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Interactions among a group of related proteins in the cellular slime mold *Dictyostelium discoideum*

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ABSTRACT

Interactions among a group of related proteins in the cellular slime mold *Dictyostelium discoideum*

Jonathan M. Gisser

In previous work, the *Dictyostelium discoideum* gene encoding the cAMP-binding protein CABP1 was isolated and cloned. Subsequent studies revealed that certain polypeptides are related in their amino acid sequence to CABP1. Each of these polypeptides possesses a region of high glycine, tyrosine, proline, glutamine, and alanine (GYPAQ) content which forms the basis for their similarity. In the present study, the genes encoding seven of these related *D. discoideum* polypeptides were cloned into the two-hybrid system vectors and subjected to the yeast two-hybrid assay.

The p34, p31, CABP1A, CABP1B, and 6C proteins each homodimerized in the assay. Furthermore, interactions were observed between the following pairs of proteins: p34 and p31, CABP1A and CABP1B, CABP1A and 6C, CABP1B and 6C, p34 and 6C, p31 and 6C, and p24 and 6C. Some of the interactions were also verified using a blot overlay assay. *Dictyostelium* annexin did not interact with any of the other related proteins, nor did it homodimerize.

To determine whether the GYPAQ-rich regions are mediating the interactions, portions of the gene encoding the 6C protein were isolated and cloned into the two-hybrid vectors and the assay was performed again. Genetically deleting the GYPAQ-rich region of 6C had the effect of abolishing or diminishing all the interactions that were observed with the intact 6C. Conversely, the GYPAQ-rich region behaved like the intact 6C. The GYPAQ-rich region therefore appears to be playing a role in the mediation of interactions with 6C.

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ABBREVIATIONS

ADH	Alcohol Dehydrogenase
amp^R	ampicillin resistance
AP	Alkaline Phosphatase
BTB/POZ	Broad complex,tramtrack and bric à brac/Pox virus and zinc finger
Ca^{++}	calcium
cAMP	Adenosine cyclic 3':5'-monophosphate
cDNA	complementary DNA
DB	DNA-binding
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EGTA	[Ethylenebis(oxyethylenenitrilo)] tetraacetic acid
ß-gal	ß- galactosidase
X-gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactoside
GST	Glutathione-S-transferase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl ß-D-thiogalactopyranoside
MCS	multiple cloning site
mU	Miller Units
NLS	nuclear localization signal
$OD_{wavelength}$	optical density
ONPG	o-Nitrophenyl ß-D-galactopyranoside
PCR	polymerase chain reaction
PKA	cAMP-dependent protein kinase
SDS	Sodium dodecyl sulfate
SH2	Src Homology module 2

SH3	Src Homology module 3
TA	transcription-activating
Te^R	Tellurium resistance
UAS	upstream activating sequence

Chapter 1

INTRODUCTION

1.1 General properties of protein-protein interactions

Many processes in many different cell types are mediated by protein-protein interactions. There are several reasons why one would wish to study these interactions. First, identifying a cascade of interacting proteins is a direct means of elucidating the elements of a metabolic pathway. Second, when the function of a protein is unknown, it can often be inferred based on the function(s) of interacting partner(s). Third, understanding the elements of a protein that facilitate its interactions with other proteins provides much insight into the structure-function relationship of the element under study. Lastly, elucidating the mechanisms of protein-protein interactions can provide an understanding of disease pathogenesis and aid in the design of new pharmaceutical agents.

Proteins physically associate with each other in many contexts. Enzymatic catalysis and inhibition, antibody-antigen recognition, assembly of protein subunits, signal transduction, cell-cell contact, to name a few, are all made possible by the occurrence of protein-protein interactions (reviewed in Davies & Cohen, 1996; Phizicky & Fields, 1995; Johnson, 1996; Cohen *et al.*, 1995). The factors that may influence the propensity for two proteins to dimerize are determined by the amino acid composition of the proteins, and their secondary and supersecondary structures (Jones & Thornton, 1996). Specifically, these structures determine which residues lie on the surface of a protein and which are hidden in its interior. They also define the shape of the protein surface. The amino acid composition of the proteins, beyond influencing the resulting secondary structure of the protein (Branden & Tooze, 1991), defines the electrostatic character of the protein surface. The tendency of hydrophobic residues to associate with one another, rather than be exposed to an aqueous environment, is one of the driving forces of protein interactions (Tsai *et al.*, 1996). Hydrogen bonds and ion pairs (electrostatic interactions between oppositely

charged residues) may also play a role in the association of two proteins, albeit a relatively minor one (Tsai *et al.*, 1996). Jones and Thornton (1996) attempted to enumerate all those characteristics of protein domains that define them as interfaces. To do so, they investigated various known protein complex interfaces from the Brookhaven Protein Databank. In addition to hydrophobic and polar interactions, they examined such interface features as their size and shape, the propensity for certain amino acid residues to occur at an interface, surface complementarity (fit), and their secondary structures. They concluded that none of the above traits alone is sufficient to differentiate an interface region from a non-interface region, even though general trends are observed. Tsai *et al.* (1996) concluded that many of the interactions that occur at interfaces mimic those that occur at protein cores, although the details differ. Kini and Evans (1996) suggest that interaction domains can be identified by the presence of flanking proline residues, although they concede that this phenomenon is a trend rather than a steadfast rule. Despite all the above efforts, it is still difficult to predict the occurrence of an interaction based solely on amino acid sequence data or the structural data of a monomer. For this reason, other empirical techniques, rather than predictive or theoretical methods, are usually employed.

The detection and study of protein-protein interactions is further complicated by the fact that proteins are seldom observed in their natural environment; most methods of detecting interactions utilize an *in vitro* system to mimic the conditions under which the interaction would be expected to occur, and even when an *in vivo* simulation is used, it is seldom in the same organism/organelle from which the proteins were derived. As such, many interactions are difficult to detect because they are transient and/or the protein components that are involved must be chemically modified before they will readily interact (Phizicky & Fields, 1995). Conversely, some proteins are erroneously deemed interactors even though they never encounter each other in the natural host cell (Midgley & Lane, 1993). For the above reasons, it is useful to approach protein interactions with a variety of different methods before assessing the physiological relevance of any results.

With the creation and rapid expansion of protein sequence databases, the existence of interaction domains within a protein, and the structure of such domains, can often be inferred based on similarity to previously characterized proteins (Bork & Gibson, 1996). Such database search programs as PROSITE and BLASTP have greatly simplified the identification of certain motifs that occur frequently in interaction domains. The Src homology modules, SH2 and SH3 (Koch *et al.*, 1991), for instance, have been implicated in the mediation of numerous signal transduction pathways, in a variety of organisms, via the binding to various specific recognition sequences (reviewed in Cohen *et al.*, 1995). Other common motifs include the leucine zipper motif (Landschultz *et al.*, 1988), basic helix-loop-helices (Hoffman *et al.*, 1991), non-Src phospho-tyrosine Binding motifs (Blaikie *et al.*, 1994), and BTB/POZ domains (Zollman *et al.*, 1994; Bardwell & Treisman, 1994). This list is far from exhaustive, but serves only to demonstrate the large variety of motifs that have been discovered to date. Despite the rapid expansion of protein databases, however, there remain many protein motifs that have not yet been characterized and therefore do not fit into any of the above categories. Moreover, there also exist numerous proteins whose dimerization domains do not adhere to a particular structural theme. Such is the case with enzymes whose binding pockets have been optimized throughout evolution for diverse but specific protein substrates; e.g. enzyme-inhibitor complexes (see Jones and Thornton, 1996). It is very possible that new, as yet uncharacterized, motifs will surface as new sequence and structural data become available.

The empirical methods that are used to detect and examine protein-protein interaction vary in terms of their sensitivity, specificity, feasibility, and range of applications. Briefly, co-immunoprecipitation utilizes specific antibodies to precipitate antigenic proteins. Non-antigenic proteins that co-precipitate are considered to be interactors. Protein affinity chromatography requires the linking of a protein to a matrix in a column. Cell extracts are then passed through the column. Those proteins that are retained in the column and eluted separately are presumed to have adsorbed to the matrix-

bound protein. A related technique, affinity blotting, has the cell extracts bound to a membrane that is subsequently incubated with a protein probe. Phage display is a method that makes use of phage's ability to display many different proteins as part of its viral coat when a cDNA library is cloned into the viral genome. Viruses that are retained by an adsorbed bait protein are extracted and transfected into bacteria so that a clone of the gene can be obtained. In chemical cross-linking experiments, chemicals that covalently link proteins together are applied to cells or to mixtures of proteins. Proteins that are interacting or are in close proximity will be fixed that way and can be identified by antibodies or a mobility shift on a two-dimensional polyacrylamide gel.

The yeast two-hybrid system differs from all the above techniques in that numerous combinations of proteins can be tested simultaneously, within a relatively short period of time.

1.2 **The yeast two-hybrid system**

The yeast two-hybrid system (Fields & Song, 1989) is a genetic assay that relies on the modular nature of yeast transcription factors to detect protein-protein interactions. Some *S. cerevisiae* transcription factors, such as GAL4, contain a functionally separate DNA binding (DB) domain and transcription activation (TA) domain (Keegan *et al.*, 1986). The former localizes the transcription factor to a specific Upstream Activating Sequence (UAS) of a specific gene in the yeast nucleus. The latter contains an acidic region that aids in the initiation of transcription. When expressed alone, the DNA binding domain localizes to its cognate UAS, but is unable to activate transcription. Likewise, when the TA domain is expressed alone, it is unable to initiate transcription because it cannot localize to the UAS. When the two domains are co-expressed and brought into close proximity, however, transcriptional activation at the transcription factor-specific UAS is reconstituted, thereby mimicking an intact transcription factor despite the fact that they are not covalently linked.

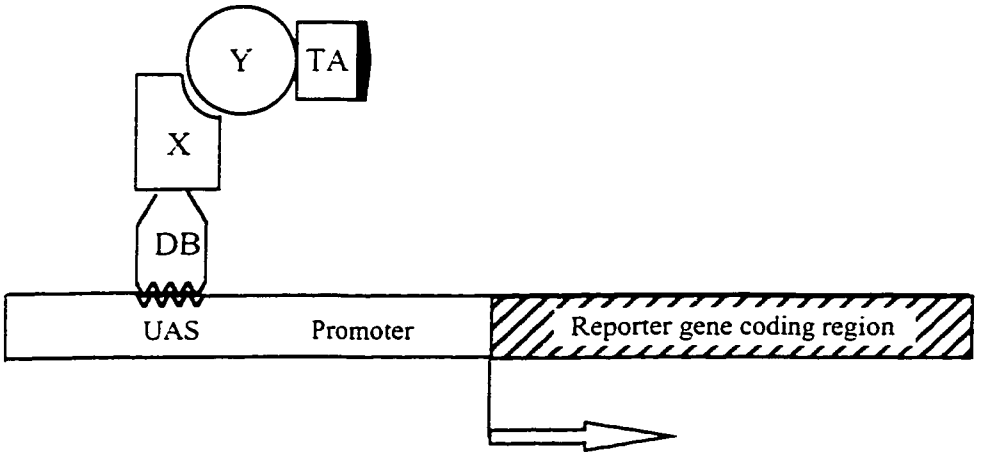
In the two-hybrid system, recombinant DNA technology is employed to generate two chimeric proteins: a protein of interest, X, fused to a DB domain, and a second protein, Y, fused to a TA domain. The genes encoding the two proteins of interest are fused, in frame, downstream of the DB- or TA-encoding portion of a yeast autonomously-replicating expression vector. The two plasmids are then co-transformed and their products expressed in the yeast nucleus. If, upon expression, a favorable interaction occurs between the two proteins, X and Y, the TA and DB domains will behave like an intact transcription factor, being joined by the two interacting proteins (Figure 1). Reporter genes, fused downstream of the UAS in the yeast genome are used to detect the activity of a reconstituted transcription factor and, hence, the interaction. Typically, the bacterial ß-galactosidase gene, *lacZ*, is used. In the presence of a chromogenic substrate, such as X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside), colonies expressing the *lacZ* gene product will change colors. Additionally, a nutritional requirement, such as histidine, is sometimes used as a selection by using a histidine-auxotrophic strain harbouring the HIS3 gene under transcriptional control of the UAS. In the absence of histidine in the growth medium only those cells that are expressing the HIS3 gene will grow.

The two-hybrid system has been used to detect interactions between proteins from a wide array of organisms and cellular processes (Fields & Sternglanz, 1994). Furthermore, the basic protocol for its use is very versatile and can be adapted for different uses. It can be used to detect or confirm an interaction between two known proteins (Fields & Song, 1989). By cloning a cDNA library into the TA domain-encoding vector, the two-hybrid system can be used to screen the library for novel proteins that interact with a "bait" protein which has been subcloned into the DB domain-encoding vector (Luban *et al.*, 1993). By deleting or mutating a segment of the gene coding for the protein being studied, one can define those regions of the protein that are responsible for

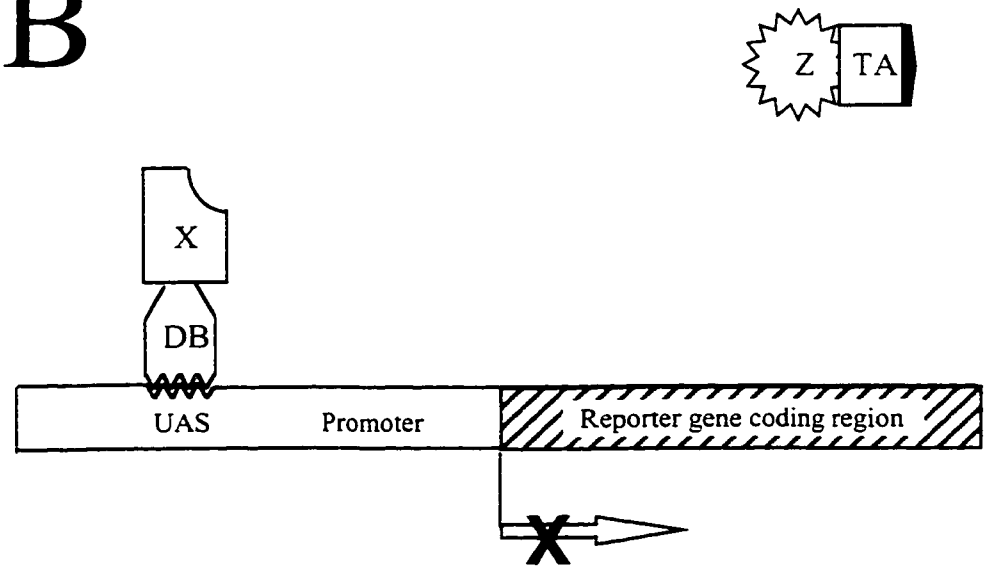
Figure 1

A cartoon illustrating the two-hybrid system for detecting protein-protein interactions. The assay takes place inside the yeast nucleus. In A, protein X and protein Y are interacting with each other, thereby bringing the two GAL4 domains (TA and DB) into close proximity. This reconstitutes the function of the transcription factor and enables transcription to occur from the yeast promoter. In B, proteins X and Z do not interact. The DB-X fusion is still capable of binding to its cognate yeast upstream activating sequence, but cannot drive transcription of the reporter gene in the absence of a properly positioned TA domain.

A


Y
TA
X
DB
UAS
Promoter
Reporter gene coding region

B


Z
TA
X
DB
UAS
Promoter
Reporter gene coding region

the interaction (Li & Fields, 1993). One of the advantages of the two-hybrid system is that it is an *in vivo* assay. Although it cannot always duplicate exactly the natural environment of many of the heterologously expressed proteins, the two-hybrid system has been shown to correlate reasonably well with *in vitro* affinity measurements (Estojak *et al.*, 1995). Nuclear, cytoplasmic, mitochondrial, extracellular, and membrane-associated proteins have all been successfully used in this system (Fields & Sternglanz, 1994). Moreover, in certain instances, the two-hybrid system has been shown to be more sensitive than immunoprecipitation and chemical cross-linking in detecting interactions (Van Aelst *et al.*, 1993).

