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Isolation and Partial Characterization of cDNAs Encoding Polypeptides that Interact with the *capB* Gene Products of *Dictyostelium discoideum*

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ABSTRACT

Isolation and Partial Characterization of cDNAs Encoding Polypeptides that Interact with the *capB* Gene Products of *Dictyostelium discoideum*

Amalia Martinez-Perez

The gene products of *capB*, p34 and p31, were previously isolated by co-purification with the cAMP binding protein CABP1 encoded by the *capB* homolog *capA*.

The GAL4 two-hybrid system was used to screen a *Dictyostelium discoideum* cDNA expression library to identify proteins that interacted with p34/31. The clones were tested to eliminate false positives. DNA sequencing analysis revealed that the cDNAs isolated encoded: p34/31; CABP1B, the smaller product of *capA*; TopA, a mitochondrial type II topoisomerase; and a previously unidentified polypeptide (clone 101) with homology to a bacterial ORF. It was also determined that the product of clone 101 interacted with the gene products of *capA* in the two-hybrid system.

The interactions between p34/31 and TopA, coupled with the results of previous mutation and localization studies, suggest a role for these polypeptides in mitochondrial function. Moreover the isolation of cDNAs encoding CABP1B and the detection of interactions between polypeptide 101 and the *capA* gene products may imply that p34/31 have more than one intracellular role.

Para Mami, Papá, Daniel y Kamil

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

1. The probes used in the DNA and RNA blot analyses were prepared by Claire Bonfils.
2. RNA blot analysis presented in Figure 10 was performed by Claire Bonfils.

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CHAPTER 1: INTRODUCTION

Dictyostelium discoideum is a cellular slime mould that inhabits the forest floor feeding on bacteria as free-living amoebae. This vegetative growth stage continues while food sources are adequate. Starvation triggers the initiation of the developmental stage in the life cycle of this organism. Ameobae begin to aggregate chemotactically in response to cyclic AMP pulses produced by aggregating cells. While this cellular movement is occurring the expression of genes needed for vegetative growth is shut down. At the same time expression of genes coding for signalling molecules needed for intracellular and intercellular communication is initiated. Following aggregation the cells proceed to the formation of a fruiting body composed of stalk and spore cells. If the conditions are not appropriate for fruiting body formation, the aggregate can migrate in the form of a slug guided by light, temperature, and chemical signals. A new stage of vegetative growth starts when scattered spores encounter a suitable environment for germination [reviewed in Mutzel, 1995].

Dictyostelium discoideum is particularly well suited to study the signalling pathways that direct the formation of multicellular organisms. Multicellular development and vegetative growth occur at separate times in the life cycle of *Dictyostelium.* This facilitates the study of developmental pathways that occur in the absence of cell division. Furthermore, development occurs over a period of 24 hours and differentiation leads to the formation of two main types of cells, namely stalk cells and spores. In addition, molecular techniques such as gene disruption and transformation have been developed to analyze these processes.

1.1 The role of cyclic AMP in *Dictyostelium* development

All living cells have evolved mechanisms to interpret signals from their environment and to respond to these signals. In addition to their response to environmental signals the cells of multicellular organisms must communicate with one another in order to ensure a

coordinated behavior that benefits the organism as a whole. This cell-to-cell communication is mediated by the binding of molecules produced by signalling cells with receptors located inside target cells or on their surface.

Cyclic AMP acts as an intracellular signalling molecule in animal cells. This ubiquitous second messenger is synthesized intracellularly from ATP by the membrane-bound enzyme adenylyl cyclase. The signal that activates adenylyl cyclase is received by cell-surface receptors linked to heterotrimeric GTP-binding proteins or G-proteins. Activation of the receptor causes the dissociation of the G-protein subunits (α, β, and γ), and the activation of the Gα subunit through the exchange of GDP for GTP. The activated G-protein interacts with adenylyl cyclase, this enzyme synthesizes cAMP until it is inactivated by the dissociation of the Gα subunit in response to GTP hydrolysis to GDP. The intracellular effects of cAMP are mediated by a cAMP-dependent protein kinase or A-kinase. Phosphorylation of specific serine or threonine residues activates target proteins which vary depending on the target cell [reviewed in Alberts *et al.*, 3rd. ed., Ch. 15].

In *D. discoideum* cAMP acts as both first and second messengers. Extracellular cAMP is the chemoattractant that effects aggregation and it also acts as a regulator of gene expression. Extracellular cAMP is synthesized by a membrane-bound adenylyl cyclase. As cAMP binds to receptors in outlying cells they in turn relay the signal. After 1-2 minutes the cells adapt to the presence of cAMP, an extracellular phosphosdiesterase degrades cAMP and allows the cells to regain responsiveness. These cycles of cAMP secretion and degradation produce the oscillation of the signal necessary for chemotaxis and induction of the genes needed for aggregation and the formation of the multicellular organism. Once the aggregate is formed the cAMP signal switches from pulses to a continuous signal that activates the expression of genes required for the multicellular stages of the life cycle [reviewed in Firtel, 1995].

There have been four different cAMP receptors (cAR1 to cAR4) characterized so far in *Dictyostelium*. Studies of null mutants have shown that cAR1 is a high affinity receptor

essential for aggregation; its effects can be mediated by cAR3 a low affinity receptor expressed during the same developmental stages as cAR1 [Insall *et al.*, 1994]. cAR2 and cAR4 are expressed during the post-aggregative stages, these receptors seem to be involved in prespore and prestalk cell differentiation [Saxe *et al.*, 1993; Louis *et al.*, 1994].

Once aggregation is completed cAMP accumulates in the multicellular mass. The presence of a continuous concentration of extracellular cAMP rather than pulses represses aggregation genes and induces the post-aggregative genes that lead to cell differentiation [Reymond *et al.*, 1995].

During development extracellular cAMP acts as a hormone-like signal that binds to G-protein-linked receptors (cARs) that in turn activate various components of intracellular signalling pathways such as CRAC, a cytosolic activator of adenylyl cyclase [Lilly and Devreotes, 1994]. There have been two adenylyl cyclase enzymes identified in *D. discoideum*, ACA expressed during aggregation and ACG expressed during spore formation [Pitt *et al.*, 1992].

A cAMP dependent protein kinase (PKA) has been identified in *D. discoideum* [De Gunzburg *et al.*, 1984], its structure is similar to that of the mammalian enzyme although it is composed of one catalytic and one regulatory subunit rather than two of each as is the case with the mammalian enzyme.

In some animal cells an increase in cAMP activates the transcription of certain genes. The regulatory region of these genes bears a DNA sequence called a cAMP response element (CRE), this sequence is recognized by a gene-regulatory protein called a CRE-binding protein (CREB). PKA activates CREB by phosphorylating it on a serine residue [reviewed in Alberts *et al.*, 3rd. ed., Ch. 15]. Although no CREB equivalent has been found in *D. discoideum* a possible target of PKA seem to be GBF, a developmentally regulated DNA-binding protein that recognizes a regulatory element present in postaggregative and cell-type specific genes called a G-box [Schnitzler *et al.*, 1994].

Many of the components of the intracellular signalling pathways involving cAMP in *Dictyostelium* have counterparts in mammalian cells. The conservation of the molecular mechanism of signal transduction across species as divergent as mammals and cellular slime moulds underlines the suitability of *Dictyostelium discoideum* as a model for the study of these intracellular processes.

