small subunit, CABP1B, is generated by a splicing mechanism which removes a 111-nucleotide

segment that codes for the extra amino acids found only in CABP1A. An alignment of the deduced amino acid sequences of p34 and p31 with those of CABP1A and CABP1B is shown in Fig 10 The similarity between p34/31 and the two subunits of CABP1 is remarkable. The amino terminal sequences are virtually identical, with only two amino acid differences in the first 45 residues. Significantly, the sequence which is specific to p34 almost exactly matches the region of CABP1 which is found only in CABP1A Furthermore, the nucleotide sequence encoding this element is flanked precisely by the eukaryotic splice consensus signals GT/AG, suggesting that p31 is generated by a splicing process very similar to the one which produces CABP1B The results obtained by expressing pDdM54 under the control of the actin 15 promoter (Fig 7) are consistent with this hypothesis

Immediately following this highly conserved amino terminus, the proteins diverge considerably This region in CABP1, which is 94 amino acids in length is very distinctive in its amino acid content It contains a high proportion of proline and glutamine residues as well as 5 copies of the 9 amino acid repeat QPAGQYGAP (Grant and Tsang, manuscript submitted) In p34/31, however, this region contains only 40 residues Even though it is considerably shorter than the same element in CABP1, its overall character remains very similar since it is also very rich in proline and glutamine residues. It also contains 2 degenerate versions of the 9 amino acid repeat found in CABP1, APAGQYSMP and QQAGQYGMP, separated by a polyglutamine tract However, in p34/31, the

Figure 10 Alignment of CABP1 and p34/31 amino acid sequences. The amino acid sequences of p34 and p31 were aligned with those of the two subunits of CABP1 using the ALIGN program (Pearson and Lipman 1988) The underlined sequences are absent from CABP1B and p31 Identical amino acids are represented by dots, while dashes indicate gaps

```
CABP1     MYNPPPPSGSQGNNNYYRQLSSTPGVSNPNPQANQFLPPQPSNTIQTPGGYPPQQQ
p34/31    .           _ ___ . .  _______ _____ . . ___ . .   P      QAPRP F   ---

          QRPPTGAPQQPGGYPIPPPPGAPCCYPPQQQPAGQYGAPPQQQPAGQYGAPQPAGQ
          --------------- -   ------ ----- ---S     PSA     . SM P PQ

          YGAPQPAGQYGAPQPAGQYGAPPPPPGGAGISLVKNQQISLTKEDPTLRKITIGLG
          ---Q Q     . M F ----- .SGIGI T.V     .D      .S      Y       V

          WDVNITPTAPFDLLAVVIMLNAQGPVRTSQDL FVNNKVSRDNSVSHQGDNLTGQG
           .       S    . ...         G.N M .QPA.. .      .Q . G IF H         A

          EGDDEVVLVNLQAVSPDVIRLVFAVTTHLADERRQNFTMVPRAFIRVANQETGRNI
          D ..     S .    ..   .      . . .    Q?EL..     G .          I. . .T

          CRYDLSQEGGPNTALIAGEVYRDPSHPNNWSFVAVGKGMQGALPGLLQIFGCQ--
          . ...TN.. T . M.V..    .  C  Q .. I   . SFP G QF C      VNAS
```

repeat has been extended to 12 amino acids by the addition of 3 proline residues to the carboxy terminus. Following this region, the similarity between CABP1 and p34/31 increases again. The carboxy terminal halves of these polypetides possess 76% identity; this value increases to approximately 85% if conservative changes are included. A comparison of the nucleotide sequences of CABP1 and p34/31 shows a similar pattern of homology.

3.2.7 Homology between p34/31 and bacterial plasmid-encoded polypeptides

It has been demonstrated that CABP1 is related to two polypeptides encoded by genes present on a bacterial plasmid which confers resistance to tellurium anions (Grant and Tsang, manuscript submitted). These two sequences, ORF4 and ORF5 of the plasmid pMER610, are presumed to have arisen by gene duplication (Jobling and Ritchie 1988). Unsurprisingly, p34 and p31 also show homology to these bacterial polypeptides. The carboxy-terminal 193 amino acids of p34/31 possess 49.7% and 48.5% identity to the proteins encoded by ORF4 and ORF5, respectively. These values are in fact slightly higher than the similarity between CABP1 and ORF4 and ORF5. An alignment of CABP1, p34/31, ORF4 and ORF5 is shown in Fig. 11. No other proteins have been found to possess significant similarity to p34 and p31.

Figure 11 Comparison of CABP1, p34/31 and two bacterial plasmid-encoded polypeptides

Screening the GENBANK database demonstrated that two polypeptides encoded by open reading frames 4 and 5 (ORF4 and ORF5) of the bacterial plasmid pMER610 are very similar to CABP1 and p34/31 Pairwise alignments of these sequences were performed using the ALIGN program (Pearson and Lipman 1988). Results of the pairwise alignments were used to generate this figure. Amino acid identities with CABP1 are represented by dots, while gaps are indicated by dashes The alignment begins at amino acid 1 for ORF4 and ORF5, amino acid 143 for CABP1, and amino acid 95 for p34/31.

```
CABP1     --ISLVKNQQISLTKEDPTLRKLTIGLGWDVNTTPTAPFDLDAVVFMLNAQGRV
P34/31    --V....D.....S....Y.....V..........S ..... . ...G.N.M
ORF4      MSV..S.GGNV..S.TA.SMKNVLV.....ARS.DGQD .   SA L A.N.K
ORF5      MAV....GGNV.....A.SMNVALV.....TRV.DGQA.   .S  LVGEN K

          RTSQDFIFYNNKVSRDNSVSHQGDNLTGQGEGDDEVVLVNLQAVSPDVTRLVFA
          .QPA........Q...G.IF.H... . A.D......S..............
          .GDA.......LK A.G..T.T...R..E.D. ..SLKIK D. PG..DKII.V
          LSDSH.V....TI.P.CA.Q.....R..E.D... Q.KID.TK AA..KK...

          VTIHLADERRQNFTMVPRAFTRVANQETGRNICRYDLSQEGGPNTALIAGEVYR
          ....QPEL.....G..... ..I.....T.........TN...T...M.V ...
          .. .D.QA...S.GO.EG. ..LV DDNQTEVA ...TEDASTE. MLF .L.
          ....E .S.K...G..SNS.M..V.ND. SE A.F...EDASTE .M F..L.

          DPSNPNNWSFVAVGKGMQCALPGLLQIFGCQ--
          ..Q..Q....I....SFP.C.QF C....VNAS
          HNGA---.K.R. .L YA.G.ASVCAQY.INAS
          HGAE---.K K...Q.FA.G AA.ATQH.INI-
```