1.3 *Dictyostelium discoideum* life cycle

The organism *Dictyostelium discoideum*, also known as the cellular slime mold, is a free-living amoeba that inhabits the upper soil layer of decaying leaves. It possesses two alternate life cycles: an asexual and a sexual cycle. In the asexual cycle, the single-celled amoeba reproduce by binary fission and, with the exception of axenic strains, feed on bacteria. Axenic strains are laboratory strains which, like their wild-type counterparts reproduce by binary fission, but differ in that they are capable of growing on a defined medium, rather than exclusively on bacteria. When a food source is depleted and/or the culture reaches a critical density, the amoeba cease vegetative growth and enter a development phase which is marked by chemotactic aggregation of the individual amoeba into a single cell mass, followed by elongation and migration of the cell mass as a multicellular slug. The slug then culminates, in a gastrulation-like fashion, to form a fruiting body consisting of a basal disk, a stalk and a spore head. Only those cells that differentiate into spores survive to repeat the cycle when the living conditions ameliorate (reviewed in Gerisch, 1987; Mutzel, 1995). There are numerous reasons why *D. discoideum* is an interesting model for the study of development. It has the ability to undergo coordinated cell movements and differentiation, it is easily manipulated and

observed in the laboratory and, because the growth phase and development phase are temporally separate, one can observe developmental processes unhindered by growth events (reviewed in Firtel, 1995).

1.4 Cyclic AMP in *Dictyostelium* development

Many of the developmental processes in *D. discoideum* are controlled by extracellular adenosine cyclic 3':5'-monophosphate (cAMP). At the onset of development, cAMP acts as a chemoattractant during aggregation. In response to starvation, cells acquire the competence to respond to, to produce, and to secrete cAMP. They also begin moving towards other cells which are generating the same pulsatile waves of cAMP (reviewed in Gerisch, 1987). This response to starvation is achieved by the actions of several factors. Cyclic AMP receptors on the cell surface, which bind extracellular cAMP, allow the cell to respond appropriately by activating a stimulatory G-protein. Adenylyl cyclase produces cAMP in response to indirect stimulation from the stimulatory G-protein. An extracellular phosphodiesterase aids in returning cells to a cAMP sensitive state allowing the next cAMP pulse to exert the same effects. To date, the cAMP secreting mechanism is unknown (reviewed in Reymond *et al.*, 1995).

Cyclic AMP also acts as an important regulator of developmental gene expression. During aggregation, genes encoding cell-cell adhesion Contact Site A Glycoprotein, cAMP receptors, adenylyl cyclase and phosphodiesterase are but a few of the genes that are induced by pulses of cAMP (reviewed in Gross, 1994). Other genes, such as Discoidin I, are repressed during aggregation in response to these pulses (Williams *et al.*, 1980). Following aggregation, normal development requires the expression of a different class of proteins whose function is to inhibit the aggregation response, adopt a different signalling cascade, and to initiate the first steps in differentiation. The genes encoding these post-aggregative proteins are expressed in response to a continuously high concentration of

extracellular cAMP, as opposed to the pulsatile, low concentration that induces expression of the aggregation-specific proteins (reviewed in Firtel, 1995).

Elucidating the role of intracellular concentrations of cAMP in developmental gene regulation has proven to be more difficult. During aggregation, the intracellular level of cAMP fluctuates (Gerisch & Hess, 1974; Devreotes, 1982) and a large rise in its concentration accompanies slug formation (Abe & Yanagisawa, 1983). In other eukaryotes, cAMP acts as a second messenger on cAMP-dependent protein kinases (PKA) (Taylor *et al.*, 1990). When a kinase bearing homology to the eukaryotic PKA was identified (De Gunzberg *et al.*, 1984), it was believed that a similar phenomenon was being observed. Indeed, strains harbouring null mutations in the catalytic subunit (Mann & Firtel, 1991) or dominant negative (Rm) mutations in the regulatory subunit of the enzyme (Simon *et al.*, 1989) display an inability to undergo aggregation (Firtel & Chapman, 1990; Simon *et al.*, 1989). Furthermore, cells expressing the Rm mutant form of PKA under the control of prespore- or prestalk-specific promoters fail to develop into mature spores or stalks (Harwood *et al.*, 1992; Hopper *et al.*, 1993).

Paradoxically, adenylyl cyclase, which would be expected to provide cAMP to activate PKA, is not essential for terminal differentiation and the increase in intracellular cAMP levels does not appear to be attributable to any of the known adenylyl cyclases expressed at this time (Pitt *et al.*, 1993). In addition, the immediate downstream substrate for PKA has not yet been found (Reymond *et al.*, 1995).

1.5 **The isolation of additional cAMP-binding proteins**

The central role that cAMP plays in all aspects of *Dictyostelium* development has prompted a search for other cAMP-binding proteins that may play a role in gene regulation and differentiation in response to changing levels of intracellular cAMP. Cyclic AMP-affinity chromatography revealed a protein consisting of two polypeptides, CABP1A and CABP1B, that displayed cAMP binding. Antibodies to the protein were raised and used

for a variety of immunoblot experiments. Of interest was the fact that the amount of CABP1 increased steadily in the cell throughout development, but it was only present at low levels during the growth phase, suggesting a role for CABP1 in development. This argument was further bolstered by the fact that certain rapid-developing strains possessed a polymorphic form of CABP1 (Tsang & Tasaka, 1986; Tsang *et al.*, 1987; Bonfils *et al.*, 1991).

Subsequent cloning and characterization of the CABP1-encoding gene, *capA*, revealed that CABP1 is encoded by a single gene whose primary transcript is alternatively spliced to yield the two polypeptides (Grant & Tsang, 1990; Grant *et al.*, 1990; Bonfils *et al.*, 1991). The predicted amino acid sequence does not resemble the sequences of previously identified cAMP-binding proteins. The amino terminal region of the polypeptide contains five repeats that are very rich in glycine, tyrosine, proline, alanine and glutamine (GYPAQ), and the carboxyl terminus is similar to bacterial plasmid-encoded genes that confer resistance to tellurium anions (Te^R) in *E. coli* (Grant & Tsang, 1990; Jobling & Richie, 1988). A similar homolog of CABP1, found in *Clostridium acetobutylicum*, has been shown to complement *rec*A defects in *E. coli* (Azeddoug & Reysset, 1994). The significance of these findings has not yet been established.

Anti-CABP1 antibodies crossreact with other *Dictyostelium* polypeptides (Tsang & Tasaka, 1986; Tsang *et al.*, 1987) and CABP1 cDNA cross hybridizes, albeit under reduced stringency conditions, with other *Dictyostelium* cDNA clones (Bain *et al.*, 1991). On the basis of this structural and sequence similarity, several genes were cloned and characterized in an attempt to deduce their relation to CABP1 (Figure 2).

The *capB* gene encodes the p34 and p31 proteins, which bear a strong resemblance to CABP1. It exhibits a developmental profile that is similar to *capA*, with a very low level of expression in vegetative cells and a gradual increase throughout development (Kay *et al.*, 1987). Also, like CABP1, p34 and p31 are the products of an alternatively spliced transcript and features a GYPAQ-rich amino terminus and a carboxyl terminus resembling

bacterial Te^R plasmids (Bain *et al.*, 1991) and a *rec*A complementing gene. While a *capA* gene disruption mutant has not yet been isolated, a stable *capB* mutant has been characterized (Bain & Tsang, 1991). In this mutant strain, a truncated p34/31 is detected at low levels and development proceeds at a slower rate. When the mutant strain is grown on bacteria the growth rate is also prolonged. This suggests a role for p34/31 in both growth and development, athough at present a function for p34/31 has not yet been elucidated.

Dictyostelium annexin, a calcium-dependent phospholipid-binding protein that is homologous to various human annexins, was also identified based on partial sequence similarity to *capA* (Greenwood & Tsang, 1991). All annexins share a common core domain with a putative calcium-channeling capacity, but differ in their N-termini (for a review see Klee, 1988). The *Dictyostelium* annexin, which exists in two isoforms, has an amino-terminus which is rich in Proline, Glutamine, Tyrosine and Glycine arranged as tandem repeats (Greenwood & Tsang, 1991). These repeats appear to be the determinants for cross-hybridization with *capA* cDNA. Like CABP1 and p34/31, the *in vivo* role of the *Dictyostelium* annexin is still unclear, although Döring *et al.* (1991; 1995) have put forth a number of possibilities based on results obtained using a disruption mutant. Only when calcium levels are reduced, via the addition of the chelator EGTA, do mutants display defects in growth and development. Wild-type strains show no such effect, suggesting that the *Dictyostelium* annexin is somehow mediating the actions of calcium.

The 6C gene, which also encodes a putative calcium-binding protein (R. Wennington & A.S. Tsang, unpublished results) may also play a role in the mediation of the effects of calcium. Sequence analysis predicts that it contains a putative calcium-binding EF-hand domain in the carboxyl terminus and a long GYPAQ-rich region at the amino terminus. FASTA protein alignment reveals that the GYPAQ-rich region is 53.4% identical to the GYPAQ-rich region of CABP1. According to immunoblot experiments, 6C is expressed in vegetative cells as well as during early aggregation.

Frequently, cAMP and calcium are regulated together in a coordinated fashion in what has been termed a "synarchic messenger system" (Rasmussen, 1983). Based on their diverse functions, it is therefore conceivable that CABP1 and its related proteins may be the mediators of such a system in *Dictyostelium*.

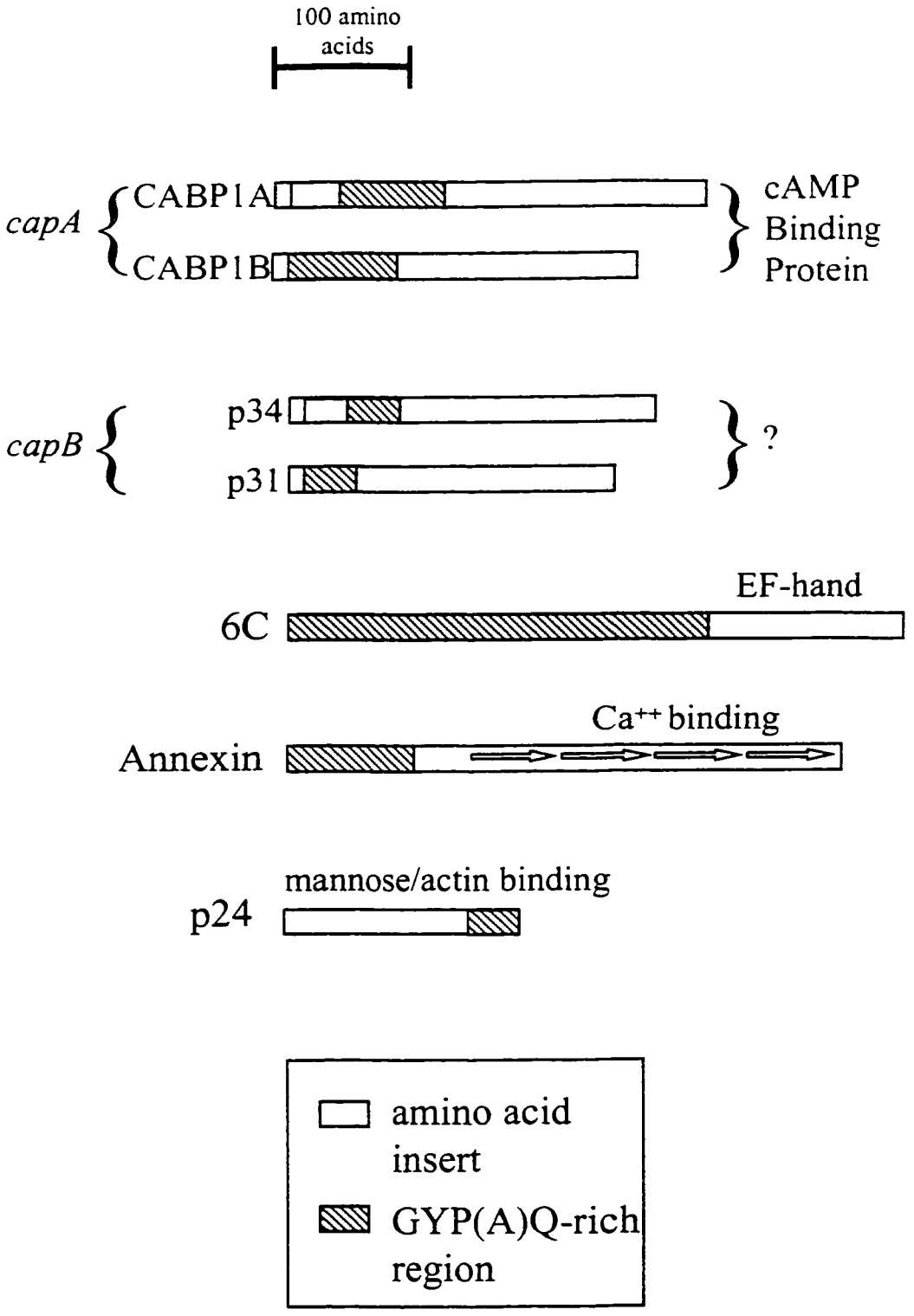
The p24 protein (Stratford & Brown, 1985; Noegel *et al.*, 1990; Greenwood & Tsang, 1992) differs from the other GYPAQ-containing proteins in that its GYPAQ domain lies in the carboxyl end of the protein. It is a 24 kDa protein that was originally isolated by virtue of its actin-binding capability in *Dictyostelium* (Stratford & Brown, 1985). Subsequent experiments have demonstrated that the protein is also capable of binding mannose residues (Jung *et al.*, 1996) and is associated with the Golgi apparatus and other vesicles (Weiner *et al.*, 1993). Based on these features, it has been suggested that the p24 protein acts as a cross-link between membrane-bound mannose and the actin cytoskeleton, and mediates vesicle transport (Jung *et al.*, 1996). Northern blotting experiments suggest that the level of p24 transcript can be down-regulated through the addition of cAMP (Greenwood & Tsang, 1992). In light of this phenomenon, it is possible that, in addition to being related in sequence, CABP1 and p24 participate in the same metabolic pathway.

1.6 **Rationale and Experimental Design**

Preliminary studies (Tsang & Tasaka, 1986) suggest that CABP1A and CABP1B associate with each other, based on the fact that they are always present in the wild-type cell in an approximate ratio of 1:1. The same pattern is observed for p34 and p31 (Bain & Tsang, 1991). As such, they too may be subunits of a single protein. Furthermore, CABP1 and p34/31 may combine with each other and with other proteins to form a multimeric structure. The evidence for this lies in the fact that partially purified CABP1 elutes near a molecular weight of M_r=250,000, according to gel filtration column

Figure 2

Features of the *D. discoideum* GYPAQ-containing proteins that were subjected to the two-hybrid assay. CABP1A (M_r=34.4 kDa) and CABP1B (M_r=30.4 kDa) are gene products of the *D. discoideum capA* gene and result from the alternative splicing of the *capA* mRNA (Grant *et al.*, 1990). CABP1B lacks the 37 residue insert that is present in CABP1A. The GYPAQ region is 83 residues long in both polypeptides. The region of the peptide wherein lies the cAMP-binding function is at present unknown. Like CABP1A and B, p34 (M_r=30.8 kDa) and p31 (M_r=27.2 kDa) are derived from a single gene, *capB*, but are alternatively spliced to yield two different products; p31 lacks a 34 amino acid insert. the p34/31 GYPAQ-rich region spans 40 amino acids and departs in homology from the CABP1 GYPAQ-rich region. 6C, the 48.9 kDa EF-hand-containing protein has the largest GYPAQ region of all the known GYPAQ-containing *D. discoideum* proteins. It extends from the amino terminus for 320 residues. There is also an abundance of alanine residues in this region. *Dictyostelium* annexin (M_r=46.5 kDa) consists of 19 QGYPPQ amino-terminal repeats and 4 direct 70-amino acid carboxyl terminal repeats (hollow arrows) that are common to many annexins. p24 (M_r=20.6 kDa) differs from the other proteins in that its 42 amino acid GYPAQ-region resides at it carboxyl rather than at its amino terminus.