1.2 Isolation of a cyclic AMP binding protein and related polypeptides

In the search for the intracellular components that mediate the effects of cAMP on gene expression Tsang and Tasaka (1986) identified a cyclic AMP binding protein composed of two subunits that they called CABP1A and CABP1B. These two polypeptides are encoded by a single gene, *capA* [Grant and Tsang, 1990]. CABP1A and CABP1B arise from the differential splicing of a single mRNA transcript. Grant *et al.* (1990) showed that the basis for this differential splicing is a degenerate 5' splicing site. When the 5' splice sequence was mutated to conform to the consensus of a *Dictyostelium* intron the mRNA was spliced constitutively to produce only the smaller polypeptide [Grant *et al.*, 1990].

Monoclonal antibodies raised against CABP1 cross-reacted with several other polypeptides, three of which co-purified with CABP1. A cDNA library was probed with *capA* in an effort to identify some of these CABP1-related polypeptides. One of the genes cloned turned out to be a homolog of *capA* and hence it was named *capB*. Its isolation and characterization are described in detail in the next section. These experiments also resulted in the identification of the genes for an actin-binding protein called p24 [Noegel *et al.*, 1990; Greenwood and Tsang, 1992], and for Annexin VII a member of a family of calcium-dependent phospholipid binding proteins [Greenwood and Tsang, 1991].

Characterization of the CABP1-related proteins revealed that they all had in common one discrete region made up of repeated sequences rich in the amino acids glycine, tyrosine, proline, and glutamine (GYPQ). Similar tandem repeats have been found in a number of proteins with diverse functions such as synapsin, a member of the annexin family; yeast

RNA polymerase II; gliadin and hordein, storage proteins found in wheat and barley respectively; and octopus rhodopsin, a photosensitive protein [Matsushima *et al.*, 1990]. The presence of a high proportion of residues capable of forming hydrogen bonds could indicate that these regions are involved in protein-protein interactions.

1.3 Isolation and characterization of *capB*

Two polypeptides with molecular masses of 34 kDa (p34) and 31 kDa (p31) were isolated by co-purification with the subunits of CABP1. Monoclonal antibodies raised against CABP1 cross-reacted with p34 and p31 [Tsang and Tasaka, 1986]. Immunoblot analysis of nuclear and cytoplasmic cell extracts from various stages of development showed that p34 and p31 were detected mainly in the nuclear fraction and that their concentration increased throughout development [Kay *et al.* 1987].

A cDNA isolated by hybridization to a *capA* probe under low stringency conditions was used to isolate complementary messenger RNAs by hybrid selection. The RNAs isolated were translated *in vitro*, and immunoprecipitation followed by SDS-PAGE showed that the translation products precipitated were two polypeptides with molecular masses of 31 and 34 kDa respectively. Subsequently full length cDNAs encoding the p31 and p34 polypeptides were isolated and sequenced. Analysis of the two clones, named pDdM31 and pDdM34, revealed that their sequences were identical except for an insert of 102 nucleotides present in the 5' end of pDdM34 [Bain *et al.*, 1991].

PolyA+ RNA blots probed with a restriction fragment bearing sequences common to both pDdM31 and pDdM34 identified three transcripts which were present throughout development in varying amounts [Bain *et al.* 1991]. Two of the transcripts with sizes of 1.15 Kb and 1.25 Kb respectively were believed to correspond to the mRNAs encoding p31 and p34. The origin and function of the third transcript, 1.4 Kb in length, are still unknown.

A comparison of the amino-acid sequences of p34/31 and the two subunits of CABP1 revealed that they shared a remarkable similarity [Bain *et al.* 1991]. The amino termini of p34 and CABP1A contain the segments that are specific to the larger proteins

encoded by *capB* and *capA*. Throughout this region p34 and CABP1A differ in two amino acids out of 45. The remainder of the amino-terminal halves of the polypeptides constitutes the GYPQ region, this segment is 94 residues long in CABP1A/CABP1B but only 46 residues long in p34/31. The carboxy-terminus halves of these polypeptides share 76% identity [Bain *et al.* 1991].

DNA blot analysis determined that pDdM31 and pDdM34 originated from a single gene. Furthermore, immunoblot analysis of metabolically labelled proteins extracted from transformants expressing pDdM34 under the control of a constitutive promoter demonstrated that the information to generate both polypeptides was present in the longer transcript [Bain *et al.* 1991].

The similarity between *capA* and *capB* suggests that the differential splicing of the main transcript occurs in a manner similar to that determined for the *capA* transcript. The 5' splice site of *capB* deviates from the consensus for *D. discoideum* introns in a way similar to that of *capA*. Given the large degree of similarity between p34/31 and the subunits of CABP1, it has been proposed that one of these genes arose by duplication [Bain *et al.*, 1991]. Furthermore, the conservation of the splicing mechanism implies that this is an important feature of these proteins.

Gene-targeted mutagenesis was used to obtain information regarding the function of *capB*, this gene was disrupted by homologous recombination. Immunoprecipitation analysis of two independent disruptants showed that these cells were expressing low levels of the truncated p34/31.

Growth analysis of the mutant cells revealed that the doubling time of disruptants grown on bacterial suspensions was 5 h, significantly longer than the 3 h doubling time of the wild-type control. However the prolonged doubling time was not observed when the disruptants were grown axenically. In addition, the mutants took 3 h longer to develop than wild-type cells [Bain and Tsang 1991].

A *D. discoideum* strain overexpressing p34/31 was used to carry out immunofluorescence studies in an attempt to visualize the intracellular localization of these polypeptides. The overexpressed proteins appeared distributed throughout the cells in a punctuate pattern [Bain, G. 1990]. More recently immunofluorescence microscopy of a strain expressing a *capB*/GFP fusion also resulted in the visualization of a punctuate pattern throughout the cytoplasm of the cells [A. Tsang, personal communication].

The amino acid sequence of the *capB* products was compared with sequences available in protein data banks. The carboxy 193 amino-acids of p34/31 were found to have 43% to 49% identity with four bacterial proteins. Two of these proteins are encoded by plasmid genes ORF4 and ORF5 from *Alcaligenes* sp., a gram-negative bacterium, and confer resistance to tellurium ions [Jobling and Ritchie, 1987]. The two other proteins encoded by ORFb and ORFc confer UV and chemical damaging resistance to *Escherichia coli recA* strains [Azeddoug and Reysset, 1994]. The genes encoding ORFb and ORFc were isolated from chromosomal DNA of *Clostridium acetobutylicum*, a gram-positive anaerobic bacterium.

The results of the disruption experiments suggested that p34/31 are involved in both growth and development. Unfortunately the finding of similar sequences in bacteria did not provide significant clues as to the function of these polypeptides. The mechanism of tellurium resistance is unknown. Moreover, the *recA*-complementing proteins from *C. acetobutylicum* have no homology with any *recA* protein discovered so far or with any gene product involved in the SOS response [Azeddoug and Reysset, 1994]. Nevertheless the degree of conservation observed in these polypeptides found in organisms as different as a cellular slime mould, a gram-positive anaerobic bacterium, and gram-negative bacterium suggests that these proteins might play an important role in the cell.

Protein-protein interactions mediate many essential cellular functions and they constitute an essential part of signal transduction mechanisms. In view of the limited information obtained so far regarding the functions of p34/31 we have turned our efforts to

the search for proteins that interact with these polypeptides. The identification of known proteins that interact with p34/31 might increase our understanding of their intracellular role.