3.3. Functional Analysis of p34 and p31 by Gene Disruption

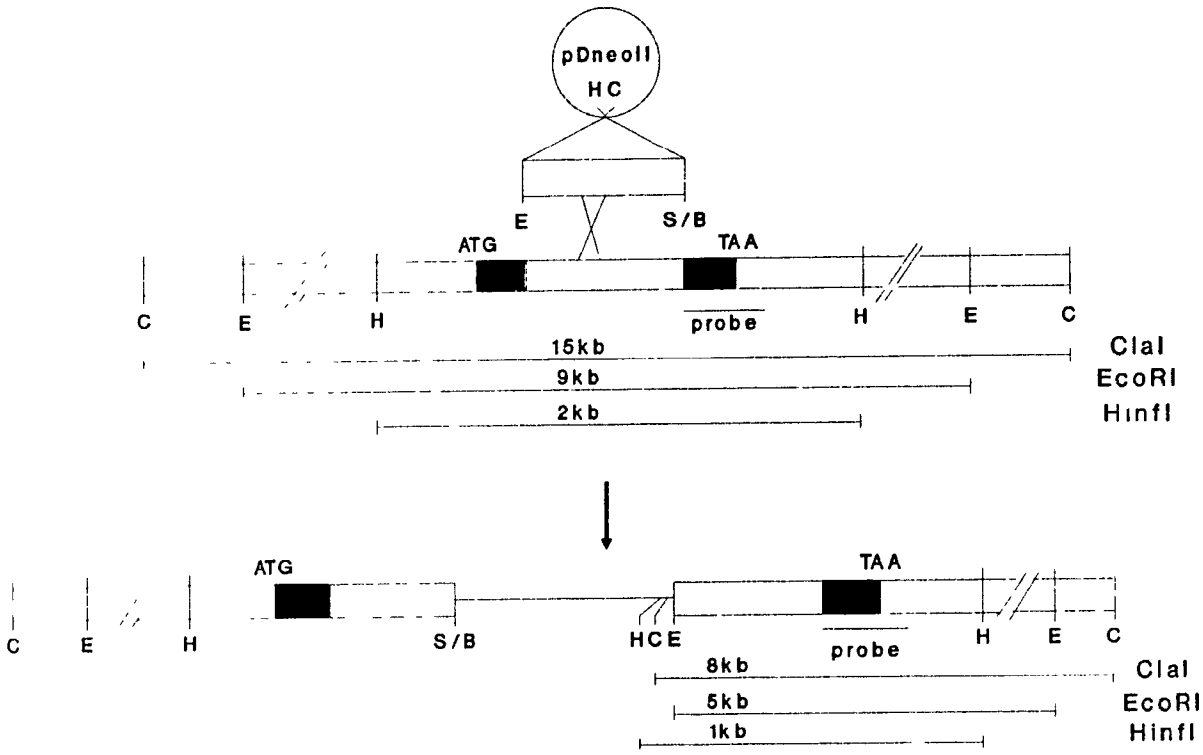
3.3.1: Generation of p34/31 mutants by gene disruption

In order to obtain clues concerning possible functions of p34 and p31 *in vivo*, the gene which encodes them has been disrupted. The strategy for this experiment is outlined in Fig. 12 A cDNA fragment lacking approximately 100 nucleotides of coding sequence from each end was inserted into the transformation vector pDneoII. The resulting construct was introduced into *Dictyostelium* cells by calcium phosphate coprecipitation. Since the transformation vector does not contain a replication origin, stable transformants can only arise when the plasmid integrates into the genome. Presumably, in at least a fraction of the transformants, integration will happen via homologous recombination between the cDNA fragment and the endogenous gene. When this occurs, two truncated copies of the gene will be generated separated by plasmid sequences One of these genes will be lacking 3' coding sequences and flanking elements while the other copy will lack a functional promoter as well as 5' coding sequences. The 3' truncated version of the gene, if expressed at all, should produce polypeptides lacking approximately 30 amino acids from the carboxy termini and should therefore differ in molecular weight compared to the wildtype molecules by about 2000-3000. The 5' truncated version of the gene should be completely inactive.

When homologous recombination occurs, it can be detected by changes in the size of specific restriction fragments. Targeted

Figure 12. Strategy for disrupting the p34/31 gene.

A partial restriction map of the region surrounding the p34/31 gene is shown. Genomic DNA is represented by rectangles, while integrated plasmid DNA is indicated by a line. The translation initiation and termination signals of the gene are marked. The black boxes represent the portions of the coding sequence that are absent from the disruption construct. The probe used to detect mutants in which homologous recombination has occurred is indicated. The sizes of various restriction fragments before and after homologous recombination are marked. Note that, for simplicity, this diagram shows only a single copy of the disruption vector integrated into the p34/31 gene. The figure is not drawn to scale. The restriction sites shown on the map include: B, BamHI; C, ClaI, E, EcoRI; H, HinfI, S, Sau3A


pDneoII
HC
E
S/B
ATG
TAA
C
E
H
probe
H
E
C
15kb
9kb
2kb
ClaI
EcoRI
HinfI
ATG
TAA
C
E
H
S/B
HCE
probe
H
E
C
8kb
5kb
1kb
ClaI
EcoRI
HinfI