100 amino acids
capA
CABP1A
CABP1B
cAMP Binding Protein
capB
p34
p31
?
EF-hand
6C
Ca++ binding
Annexin
mannose/actin binding
p24
amino acid insert
GYP(A)Q-rich region

chromatography markers (Tsang & Tasaka, 1986). In addition, when extracted by differential ultracentrifugation, CABP1 co-purifies with annexin in a calcium-dependent manner (Bonfils, 1992), possibly suggesting an interaction between CABP1 and annexin or some other calcium-binding protein. Furthermore, the 6C putative calcium-binding protein is expected to interact with itself because it only possesses one EF-hand, in spite of the fact that EF-hand proteins usually possess a minimum of two adjacent EF-hand domains within the same polypeptide (reviewed in Kawasaki & Krestsinger, 1994). Multimerization may therefore be necessary for 6C to function. The hydrophobicity of alanine, the partial hydrophobic character of proline and the tendency of glutamine and tyrosine residues to form hydrogen-bonds suggests that the GYPAQ regions may be mediating specific interactions between some, or all, of the GYPAQ-rich proteins.

The question of whether the GYPAQ-rich polypeptides combine to form multimeric structures has not yet been addressed directly. The aim of the present study, therefore, is to determine whether any of the CABP1-related proteins interact with each other. To this end, the two-hybrid system is employed. The genes encoding the various GYPAQ-rich proteins were subcloned into the two-hybrid vectors to create either DB or TA domain fusions. All pairwise combinations of the DB and TA domain fusion plasmids, including various controls, were co-transformed into yeast strain PCY3 and the two-hybrid assay was performed. In the event of an interaction, an effort was made to isolate the domain that mediates the interaction, by deleting part of the gene and repeating the assay to establish whether the interaction is preserved using the truncated proteins.

To verify the interactions by an independent means, affinity blotting was attempted. The genes encoding the interacting proteins were expressed heterologously as Glutathione-S-transferase fusions in *E. coli* and metabolically labelled with [^{35}S]methionine. The resulting proteins are readily purified based on their glutathione-binding capabilities. The purified proteins were used to probe putative interacting proteins bound to nitrocellulose membranes.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial and yeast strains

All strains used are listed in Table I. The *Escherichia coli* strain XL1-Blue was used for all molecular cloning procedures as well as for the heterologous expression of *capA*, *capB* and 6C genes. In order to determine strain differences affecting expression, some of the clones were expressed in the *E. coli* strain Top Ten in addition to XL1-Blue. For all two-hybrid assays, the *Saccharomyces cerevisiae* strain PCY3 was used.

2.2 Construction of vectors

2.2.1 Features of the two-hybrid system vectors

The plasmid pDB62 (Figure 3) harbours the GAL4 nuclear localization signal (NLS) and DNA-binding domain. pTA86 (Figure 4) contains the simian virus 40 large T antigen NLS and GAL4 transcriptional activator domain. In addition to the GAL4 domains, each vector also possesses the ß-lactamase ampicillin resistance (amp^R) gene. The yeast selectable marker expressed by pDB62 is the *TRP1* gene product which confers tryptophan prototrophy to cells expressing it. pTA86 expresses the *LEU2* gene product that confers leucine prototrophy. Each vector contains the yeast alcohol dehydrogenase (ADH) promoter region, P_{ADH1}, upstream of the GAL4 coding sequence and the ADH terminator, T_{ADH1}, downstream of the multiple cloning site (MCS). Bacterial replication is initiated at the *ori* bacterial replication origin found in each vector, and the yeast replication system is based on the yeast 2µ plasmid.

pDB62 was constructed by replacing the MCS of pGBT9 (Bartel *et al.*, 1993) with that of pPC62 (Chévray & Nathans, 1992). Briefly, an *Xho*I-*Pst*I fragment spanning part of the GAL4 DNA-binding domain and the MCS of pPC62 was sub-cloned into the *Xho*I-*Pst*I sites of pGBT9, thereby replacing its MCS. To obtain pTA86, the MCS of pGAD424

TABLE I: BACTERIA AND YEAST STRAINS USED FOR CLONING AND EXPRESSION

Strain	Organism	Genotype	Reference or Source
XL1-Blue	*Escherichia coli*	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*⁻ F' [*pro*AB⁺ *lacI*^{q} *lacZ*ΔM15 Tn*10* (*tet*^{r})]	Bullock *et al.*, 1987
Top10	*Escherichia coli*	*mcrA* Δ(*mrr*-hsdRMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lacX*74 *deoR* *recA*1 *ara*D139 Δ(ara- leu)7697 *galU* *galK* *rpsL*(streptomycin^r) *endA*1 *nupG*	Invitrogen Corporation
PCY3	*Saccharomyces cerevisiae*	*Matα* Δ*gal4* Δ*gal80* *URA3::GAL1-lacZ* *LYS2::GAL1-HIS3* (*his3*-Δ200) *trp1*-Δ*63* *leu2* *ade2-101*^{oc}	Based on PCY2 in Chévray & Nathans, 1992

(Bartel *et al.*, 1993) was replaced with the MCS of pPC86 (Chevray & Nathans, 1992) in a similar cloning strategy to that of pDB62. An *Sph*I fragment from pGAD424, extending from the ADH (alcohol dehydrogenase) promoter to the ADH terminator, including the MCS, was replaced with a similar 1.2 Kb *Sph*I fragment from pPC86. All ligations were carried out using T4 DNA ligase. DNA was electroporated into *E. coli* strain XL1-blue (see section 2.6.2) and transformants were analyzed by rapid plasmid preparation followed by restriction analysis of putative positive clones.

2.2.2. Preparation of insert for subcloning into the two-hybrid vectors

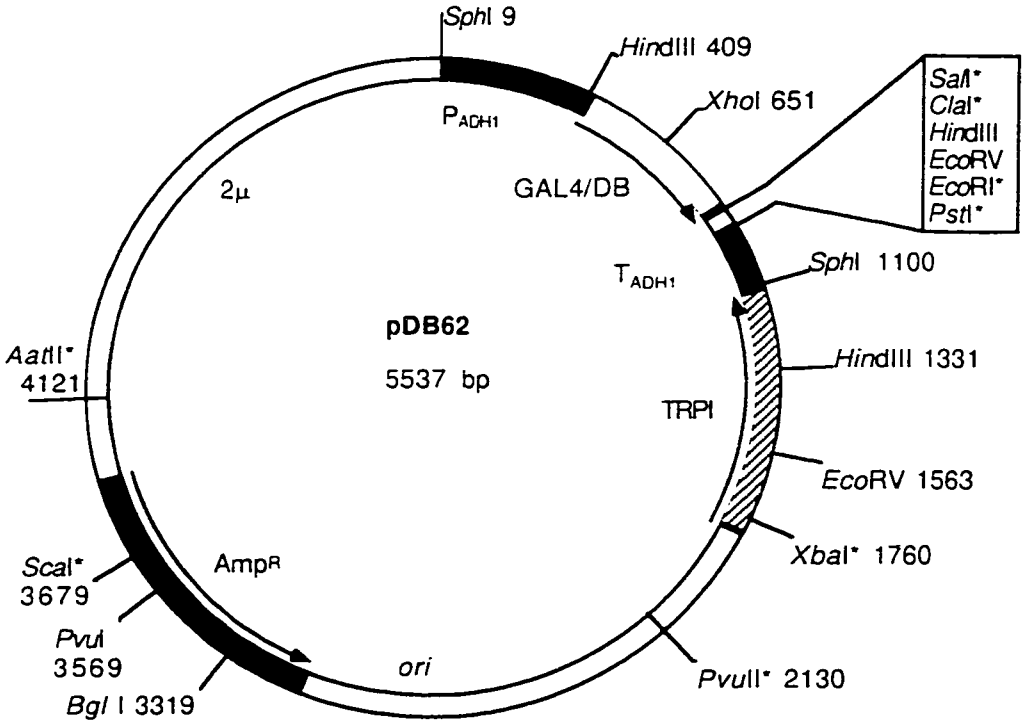
The various inserts used for subcloning into the two-hybrid vectors are listed in Table II. In order to sub-clone some of the inserts in the correct frame, into available restriction sites, compatible restriction sites were introduced by polymerase chain reaction (PCR). The inserts were amplified using oligo-deoxynucleotide primers (Sheldon Biotechnology Centre of McGill University, Montreal, Quebec) containing the desired restriction sites, which differed from complementarity to the template strand by no more than 4 bases (Table III). *Pfu* DNA polymerase (STRATAGENE) was chosen for PCR amplification due to its low misincorporation rate during polymerization. Following amplification, the PCR product was extracted with phenol-chloroform and ethanol precipitated to eliminate unincorporated nucleotides, the enzyme, and salts. The PCR product was then cleaved with the appropriate restriction enzymes, and cloned into pDB62 and pTA86 via standard cloning techniques (Sambrook et al., 1989).

2.2.3 Features of pGEX-1 and sub-cloning strategies

The bacterial expression vector pGEX-1 (Figure 5) (Smith & Johnson, 1988), which contains the gene encoding Glutathione-S-Transferase upstream of the MCS, was used for heterologous expression of the *D. discoideum* 6C, *capA*, and *capB* genes in *E.*

Figure 3

pDB62 is the two-hybrid system vector encoding the GAL4 nuclear localization signal (not indicated) and the DNA-binding domain of the GAL4 transcription factor (GAL4/DB). This region is flanked by the yeast alcohol dehydrogenase promoter and terminator (P_{ADH1} and T_{ADH1}, respectively). Asterisks denote unique restriction sites. The boxed sites comprise the multiple cloning site (MCS) of the vector and enable the in-frame fusion of a gene of interest.



Multiple Cloning Site

GAL4

146 147

VAL SER SER ARG SER THR VAL SER ILE SER LEU

GTA TCG TCG AGG TCG ACG GTA TCG ATA AGC TTG

*Sal*I *Cla*I *Hin*dIII

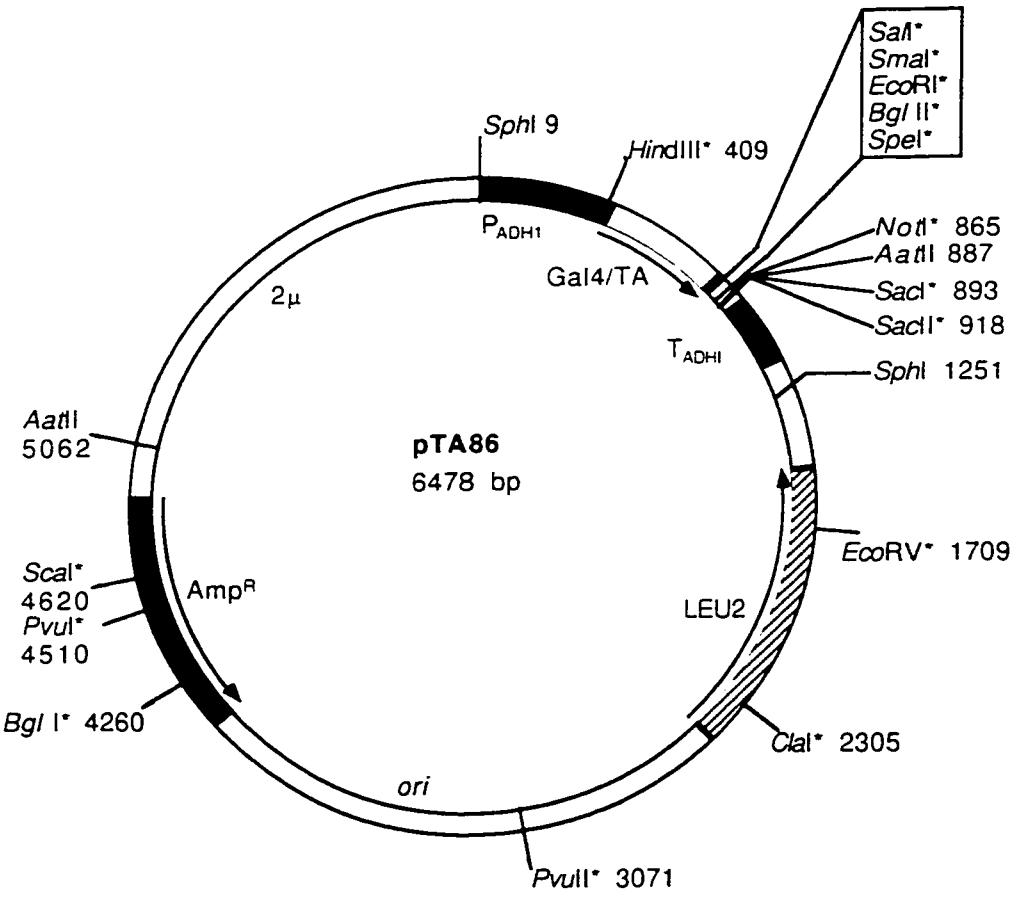
ILE SER ASN SER CYS SER

ATA TCG AAT TCC TGC AGC

*Eco*RV *Eco*RI *Pst*I

Figure 4

pTA86 is the two-hybrid system vector that encodes the T antigen nuclear localization signal (not indicated) and the Transcriptional Activator domain of the GAL4 transcription factor (GAL4/TA). This region is flanked by the yeast alcohol dehydrogenase promoter and terminator (P_{ADH1} and T_{ADH1}, respectively). Asterisks denote unique restriction sites. The boxed sites comprise the multiple cloning site (MCS) of the vector and enable the fusion of a gene of interest. A doubly underlined base in the multiple cloning site indicates an overlapping restriction site.



Multiple Cloning Site

GAL4

880 881

LYS GLU GLY GLY SER THR PRO GLY ILE GLN ILE

AAA GAG GGT GGG TCG ACC CCG GGA ATT CAG ATC

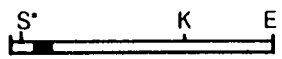
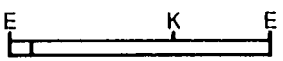
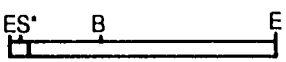
*Sal*I *Sma*I *Eco*RI *Bgl*II

TYR END -

TAC TAG T

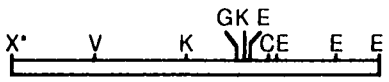
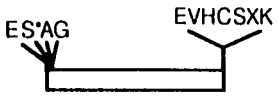
*Spe*I

TABLE II: THE DNA INSERTS USED FOR SUBCLONING

Insert Source	Relevant Restriction sites	Description	Reference
pDdM100		• A genomic clone containing the entire*D. discoideum capA* coding region as well as the gene encoding the ribonucleotide reductase small subunit (not shown) and its regulatory region (not shown) • encodes CABP1A • grey region is 111 bp intron	Caroline Grant, unpublished data
pCX		• A cDNA clone of *D. discoideum capA* lacking 111 bp intron • encodes CABP1B	Caroline Grant, unpublished data
pDdM34		• The entire*D. discoideum capB* cDNA coding region • encodes p34 • grey region is 102 bp intron	Bain *et al.*, 1991
pDdM31		• *D. discoideum capB* cDNA lacking 102 bp intron • encodes p31	Bain *et al.*, 1991

Restriction sites: A, *Xba*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; V, *Eco*RV; X, *Xho*I; Sites indicated with an "*" were engineered into the clone by PCR for cloning purposes and are not present in the plasmid (see text, section 2.2.2). N.B. The scale of the diagrams may not be proportional.