1.4 Use of the two-hybrid system to detect protein-protein interactions

The two-hybrid system is a genetic system developed to detect protein-protein interactions *in vivo* (Fields and Song, 1989). It takes advantage of the functional independence of two domains of the GAL4 protein of *Saccharomyces cerevisiae*, namely the DNA-binding domain and the transcriptional activation domain. Genes for each of these domains have been cloned into *S. cerevisiae* shuttle vectors (i.e. they can be maintained in both *E. coli* and *S. cerevisiae*).

Genes for two proteins of interest are fused to each of the GAL4 domains. These vectors are then co-transformed into a *S. cerevisiae* strain that contains a reporter gene (*lacZ*) under the control of upstream activating sequences specific for GAL4. In this manner, two hybrid proteins are expressed each bearing a GAL4 domain. Interaction between the two proteins brings the GAL4 domains together and the reporter gene is activated (Figure 1). The activity of the reporter gene can be detected using filter or liquid assays for β-galactosidase activity.

The two-hybrid systems allows us to screen cDNA libraries to search for proteins that interact with a protein of interest. The cDNA library is constructed in the activation domain vector and the protein of interest (the "bait") is cloned into the DNA-binding domain vector.

The use of any system to detect intracellular molecular interactions raises questions regarding the significance of the results *in vivo*. The two-hybrid system is a library based method that provides qualitative results of the interactions between proteins expressed in yeast cells and transported to their nucleus.

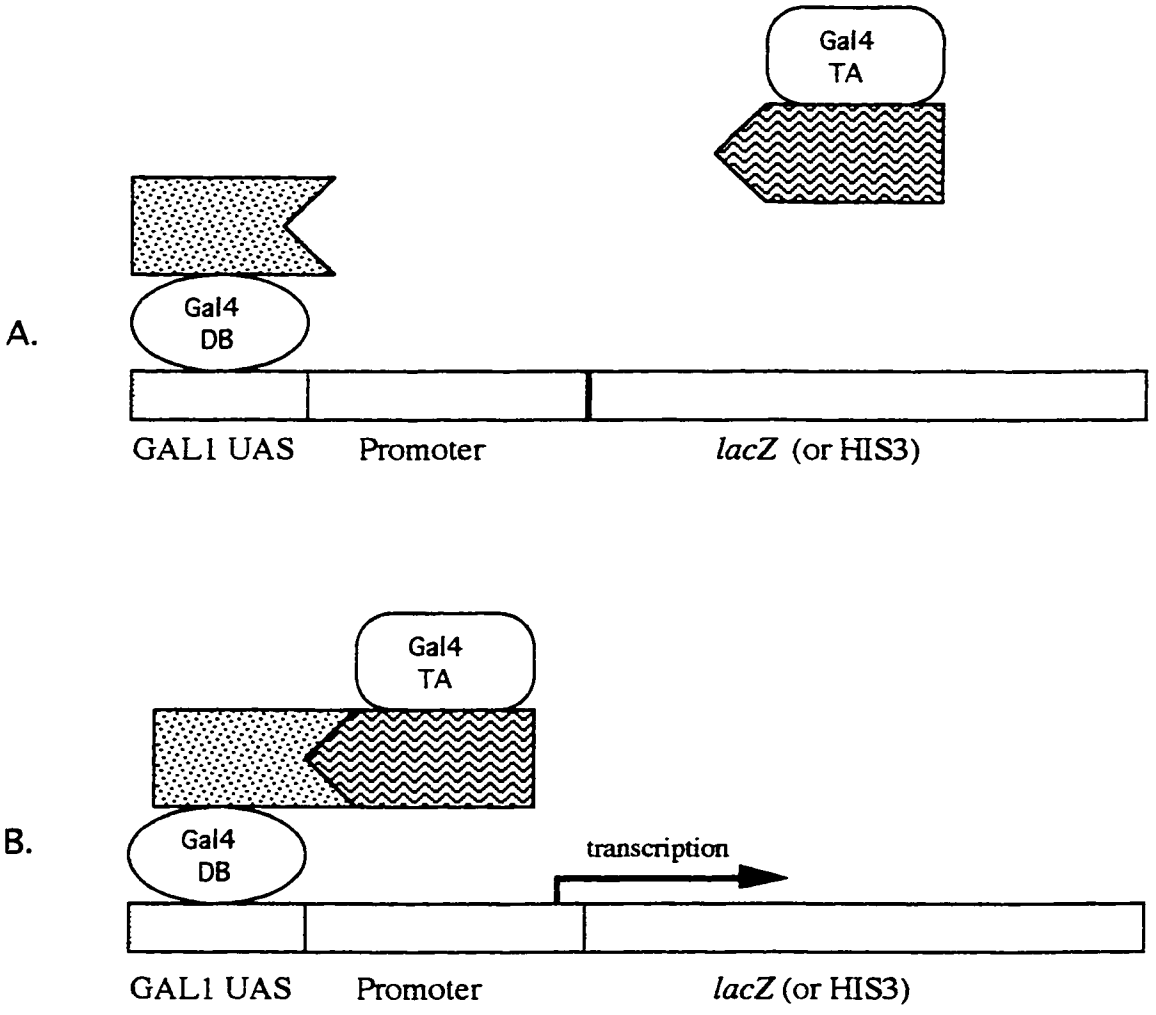


Figure 1. The two-hybrid system. (A) The hybrid proteins are expressed in the yeast cell. The GAL4 DNA-binding domain hybrid binds to the GAL1 upstream activating sequences (UAS). (B) Interaction between the two proteins bring the transcriptional activation domain close to the GAL1 UAS/promoter region and the reporter gene (*lacZ* or HIS3) is activated.

Estojak *et al.* (1995) performed a systematic study to assess the strength and significance of the interactions detected with two-hybrid trap systems. They studied the interactions between proteins whose associations have been estimated *in vitro*. Their findings indicated that the strength of activation of a particular reporter gene allowed them to discriminate between high-, intermediate-, or low-affinity interactions for proteins known to dimerize *in vitro* with K_ds ranging from 20μM to 2 nM [Estojak *et al.*, 1995]. However they report that the differences in affinity of particular pairs of proteins did not correspond linearly to the affinities reported *in vitro*, hence we cannot infer quantitative differences in affinity based on two-hybrid data. In addition the minimum threshold of responsiveness varied with different reporters, they also found that different fusion proteins had different thresholds for a given reporter. Estojak *et al.* (1995) observed that in some cases the fusion proteins exhibited directionality, that is an interaction detected with a particular DNA-binding domain/bait fusion was not detected when the bait protein was fused to the activation domain and the protein isolated was fused to the DNA-binding domain. Estojak *et al.* concluded that although two-hybrid trap systems do not provide a quantitative measure of affinity for the proteins studied they represent useful and powerful tools in the identification and analysis of protein-protein interactions.

This thesis describes the use of the GAL4 two-hybrid system [Fields and Song 1989] to identify cDNA clones encoding proteins that interact with p31 and p34. The cDNA clones isolated were screened for false positives and sequenced. Some of the positive clones encoded proteins that had already been characterized while others encoded putative novel proteins whose function is yet to be determined.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains

Saccharomyces cerevisiae strains Y153 and HF7c, and *Escherichia coli* strains MC1066, DH10B, and XL1-Blue were used in this work. Their genotypes are listed in Table I.