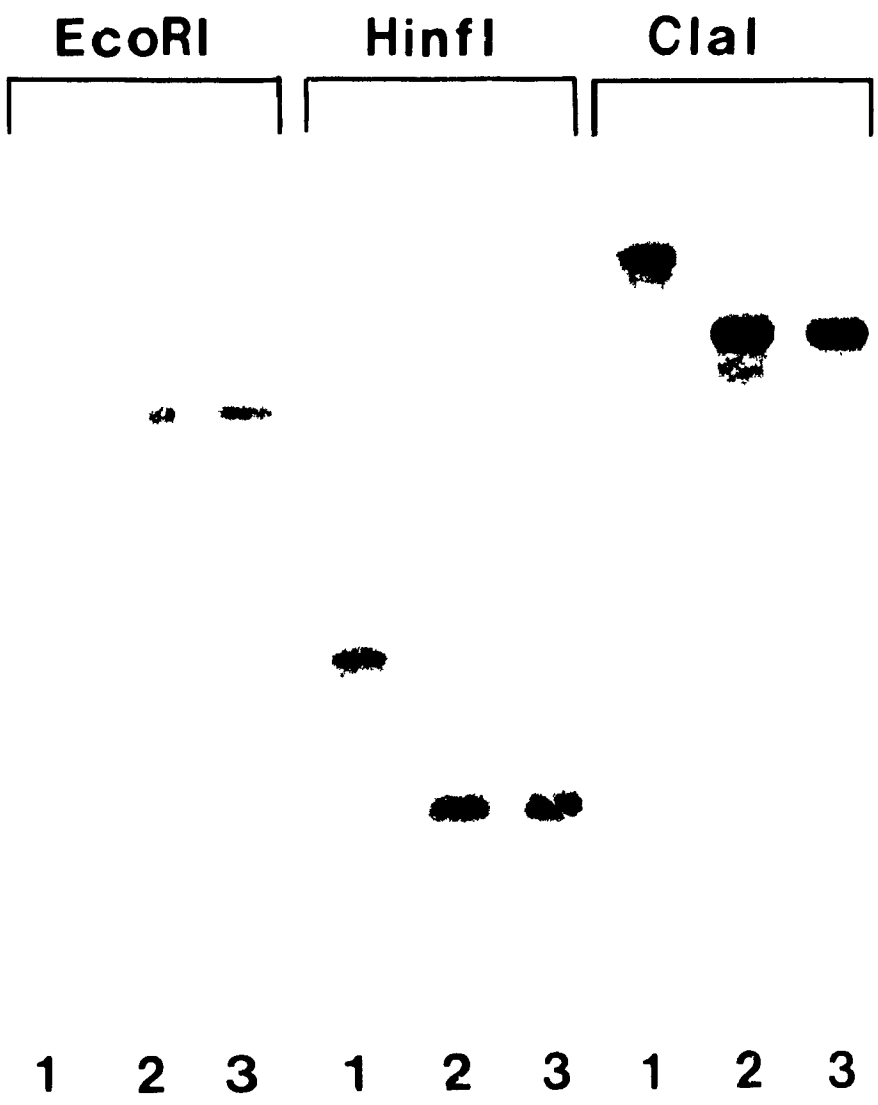
disruption of the p34/31 gene can be predicted to alter the size of the genomic EcoRI restriction fragment that will hybridize to a cDNA probe. This probe contains sequences specific for the extreme 3' end of the p34/31 gene and will not hybridize to the cDNA fragment present in the disruption construct. Therefore, it will only detect the 3' end of the endogenous gene. As shown in Fig. 12, this probe hybridizes to a 9kb EcoRI fragment in genomic DNA from wildtype strains but will detect a 5kb fragment when homologous recombination has disrupted the p34/31 gene. This alteration in the size of the EcoRI fragment formed the basis for the screen to detect mutants in which the p34/31 gene has been disrupted. The effects of homologous recombination on the sizes of some other restriction fragments is also indicated in Fig. 12.

It should be noted that transformed Dictyostelium cells often carry multiple copies of the transforming plasmid integrated into a single site in the genome arranged in a tandem head-to-tail array. Since the probe used to screen for the p34/31 mutants does not recognize sequences present in the disruption construct, the alterations described above will be detected regardless of the number of copies of the vector which integrate into the p34/31 gene.

Genomic DNA was extracted from a total of 80 independently-isolated stable transformants. After digestion with EcoRI, the DNA was fractionated by agarose gel electrophoresis, transferred to a membrane and then probed with the cDNA fragment described above. While most of the transformants possessed the wildtype 9kb

Figure 13. Southern analysis of mutants.

Genomic DNA isolated from wildtype cells and two putative p34/31 mutants identified during the initial screening process was digested with ClaI, EcoRI, or HinfI, fractionated by agarose gel electrophoresis, and transferred to a filter The blot was then probed with a cDNA fragment corresponding to the extreme 3' end of the p34/31 gene. Lanes 1, genomic DNA from wildtype AX2 cells; Lanes 2, genomic DNA from transformant AX-2T133-40; Lanes 3, genomic DNA from transformant AX-2T133-43.


EcoRI
HinfI
ClaI
1 2 3 1 2 3 1 2 3

EcoRI fragment, 4 of them contained the 5kb fragment predicted to be present in p34/31 mutants. Two of these transformants, AX-2T133-40 and AX-2T133-43, were chosen for further analysis.

Genomic DNA was prepared from these strains by CsCl density gradient centrifugation and examined by blot analysis. As Fig. 13 shows, both of these strains do indeed contain the predicted 5kb EcoRI fragment instead of the wildtype 9kb fragment. Furthermore, wildtype DNA digested with HinfI contains a 2kb fragment which hybridizes to the probe while the two transformants possess a 1kb fragment. Similarly, the probe identifies a 15kb fragment in ClaI-digested wildtype DNA in contrast to an 8kb fragment in both transformants These results clearly indicate that homologous recombination has disrupted the p34/31 gene in AX-2T133-40 and AX-2T133-43

3.3.2. Expression of p34 and p31 in AX-2133-40 and AX-2T133-43

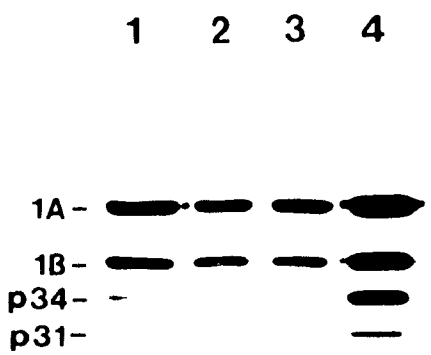
To examine the expression of p34 and p31 in the disrupted strains, developing cells were metabolically labelled with [^{35}S]methionine and then lysed A monoclonal antibody raised against CABP1 which also recognizes additional polypeptides including p34 and p31 (Tsang and Tasaka 1986) was used to immunoprecipitate these molecules, which were then resolved by electrophoresis through SDS polyacrylamide gels and detected by fluorography Both subunits of CABP1 as well as p34 and p31 are present in wildtype cells (Fig. 14A). Furthermore, the same polypeptides are also present in AX-2T133-29, a control transformant carrying the disruption construct which has