TABLE II (*continued*)

Insert Source	Relevant Restriction Sites	Description	Reference
p6CZAP-2		• Fragment of *D. discoideum* cDNA encoding the 6C protein • isolated from λ-zap cDNA library • hatched line is Bluescript™ vector and cluster of restriction sites is Bluescript™ MCS	Michael Greenwood, unpublished data
pNXN7ZAP-8		• cDNA encoding *D. discoideum* annexin • isolated from λ-zap cDNA library	Michael Greenwood, unpublished data
pDdAB7		• cDNA encoding the *D. discoideum* p24 actin/mannose-binding protein • isolated from λgt11 cDNA expression library • cluster of restriction sites is Bluescript™ MCS	Greenwood & Tsang, 1992

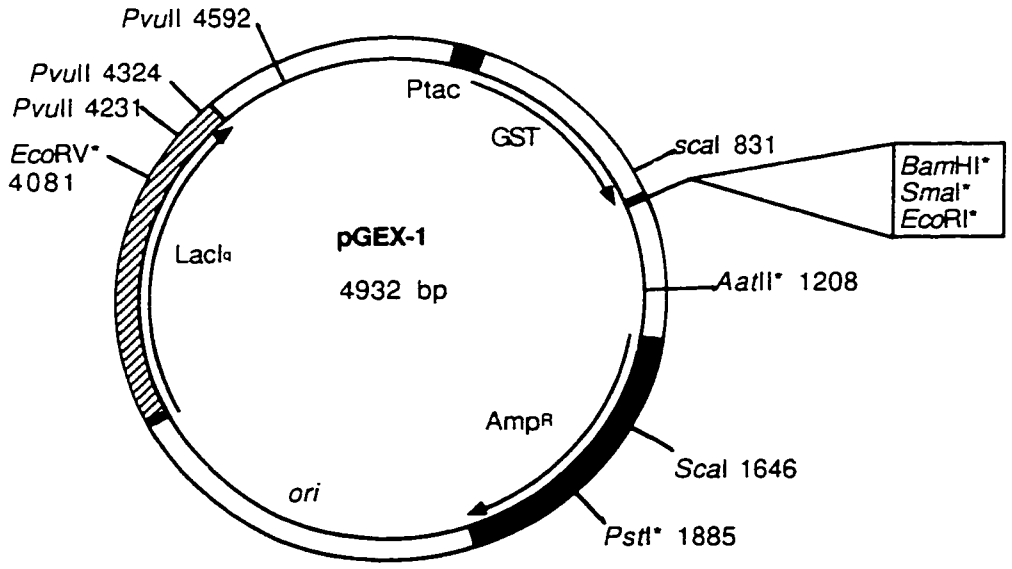
Restriction sites: A, *Xba*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; V, *Eco*RV; X, *Xho*I; Sites indicated with an "*" were engineered into the clone by PCR for cloning purposes and are not present in the plasmid (see text, section 2.2.2). N.B. The scale of the diagrams may not be proportional.

TABLE III: OLIGONUCLEOTIDE PRIMERS USED FOR AMPLIFICATION AND INCORPORATION OF SPECIFIC IN-FRAME RESTRICTION SITES

Primer	Sequence	Purpose
CASAL	5'-GTATAACCCACC*GTCGAC*ATCTGG-3' *Sal*I	Introduce in-frame *Sal*I site 15 bases downstream of *capA* ATG in pDdM100 (Caroline Grant, unpublished data)
34SAL	5'-GTATAATCCACC*GTCGAC*ATCCGG-3' *Sal*I	Introduce in-frame *Sal*I site 15 bases downstream of *capB* ATG in pDdM34 or pDdM31 (Bain *et al.*, 1991)
NXHO	5'-AC*CTCGAG*CATCAAAATGTCC-3' *Xho*I	Introduce in-frame *Xho*I site 12 bases upstream of *nxnA* ATG in PNXN7ZAP-8 (Michael Greenwood, unpublished data)
ACBSAL	5'-CAAGGTGAACACTTACA*GTCGAC*C-3' *Sal*I	Introduce in-frame *Sal*I site 33 bases downstream of p24 ATG in pDdAB7 (Greenwood & Tsang, 1992)
6CXHO	5'-CTCCATATT*CTCGAG*CCGCTGC-3' *Xho*I	Introduce in-frame *Xho*I site 1,145 bases downstream of 6C ATG (originally an *Xba*I site) in p6CZAP-2 (Michael Greenwood, unpublished data)

Figure 5

The pGEX-1 bacterial expression vector (Smith & Johnson, 1988). Immediately upstream of the MCS is the gene encoding glutathione-S-transferase (GST). Expressing a protein as a fusion with GST permits the rapid and convenient purification of the protein with a Glutathione-Sepharose column. A doubly underlined base in the multiple cloning site indicates an overlapping restriction site. For additional features of the vector see section 2.2.3.



Multiple Cloning Site

PRO LYS SER ASP PRO ARG GLU PHE ILE VAL THR ASP END
CCA AAA TCG GAT CCC CGG GAA TTC ATC GTC ACT GAC TGA
*Bam*HI *Sma*I *Eco*RI

coli. Expression is driven by the *tac* promoter and repressed by the *lac I*^{q} repressor. The vector also possesses the ß-lactamase (amp^{R}) selection marker.

The 6C gene was obtained from p6CZAP-2, a Bluescript™-based plasmid containing the 6C cDNA (R. Wennington & A.S. Tsang, unpublished data). A 1.7 Kb *Pvu*II DNA fragment (see Table II for a restriction map) was purified (see section 2.6.3) from the rest of the vector and sub-cloned into the lone *Sma*I site in pGEX-1. Because the *Sma*I site at the *Sma*I-*Pvu*II junctions is destroyed following ligation, the *Sma*I restriction enzyme was used to digest any re-circularized vector prior to transformation.

The genes encoding CABP1A and p34 were sub-cloned from pTrcHis into pGEX-1. pTHcapA1 and pTHcapB1 are the pTrcHis-derived (Invitrogen Corporation) bacterial expression vectors that were originally used, unsuccessfully, for heterologous expression and purification of CABP1A and p34, respectively. To generate pTHcapA1, the *Sal*I-*Eco*RI fragment from pDBcapA1 was ligated to the *Xho*I and *Eco*RI sites of the pTrcHisB vector. For sub-cloning *capA* into pGEX-1, the pTHcapA1 *Bam*HI-*Eco*RI fragment encompassing the *cap*A gene was ligated to the *Bam*HI-*Eco*RI sites of pGEX-1. Digestion with *Hin*dIII and *Sma*I following ligation eliminates the parent vector and re-circularized host vector. All but the first five residues of CABP1A are expressed by the pGEXcapA1 plasmid.

For the construction of pTHcapB1, pDBcapB1 was digested with *Sal*I and *Eco*RI, and ligated to the *Xho*I and *Eco*RI sites of the pTrcHisB vector. The *capB* gene was then sub-cloned into pGEX-1 by digesting pTHcapB1, with *Eco*RI and by partially digesting it with *Bam*HI (*capB* has an internal *Bam*HI site and must therefore be only partially digested with *Bam*HI to preserve the entire coding region). The 0.95 Kb *Bam*HI-*Eco*RI fragment (containing the internal *Bam*HI site) was then purified from an agarose gel (see section 2.6.3) and ligated to *Bam*HI/*Eco*RI-digested pGEX-1. Again, *Sma*I was used prior to

TABLE IV: Plasmids used in the two-hybrid assays

Plasmid Name	Two-Hybrid Vector	Insert Derivation	Cloning Strategy
pTAcapA1	pTA86	pDdM100	• PCR amplified using CASAL and reverse primers • Amplified product digested with *Sal*I and *Eco*RI and sub-cloned into dephosphorylated *Sal*I/*Eco*RI-digested vector
pDBcapA1	pDB62	pTAcapA1	• *Sal*I/*Eco*RI-digested pTAcapA1 subcloned into dephosphorylated *Sal*I/*Eco*RI-digested pDB62 • Ligation mixture digested with *Cla*I to remove unwanted products
pDBcapA2	pDB62	pCX	• *Eco*RI-digested pCX sub-cloned into *Eco*RI-digested, dephosphorylated pDB62 • Ligation mixture digested with *Bam*HI to remove unwanted products
pTAcapA2	pTA86	pDBcapA2	• Large fragment purified from *Sal*I/*Kpn*I-digested pTAcapA1 • Insert fragment of *Sal*I/*Kpn*I-digested pDBcapA2 sub-cloned into pTAcapA1 • Ligation mixture digested with *Pst*I to remove unwanted products

TABLE IV (*Continued*)

Plasmid Name	Two-Hybrid Vector	Insert Derivation	Cloning Strategy
pDBcapB1	pDB62	pDdM34	• PCR amplified using 34SAL and reverse primers • Amplified product digested with *Sal*I and *Eco*RI and sub-cloned into *Sal*I/*Eco*RI-digested vector • Ligation mixture digested with *Cla*I to remove unwanted products
pTAcapB1	pTA86	pDdM34	• PCR amplified using 34SAL and reverse primers • Amplified product digested with *Sal*I and *Eco*RI and sub-cloned into *Sal*I/*Eco*RI-digested vector • Ligation mixture digested with *Sma*I to remove unwanted products
pDBcapB2	pDB62	pDdM31	• PCR amplified using 34SAL and reverse primers • Amplified product digested with *Sal*I and *Eco*RI and sub-cloned into dephosphorylated *Sal*I/*Eco*RI-digested vector
pTAcapB2	pTA86	pDdM31	• PCR amplified using 34SAL and reverse primers • Amplified product digested with *Sal*I and *Eco*RI and sub-cloned into dephosphorylated *Sal*I/*Eco*RI-digested vector

TABLE IV (*Continued*)

Plasmid Name	Two-Hybrid Vector	Insert Derivation	Cloning Strategy
pDB6C	pDB62	p6CZAP-2	• pDB62 digested with *Sal*I followed by dephosphorylation and end-filling with Klenow Fragment • 1.7 Kb fragment purified from *Pvu*II-digested p6CZAP-2 and sub-cloned into blunt-ended vector
pTA6C	pTA86	p6CZAP-2	• pTA86 digested with *Sal*I followed by dephosphorylation and end-filling with Klenow Fragment • 1.7 Kb fragment purified from *Pvu*II-digested p6CZAP-2 and sub-cloned into blunt-ended vector
pDBnxnA	pDB62	pNXNZap-8	• PCR amplified using NXHO and reverse primers • Amplified product digested with *Xho*I and *Eco*RI and sub-cloned into *Sal*I/*Eco*RI-digested vector
pTAnxnA	pTA86	pNXNZap-8	• PCR amplified using NXHO and reverse primers • Amplified product digested with *Xho*I and *Bam*HI and sub-cloned into *Sal*I/*Bgl*II-digested vector

TABLE IV (*Continued*)

Plasmid Name	Two-Hybrid Vector	Insert Derivation	Cloning Strategy
pTAp24	pTA86	pDd5C2	• PCR amplified using ACBSAL and reverse primers • Amplified product digested with *Sal*I and sub-cloned into *Sal*I-digested vector • cloned by P. Gaudet, unpublished data
pDBp24	pDB62	pTAp24	• pTAp24 digested with *Sal*I and *Eco*RI, and sub-cloned into dephosphorylated *Sal*I/*Eco*RI-digested vector • cloned by P. Gaudet, unpublished data
pGBT9/actin	pGBT9	pMB9/actin	• pMB9/actin digested with *Hin*dIII, end-filled using Klenow Fragment, and digested with *Bgl*II • Vector digested with *Sma*I and *Bam*HI • Insert subcloned into compatible vector sites • cloned by P. Gaudet, unpublished data
pGAD424/actin	pGAD424	pMB9/actin	• pMB9/actin digested with *Hin*dIII, end-filled using Klenow Fragment, and digested with *Bgl*II • Vector digested with *Sma*I and *Bam*HI • Insert subcloned into compatible vector sites • cloned by P. Gaudet, unpublished data

TABLE IV (*Continued*)

Plasmid Name	Two-Hybrid Vector	Insert Derivation	Cloning Strategy
pDB6CΔ3	pDB62	pTA6C	• pTA6C digested with *Xba*I, end-filled, and digested with *Sal*I • Vector digested with *Eco*RI, end-filled, and digested with *Sal*I • *Sal*I-*Xba*I(end-filled) 6C fragment subcloned into prepared vector • Ligation products digested with *Cla*I to remove unwanted products
pTA6CΔ3	pTA6C	None	• pTA6C digested with *Xba*I and *Spe*I and re-ligated • ligation products digested with *Xba*I to remove non-deleted plasmid
pDB6CΔ5	p6CZAP-2	pGBT9	• PCR amplified using 6CXHO and universal primers • PCR product digested with *Sal*I and *Xho*I and sub-cloned into dephosphorylated *Sal*I-digested vector
pTA6CΔ5	p6CZAP-2	pGAD424	• PCR amplified using 6CXHO and universal primers • PCR product digested with *Sal*I and *Xho*I and sub-cloned into dephosphorylated *Sal*I-digested vector

transformation in order to eliminate re-circularized host vector. The fusion protein that results from pGEXcapB1 lacks only the first five residues of the *capB* gene.

All ligation products were transformed into XL1-blue. Ampicillin-resistant colonies were selected for rapid plasmid preparation and subsequent restriction analysis.

2.3 **The two-hybrid system and supporting procedures**

2.3.1 Yeast co-transformations

Transformation of yeast cells was performed according to the method of Gietz *et al.* (1992), whose protocol is a modification of the method developed by Ito *et al.* (1983). To prepare transformation-competent cells, a fresh colony of PCY3 was inoculated into 50 ml of YEPD medium (2% bacto peptone; 1% yeast extract; 2% glucose), and incubated with shaking overnight at 30°C. Part of this saturated culture was then diluted into 300 ml of fresh YEPD to a final OD_{600} of 0.2 and grown to an OD_{600} of approximately 0.5. The culture was centrifuged at 5,000 rpm in a Beckman JA-14 rotor for 5 minutes and the resulting pellet was washed in 10 ml of sterile H_2O. Following a second centrifugation, the pellet was resuspended in 1.5 ml of TE/lithium acetate (10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 100 mM lithium acetate) and the resulting cells were ready to be transformed. For the actual transformation procedure, 0.2 ml of this cell suspension were added to a tube containing approximately 1-5 μg of each plasmid and 200 μg of single-stranded carrier salmon sperm DNA (see section 2.6.4 for preparation) in TE/lithium acetate solution. Polyethylene glycol 3350, 40% in TE/lithium acetate, was added and the suspension was incubated for 30 min at 30°C. Following the incubation, dimethyl sulfoxide (DMSO) was added to a final concentration of 10%, and the cells were heat shocked at 42°C for 15 min. The suspension was then chilled, centrifuged, and washed in TE (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). Selection for the presence of both pDB62 and pTA86 plasmids was carried out on Leu-/Trp- sucrose dropout plates (0.13% amino acid dropout powder (without

leucine and tryptophan); 0.17% Difco yeast nitrogen base; 37.8 mM $(NH_4)_2SO_4$; 2% sucrose). Plates were incubated at 30°C until colonies appeared.

2.3.2 ß-galactosidase filter assays

Co-transformants (Tables V and VI) were restreaked on Whatman #1 filters overlaid on dropout plates. The filters were incubated overnight at 30°C to allow for further growth and expression of the *lacZ* gene in the event of an interaction. The filters were then dipped for five seconds in liquid nitrogen to permeabilize the cells, and overlaid over a filter which had been soaked in a 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) solution (1.67% X-gal stock solution (20 mg/ml in N,N-dimethylformamide); 0.27% ß-mercaptoethanol in Z-buffer (60 mM $Na_2HPO_4 \cdot 7H_20$; 40 mM $NaH_2PO_4 \cdot H_2O$; 10 mM KCl; 1 mM $MgSO_4 \cdot H_2O$)). Filters were observed for a colorimetric change intermittently for 30 hours.