2.2 The two-hybrid system

2.2.1 Construction of a two-hybrid system *D. discoideum* cDNA library

A two-hybrid system cDNA library was constructed in our laboratory by Bruce Williams and Claire Bonfils. A ZAP-cDNA® synthesis kit (Stratagene) was used to synthesize cDNA from poly(A)+ RNA extracted from *D. discoideum* vegetative cells and cells that had been developed for 8 hours and 16 hours. The uneven ends of the cDNA were blunted with Klenow and *Eco*RI adaptors were ligated to the blunted ends. The cDNAs were directionally cloned into the *Eco*RI-*Sal*I sites of the two-hybrid vector pGAD424 (Figure 2). The *Sal*I site is compatible with an *Xho*I site introduced in the 3' end of the cDNA clones by the Poly(dT) primer provided with the ZAP-cDNA® synthesis kit. The library was estimated to contain 2×10^6 independent clones with an average insert size of 1.2 Kb.

2.2.2 The bait constructs

The cDNA encoding the bait proteins used for this work were cloned into the DNA-binding domain plasmid by Jonathan Gisser as part of his M.Sc. work. The cDNA library was screened with the constructs pDB62nxnA, pDB62capB1 and pDB62capB2. The maps of these plasmids are shown in Figures 3 to 5.

2.2.3 Library screening

Yeast strain Y153 (Table I) was transformed sequentially first with the DNA-binding domain bait construct, then with the cDNA library. The transformation protocol used is detailed in the next section.

Table I: Strains used

STRAIN	ORGANISM	GENOTYPE	REFERENCE
Y153	*S. cerevisiae*	MATa ura3-52 leu2-3,112 his3-200 ade2-101 trp1-901 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL1::GAL1-*lacZ* GAL1-*lacZ* (unknown location)	ATCC[a] No. 963948
HF7c	*S. cerevisiae*	MATa ura3-52his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)$_3$ CYC1-*lacZ*	Fellotter *et al.*, 1994
MC1066	*E. coli*	F$^-$ *hsdR*$^-$ ($r_k^-m_k^+$) *leuB6* *trpC9830 pyrF674*::Tn*5*(Km^r) *lacX74 galK galU* strA^r	Casadaban, *et al.*, 1983
DH10B	*E. coli*	F$^-$ *mrcA* Δ(mrr-*hsdRMS-mcrBC*) ϕ80*dlacZΔM15* *ΔlacX74* endA1 *recA1* *deoR* Δ(*ara,leu*)7697 *araD139* *galUgalK nupG rpsL*	GIBCO BRL Catalog and Reference Guide 1995-1996 p.R-40
XL1-Blue	*E. coli*	F' ::Tn*10proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15/recA1 endA1* *gyrA96* (Nal^r) *thi hsdR17* (r_k^- m_k^+) *supE44 relA1 lac*	Bullock *et al.*, 1987

[a] American Type Culture Collection

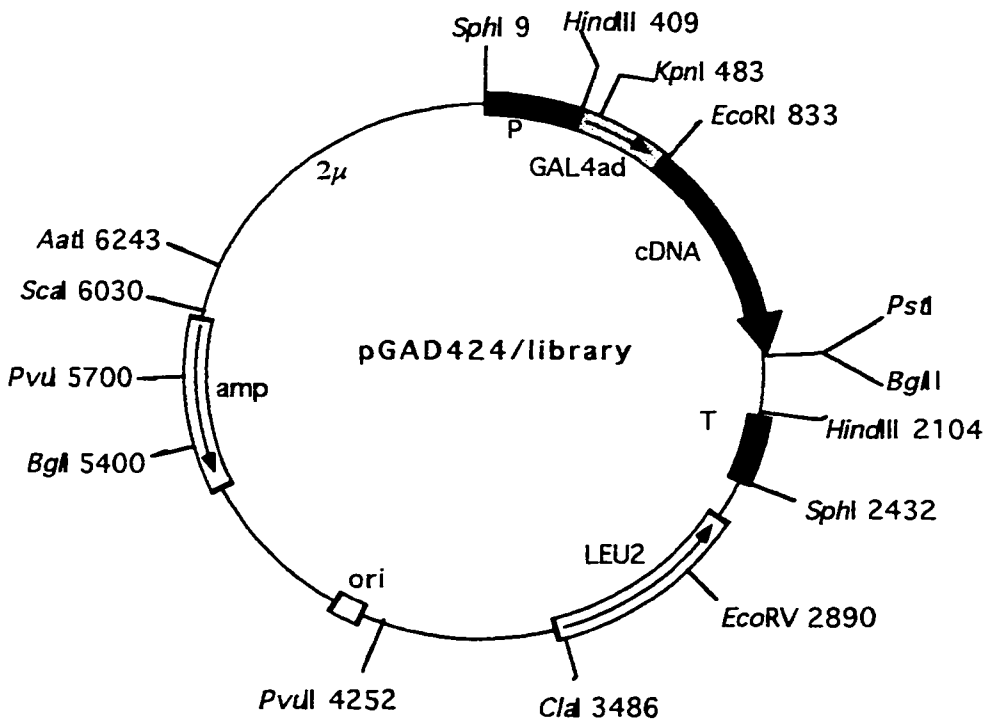


Figure 2. Plasmid pGAD424 [Bartel *et al.*, 1993a] with a cDNA insert.

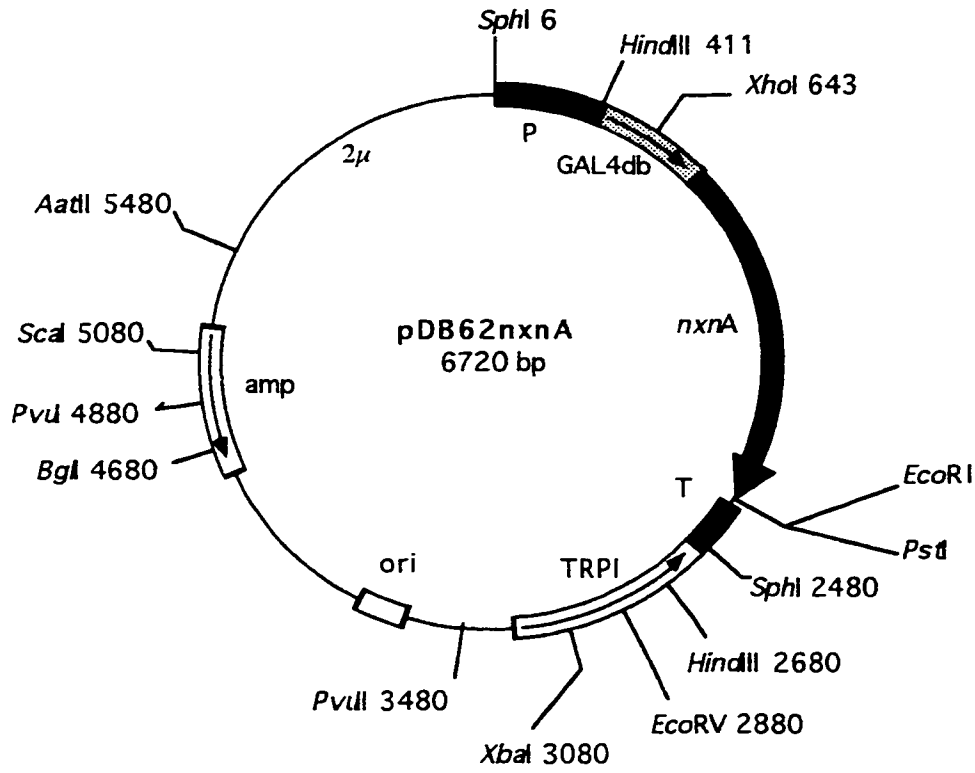


Figure 3. Plasmid pDB62nxnA. A 1.38 Kb *Xho*I-*Eco*RI fragment comprising the coding sequence of *nxnA* was cloned into the *Sal*I-*Eco*RI sites of the bait plasmid pDB62 [J. Gisser, personal communication]. J. Gisser constructed plasmid pDB62 by replacing the multicloning site of pGBT9 [Bartel *et al.*, 1993a] with that of pPC62 [Chevray and Nathans, 1992].