Figure 14. Expression of p34 and p31 in mutants

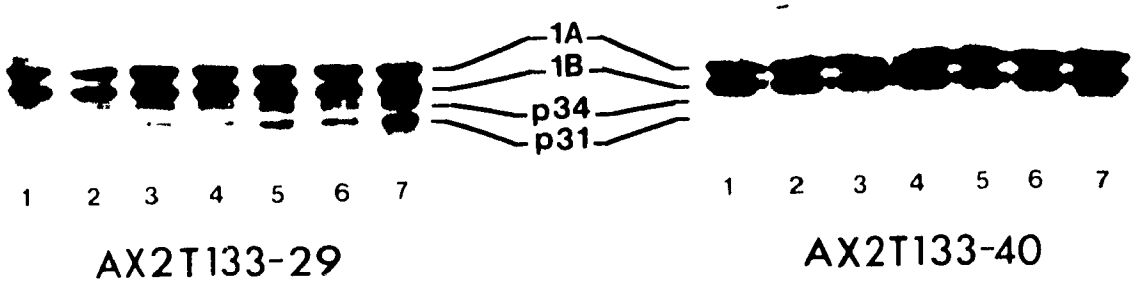
A Immunoprecipitation analysis. Wildtype and mutant cells were metabolically labelled with [^{35}S]methionine and then lysed. CABP1 and its related polypeptides were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography. The two subunits of CABP1 as well as p34 and p31 are indicated. The immunoprecipitated products from wildtype cells (Lane 1), AX-2T133-40 cells (Lane 2), AX-2T133-43 cells (Lane 3), and AX-2T133-29, a wildtype transformant in which the p34/31 gene is intact (Lane 4), are shown.

B. Immunoblot analysis. Protein extracts isolated at 4h intervals from vegetative and developing cells of AX-2T133-29 and AX-2T133-40 were fractionated by SDS-PAGE and then transferred to filters. The blots were then probed with the same antibody used for the above immunoprecipitation. The two subunits of CABP1 as well as p34 and p31 are labelled. Lane 1, vegetative extracts, Lane 2, 4h extracts; Lane 3, 8h extracts, Lane 4, 12h extracts, Lane 5, 16h extracts, Lane 6, 20h extracts, Lane 7, 24h extracts.

A


1 2 3 4
1A -
1B -
p34 -
p31 -

B


1A
1B
p34
p31
1 2 3 4 5 6 7
1 2 3 4 5 6 7
AX2T133-29
AX2T133-40

integrated randomly into the genome; DNA blot analysis indicates that the p34/31 gene is intact in this strain In contrast, while the two subunits of CABP1 are present in both AX-2T133-40 and AX-2T133-43, no wildtype p34 or p31 polypeptides are detected in these strains. Instead, very low levels of two slightly smaller molecules are present (Fig. 14A, lanes 2 and 3) These two polypeptides probably represent truncated versions of p34 and p31 generated by the 3' deleted copy of the gene generated by homologous recombination (see Fig 12)

Immunoblot analysis was also performed to determine the expression of p34 and p31 in one of the disrupted strains Cells were developed on filters as described in section 2 1 Protein samples were taken at 4h intervals, fractionated by SDS-PAGE, and transferred to filters. The blots were then probed with the same antibody used for the immunoprecipitation analysis described above. As Fig 14B shows, the control transformant in which the p34/31 gene is intact displays the previously described profile of expression for these two polypeptides (Kay et al 1987) Both molecules are absent from vegetative cells and appear by 4h of development. Their levels then remain relatively constant throughout the rest of the developmental cycle In AX-2T133-40, however, the truncated forms of p34 and p31 can just barely be detected during development

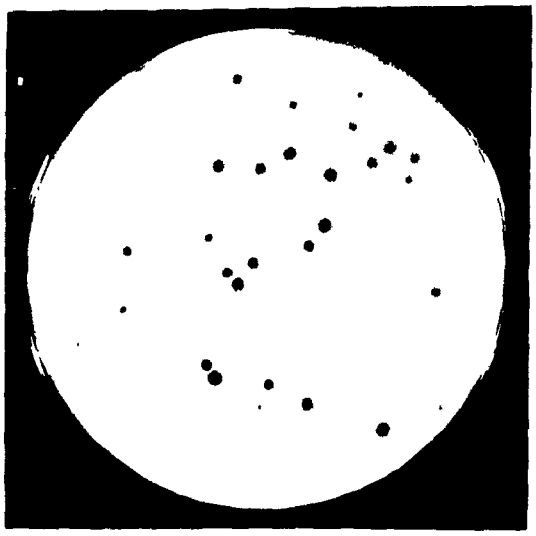
3 3 3: Growth of AX-2T133-40 and AX-2T133-43

Phenotypic analysis has revealed a growth defect in the two p34/31 mutants When Dictyostelium cells are mixed with a bacterial suspension and then spread on agar plates, uniform lawns soon appear as the bacteria grow. A short time later, plaques appear in the bacterial lawns as the Dictyostelium cells grow and ingest surrounding bacteria. Fig. 15 shows plaques that are produced by a control transformant in which the p34/31 gene is intact compared to those that are formed by AX-2T133-40. Very tiny plaques are produced by the mutant cells compared to the wildtype control, indicating a slower rate of growth. Identical results are obtained with AX-2T133-43

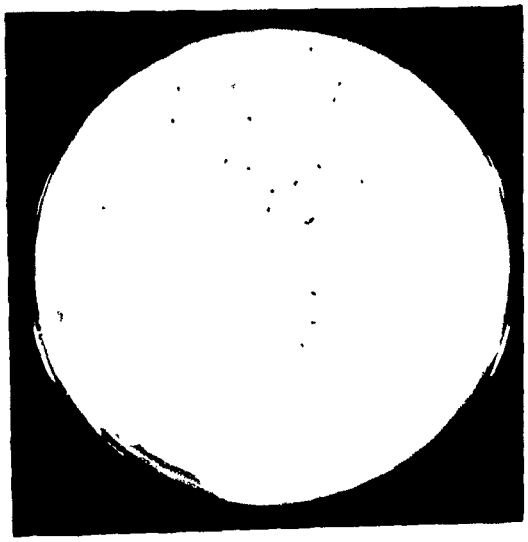
A quantitative analysis of growth in these strains has also been performed (Fig 16A). Cells of each strain were inoculated into bacterial suspensions and then agitated. At regular intervals, small aliquots were removed from each culture and the Dictyostelium cell density was determined directly using a hemocytometer While wildtype AX-2 cells and the control transformant grew with a doubling time of approximately 3h, both AX-2T133-40 and AX-2T133-43 grew significantly slower with a generation time of about 5h Intriguingly, this growth defect was seen only when the cells were fed bacteria. When grown axenically in HL5 medium, all 4 strains grew with a generation time of about 8h (Fig. 16B)

Figure 15. Plaque formation by mutants.