2.3.3 ß-galactosidase liquid assays

The liquid assay was modified from a protocol by Miller (1972). For the ß-galactosidase liquid assay, single colonies were inoculated into Leu-/Trp- sucrose dropout medium, and grown overnight to an OD_{600} of 0.4 - 1.2. The cells were then pelleted by centrifugation, immersed in liquid nitrogen, and resuspended in Z-buffer/ß-mercaptoethanol (0.27% ß-mercaptoethanol in Z-buffer). 0.16 ml of an o-nitro-phenyl-ß-D-galactopyranoside (ONPG) solution (4 mg/ml ONPG in 0.1M phosphate buffer (82 mM Na_2HPO_4 18 mM NaH_2PO_4, pH 7.0)) was added to each tube. The cells were then incubated until a yellow color was observed, at which point the chromogenic reaction was quenched by the addition of 0.4 ml of 1M Na_2CO_3. The OD_{420} of each sample was taken, and the ß-galactosidase activities, expressed in Miller Units (Miller, 1972), were calculated using the following equation:

$$\text{ß-Galactosidase activity} = \frac{(1000 \times OD_{420})}{(t \times v \times OD_{600})}$$

Where OD_{420} is the optical density of the quenched reaction, t is the reaction time in minutes, v is the volume of the reaction in milliliters, and OD_{600} is a measure of the cell density of the culture used (see Appendix).

2.4 **Heterologous expression and purification of *D. discoideum* proteins**

2.4.1 Optimization of induction of protein expression

To assay for optimal expression of inserts, pilot experiments were performed whereby incubation temperature, length of incubation, and host strains were varied. Plasmid DNA was transformed into *E. coli* strains XL1-Blue or Top 10. Typically, single colonies were inoculated into LB broth (1% Bacto-tryptone; 0.5% Bacto-yeast extract; 1% NaCl; 100 µg/ml ampicillin) or modified M9 medium (40 mM Na_2HPO_4; 20 mM KH_2PO_4; 8 mM NaCl; 18.7 mM NH_4Cl; 2 mM $MgCl_2$; 1% glucose; 0.1 mM $CaCl_2$; 0.05 mM thiamine; 100 µg/ml ampicillin) and grown overnight. The following morning the cultures were subcultured into the same medium and allowed to grow for a variable period of time following which 1 ml was removed to a separate tube and isopropyl ß-D-thiogalactopyranoside (IPTG) was added to the remaining culture to a final concentration of 1 mM. The cultures were again left to incubate for a variable period of time (2 -24 h) at 30°C or 37°C, transferred to microcentrifuge tubes, centrifuged at 5,000 rpm for 5 min, and the pellet (from 1 ml of original culture) was resuspended in 100 µl SDS sample buffer (62.5 mM Tris-Cl, pH 6.8; 2.3% SDS; 10% glycerol; 5% ß-mercaptoethanol). These bacterial lysates were then boiled for 10 minutes and centrifuged for 10 minutes at 13,800 x g prior to electrophoresis on a 10% SDS-PAGE gel for analysis (Laemmli, 1970).

2.4.2 Expression and metabolic labelling of proteins

Protein expression and purification protocols were based on those by Frangioni & Neel (1993). The metabolic labelling protocol was adapted from Lydan and O'Day (1993). Once expression conditions had been optimized (see above), the desired expression constructs were transformed into the *E. coli* strain XL1-blue. Single colonies were picked for inoculation into LB (100 μg/ml ampicillin) broth for overnight growth. Four milliliters from the culture were then transferred to 50 ml of pre-warmed fresh medium until an OD_{550} of 0.7-1.0 was reached.

For the preparation of methionine-labelled protein, the above culture was centrifuged prior to the addition of IPTG, washed and resuspended in modified M9 medium lacking $MgSO_4$ so as to enhance [^{35}S]methionine incorporation. After a 30 min. incubation period, 1 mCi of TransLabel [^{35}S]methionine label (ICN), and the IPTG (final concentration 1 mM) were added.

For the preparation of unlabelled protein, isopropyl-ß-D thiogalactopyranoside (IPTG) was added directly to the culture to a final concentration of 1 mM, and the culture was incubated over night at 30°C. Incubation proceeded as with the labelled culture - shaking overnight at 30°C.

All subsequent treatments were carried out at 4°C. Induced cultures were centrifuged, resuspended in lysis buffer (10% sucrose; 100 mM Tris-Cl, pH 8.0; 1.5 mM EDTA) and treated with lysozyme (40 mM Tris-Cl, pH 7.5; 1 mM EDTA; 150 mM NaCl; 0.5% w/v lysozyme) to a final concentration of 100 μg/ml for 30 minutes. Prior to extraction by sonication, 5 ml of extraction buffer (10 mM Tris-Cl, pH 8.0; 0.16 mM EDTA; 1% sucrose; 5 mM DTT; 50 mM NaCl; and 0.2% sarkosyl) were added to the cells. The suspension was sonicated with a large (1 cm) horn at medium strength (Amplitude = 4-5 microns) to prevent frothing. The sonicate was then centrifuged to remove insoluble material. Triton X-100 was added to the supernate to a final concentration of 1.0%.

2.4.3 Purification of heterologously expressed proteins

A Glutathione-Sepharose® 4B column (Pharmacia) was equilibrated with 1% Triton X-100 in phosphate buffered saline (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 pH 7.3) and connected to a peristaltic pump to regulate the flow to 2.5 ml/hr. The cleared lysate, prepared as described above, was applied to the column. Elution was carried out with 5 bed volumes of 5 mM reduced glutathione in 100 mM Tris-Cl. The eluate was aliquoted and stored at -70°C.

2.5 **Blot overlay assay**

The blot overlay assay was adopted from Crawford & Beckerle (1994). Crude and purified protein extracts were applied to a 10% polyacrylamide gel (Laemmli, 1973) and electrophoretically transferred to a nitrocellulose (BA-85) membrane at 4°C in transfer buffer (50 mM Tris-glycine, pH 8.3; 0.1% SDS; 20% methanol). Following transfer, the membrane was blocked overnight at 4°C in 50 mM Tris-Cl pH 8, 150 mM NaCl, 5% non-fat milk, 0.2 % Tween 20, and 0.02% NaN_3. For the overlay assay, the membrane was placed in a heat sealable bag with 2.5 X 10^5 cpm/ml of labelled protein in 2 ml of overlay buffer (20 mM HEPES, pH 7.5; 10 mM NaCl; 1% non fat milk; 1 mM EDTA; 0.05% Tween 20; 5 mM ß-mercaptoethanol), and incubated overnight at 4°C. The blot was rinsed 4 times in washing buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl; 0.05% Tween 20), dried, and exposed to a Biorad CS phosphor-imager screen for 70-92 hours.

2.6 **Other Procedures**

2.6.1 Preparation of electrocompetent cells and electroporation

A single colony of bacterial strain XL1-blue (Table I) was inoculated into 2.5 ml of LB medium and incubated overnight at 37°C. The following morning, this starter culture was used to inoculate 500 ml of LB. At OD_{600}=0.5, the culture is chilled in ice water for 15-30 min. The chilled cells were pelleted by centrifugation at 5000 rpm in a Beckman JA-14 rotor for 15 min at 4°C and washed once in 500 ml of sterile H_2O. A second wash was

performed with 250 ml of sterile H_2O and the cells were resuspended in a final volume of 1.5 ml with 10% glycerol. Cells were aliquoted into Eppendorf tubes by flash freezing in dry ice/ethanol.

Electroporation was performed using a BioRad Gene Pulser™ and Pulse controller™ set to 1.3 kV, 25 μF capacitance, and 200 Ω resistance for a 0.2 cm gap width electroporation cuvette. Electrocompetent cells were thawed, mixed with DNA inside the cuvette and pulsed. Cells were then quickly resuspended in SOC (2% Bacto tryptone; 0.5% Bacto-yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) and allowed to express selection markers for 1 hour. One to 200 μl of this suspension was then plated on LB agar containing 100 μg/ml of ampicilin.

2.6.2 Harvesting and analysing plasmid DNA

To obtain plasmid DNA the alkali lysis method of plasmid DNA preparations (Sambrook *et al.*, 1989) was used. Electrophoresis and restriction analysis of plasmid DNA was performed using 0.9% agarose gels and commercially available restriction enzymes and buffers (Bethesda Research Laboratories, Boehringer Mannheim).

2.6.3 Band Purification of DNA fragments

Certain cloning strategies were facilitated by the purification of specific DNA fragments. Typically, digested DNA was electrophoresed on an agarose gel in 1 X TAE buffer (40 mM Tris acetate, pH 8.0; 2 mM EDTA). Following a brief staining in 0.5 μg/ml ethidium bromide, the desired band of agarose (containing the DNA) was viewed and excised from the gel and placed in a pre-weighed Eppendorf tube. The Geneclean II DNA purification protocol (Bio 101 Inc.) was then utilized to purify the DNA from the excised gel slab.

2.6.4 Preparation of salmon sperm DNA

Preparation of salmon sperm carrier DNA was performed by A. Martinez. Salmon sperm DNA obtained from Sigma Corporation (catalog # D-1626) was sheared by sonication to a size ranging from 0.5-5.0 Kb, as verified by agarose gel electrophoresis. The resulting sonicate was filter sterilized, boiled for 10 minutes in a boiling water bath, and chilled on ice immediately afterwards so as to avoid reannealing.

2.6.5 Immunoblotting

Proteins to be probed with the antibody were electrophoresed on a 10% polyacrylamide gel using a BioRad mini-gel apparatus, and electrophoretically transferred in transfer buffer (25 mM Tris-glycine, pH 8.0; 15% methanol pH 8.2) to a pre-wet Immobilon™ PVDF membrane (Millipore). The blot was then blocked overnight at 4°C in Tris buffered saline (1 X TBS) (50 mM Tris-Cl, pH 8.0; 150 mM NaCl), 0.5% Tween 20, and 5% non-fat milk. The following day the primary antibody is diluted 1:1000 to 1:2000 in 1 X TBS, 0.5% Tween 20, and 3% non-fat milk. For all experiments in this thesis, the antibody used was B9 (Tsang & Tasaka, 1986), an anti-CABP1 monoclonal mouse IgG which cross-reacts with other GYPAQ-bearing proteins depending on the wash stringency. The blot was incubated with the antibody at room temperature for two hours, and was washed four times in 0.5% Tween 20 in 1 X TBS. The secondary antibody in these experiments was goat anti-mouse IgG coupled to alkaline phosphatase (AP), pre-adsorbed with methanol-fixed cells and diluted as above. The membrane was incubated for two hours at room temperature with the secondary antibody and washed as above. The membrane was next rinsed in AP buffer (100 mM Tris-Cl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) and incubated in the substrate mixture (10 ml of AP buffer, 44 µl of nitroblue tetrazolium chloride, 33 µl of 5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt) (GIBCO BRL Life Technologies) until color appeared, after which the reaction was stopped with distilled water.

Chapter 3

RESULTS

3.1 Cloning of Inserts

In order to generate convenient restriction sites for fusing the various genes into the two-hybrid vectors, the MCS of pGBT9 was replaced with the MCS of pPC62, resulting in the vector pDB62 (Figure 3). Likewise, the MCS of pGAD424 was replaced with the MCS of pPC86 to generate pTA86 (Figure 4). Next, the genes encoding the various GYPAQ-rich proteins were sub-cloned into pDB62 and pTA86 (Table IV). In each case, extensive restriction analysis of the potential positives was performed to verify authenticity of the constructs.

3.2 Two hybrid ß-galactosidase filter assays

All the possible pairwise combinations of pDB and pTA plasmids (summarized in Tables V and VI) were co-transformed into *S. cerevisiae* strain PCY3, and assayed for the presence or absence of an interaction. Henceforth, the term "combination" refers to a co-transformant harbouring a DNA-binding domain fused to X, and a transcription activation domain fused to Y. The "reciprocal" or "opposite orientation" of this combination, conversely, refer to a transformant containing the DNA-binding domain fused to Y, and the transcription activation domain fused to X.

As a positive control, pDB and pTA plasmids encoding actin were co-transformed and subjected to the filter assay (Breeden & Nasmyth, 1985). Actin multimerizes to form bundles in microfilament assemblies (for reviews see Korn, 1982; Korn *et al.*, 1987) and has previously been established as an interacting combination in the two-hybrid system by P. Gaudet of our lab. As the filter assay is primarily a qualitative assay, positives were scored as being roughly equivalent to or greater than an actin-actin response in blue-

intensity (+++), approximately 2/3 the intensity (++) or approximately 1/3 the intensity (+). See Figure 6 for a representative filter assay result.

Several negative controls were employed. The pDB62 and pTA86 vectors alone, lacking inserts, were co-transformed into yeast and assayed. In addition, every fusion was assayed in combination with the pDB or pTA vectors alone. All positive and negative controls behaved as expected, except pTA co-transformed with pDBcapA2, which will be discussed below.

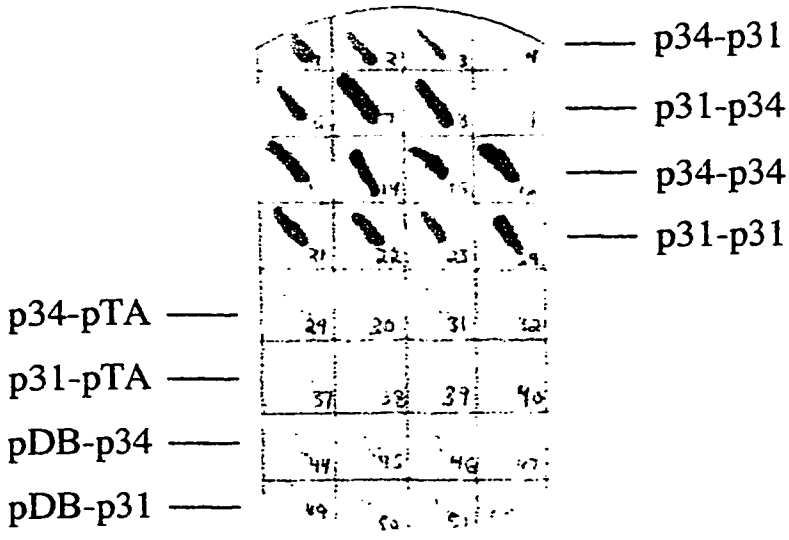
The initial filter assay detected several putative interactions, as summarized in Table V. First, the pDBcapA1/pTAcapA1 and pDBcapA2/pTAcapA2 combinations each displayed elevated reporter activity, suggesting that each protein is capable of forming homodimers. Reporter gene expression, as reflected by the shade of blue attained during incubation of the colonies, was stronger for the CABP1A homodimer than the CABP1B homodimer. When pDBcapA1 was co-transformed with pTAcapA2 a moderate color reaction occurred, revealing a potential association of CABP1A with CABP1B, as predicted by Tsang and Tasaka (1986). The combination of pDBcapA1 and pTAcapA2, however, displayed a higher level of reporter activity than did its reciprocal. Like capA, all pairwise combinations of capB produced a ß-galactosidase positive reaction, implying that p34 and p31 can also homo- and heterodimerize. Again, different combinations displayed different degrees of reporter activity. Surprisingly, no capA/capB combinations elicited a true reporter gene response, in spite of their high homology.