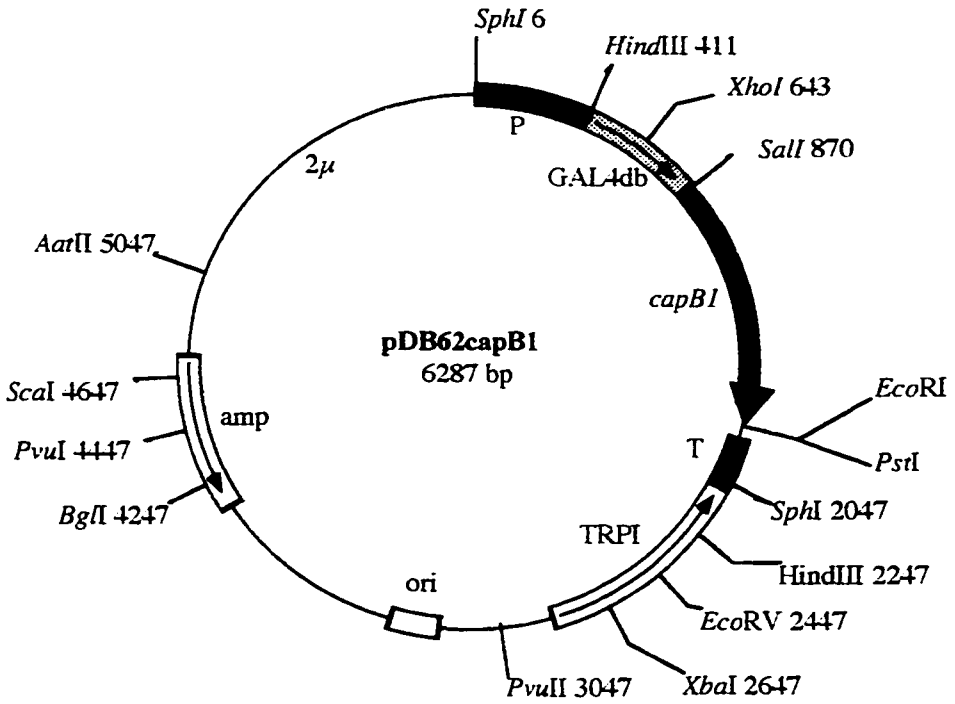


Figure 4. Plasmid pDB62capB1. A .947 Kb *SalI-EcoRI* fragment comprising the coding sequence of *capB1* was cloned into the *SalI-EcoRI* sites of the bait plasmid pDB62 [J. Gisser, personal communication].

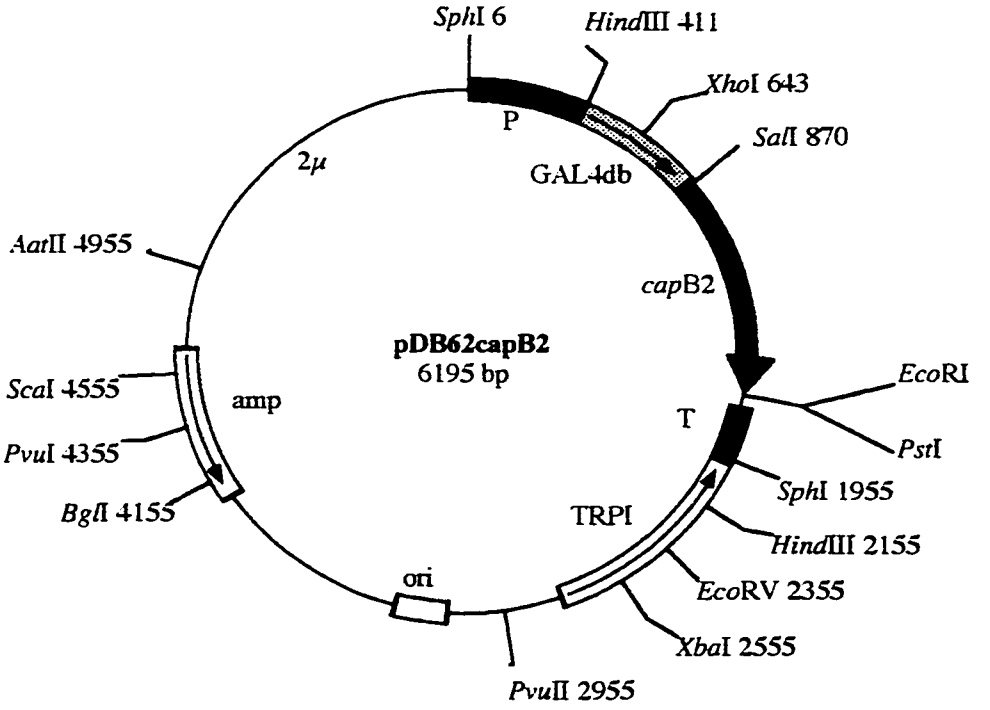


Figure 5. Plasmid pDB62capB2. A .855 Kb *SalI-EcoRI* fragment comprising the coding sequence of *capB2* was cloned into the *SalI-EcoRI* sites of the bait plasmid pDB62 [J. Gisser, personal communication].

2.3 Yeast transformation

2.3.1: Large scale yeast transformation for library screening

The method used for large scale library screenings was the lithium acetate protocol developed by Ito *et al.* (1983) and modified by Schiestl and Gietz (1989), Hill *et al.* (1991) and Gietz *et al.* (1992). Cells from an overnight culture, harbouring the bait plasmid, were inoculated into 50 ml of synthetic dropout (SD) medium lacking tryptophan (-W) (Table II). This culture was grown overnight at 30°C, shaking at 300 rpm. The next morning enough culture was transfered to 300 ml warm SD (-W) medium to produce an OD_{600} = 0.2 (approximately 2-8 x 10^6 cells/ml). Incubation at 30°C with shaking was resumed. After reaching an OD_{600} of 0.5-0.7 the cells were washed by centrifugation at 1000 x g for 5 min at room temperature once with 150 ml of sterile water, once with 10 ml of 1 x TE/0.1 M lithium acetate solution, and then resuspended in 8 ml of the TE/LiAc solution. The resuspended cells and 60 ml of a 40% PEG/0.1 M LiAc solution were added to a 500-ml flask containing 400 μg of library DNA and 20 mg of single-stranded carrier DNA. The mixture was incubated at 30°C with shaking at 200 rpm for 30 min. Dimethyl sulfoxide was added to 10%. The cells were heat shocked for 15 min at 42°C, chilled on ice, and harvested by centrifugation. The cell pellet was resuspended in 10 ml of TE buffer and plated (200 μl) on 150 mm agar plates containing SD (-L -H -W) medium and 3-amino-1,2,4-triazole (3AT) at a concentration of 20 mM. The plates were incubated for 4-10 days at 30°C. The omission of tryptophan and leucine selected for the two plasmids, 3AT was added to inhibit the basal level of HIS3 activity from the reporter gene. To estimate transformation efficiency three dilutions of the transformation mixture were plated onto SD (-L -W) medium agar plates.

2.3.2 Other yeast transformations

Small and medium scale yeast transformations were performed using the protocol outlined above, scaled down to the appropriate volumes.