Approximately 50 cells of strains AX-2T133-29 and AX-2T133-40 were mixed with bacteria and then spread on agar plates. The plates were incubated at 22°C for 72h to allow plaques to form in the bacterial lawns.



AX2T133-29



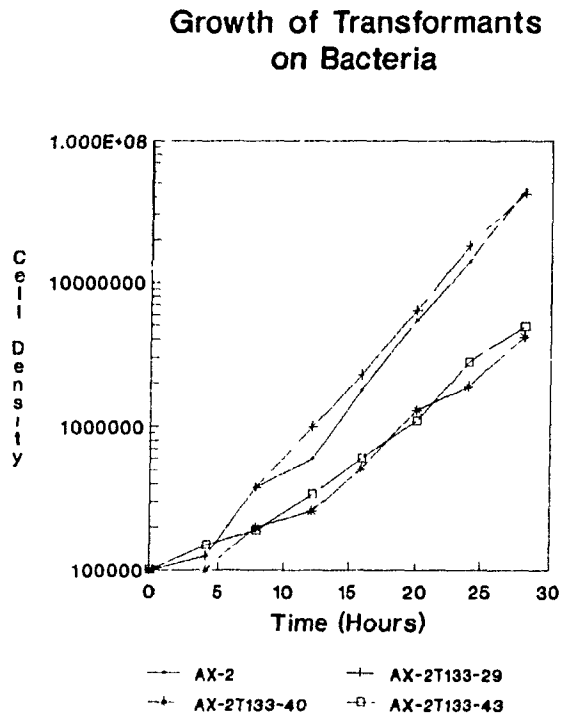
AX2T133-40

Figure 16. Growth curves for wildtype and mutant strains

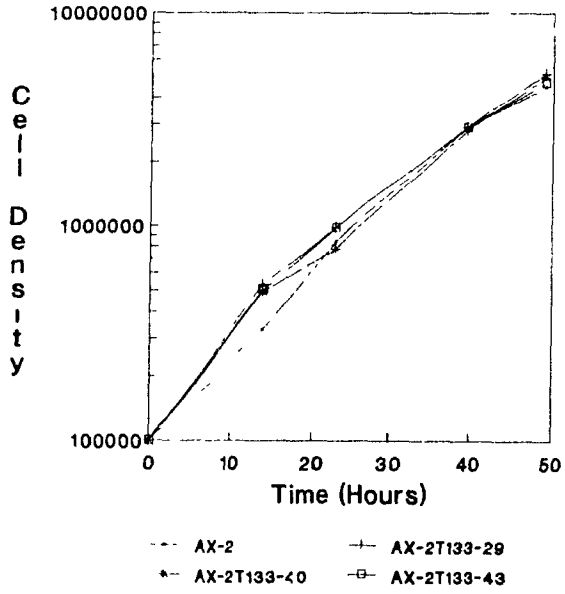
A. Growth on bacteria. Wildtype and mutant cells were inoculated into bacterial suspensions and incubated at 22°C with agitation Cell density was determined at 4h intervals using a hemocytometer

B. Axenic growth. The same strains analyzed above were inoculated into HL5 medium and grown axenically at 22°C Cell density was determined every 8h.

A


Growth of Transformants
on Bacteria
1.000E+08
10000000
1000000
100000
Cell Density
0
5
10
15
20
25
30
Time (Hours)
AX-2
AX-2T133-29
AX-2T133-40
AX-2T133-43

B


Growth of Transformants
in Axenic Culture
10000000
1000000
100000
Cell Density
0
10
20
30
40
50
Time (Hours)
AX-2
AX-2T133-29
AX-2T133-40
AX-2T133-43

3.3.4: Development of AX-2T133-40 and AX-2T133-43

To assess the developmental profile of AX-2T133-40 and AX-2T133-43, bacterially-grown cells were plated on filters as described in section 2.1 They were monitored at 1-2h intervals and compared to wildtype AX-2 and the control transformant AX-2T133-29. Although the mutants form normal fruiting bodies, they possess a minor defect in development. They complete development about 1h later than normal strains. Interestingly, the defect responsible for this delay occurs at a specific developmental stage. The mutants behave identically to wildtype controls throughout most of the developmental cycle, including aggregation, tip formation, and establishment of a migratory slug. They then remain in the slug phase for approximately 1h longer than the wildtype controls before going on to complete terminal development in a normal manner.

Chapter 4: Discussion

As described in chapter 1, the molecule cAMP plays a central role in the lifecycle of Dictyostelium discoideum. It acts as a chemoattractant to direct the formation of multicellular aggregates after development has been initiated by starvation (Konijn et al 1967), regulates the differentiation of both spore and stalk cells (Williams 1987), and controls much of the gene expression which occurs during development (Firtel et al. 1989). While it appears that most of the effects of cAMP on development are the result of an interaction between extracellular cAMP and a cell-surface receptor in which a series of G-protein linked signal transduction events play a key role (Firtel et al. 1989), there is also compelling evidence which suggests that intracellular cAMP is important in at least some of these events (Kimmel 1987; Kay 1989; Riley et al. 1989; Simon et al. 1989; Chapman and Firtel 1990).

The isolation of the novel intracellular cAMP binding protein CABP1 from D. discoideum (Tsang and Tasaka 1986) has identified a possible pathway through which the effects of intracellular cAMP may be mediated. The observation that CABP1 accumulates in the nuclei of developing cells suggests that it may play an important regulatory role (Kay et al 1987). In addition, monoclonal antibodies raised against CABP1 crossreact with several polypeptides (Tsang and Tasaka 1986) while CABP1 cDNA probes recognize a number of genes in the genome of D. discoideum (Tsang et al 1988). These observations suggest that CABP1 may belong to a family of related molecules.