Preliminary examination suggested that CABP1B interacts with itself and with CABP1A (see above), as well as with p34, p31, annexin, 6C, and p24. However, these finding were called into question based on the following observations. First, apart from the interactions with CABP1, the reciprocal interactions were not observed. Second, when pDBcapA2 was co-transformed with the TA domain vector (pTA86), the combination produced a blue reaction where none was expected. Finally, the reporter expression level was low for each of these combinations, with the exception of the interaction with 6C. All

Figure 6

A typical two-hybrid system filter assay. Yeast co-transformants (PCY3) are grown overnight on a filter which has been overlaid on a dropout plate. The cells are permeabilized in liquid nitrogen and the filter is then incubated in the chromogenic X-gal solution. Each row represents one pairwise combination, with multiple colonies streaked from a single plate of transformants. In this assay, p34 and p31 are self-associating as well as interacting with each other in all pairwise combinations. In order to rule out the possibility that p34 or p31 are capable of activating transcription alone, by virtue of an intrinsic TA activity or an ability to interact with components of the GAL4 transcription system, each plasmid is co-transformed with the pGB or pGAD vectors as a negative control.

p34/31 Interactions



these observations, taken together, suggest that CABP1B interactions with p34, p31, annexin, p24, and possibly itself are artefactual (see discussion). The interactions with CABP1A were preserved in the reciprocal combination and will be considered further. Likewise, the interaction with 6C, while lacking a reciprocal response, yielded a much stronger activity than the other combinations and merits further study.

The combination of pDB6C-pTA6C displayed the highest level of reporter gene expression in the two-hybrid filter assay. The 6C protein also interacted with CABP1A, CABP1B, p34, p31, and to a lesser extent, p24 in the two-hybrid assay. However, elevated reporter activity was only observed in one orientation: when 6C was fused to the TA domain and the other proteins were fused to the DB domain. Such directionality of interactions is occasionally encountered although the underlying molecular basis for its occurence remains unknown (Estojak *et al.*, 1995).

$6C^{\Delta}3$, a 6C protein lacking the carboxy-terminal 85 residues, behaves in a similar fashion to the intact 6C protein in the two-hybrid system (Table VI): it self-associates, and interacts with CABP1A, CABP1B, p34, p31, and p24, albeit in only one orientation. As with the entire 6C protein, a heterologous interaction was only observed when 6C was fused to the GAL4 TA domain and not the DB domain. $6C^{\Delta}3$ also interacts strongly with the complete 6C protein in both orientations.

The $6C^{\Delta}5$ protein consists exclusively of the carboxy-terminal 85 amino acid residues of the 6C protein. It lacks the entire GYPAQ-rich region that is present in the intact protein. In the two-hybrid system, $6C^{\Delta}5$ interacted very weakly with the intact 6C protein, and only in one orientation (Table VI). It did not interact with itself and only produced a very weak response in one orientation with CABP1B and p24 (Table VI).

3.3 **Two-hybrid ß-galactosidase liquid assays**

The ß-galactosidase liquid assays (Miller, 1972) were performed to verify and quantify the results obtained in the filter assays. The ß-galactosidase reporter activity of the

TABLE V: PAIRWISE COMBINATIONS OF CO-TRANSFORMANTS AND FILTER ASSAY RESULTS

DB domain \ TA domain	capA1	capA2	capB1	capB2	nxnA	6C	p24	Actin	pTA
capA1	++	++				++			
capA2*	+	+	+	+	+	+++	+	+	+
capB1			+++	++		++			
capB2			+++	++		++			
nxnA									
6C						+++			
p24						+			
Actin								+++	
pDB									

* When fused to the DB-domain of GAL4, CABP1B produced a color reaction almost indiscriminately, but most notably with the TA vector alone. The reaction with 6C, however, is most likely not artefactual because it is much stronger than all the others.

TABLE VI: FILTER ASSAY RESULTS - TRUNCATED 6C AND OTHER PROTEINS

DB domain

TA Domain	6C	6CΔ3	6CΔ5	pTA
6C	+++	+++	+	
6CΔ3	+++	+++		
6CΔ5				
pDB				

	capA1	capA2	capB1	capB2	P24
*6CΔ3	+	+++	+	+	++
*6CΔ5		+			+

* Reciprocals are not shown because no ß-galactosidase activity was observed for the reciprocal combinations

TABLE VII: LIQUID ASSAY ß-GALACTOSIDASE ACTIVITIES OF SELECTED CO-TRANSFORMANTS

Combination	†Avg ß-gal Activity (Miller Units)	††Times greater than Background
pDBcapA1-pTAcapA1	0.145	1.5
pDBcapA2-pTAcapA2	0.37	2.7
pDBcapA1-pTAcapA2	0.245	2.4
pDBcapA2-pTAcapA1	0.21	1.8
pDBcapA1-pTA	0.10	
pDB-pTAcapA1	0.085	
pDBcapA2-pTA	0.15	
pDB-pTAcapA2	0.12	
pDBcapB1-pTAcapB1	3.96*	28
pDBcapB2-pTAcapB2	0.99	8
pDBcapB1-pTAcapB2	0.295	2.4
pDBcapB2-pTAcapB1	4.5	32
pDBcapB1-pTA	N/A	
pDB-pTAcapB1	0.14	
pDBcapB2-pTA	N/A	
pDB-pTAcapB2	0.125	
pDB6C-pTA6C	110.56**	960
pDB6C-pTA	0.11	
pDB-pTA6C	0.12	

TABLE VII (*Continued*)

Combination	†Avg ß-gal Activity (Miller Units)	††Times Greater Than Background?
pDBcapA1-pTA6C	0.78	7
pDBcapA2-pTA6C	2.08	15.4
pDBcapB1-pTA6C	0.73	6
pDBcapB2-pTA6C	0.33	2.75
pDBp24-pTA6C	0.62	5
pDB6C-pTAcapA1	0.09	-
pDB6C-pTAcapA2	0.18	1.6
pDB6C-pTAcapB1	0.27	2.5
pDB6C-pTAcapB2	0.17	1.5
pDB6C-pTAp24	0.18	1.6
pDB-pTA	0.075	

† ß-galactosidase activities are averages of the activities of two separate yeast colonies.

†† To derive the "Times Greater Than Background?" value, the average ß-gal activities of the negative controls for that combination were divided by the average ß-gal activity of a particular combination. For example, the pDBcapA2-pTAcapA2 combination has as its negative controls pDBcapA2-pTA and pDB-pTAcapA2. The average ß-galactosidase activity of these controls is 0.135. The average ß-galactosidase activity of pDBcapA2-pTAcapA2 (0.37) is therefore 2.74 times greater than background. These values are only to be used as a rough estimate because not all negative control experiments were performed in the liquid assay.

* Only one value available, therefore the result is not an average of two trials

** The OD_{600} of the 6C-6C culture was not in the range of 0.4 - 1.2 when the experiment was attempted (see Appendix), but the ß-galactosidase activity for this co-transformant was so exceptionally high that it was included in the data anyway

various positive combinations, and their respective negative controls, were measured and recorded (see Appendix).

For each pairwise combination, the reporter activities of two separate colonies (from a single transformation) were calculated. The ß-galactosidase activities of each combination, measured in Miller Units (mU), is shown in Table VII as an average of the two trials, except in the case of the capB1/capB1 transformant, where one culture was lost during the procedure. Negative controls ranged in reporter activity from 0.075 to 0.15 mU. The plasmid pADHGVFA (a gift from M. Greenwood), harbouring the fused GAL4 TA and GAL4 DB domains, was used as a positive control and had an average ß-galactosidase activity of approximately 250 mU.

As in the filter assay, the combination of pDB6C/pTA6C elicited the strongest ß-galactosidase response, in this case 110.56 mU. Moreover, a directionality was observed when 6C was co-transformed with either capA1, capA2, capB1, capB2, or p24. ß-galactosidase activities ranged from 2.75-15.4 times greater than background, but only when 6C was fused to the TA domain of GAL4. Reciprocal transformations, with the exception of pDB6C/pTAcapB1, yielded activities that were lower or only slightly higher than background levels (1.5-1.6 times background).

ß-galactosidase levels from transformants harbouring capB1 and capB2 in all pairwise combinations also bore a resemblance to the results obtained in the filter assay. pDBcapB2/pTAcapB1 yielded a ß-galactosidase activity (4.5 mU) that was more than 30 times greater than its controls. Its reciprocal, pDBcapB1/pTAcapB2, gave an activity that was twice the activity of the negative control (0.30 mU). Also suggested by these results are the abilities of p34 and p31 to each homodimerize, with reporter activities of pDBcapB1/pTAcapB1 and pDBcapB2/pTAcapB2 exceeding controls by 28 and 8 times, respectively. All the responses in capA1/A2 pairwise combinations were low, although still above background. The activities of capA1/capA1 and capA2/capA1 transformants

were 1.5 and 1.8 times background, respectively, and the activities of capA2/capA2 and capA1/capA2 exceeded background levels by approximately 2.5 times.

3.4 **Expression and purification of fusion proteins**

Before expressing the desired proteins for purification purposes, it was first necessary to define the growth conditions that would permit optimal expression of the cloned genes. pGEXcapA1, pGEXcapB1, and pGEX6C were transformed into XL1-Blue or Top Ten bacteria (Table I), the bacteria were induced with IPTG, and the cultures were incubated at different temperatures and/or for different lengths of time. Cells were also sonicated under non-denaturing conditions to determine whether the desired proteins were soluble or not.

Figure 7 depicts crude bacterial lysates that were isolated after various lengths of incubation in modified M9 medium and electrophoresed on an SDS-PAGE. It should be noted that although the intensity of the desired protein band steadily increases and plateaus at 20-24 hours, the relative intensity of the remaining bands stays approximately the same regardless of the duration of induction. This suggests that, at least for CABP1A and p34, an overnight induction is sufficient for optimal expression. The acrylamide gels portrayed in Figure 8 suggest that 30°C and 37°C are both adequate incubation temperatures for expression of the fusion genes. Incubation at 30°C was adopted for all subsequent experiments to prevent the potential formation of insoluble inclusion bodies, as suggested by Schein and Noteborn (1988). Although expression of 6C was assayed in a similar manner, a band corresponding to the 6C protein was not discernible and could not be distinguished from any bands in un-induced lysates (not shown).

GST-CABP1A and GST-p34 remain soluble following sonication, when the optimal growth conditions (determined above) are used (Figures 9A and 9B, lanes S). The same growth conditions were applied to the expression of GST-6C and, although expression was observed, the desired protein was insoluble (Figure 9C, lane I) and could

Figure 7

SDS-PAGE gels depicting expression of A) GST-CABP1A and B) GST-p34 as a function of post-induction incubation time at 30°C. 2% of each crude lysate, corresponding to roughly 1-2.5 μg each, were applied to the gel. The gel was subsequently stained with coomassie blue. Uninduced (UI) and induced cells were lysed following 2, 4, 6, 20, and 24 hours of induction with IPTG. As this was a pilot experiment for metabolic labelling of proteins (see Section 2.4.2), cells were first grown in LB (100 μg/ml Amp) centrifuged and resuspended in modified M9 medium (supplemented with 0.3 mM methionine) just prior to induction. Lane M corresponds to the Low Molecular Weight marker (Pharmacia).

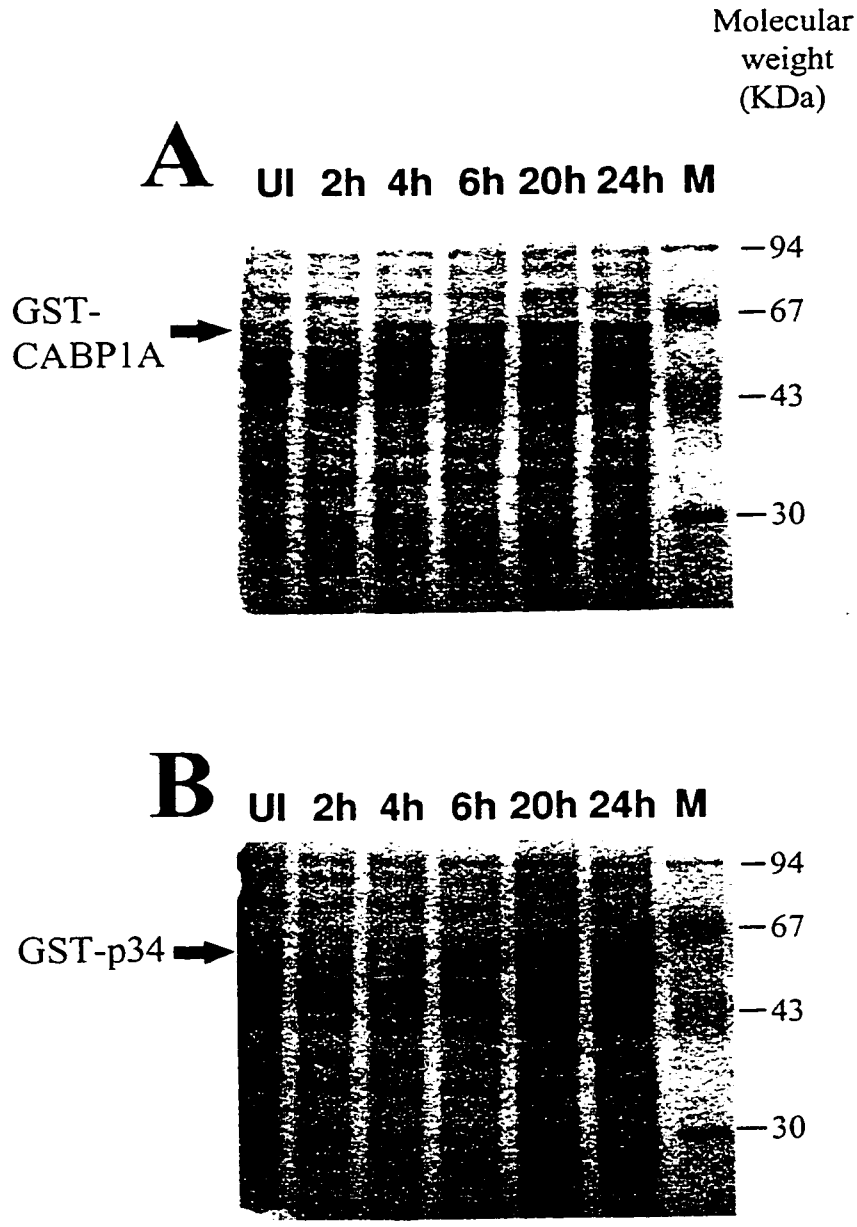

Molecular weight (KDa)
A
UI 2h 4h 6h 20h 24h M
GST-CABP1A
94
67
43
30
B
UI 2h 4h 6h 20h 24h M
GST-p34
94
67
43
30

Figure 8

SDS-PAGE gels portraying expression of A) GST-CABP1A and B) GST-p34 as a function of temperature. Approximately 2.5-5 μg of each crude lysate were loaded onto the gel. Uninduced cultures were incubated for 6 hours at 30°C or 37°C (lanes 1 and 5, respectively). Induced cultures were incubated for 2, 4, or 6 hours at 30°C (lanes 2-4) and at 37°C (lanes 6-8).