Table II: Growth media

ORGANISM	GROWTH MEDIUM	MEDIUM COMPOSITION
E. coli	L-Broth	10% Bacto-tryptone, 5% Bacto-yeast extract, and 10% NaCl adjusted to pH 7.
E. coli	SOC	2% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 0.25 mM KCl, 0.01 M $MgCl_2$, and 20 mM glucose.
E. coli	M9	0.2 % glucose, 1 mM MgSO4, 100 μM CaCl2, 20% M9 salts (30 g/l Na2HPO4, 15 g/l KH2PO4, 5 g/l NH_4Cl, 2.5 g/l NaCl).
S. cerevisiae	YPD	1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose
S. cerevisiae	SD (synthetic dropout)	1.3 g/l dropout powder to provide amino acids[a], 1.7 g/l yeast nitrogen base (lacking ammonium sulfate and amino acids), and 5 g/l ammonium sulfate.

[a] Prepared as described in Ausubel *et al.* (1989).

2.4 β-galactosidase filter assays

The filter assays for β-galactosidase activity were performed according to the protocol of Bartel *et al.* (1993a). A sterile 12.5 cm filter (Whatman No. 1) was placed onto a transformation plate to adsorb the yeast colonies. The filter was lifted and transferred to a pool of liquid nitrogen for 5 sec. The thawed filter was layered, colony side up, over another 12.5 cm filter presoaked with 4 ml of a solution containing 100 ml of Z-buffer (16.1 g/l $Na_2HPO_4.7H_2O$, 5.5 g/l $NaH_2PO_4.H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4.7H_2O$, pH 7), 0.27 ml of β-mercaptoethanol, and 1.67 ml of X-gal (20 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside in N,N-dimethylformamide). Assays using standard 100 mm petri dishes required 1.5 ml of the X-gal/Z-buffer solution. Colonies that turned blue within 24 hours were picked from the original plates, re-streaked on to SD (-L, -W, -H) medium, and assayed again for β-galactosidase activity.

To test for false positives colonies were patched, along with positive and negative controls, on Whatman No. 1 filters layered directly onto selective agar medium. The patches were grown overnight, and the filters assayed as described above.

2.5 Isolation of library plasmids

2.5.1 Plasmid extraction from yeast colonies

Plasmid DNA was extracted from yeast colonies expressing β-galactosidase using the following modification of a protocol posted by james_milligan@merck.com (Newsgroup: bionet.molbio.methds-reagnts, Article 32109, Nov. 2, 1995). A single yeast colony was picked directly from an agar plate, resuspended in 10 μl of sterile water, and mixed with 100 μl of lysis buffer (2.5 M LiCl, 50 mM Tris-HCl pH 8.0, 4% Triton X-100 and 62.5 mM EDTA). An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to this mixture along with 0.2 g of glass beads (.45-.5 mm). The mixture was vortexed for 2 min and centrifuged in a microfuge (12,000 x g) for 1 min. The aqueous

phase was transferred to a fresh microfuge tube and precipitated with 2.5 volumes of 95% ethanol at -20°C for 20 min. After centrifugation for 15 min at 12,000 x g the pellet was washed with cold 70% ethanol, dried, and resuspended in 20 μl of sterile water.

2.5.2 Recovery of library plasmid.

To recover the library plasmid an *E. coli* strain with a *LeuB* mutation (MC1066, Table I) was transformed with the plasmid DNA isolated from the yeast cells. Bacterial cells were transformed using the $CaCl_2$ method described in subsection 2.8.1.

Transformants were replica patched onto M9 (-L, +W, +ura) and M9 (+L, -W, +ura) plates and incubated overnight at 37°C. Plasmid DNA was extracted from +L -W colonies using the alkaline lysis miniprep protocol described in Maniatis *et al.* (1989).

2.6 DNA sequencing

DNA sequencing was performed using a Pharmacia "T7-Sequencing Kit" according to the manufacturer's protocol. The templates used were double stranded plasmid DNA prepared using a modified mini alkaline lysis/PEG precipitation procedure described in Section 2.7. Polyacrylamide gels (6%) with urea were prepared according to protocols in Maniatis *et al.* (1989)

Primers LibrA and LibrB were designed to sequence inserts cloned into the two hybrid vector pGAD424 (Figure 2). Restriction fragments subcloned into the plasmid Bluescript KS+ (Stratagene) were sequenced using the primers Reverse, Universal, T3, and T7 (all from Stratagene). Additional primers were designed to extend the sequencing of clones 101 and 302. Table III shows the sequence and annealing sites of all primers designed for the work reported in this thesis.

2.7 Isolation of plasmid DNA for sequencing

E. coli strains XL1-Blue or DH10B were transformed with library plasmid DNA isolated from strain MC1066. Plasmid DNA, from a 3-ml overnight culture, was isolated

Table III: Primers designed for DNA sequencing and PCR

PRIMER	SEQUENCE	ANNEALING SITE	USE
LibrA	5'-GCGTTTGGAATCACTACAGG-3'	Bases -87 to -106 upstream from the EcoRI cloning site. Coding strand of plasmid pGAD424 .	Sequencing and PCR
LibrB	5'-GCACGATGCACAGTTGAAGT-3'	Bases 41 to 60 downstream from the PstI cloning site. Non-coding strand of plasmid pGAD424 .	Sequencing and PCR
Repl	5'-CAGAGTTTGGAATACCG-3'	Bases 958 to 974. Non-coding strand of cDNA clone 101.	Sequencing
Topo1	5'-CTATCATCATGGTGAGC-3'	Bases 265 to 280. Coding strand of cDNA clone 302/403.	Sequencing and PCR
Topo2	5'-CGAGTACCAAACTGACCACTTGG-3'	Bases 353 to 375. Non-coding strand of cDNA clone302/403.	Sequencing
Topo3	5'-CATTCCAAGAATCAAATACTG-3'	Bases 840 to 860. Coding strand of cDNA clone 302/403.	Sequencing
Topo4	5'-CTTCCATTTGTTCCAATTG-3'	Bases 899 to 917. Non-coding strand of cDNA clone302/403.	Sequencing
Topo5	5'-CGTAGAGAATACCTCTTG-3'	Bases 1076 to 1093. Coding strand of cDNA clone 302/403.	Sequencing
Topo6	5'-CTCTGGTGGATGAGTTCC-3'	Bases 1244 to1261. Non-coding strand of cDNA clone302/403.	Sequencing

using the alkaline lysis miniprep technique (Maniatis *et al.*, 1989). After the final precipitation step the air-dried DNA pellet was resuspended in 50μl of TE + 10 μg/ml RNase.

Thirty microlitres of a 20% PEG 8000/2.5 M NaCl solution were added to the resuspended plasmid and the mixture was incubated on ice for 1 hour. Plasmid DNA was precipitated at 12,000 x g in a microcentrifuge for 20 min at room temperature. The DNA pellet was rinsed with 500 μl of 70% ethanol, air dried, and resuspended in 50 μl of TE. Sequencing reactions were prepared using 10 μl of the plasmid preparation.

2.8 Genomic DNA blot analysis

Genomic DNA from *D. discoideum* strain AX2 was digested with various restriction enzymes according to manufacturer's recommendations (Promega or Gibco/BRL). One microgram of each digest was fractionated by electrophoresis through a 0.9% agarose gel, and transfered to Nytran membranes (Schleicher & Schuell) according to the manufacturer's protocol. DNA was cross-linked to the membrane by UV irradiation.