I have analyzed the mechanism by which the two subunits of CABP1 are produced from a single gene. In addition, I have isolated and characterized cDNA clones encoding two polypeptides which crossreact with anti-CABP1 monoclonal antibodies and have disrupted the gene which codes for these molecules. The conclusions based on these experiments are presented here.

4.1: An Unusual Splicing Mechanism Generates the Two Subunits of CABP1 from a Single Gene

The two subunits of CABP1 are encoded by a single gene. The evidence for this conclusion includes DNA blot analysis (Grant and Tsang, manuscript submitted), RNA blot analysis (Grant et al., manuscript submitted), and comparison of genomic and cDNA sequences (Grant et al., manuscript submitted). Finally, the observation that the expression of a full-length CABP1A cDNA in Dictyostelium cells leads to the synthesis of both CABP1A and CABP1B directly demonstrates that the two polypeptides are encoded by one gene (this thesis, section 3.1.1).

The two subunits of CABP1 appear to be produced by an unusual splicing mechanism. These two subunits are identical except for the presence of an additional 37 amino acids inserted near the amino terminus of CABP1A (Grant and Tsang, manuscript submitted). The sequence of a cDNA clone encoding CABP1A is absolutely identical to that of the CABP1 gene, while CABP1B-specific cDNAs are lacking a 111-nucleotide element located just downstream of the translation initiation codon in the gene (Grant and Tsang, manuscript submitted; Grant et al., manuscript submitted). Since this 111-nucleotide segment is flanked precisely by GT/AG eukaryotic splice consensus signals, it is probable that the mRNA encoding CABP1B is generated by a splicing mechanism which sometimes removes a portion of the coding region from the CABP1 primary transcript while CABP1A mRNAs consist of unspliced transcripts. It has been suggested that the splicing reaction is

regulated by a 2-nucleotide deviation in the 5' splice site present in the CABP1 gene compared to the consensus sequence derived from several D. discoideum introns (Grant 1990). This deviation, which occurs at positions 5 and 6, may cause splicing to be relatively inefficient, thereby ensuring that both subunits are synthesized.

To determine whether the two subunits of CABP1 are in fact generated by a splicing mechanism, and that this splicing is regulated by the deviation from the consensus 5' splice sequence, gene fusion and site-directed mutagenesis experiments were performed (section 3.1). The results from these experiments strongly support the above hypotheses. First, expression of a full-length CABP1A cDNA under the control of the actin 15 promoter generates 2 mRNAs, one of which codes for CABP1A and the other of which codes for CABP1B (Figs 1 and 2) This result demonstrates directly that the two subunits are the products of a single gene and that the information required to generate the CABP1B mRNA is present within the CABP1A cDNA. Second, when the 5' splice site present in CABP1 (GTAATA) is converted to the D. discoideum consensus (GTAAGT), only the CABP1B mRNA is generated and only CABP1B is expressed (Figs. 1 and 2) This result demonstrates that RNA splicing is clearly responsible for generating the CABP1B mRNA, and that the 2-nucleotide deviation present in the 5' splice site of CABP1 compared to the consensus ensures that splicing does not occur constitutively, thereby preventing the synthesis of only CABP1B.

There are at least two explanations for the decreased amount of the actin15-CABP1B fusion polypeptide present in cells transformed with the mutated CABP1A expression construct compared to control transformants carrying the wildtype expression plasmid (Fig. 1) First, the amount of the corresponding mRNA present in the cells could be different. Since the PCR experiments shown in Fig. 2 were nonquantitative, it is not possible to determine the relative amounts of the transcript present in the two populations of transformants. Alternatively, the individual subunits of CABP1 may not be stable and might have to interact with one another in order to prevent degradation If this is true, it would imply that the splicing event plays an important role in regulating the expression of CABP1.

As described in section 3.1.1, cells carrying the mutated CABP1A expression construct occasionally contain an additional polypeptide which can be immunoprecipitated with an anti-CABP1 monoclonal antibody This polypeptide migrates slightly faster than the wildtype actin 15-CABP1A fusion. While the precise origin of this molecule is not known, its size suggests that it may represent the product of a second spliced mRNA. Two potential 3′ splice sites (AG) are located between the 5′ and 3′ splice junctions which are normally used to generate the CABP1B transcript The use of one of these two sites could be responsible for the generation of the extra polypeptide. However, only one of them maintains the appropriate reading frame and the resulting polypeptide would be smaller than the molecule which is

actually observed. Thus, this polypeptide may be produced by aberrant splicing or by the use of cryptic splice sites. Attempts to determine its precise origin by using the polymerase chain reaction to isolate a cDNA which encodes it have not been successful, perhaps due to its apparently transient nature. It is important to stress the fact that, in these cells, no unspliced product corresponding to CABP1A has ever been detected at either the protein or mRNA levels. Thus, the results strongly support the hypothesis that the 2-nucleotide deviation in the 5' splice site of the CABP1 gene compared to the Dictyostelium consensus plays a major role in generating the two transcripts which encode CABP1A and CABP1B.

These results are consistent with a number of other studies which have examined the effects of specific mutations within the 5' splice site of introns in another lower eukaryote, the yeast Saccharomyces cerevisiae. In this organism, a strongly conserved 5' splice consensus sequence of GTATGT has been observed (Teem et al. 1984). Mutations at position 5 which convert the G residue to C or A have been found to significantly reduce the efficiency of splicing and lead to the accumulation of unspliced as well as spliced transcripts (Fouser and Friesen, 1986, Parker and Guthrie, 1985).

For introns in mammalian genes, a slightly different 5' splice consensus sequence, GT(A/G)AGT, has been found (Smith et al. 1989). This sequence is much less highly conserved than the corresponding element in yeast and Dictyostelium introns. Only

about 5% of all mammalian introns examined to date possess a perfect match to this consensus (Smith et al. 1989). In general, mutation of the 5' splice site towards the consensus can make it more efficient (Smith et al. 1989).