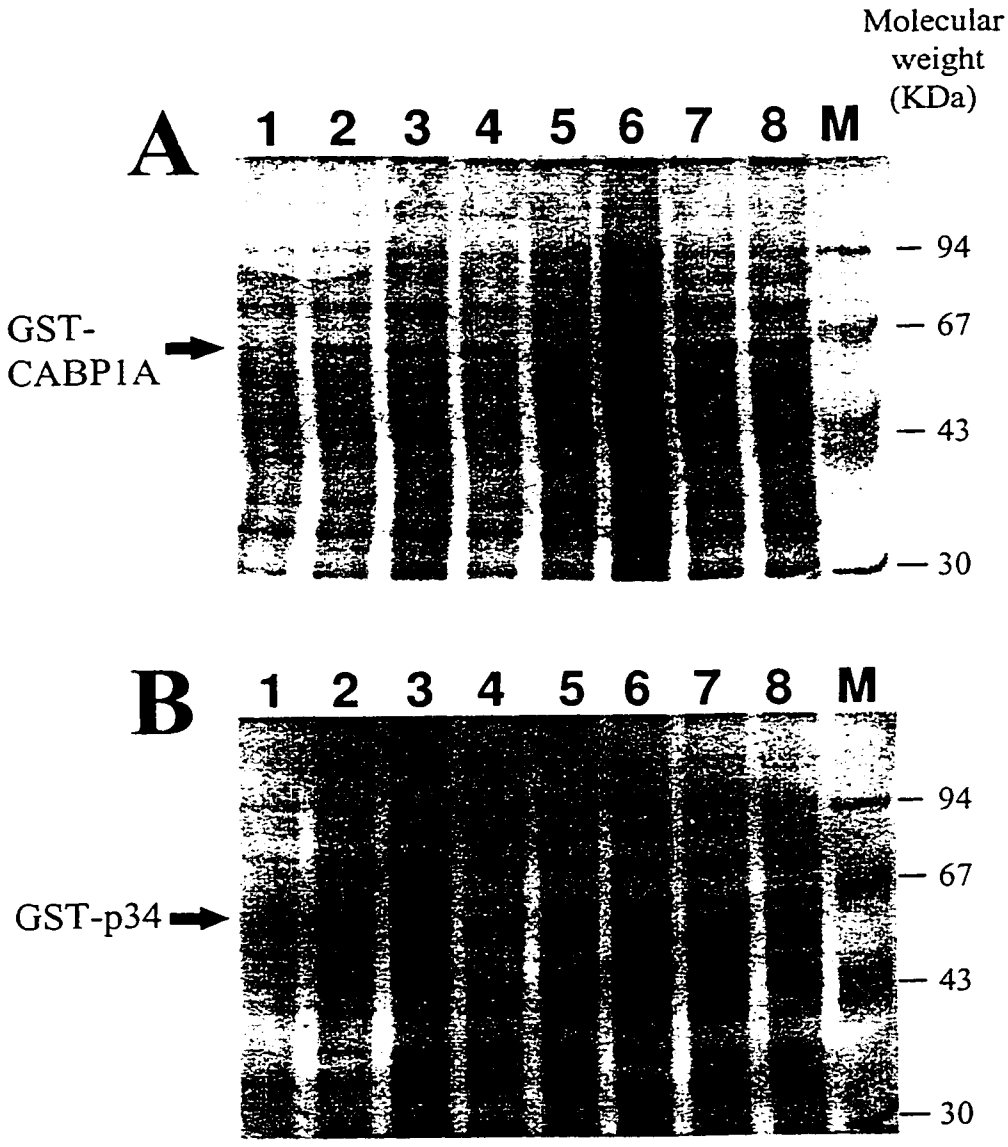
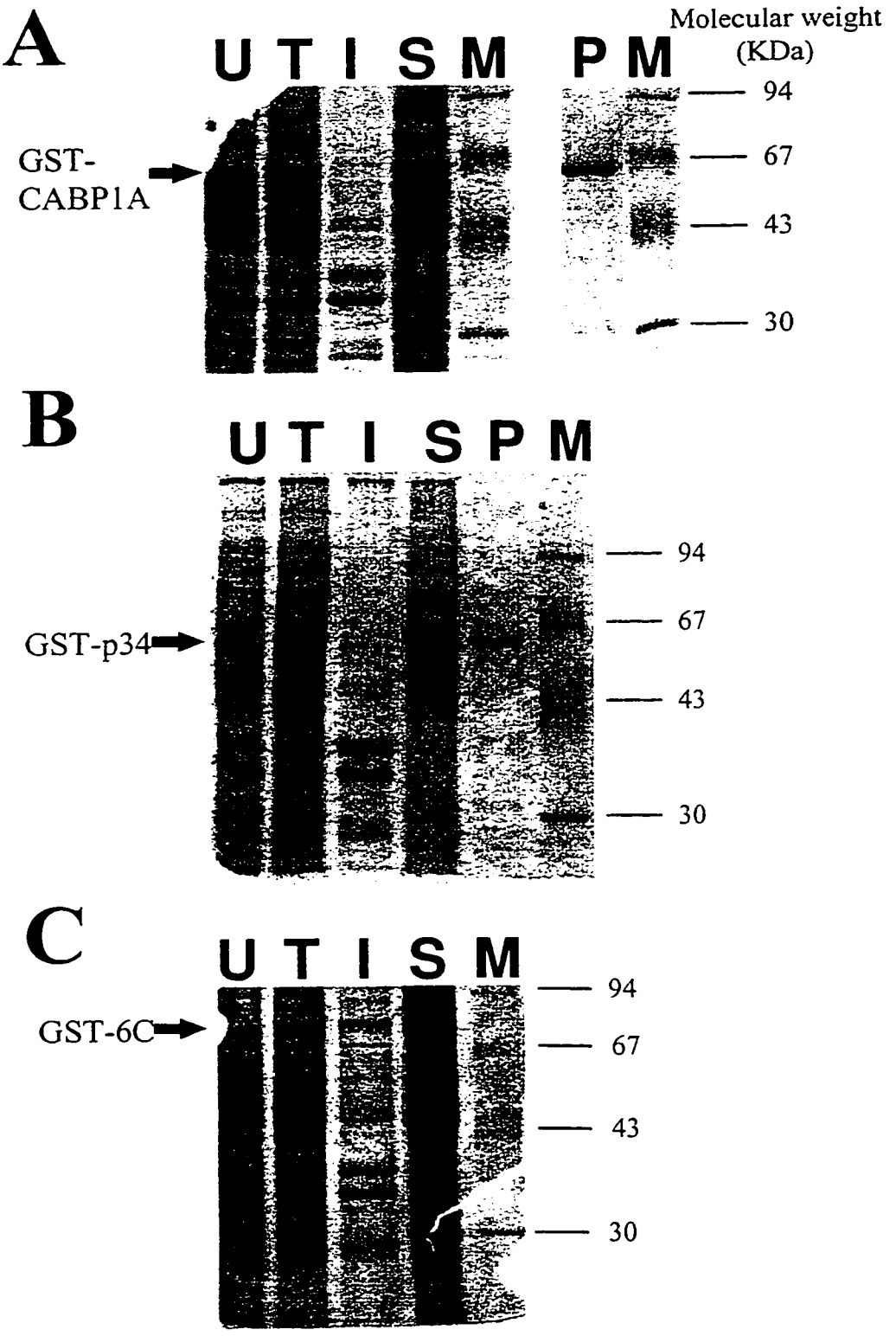

Molecular weight (KDa)
A
1 2 3 4 5 6 7 8 M
GST-CABP1A
94
67
43
30
B
1 2 3 4 5 6 7 8 M
GST-p34
94
67
43
30

Figure 9

SDS-PAGE gels illustrating the various stages in the purification of A) GST-CABP1A, B) GST-p34, and C) GST-6C. The uninduced lysate (U), was prepared in the same manner as the total crude induced lysate (T), but without IPTG. The soluble fraction (S) was derived from the soluble post-sonication supernatant, whereas the insoluble fraction (I) is derived from the post-sonication pellet, which is detergent-solubilized with SDS. GST-CABP1A and GST-p34 were soluble when lysed under non-denaturing conditions, and could therefore be purified on a glutathione-Sepharose column. GST-6C resides in the insoluble pellet following sonication and cannot be purified via the same column. Lane P corresponds to 5% of a collected fraction of the purified protein (approximately 1-2 μg).


A
U T I S M
P M
Molecular weight
(KDa)
GST-
CABP1A
94
67
43
30
B
U T I S P M
GST-p34
94
67
43
30
C
U T I S M
GST-6C
94
67
43
30

not be used for subsequent purification.

Purified GST-CABP1A and GST-p34 is shown in Figures 9A and 9B, lanes P. Purification was also carried out with metabolically labelled lysates in order to prepare [^{35}S]methionine-radiolabelled proteins for the blot overlay assay (Figure 10).

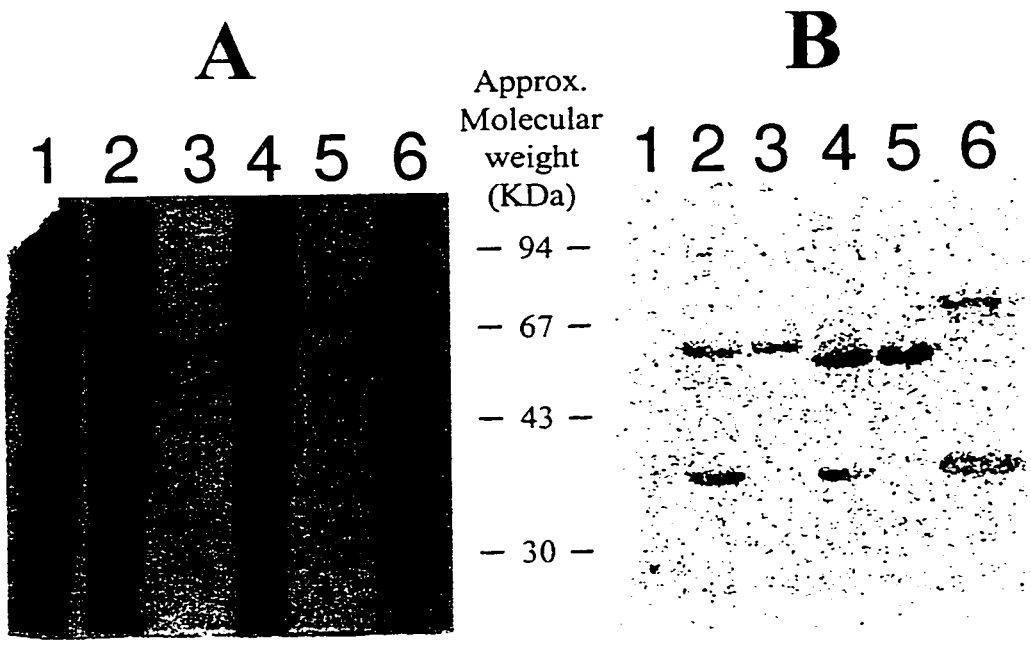
3.5 **Blot overlay assay**

In order to verify some of the observed two-hybrid system interactions, a blot overlay assay was performed. First, a radiolabelled protein probe was generated by growing induced cultures in a medium containing [^{35}S]methionine and purifying the desired protein on a glutathione-Sepharose column. Next the labelled protein was used to probe potential interacting partners on a nitrocellulose membrane.

The overlay assay was perfomed using GST-p34 as the radiolabelled probe. Figure 10A depicts a replica of the SDS-polyacrylamide gel that was subsequently transferred to nitrocellulose and probed. Figure 10B is the resulting autoradiogram, following a 92 hour exposure. For presentation purposes, the contrast and brightness of the autoradiogram was adjusted using Biorad™ Molecular Analyst and Adobe™ Photoshop. As such, the intensity of the bands on the autoradiogram are not a reflection of probe strength or interaction affinities. The GST-p34 probe does appear to be exhibiting specificity for particular bands in the extract. The nature of the lower molecular weight bands in lanes 2, 4, and 6 is unknown, although the larger bands correspond to CABP1, p34, and 6C. To ensure that the GST portion of the labelled fusion-protein probe was not binding randomly to proteins on the membrane, a crude bacterial lysate derived from *E. coli* expressing GST alone was also electrophoresed along with the other extracts (Lane 1). The radiolabelled GST-p34 probe did not bind to any region in this lane.

Figure 10

The blot overlay assay for testing protein protein interactions. Figure A is a duplicate of the SDS-polyacrylamide gel that was subsequently transferred to a nitrocellulose membrane for the assay. Figure B is the phosphorimage (autoradiogram) that results from the probing of the same nitrocellulose membrane with [^{35}S]methionine-radiolabelled GST-p34 protein. Lane 1, a crude bacterial lysate derived from cells expressing Glutathione-S-transferase (induced for 4 hours) (negative control); Lanes 2 and 4, crude lysates from cells expressing GST-CABP1A and GST-p34, respectively; Lane 3, purified GST-CABP1A; Lane 5, purified GST-p34; Lane 6, Insoluble fraction of bacterial lysate expresing GST-6C.


A
1 2 3 4 5 6
Approx.
Molecular
weight
(KDa)
— 94 —
— 67 —
— 43 —
— 30 —
B
1 2 3 4 5 6

Chapter 4

DISCUSSION

Tsang and Tasaka (1986) first suggested that CABP1A and CABP1B are subunits of the same protein because the two polypeptides co-purify, they exhibit a similar developmental expression profile, and they always appear to be present in a 1:1 ratio. Studies of p34 and p31 (Bain *et al.*, 1991) suggest that they too are subunits of a single functional protein, for similar reasons. Here, the two-hybrid system was employed a) to determine whether these proteins are indeed interacting, b) to characterize any other interactions that are occurring among the group of CABP1-related proteins, and c) to determine whether the GYPAQ-rich regions within the peptides are mediating such interactions, if they are indeed occurring, by virtue of their partial hydrophobicity and ability to hydrogen-bond. The genes encoding the various proteins were subcloned into the two hybrid vectors, pDB62 and pTA86, and assayed for the occurrence of *in vivo* interactions in yeast.

One of the pitfalls that is encountered when using the two-hybrid system lies in the fact that reporter activity, as measured by the shade of blue recorded in a filter assay, or by ß-galactosidase units in a liquid assay, does not necessarily measure, or correlate with the strength of an interaction. Low activity may reflect poor expression or stability of the fusion proteins in question, or a variance in the geometry of an interaction, for example. Furthermore, He *et al.* (1996) reported that their results, upon repeating the liquid assay, fluctuated by as much as ten per cent from day to day. When the results from different investigations are compared, where different strains or conditions may have been used, the differences are even more pronounced. Of those investigations that used comparable methods to quantify their results, some have considered 27.9±12.4 mU to be a non-interacting pair (Shen *et al.*, 1996), whereas others found 12.5 and 20 mU to be their strongest interactors (De Vries *et al.*, 1996). As such, caution must be exercised before

two proteins can be deemed interactors. The difficulty lies in choosing an appropriate reporter activity threshold, above which a combination is considered to be an interaction pair. Currently, a standard, widely-used, activity threshold does not exist. For the present study, the criteria used by Voelker *et al.* (1996) were adopted, where each combination was compared to its own negative controls, rather than being ranked alongside different combinations (see Table VII).

4.1 **The p34 and p31 proteins**

4.1.1 Interactions are observed in all pairwise combinations

The two-hybrid system data presented here suggest that the p34 and p31 polypeptides are capable of associating in all pairwise combinations in *D. discoideum*. The four possible combinations of capB1 and capB2 co-transformants elicited a ß-galactosidase response that was, in all cases, greater than the background levels of the negative controls (see Figure 6, Tables V and VII). According to the liquid assay results, for example, capB2/capB1 had a reporter activity that was roughly 30 times greater than the pDB/capB1 negative control, the GAL4 TA-p34 fusion co-expressed with a GAL4 DB domain. The reciprocal combination of capB1/capB2 had an activity that was still higher than background, albeit only two-fold. As previously mentioned, such directionality of interactions is common in the two-hybrid system and may be the manifestation of one chimeric transcription factor possessing a distinct geometry from its reciprocal counterpart (Estojak *et al.*, 1995). In general, the filter assay results, although qualitative, roughly corroborate those of the liquid assay, with p34-p34 and p31-p34 producing a stronger response than the remaining combinations. The results of the *in vitro* p34 overlay assay were somewhat inconclusive with respect to a p34-p34 interaction. While the radiolabelled p34 probe did adhere to the membrane-bound GST-p34, the autoradiogram of the probed membrane also revealed the non-specific binding of the probe to a smaller protein of approximately 40 kDa which resides in the crude extract (Figure 10, lane 4). Western

blotting with anti-p34 antibody (not shown) did not expose the identity of this band as a breakdown product of GST-p34. Taken together with the two-hybrid results, however, the assay supports the finding that p34 can self-associate.