The probes used were prepared using a "Random Primers DNA Labelling System" from BRL Life Technologies Inc. according to the product's protocol. The membranes were pre-hybridized in a solution composed of 5 x Denhardt's, 6 x SSC, 50% formamide, 1.0% SDS, and 200 μg/ml of denatured salmon sperm DNA for 2 hours at 42°C. The probe was added and hybridization proceeded for 20 hours at 42°C.

The membranes were washed four times, 15 min each, twice at room temperature and twice at 65°C. Washing solution was 2 x SSC/0.1% SDS for low stringency washes, and 0.4 x SSC/0.1% SDS for moderate stringency washes. Blots were exposed to Kodak X-Omat films at -70°C with intensifying screens.

2.9 RNA blot analysis

Total cellular RNA was size-fractionated by electrophoresis on a 1.4 % agarose gel as described [Fourney *et al.*, 1988]. Equal loading of the lanes was confirmed by

visualization and photography in a UV-light trans-illuminator. RNA was transferred to Nytran membranes (Schleicher & Schuell) according to the manufacturer's protocol, and cross-linked to the membrane by UV irradiation.

The hybridization procedures were the same as those used for genomic DNA blot analysis described above except for a hybridization temperature of 37°C. Hybridization bands were visualized using the Phosphoimaging Analysis System (Bio-Rad Laboratories).

2.10 Bacterial transformation

2.10.1 $CaCl_2$ transformation

Cells from a saturated overnight culture were inoculated into L-broth (inoculum volume 1/100 of total) and grown at 37°C until OD_{550} was 0.2 to 0.25. The culture was cooled on ice for 10 min and the cells were harvested by centrifugation at 4000 x g for 5 min at 4°C. After resuspension in 15 ml of ice cold 50 mM $CaCl_2$, the cells were incubated on ice for 30 min, centrifuged again, and resuspended in 3 ml of ice-cold 50 mM $CaCl_2$.

A mixture of 200 μl of the above competent cells and 10 μl of a yeast fast mini-prep (section 2.4.1) was heat shocked at 42°C for 1.5 min, and cooled on ice for a few seconds. After adding 800 μl of SOC medium (Table II), the mixture was incubated at 37°C with shaking for 30 min. Ampicillin was added to a concentration of 100 μg/ml, and the cells were incubated as before for another hour to allow expression of the selection gene (LEU2 in the library plasmid). Cells were harvested by centrifugation, resuspended in 200 μl of M9 medium, spread on M9 agar supplemented with appropriate amino acids and nucleotides, and incubated until colonies appeared (36-48 hours).

2.10.2 Transformation by electroporation

High voltage electroporation was the method of choice when high transformation efficiency was required. Electrocompetent cells were prepared by inoculating 1 liter of half-salt L-broth with 1/100 of a fresh overnight culture. After reaching an OD_{600} of 0.5-0.6 the cells were chilled on ice for 15 min, and harvested by centrifugation at 4000 x *g* at 4°C. The cells were washed by centrifugation once with 1 liter of cold water, once with

0.5 liter of cold water, and once in 20 ml 10% glycerol. After the last wash the pellet was resuspended in 10% glycerol to a final volume of 2 ml, and frozen on dry ice in 50 μl aliquots.

Electrotransformation was performed using a BioRad Gene Pulser^{TM}. In a cold 0.1-cm electroporation cuvette, 20 μl of competent cells were mixed with 1 μl of plasmid DNA (1-2 ng). The Gene Pulser was set to the 25μF capacitor, 1.25 kV, and the Pulse Controller unit to 200 Ω. The cell mixture was pulsed once, 1 ml of SOC was added immediately to the cuvette, and the cell suspension was incubated in a test tube at 37°C for 30 min. Appropriate aliquots were plated on selective medium.

2.11 Other procedures

2.11.1 Polymerase chain reaction

PCR amplifications were carried out in a Techne PHC-2 thermal cycler. Reaction components were 2.5 units of Taq polymerase (Gibco/BRL) and its corresponding buffer, 200 μM dNTPs, 300 nM of each primer, 20 ng of template DNA, and water to a final volume of 100 μl.

Library cDNA inserts in plasmid pGAD424 (Figure 2) were amplified using the primers LibrA and LibrB (Table III). Twenty five cycles were programmed with the following conditions: denaturation at 95°C for 30 seconds, annealing at 45°C for 30 seconds, and polymerization at 72°C for 1.5 min.

2.11.2 Preparation of single stranded carrier DNA

Lyophilized salmon sperm DNA (Sigma D1626) was rehydrated in water to a concentration of 10 mg/ml, and sonicated to produce fragments of 0.5-5 Kb long. The solution was filter sterilized, boiled in a water bath for 10 minutes, immediately chilled on ice, and stored at -20°C in 1-ml aliquots.

CHAPTER 3: RESULTS

3.1 Screening for Annexin VII interacting clones

A *Dictyostelium discoideum* cDNA library constructed in the two-hybrid system vector pGAD424 (Figure 2) was screened with the bait construct pDB62nxnA (Figure 3). Annexin VII is the product of the *nxnA* gene and a member of a family of proteins known to bind phospholipid membranes in a Ca^{2+}-dependent manner. The biological function of these proteins is not known. Three medium-scale screenings (~10^5 colonies per transformation) and four large-scale screenings (10^6-10^7 colonies per transformation) with pDB62nxnA yielded 25 *lacZ*+ colonies. These clones turned out to be false positives when streaked for single colonies and assayed for β-galactosidase expression a second time.

We estimated that more than 12 million colonies were screened with annexin VII with negative results. Considering that the library has 2 million independent clones, we believed that any interactions between annexin VII and other proteins could not be detected using this plasmid and library combination in the two-hybrid system.

3.2 Screening for p34 and p31 interacting clones

The *capB* gene generates two transcripts by alternative splicing of a retained intron [Bain *et al.*, 1991]. These transcripts encode two polypeptides 34,000 Da and 31,000 Da in molecular mass that are referred to as p34 and p31.

I screened the library with bait plasmids pDB62capB1 (Figure 4) and pDB62capB2 (Figure 5) which express proteins p34 and p31 respectively as GAL4 DNA-binding domain hybrids. Two medium-scale and one large-scale library screenings using pDB62capB1 as bait yielded 33 *lacZ*+ colonies from screening over 10^7 transformants (Table IV). Two large-scale screenings using pDB62capB2 yielded three *lacZ*+ colonies from a total of approximately 2 x 10^7 transformants (Table IV). Single colonies were obtained from the

Table IV: *Dictyostelium discoideum* cDNA library screenings using the two-hybrid system

BAIT PLASMID	NUMBER OF COLONIES SCREENED	NUMBER OF COLONIES EXPRESSING *lacZ*	CLONE NUMBER
pDB62nxnA	>1.2 x 10^7 [a]	25	-
pDB62capB1	6.0 x 10^5	2	101-102
pDB62capB1	n/d	2	201-202
pDB62capB1	1.0 x 10^7	29	301-329
pDB62capB2	9.0 x 10^6	2	401-402
pDB62capB2	1.0 x 10^7	1	403

[a] Total of seven independent library screenings.

putative positive and these were re-tested for β-galactosidase expression. I extracted and analyzed library plasmid DNA from colonies that tested positive for *lacZ* expression.

3.3 Analysis of putative positives

3.3.1 Restriction digest of cDNA clones extracted from colonies expressing *lacZ*

Extraction of plasmid DNA from yeast colonies expressing *lacZ* and isolation of the library plasmids harbouring the cDNA inserts was performed as described in Section 2.4.

The library had 2 x 10^6 independent clones and considerable redundancy was expected in a screen of over 3 x 10^7 transformants. Restriction digest analysis was used to classify the putative positives into groups of clones that appeared to be copies of the same transcript.