Thus, the two subunits of CABP1 appear to be produced by an unusual splicing event which sometimes removes a portion of the coding sequence present in the primary transcript. Spliced mRNAs encode CABP1B while unspliced transcripts code for CABP1A. Splicing does not occur constitutively due to a two-nucleotide deviation in the 5' splice site compared to the consensus sequence

A similar mechanism is used to generate two isoforms of γ-fibrinogen in rats and humans from a single gene (Crabtree and Kant 1982; Fornace et al. 1984). In these cases, however, the retained intron contains an in-frame stop codon which causes translation of the two mRNAs to terminate at different sites. Similarly, the presence of a retained intron in somatic cells of the Drosophila P-element transposase generates a truncated and nonfunctional protein due to the presence of a stop codon (Laski et al 1986). More recently, it has been suggested that the two human high mobility group proteins HMG-Y and HMG-I are produced from a single gene in a manner identical to that which generates CABP1A and CABP1B (Johnson et al. 1989). Thus, this mechanism, or slight variations of it, appears to be relatively common in eukaryotes

4.2: The Two CABP1-Related Polypeptides p34 and p31

In order to understand the mechanisms which mediate the effects of cAMP on the development of D. discoideum, it is necessary to identify all the cellular components which directly interact with this molecule. Two cAMP-binding proteins have been intensively studied in this organism. These molecules consist of an integral membrane protein involved in chemotaxis and gene expression (Klein et al 1988) and the regulatory subunit of the cAMP-dependent protein kinase (Mutzel et al. 1987). A novel cAMP-binding protein designated CABP1 has recently been characterized in this organism (Tsang and Tasaka 1986, Kay et al. 1987, Tsang et al. 1987; Tsang et al. 1988). In the course of these studies, it has been found that CABP1 appears to be a member of a family of related polypeptides (Tsang et al 1988). This hypothesis has been confirmed by the isolation of cDNA clones which encode two CABP1-related molecules, p34 and p31

Based on the data presented in chapter 3, it is very likely that the cDNA clones pDdM34 and pDdM31 respectively encode p34 and p31. First, hybrid selection analysis of the cDNA clone pC5D9 (Fig. 3) indicates that this sequence is complementary to mRNAs which encode these two polypeptides When a cDNA library was screened using this clone as a probe, two different classes of cDNAs were isolated, represented by pDdM34 and pDdM31 Second, the sizes of these two clones agree well with the sizes of two of the transcripts which are detected by Northern blotting (Fig 9) Third, the polypeptides encoded by pDdM34 and pDdM31 appear to be the products of a single gene (Figs. 6 and 7) and are very

similar to the two subunits of CABP' (Fig. 10), which are also produced from a single gene by an alternative splicing mechanism (Figs. 1 and 2). Finally, when a truncated fragment of one of the cDNAs was used to disrupt its cognate gene by homologous recombination, the levels of p34 and p31 were drastically reduced (Fig. 14) Taken together, these data strongly suggest that pDdM34 and pDdM31 are complementary to the mRNAs which encode p34 and p31.

The results described in section 3.2 indicate that p34 and p31 are the products of a single gene. The two subunits of CABP1 are also encoded by one gene (Grant and Tsang, manuscript submitted, Grant et al. manuscript in preparation; this thesis, section 3.1). Based on the high similarity between the 4 polypeptides (Fig 10), it is probable that these two genes arose by duplication. Subsequent sequence divergence has led to a major change in only one domain, with the remainder of the two genes maintaining close similarity, especially at their 5' ends. The virtual identity of the amino termini of p34/31 and CABP1 in contrast to the relatively weaker similarity displayed by the remainder of the proteins suggests that this region of the polypeptides is crucial for function. Furthermore, this area contains the segments which are removed from the CABP1 and p34/31 primary transcripts to generate CABP1B and p31. It is very likely that p34 and p31 are generated from a single gene by the same mechanism which produces CABP1A and CABP1B. Similar to CABP1, the 5' splice site in the p34/31 gene (GTAACA) deviates from the D.

discoideum consensus at positions 5 and 6. The strong conservation of this feature between the two genes implies that the abili to generate two polypeptides is functionally very important.

The high degree of similarity between p34/31 and CABP1 raises the question of whether these molecules are functionally identical. It is not yet known whether p34 and p31 possess the cAMP binding activity displayed by CABP1. However, due to the extensive homology between these polypeptides, it is very probable that p34 and/or p31 will also bind cAMP On the other hand, gene disruption experiments indicate that these molecules probably do not perform exactly the same functions in the cell (see section 4.3).

The functional relationship between p34 and p31 is not clear. Since CABP1 operates as a heterodimer between CABP1A and CABP1B (Tsang and Tasaka 1986), it is possible that p34 and p31 also associate to form one functional protein. Alternatively, all four polypeptides, or various combinations of them, may form a complex Consistent with this hypothesis, gel chromatography experiments have revealed that native CABP1 elutes in a high molecular weight fraction which also contains p34 and p31 along with a number of other polypeptides which are antigenically related to CABP1 (Tsang and Tasaka 1986). Further analysis is required to resolve this question.

Because both the CABP1 and p34/31 genes encode two polypeptides, they represent two of the most complicated

Dictyostelium genes analyzed to date It appears that the p34/31 gene is even more complex Northern analysis using p34/31 cDNA probes detects transcripts with sizes of 1.15kb, 1.25kb and 1.4kb (Fig 9). The two smaller mRNAs probably encode p34 and p31 and most likely correspond to the cDNAs pDdM34 and pDdM31. The nature of the largest mRNA is not clear at present It does not appear to represent the transcript of a second closely related gene. First, Southern analysis clearly indicated that only a single gene encodes p34 and p31 (Fig. 6A). Second, under low stringency conditions, p34/31 probes crossreacted with only one additional gene, that which encodes CABP1 (Fig. 6B). CABP1 probes, however, do not hybridize to the 1.4kb transcript under conditions of high stringency (Grant and Tsang, manuscript submitted). Finally, this transcript was recognized by probes derived from all regions of the p34 and p31 cDNAs, even when very high stringency conditions were employed. Therefore, the 1.4kb transcript probably represents a third message which is produced by the p34/31 gene. The determination of its precise origin and function will require additional analysis

The discovery of very high similarity between CABP1, p34/31 and two bacterial polypeptides encoded by a plasmid which confers resistance to tellurium anions (Fig. 11) was completely unexpected. This close similarity suggests that these molecules have a common evolutionary origin. It also implies that they probably have similar functions. Unfortunately, the mechanism which imparts tellurium resistance in bacteria is not known, so we

can gain no clues as to the function of p34/31 and CABP1. It is also not known whether these bacterial polypeptides can bind cAMP.