4.1.2 Implications of the p34/31 interactions on the function of the GYPAQ-rich regions

On the basis of their partial hydrophobic nature, and ability to hydrogen-bond, the GYPAQ-rich regions of the various proteins were expected to be mediating any interactions that occur among the protein. It was also expected that the variability in the GYPAQ-rich sequences would dictate the specificity of the various interactions. Interestingly, the retained intron at the *capB*1 5'-end, which encodes a 34 amino acid insert that is not present in p31 (see Figure 2), does not appear to impose a specificity on p34/p31 interactions, since all combinations of p34/31 associate in the two-hybrid system, regardless of whether the polypeptides possess this insert or not. It is still, however, possible that one part of the insert is the determinant for one type of interaction (e.g. p34-p31), and another region of the insert mediates the other types of interaction (e.g. p34-p34 and p31-p31). This possibility could only be confirmed by internally inserting or deleting particular regions of the proteins and verifying whether this alters the specificity of interactions. Alternatively, the insert may serve an entirely different, as yet unknown, purpose. For instance, it could serve as a signal sequence or a site for chemical modification of the protein. The data obtained are not yet sufficient to implicate the insert in any of these functions.

4.1.3 Stoichiometry and stability of the p34/31 protein

Although p34 and p31 interact in all combinations tested, the two-hybrid system does not provide any information concerning the stoichiomety, or transience/stability of these interactions. As such, one cannot determine how many subunits are in the wild-type protein. One can, however, infer certain possible conformations based on the fact that all combinations of the polypeptides can interact in the two-hybrid system. One possibility is

that there are a multiple of four subunits in the fully functional protein: two p34 polypeptides and two p31 polypeptides. This scenario would have a given subunit associating with one identical subunit and one different one. Alternatively, the *capB* proteins could exist transiently as homo- and heterodimers, with each type of dimer possessing a different function in the cell. Both of these structures are equally plausible because both account for the ~1:1 ratio of the two polypeptides in the cell and the capability of p34/31 to form homo- and heterodimers in the two-hybrid system. A third, less likely, possibility stems from the fact that the two-hybrid system is configured such that, per yeast nucleus, a fusion protein is challenged with only one other species of fusion protein (assuming local yeast proteins do not interfere in the assay). In the case of p34/31, for instance, the interaction is being tested in a foreign nucleus, in the absence of other *D. discoideum* cognate proteins. Without competing proteins, which could affect local concentrations of p34 and p31, one cannot confirm that an interaction observed in the two-hybrid system would indeed occur in *Dictyostelium*. In this case, other, more direct, means of detecting interactions, such as chemical cross-linking, would be needed to establish the true stoichiometry of the various subunits.

The significance of the p34/p31 interactions is difficult to ascertain because the biochemical and physiological functions of the protein remain unknown. It is unclear whether p34 localizes to the mitochondrion, however recent evidence suggests that it is capable of interacting with a mitochondrial topoisomerase (Martinez, 1997). This implies a possible role for p34/31 in replication of the mitochondrial genome. Mutants which are disrupted in p34/31 exhibit slower growth when grown on bacteria, and develop more slowly than wild type cells (Bain & Tsang, 1991). It would be interesting to determine whether these defects are the result of a reduced number of mitochondria. By analogy, yeast petite strains that are deficient in mitochondrial oxidative phosphorylation activity display slower growth (Whittaker, 1979).

4.2 **The CABP1 protein interactions**

Although the evidence for an interaction between CABP1A and CABP1B is not as striking as the data obtained in support of an interaction between p34 and p31, it is still very likely that these two polypeptides also associate *in vivo*. The filter assay results (Table V) demonstrate quite clearly that CABP1A can homodimerize and that CABP1A interacts in at least one orientation with CABP1B (pDBcapA1-pTAcapA2) in the two-hybrid system. However, in the liquid assay, the pDBcapA1-pTAcapA1's ß-galactosidase response was quite low (1.5 times background) (Table VII) and implies that CABP1 may not self-associate. Furthermore, interpretation of the remaining CABP1 pairwise filter assay results is complicated by the fact that CABP1B, when fused to the DNA-binding domain of GAL4, is capable of activating transcription of the reporter gene indiscriminately, regardless of which fusion is being co-expressed with it. For instance, the combination of pDBcapA2-pTA yielded a blue response that was greater than that of pDB-pTA and equal to that of pDBcapA2-pTAcapA1. Consequently, it is difficult to distinguish between a true positive and artefactual reporter activity when the former has an activity only slightly above background. This artefactual activity is not seen in the liquid assay, perhaps because the liquid assay is, in general, not as a sensitive as the filter assay. Although the relative strengths of the liquid assay responses corroborated well with those of the filter assay, the overall strengths of the responses in the liquid assay may have been lower, or less striking compared to background levels, than the filter assay responses. The diminished sensitivity of the liquid assay would also explain the absence of a CABP1A-CABP1A interaction that is otherwise seen in the filter assay.

4.3 **Conflicting evidence for an interaction between p34/31 and CABP1**

All interactions between CABP1 and p34/31 are conspicuously absent from the two-hybrid filter and liquid assays. The evidence regarding such an interaction is conflicting. Immunoprecipitation experiments using CABP1-specific antibodies failed to

co-precipitate p34 or p31 (C. Bonfils, M.Sc. thesis), but a recent two-hybrid cDNA library screen detected CABP1B as interacting with p34 or p31 (Martinez, 1997). The capB2 cDNA, or portions thereof, was isolated 10 separate times out of > $3.0x10^7$ clones assayed. The overlay assay results also suggest that p34 and CABP1A interact (Figure 10). When the membrane was probed with [^{35}S]methionine-labelled GST-p34, the autoradiogram identified three known proteins as potential interactors: CABP1A, p34, and 6C. However, an unknown ~40 kDa protein (mentioned above), probably bacterial in origin, also bound the probe and therefore casts some doubt on the specificity of the assay. As such, the overlay assay results, in the absence of certain controls, are inconclusive. Ideally, one would re-probe the membrane with radiolabelled unfused GST, or with an unrelated protein. In summary, the evidence in support of a p34-CABP1B interaction remains inconclusive.

4.4 No *Dictyostelium* Annexin associations are observed

Dictyostelium discoideum annexin failed to interact with any of the other GYPAQ-containing proteins in the two-hybrid system. Furthermore, a two-hybrid system *Dictyostelium* cDNA library screen using annexin as the bait (conducted by A. Martinez of our lab) did not identify any proteins that were capable of associating with annexin. Initially, an interaction between annexin and CABP1 was predicted because differential ultra-centrifugation experiments suggest that CABP1 and annexin co-purify with the detergent-insoluble fraction in a calcium-dependent fashion (Bonfils, 1992). Specifically, when *Dictyostelium* lysates were resuspended and centrifuged in the presence of calcium, both annexin and CABP1 would be found in the insoluble pellet. Conversely, when the lysates are resuspended with the calcium chelator EGTA, the two peptides were detected in the soluble fraction. A physical interaction between CABP1 and annexin would have been expected in light of this pattern. Another explanation is that CABP1 is co-purifying (or interacting) with another, as yet unidentified, calcium-dependent protein. It is unlikely that

unlikely that CABP1 is, by itself, being purified in a calcium-dependent manner because no putative calcium-binding domains have been identified in CABP1.

4.5 **The 6C protein - a common factor among most of the proteins tested**

4.5.1 The 6C protein self-associates and heterodimerizes

The 6C protein may potentially explain CABP1's calcium-dependent behaviour in the ultra-centrifugation experiments. 6C contains an EF-hand domain which may bind calcium. The 6C protein, like p34/31 and CABP1A/B, is also capable of self-associating. This combination produced the strongest reporter response in both the filter and liquid assay. The 6C-6C interaction was somewhat expected because EF-hand proteins typically occur in pairs within the same polypeptide chain (reviewed in Kawasaki & Krestsinger, 1994). Although 6C has not yet been studied in the context of differential ultra-centrifugation, it does associate with CABP1A, CABP1B, p34, p31, and p24 in the two-hybrid system. In the liquid and filter assays, all the above proteins interacted with 6C when fused to the DB domain. Again, the reciprocal combinations produced no ß-galactosidase response. Still, it would appear that 6C is a common factor among the GYPAQ proteins that were tested, having interacted with many of them. At present, our lab is attempting to determine whether 6C possesses the calcium-binding activity that is implied by the presence of an EF-hand domain.

4.5.2 Defining a role for the 6C GYPAQ-rich region

The 6C protein was chosen for further interaction analysis because it appears to interact with many of the other GYPAQ-containing proteins. Two-hybrid plasmids harbouring 3'- or 5'-deletions in the 6C coding region were constructed and transformed into yeast in all pairwise combinations. The full-length 6C protein is 467 residues. The first 320 residues consist mainly of glycine, tyrosine, proline, alanine, glutamine, while the latter 147 residues harbour the putative EF-hand calcium-binding domain (residues 422-

433). The plasmids pDB6C$^{\Delta}$3 and pTA6C$^{\Delta}$3 express a 6C protein that is truncated at its carboxyl terminus by 87 residues, whereas pDB6C$^{\Delta}$5 and pTA6C$^{\Delta}$5 express only the carboxy-terminal 87 residues of 6C. The 6C$^{\Delta}$3 proteins lack the EF-hand domain, and the 6C$^{\Delta}$5 proteins, conversely, lack the entire GYPAQ domain. In the two-hybrid system, the 6C$^{\Delta}$3 protein behaved much like the complete 6C protein (Table VI). Interactions with CABP1, p34/31, and p24 were all preserved. The 6C$^{\Delta}$3 protein also associated with the entire 6C protein in all pairwise combinations. Furthermore, 6C$^{\Delta}$3 was capable of homo-dimerizing and yielded a reporter response that was comparable in strength to that of the intact protein. When the 6C$^{\Delta}$5 protein was assayed in the two-hybrid system, all of the 6C interactions were severely diminished or abolished altogether. A very limited amount of ß-galactosidase activity is detected in combination with capA2, p24 and the intact 6C protein, albeit in only one orientation (see Table VI). The 6C$^{\Delta}$5 protein is also incapable of homodimerizing. These findings support the likelihood that the GYPAQ-containing region of the protein mediates most, if not all, of the 6C protein-protein interactions, and that the EF-hand domain alone is insufficient to maintain these interactions. It remains unclear whether these findings can be applied to the GYPAQ domains of the other proteins.

Further study is required to determine whether the GYPAQ region is solely an interaction domain or whether it serves other purposes such as defining the specificity of the interaction. It is possible that a region outside the GYPAQ domain dictates which two proteins can come together *in vivo* and that particular combinations of proteins are incompatible despite the fact that they possess very similar GYPAQ-rich domains. To study the intrinsic qualities of the GYPAQ-rich domains in the absence of flanking regions, one could subclone only those regions encoding the GYPAQ domains into the two-hybrid vectors.

4.6 **General considerations on the two-hybrid system**

The two-hybrid system has proven to be a reliable assay for detecting protein-protein interactions. It has already been used to study interactions from a wide array of organisms and biochemical pathways (see Fields & Sternglanz, 1994 for a review). One of the general observations that has arisen from its repeated use is that a negative result does not necessarily imply that two proteins are not capable of interacting. For instance, a false negative can result if the interaction domain of the protein lies in the amino terminus of the protein and is blocked by the transcription factor domain in the fusion (Fields & Sternglanz, 1994). This observation is manifest in situations where other biochemical evidence conflicts with a negative two-hybrid result. Such is the case with p24, for instance. Jung *et al.* (1996) found p24 capable of dimerizing in chemical cross-linking experiments, but no interaction was observed in the two-hybrid system (Table V; P. Gaudet, unpublished data).

Conversely, once all the requisite controls have been performed, a positive result usually does imply that an interaction is occuring. As such, positive results usually correlate well with those obtained via other techniques, except in cases where the two-hybrid system has proven to be more sensitive than the alternative technique. Still, one would ideally like to corroberate the results obtained in the two-hybrid system with other evidence that two proteins are interacting *in vivo*. Results are more convincing when the two proteins in question are known to co-localize to the same cellular compartment or organelle, or possess synchronous expression patterns.

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Appendix - ß-galactosidase liquid assay (Oct. 13, 1995) - raw data

COMBINATION	*OD_{600}a	*OD_{600}b	OD_{420}a	OD_{420}b	†TIMEa	†TIMEb	ß-GAL UNITSa	ß-GAL UNITSb
pDBcapA1-pTAcapA1	0.8	0.715	0.048	0.023	207.5	207.5	0.193	0.103
pDBcapA1-pTAcapA2	0.671	0.765	0.041	0.069	207.5	207.5	0.196	0.290
pDBcapA1-pTA6C	0.614	0.504	0.124	0.144	207.5	207.5	0.649	0.918
pDBcapA2-pTAcapA1	0.707	0.68	0.056	0.035	207.5	207.5	0.254	0.165
pDBcapA2-pTAcapA2	0.725	0.691	0.122	0.043	207.5	207.5	0.541	0.200
pDcapA2-pTA6C	0.599	0.665	0.295	0.358	190	148	1.728	2.425
pDBcapB1-pTAcapB1	0.511	0.67	0.483	SPILL	159	SPILL	3.963	SPILL
pDBcapB1-pTAcapB2	0.665	0.794	0.062	0.072	207.5	207.5	0.300	0.291
pDBcapB1-pTA6C	0.41	0.44	0.097	0.096	207.5	207.5	0.760	0.701
pDBcapB2-pTAcapB1	0.568	0.7	0.379	0.467	88	113	5.055	3.936
pDBcapB2-pTAcapB2	0.783	1.07	0.174	0.389	207.5	193	0.714	1.256
pDBcapB2-pTA6C	0.465	0.46	0.053	0.041	207.5	207.5	0.366	0.286
pDBp24-pTA6C	0.541	0.657	0.105	0.124	207.5	207.5	0.624	0.606
pDB6C-pTA6C	0.077	0.103	0.456	0.657	53	29	74.492	146.635
GAL4	0.031	0.02	0.541	0.526	46	74.5	252.922	235.347
pDB-pTA	0.939	1.104	0.024	0.025	207.5	207.5	0.082	0.073

* Two different cultures were assayed for activity (a and b)

† in minutes

ß-gal activity = $(1000*OD_{420})/(t * v * OD_{600})$

t = time in minutes

v = volume in ml (1.5 ml for all samples)

A

Appendix - ß-galactosidase liquid assay (Oct. 20, 1995) - raw data

COMBINATION	*OD_{600}a	*OD_{600}b	OD_{420}a	OD_{420}b	†TIMEa	†TIMEb	ß-GAL UNITSa	ß-GAL UNITSb
pDB6C-pTAcapA1	0.741	0.972	0.016	0.026	180	180	0.080	0.099
pDB6C-pTAcapA2	0.657	0.578	0.031	0.029	180	180	0.175	0.186
pDB6C-pTAcapB1	0.615	0.62	0.05	0.04	180	180	0.301	0.239
pDB6C-pTAcapB2	0.753	0.853	0.038	0.035	180	180	0.187	0.152
pDB6C-pTAp24	0.095	0.708	0.006	0.025	180	180	0.234	0.131
pDB6C-pTA	1.217	1.205	0.048	0.024	180	180	0.146	0.074
pDB-pTAcapA1	1.256	1.151	0.02	0.033	180	180	0.059	0.106
pDB-pTAcapA2	1.192	0.944	0.045	0.035	180	180	0.140	0.137
pDB-pTAcapB1	1.15	1.118	0.046	0.04	180	180	0.148	0.133
pDB-pTAcapB2	1.086	0.945	0.036	0.032	180	180	0.123	0.125
pDB-pTA6C	0.971	0.998	0.037	0.028	180	180	0.141	0.104
pDBcapA1-pTA	1.127	0.737	0.031	0.02	180	180	0.102	0.101
pDBcapA2-pTA	1.077	0.772	0.051	0.026	180	180	0.175	0.125
GAL4	lost pellet	0.037	lost pellet	0.534	lost pellet	37	lost pellet	260.044

* Two different cultures were assayed for activity (a and b)

† in minutes

ß-gal activity = $(1000*OD_{420})/(t * v * OD_{600})$

t = time in minutes

v = volume in ml (1.5 ml for all samples)

B