In summary, the results presented in section 3.2 demonstrate that p34 and p31, two of the polypeptides which crossreact with anti-CABP1 antibodies, are in fact very closely related to CABP1. These polypeptides, like the two subunits of CABP1, appear to be the products of a single gene. The CABP1 and p34/31 genes probably arose by duplication followed by sequence divergence It is likely that both genes use the same mechanism to generate two polypeptides. Based on these results, it is clear that CABP1 belongs to at least a small gene family. Because anti-CABP1 antibodies crossreact with a number of polypeptides in addition to p34 and p31 (Tsang and Tasaka 1986) while CABP1 cDNA probes hybridize to several genomic DNA fragments (Tsang et al 1988), several more genes may belong to this family The total size of the CABP1 gene family will not be known until all the genes identified by crosshybridization with CABP1 probes have been characterized.

4.3. p34 and p31 Appear to Play Important Roles in Both Growth and Development

One of the most powerful approaches available to determine the function of a protein is to isolate mutants which express altered amounts of the molecule in question. The recent demonstration that homologous recombination can be employed to specifically disrupt genes in D. discoideum (De Lozanne and Spudich 1987) makes this a very powerful system with which to analyze the role of specific polypeptides in growth and differentiation. In order to determine possible functions for p34 and p31, the gene which codes for them has been disrupted.

After transforming cells with a plasmid containing a p34/31 cDNA fragment truncated at both ends, 80 independent transformants were analyzed by Southern blotting. A total of four mutants were obtained. While the frequency with which homologous recombination occurred in this case was not particularly high (4/80 or 5%), it agrees well with results obtained for the few other Dictyostelium genes that have been disrupted in this manner. For four other genes, homologous recombination has occurred in 5-30% of all transformants analyzed (De Lozanne and Spudich 1987; Witke et al. 1987; Harloff et al. 1989; Jung and Hammer 1990).

Two of the p34/31 mutants, AX-2T133-40 and AX-2T133-43, have been analyzed for the expression of p34 and p31. Immunoprecipitation and immunoblotting experiments (Fig. 14) clearly indicate that, although a null allele has not been isolated, the disrupted strains express drastically reduced levels of truncated versions of p34 and p31. The residual amounts of

these two molecules are probably produced by the 3' truncated version of the p34/31 gene which is generated as a consequence of homologous recombination (see Fig. 12). This gene contains a functional promoter as well as most of the coding sequence. It lacks the last 30 codons and all 3' flanking sequences. Since transcripts from this gene will not contain a polyadenylation signal and probably will not terminate properly due to the absence of a specific transcription termination sequence, they are unlikely to be very stable. This would explain the very low but detectable levels of truncated p34 and p31 found in the disrupted strains.

The observation that AX-2T133-40 and AX-2T133-43 form very small plaques on bacterial lawns compared to a wildtype control (Fig. 15) suggested that these mutants grow slowly. This hypothesis was confirmed by analyzing the growth rates of the mutants (Fig. 16A). Intriguingly, this defect was seen only when the cells were grown on bacteria. When cultured axenically in HL5 broth, AX-2T133-40 and AX-2T133-43 grew at the same rate as wildtype controls (Fig. 16B). There are at least two possible explanations for this difference. First, the defect responsible for slow growth in the p34/31 mutants may be specific to a process which occurs only in cells which feed upon bacteria. Alternatively, since axenic cells grow approximately 3 times slower than those grown on bacteria (compare Fig. 16A and 16B for wildtype controls), it is conceivable that the growth defect is simply masked by the already extended generation time of

axenically growing cells

These results indicate that, directly or indirectly, p34 and p31 must play an important role in growth, at least in cells which feed on bacteria. This is very surprising because these two polypeptides are not detected by Western blotting in vegetative cell extracts (Kay et al. 1987) although the transcripts which encode them are clearly present at this time (Fig. 9). However, since two independent mutants behave the same, it is highly unlikely that this growth defect is an artifact. This suggests that p34 and p31 are in fact present and play an important role in growing cells. The inability to detect them may be explained several ways Perhaps only very small amounts of these molecules are produced during growth. Alternatively, they may only be synthesized at very specific times in the cell cycle. Finally, p34 and p31 may be unstable in growing cells. Whatever the case, interfering with the normal production of these polypeptides in vegetative cells by disrupting the gene which encodes them appears to lead to a decreased growth rate.

In addition to the growth defect described above, AX-2T133-40 and AX-2T133-43 possess a minor developmental defect. Both mutants exhibit a slight increase in the length of the slug phase. All other stages of development appear to be normal. These results suggest that p34 and p31 perform a specific function late in development.

Furthermore, this analysis suggests that p34 and p31 possess functions distinct from those of CABP1. Both p34/31 mutants

described here express normal levels of CABP1 (Fig. 14). Therefore, although p34 and p31 are very closely related to CABP1, they are not functionally equivalent to it, at least not completely.

Because a null mutation has not been obtained, other interpretations of the above data are also possible. It is conceivable that all of the observed abnormalities present in the mutant strains are not caused by a lack of normal p34/31. Instead, they could be the result of the presence of the truncated forms of p34 and p31 in the cells. For example, the altered polypeptides could interact incorrectly with other intracellular factors to change growth and developmental properties, or they could somehow antagonize the activity of CABP1. Thus, the isolation of a null mutation in the p34/31 gene will be required to clarify these results.

4.4: Concluding Remarks

The results described in this thesis have increased our knowledge concerning CABP1 and the closely-related p34 and p31 polypeptides. However, there is still much to be done. First, the isolation and basic characterization of the remaining CABP1-related genes present in the genome of D. discoideum should be performed. This analysis could provide valuable information concerning the evolution, structure and function of CABP1 and its related polypeptides. Second, the generation of antibodies with greater specificity than those presently available would greatly simplify the task of determining the expression and intracellular localization of these proteins. It would also provide a way to examine the interactions, if any, between these different molecules. Finally, the generation of mutations in all of these genes by homologous recombination or gene replacement should yield valuable data concerning the functions of the individual polypeptides. It is only by performing these, and other, experiments that we can fully evaluate the roles of CABP1, p34/31, and their related polypeptides in the growth and development of D. discoideum

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