The plasmids were cut with *Hin*dIII, an enzyme that digested sites flanking the multiple cloning site of pGAD424 (Figure 2). In addition, the plasmids were analyzed with *Bgl*II, *Bam*HI, *Eco*RV, *Eco*RI, *Cla*I, and *Xba*I. The clones were classified by restriction analysis and insert-size into seven groups (Table V).

3.3.2 Preliminary DNA-sequencing analysis

Sequencing information from the 5' end of the cDNA clones was obtained using the primer LibrA. LibrA is one of a pair of primers designed to sequence or PCR-amplify inserts cloned into the vector pGAD424 (Figure 2 and Table III).

Analysis of the 5' end sequences of the cDNA clones identified six different cDNAs. As we expected, one of the genes identified was *capB* itself. This gene codes for the bait proteins used in the library screenings. The gene products of *capB* (p34 and p31) have been shown to interact with each other and with themselves in the two-hybrid system [J. Gisser, personal communication]. Thirteen *capB* cDNA clones were isolated, they were classified in Tables V and VI as Group C. We obtained 5'-end sequencing information from five of these clones. The remaining 8 clones in Group C were classified based on the size of the inserts and on the presence of a *Bam*HI site approximately 0.7 Kb from the 3' end of the cDNA.

TABLE V: CLASSIFICATION OF PUTATIVE POSITIVE cDNA CLONES BY SIZE AND RESTRICTION SITES

GROUP	CLONES	SIZE (Kb)[a]	RESTRICTION SITES[b]					
			*Bgl*II	*Bam*HI	*Eco*RV	*Eco*RI	*Cla*I	*Xba*I
A	302, 403	1.6	-	-	1.0 Kb	-	1.0 Kb	1.2 Kb
B	101, 303, 321	1.0-1.2	-	-	1.1 Kb[c]	-	0.7 Kb	0.5 Kb
C	301, 304, 306, 307, 309, 312, 317, 318, 319, 325, 328, 329, 401	0.9 - 1.0	-	0.7 Kb	-	-	-	-
D	102 ,308, 311, 324	1.0-1.1	0.3 Kb	-	-	1.1 Kb	-	-
E	305, 313, 315	1.0	-	-	-	-	-	-
F	201, 202, 310, 314, 320, 322, 402	0.5-0.6	-	-	-	-	-	-
G	316, 323, 326, 327	0.3	-	-	0.3 Kb	-	-	-

[a] Approximate sizes based on restriction analysis data.

[b] The approximate distance of the restriction site from the 3' end of the clone is indicated.

[c] Site was not present in cDNA clone 321, the 5' end of this clone starts 21 bases downstream from the *Eco*RV site.

Table VI: Grouping of putative positive cDNA clones based on 5'-end sequencing information

GROUP	CLONES	COMMENTS
A	302, 403	Identical copies of the same extension product, 1.6 Kb long.
B	101, 303, 321	Different extension products of the same gene 1.1-1.2 Kb long.
C	301, **304**, **306**, **307**, **309**, **312**, 317, 318, **319**, 325, **328**, 329, **401**	Identified as *capB*. Clones 301 and 325 were different extension products of *capB*1. Clones 317, 318, and 329 were the same extension product of *capB*, these cDNAs stopped short of the 5' region that differentiates *capB*1 from *capB*2. Clones in bold face were not sequenced, they were included in this group based on the presence of a *Bam*HI site located 0.7 Kb from the 3' end of the cDNA insert.
D	102, 308, 311, 324	Different extension products of the same gene 1.0-1.1 Kb long.
E	305, 313, 315	Identified as *capA* approximately 1 Kb long. Identical copies of the same extension product. Sequencing data from both ends of these clones suggest that they encode the full sequence minus the first eight bases of the *capA* product CABP1B.
F	201, 202, 310, 314, 320, 322, 402	Identified as *capA* approximately 0.56 Kb long. Identical copies of the same extension product. Encode amino acids 10-196 of the *capA* product CABP1B.
G	316, 323, 326, 327	Identical copies of the same extension product, 0.3 Kb long.

The other gene identified by 5'-end sequencing was *capA* [Grant and Tsang, 1990]. As described in Chapter 1, *capA* encodes a cyclic AMP-binding protein with extensive similarity to *capB* [Bain *et al.*, 1991]. This gene was isolated ten times. The three *capA* clones classified as group F in Table VI were approximately 1 kb in length. Based on sequencing data obtained from both ends of the clones it appears that they encode the full sequence, minus the first three residues, of the *capA* product CABP1B. Seven *capA* clones were .56 Kb long and encoded residues 10-196 of CABP1B (296 residues long), they comprised Group F in Table VI. The .56 Kb clones seem to be the product of a recombination event since they are missing the 3' end of the coding sequence of *capA*.

In summary, the results obtained from partial sequence analysis complemented the restriction analysis data in the classification of the interacting clones. Preliminary sequencing revealed the identity of 23 of the 36 putative positive clones. Clones in Group C (Tables V and VI) were identified as *capB* and clones in Groups E and F were identified as *capA*. The remaining thirteen cDNAs, classified into groups A, B, D, and G (Tables V and VI), required further sequencing in order to obtain enough information to perform data-base searches.

3.4 Screening of cDNA clones for false positives

Representatives of each of the gene groups identified above were tested in order to eliminate clones that turned on the reporter genes through non-specific interactions. In the two-hybrid system false positives generally fall into two classes. The first class of false positives is believed to arise through the interaction of the activation domain hybrid with the DNA sequence of the promoter. The second class of false positives activate the reporter gene non-specifically when they are co-expressed with any DNA-binding domain hybrid. [Bartel *et al.*, 1993b].

In the first test for false positives a representative cDNA construct from groups A, B, D, E, and G (Table VI) was co-transformed with the bait plasmid pDB62capB1 into strain HF7c. It should be noted that clones from group C, identified as *capB*, were not

tested further. The interactions of the bait proteins with each other and with themselves in the two-hybrid system had been previously demonstrated [J. Gisser, personal communication]. As reported in Section 3.3.2 clones in groups E and F were identified as *capA*; clones from group F were missing the 3' end whereas clones from group E seemed to contain most of the coding sequence of the gene, therefore a clone from group E was chosen to represent both groups.

Several yeast strains are available for library screening and testing. Some of these strains carry different promoter/reporter constructs. For example the *lacZ* reporter in strain Y153 is controlled by the GAL1 promoter, while the *lacZ* gene in strain HF7c is under the control of the CYC1 promoter that has had its own activating sequence replaced with three copies of the GAL4 17-mer consensus binding sites. Since the library screening was conducted in strain Y153, testing the putative positives in strain HF7c would allow us to eliminate a class of false positives that arise through spurious interactions between the transcriptional activation hybrid and the promoter region of the reporter. The putative positives were individually cotransformed with the bait plasmid pDB62capB1 into strain HF7c.

Each set of transformants was grown on two different types of selective agar plates: SD (-H-L-W) and SD (-L-W). The absence of leucine and tryptophan in the medium selected for the two plasmids while histidine selection was for the HIS3 reporter present in the strain. Transformants were patched onto filters, along with positive and negative controls, and assayed for β-galactosidase expression.

As shown in Table VII, four of the five clones tested expressed *lacZ* and grew on selective medium lacking histidine. Clone 308 from group D failed to grow under histidine selection and tested negative for β-galactosidase expression. In consequence clones from group D were scored as false positives and were not examined further.

Table VII: First test to eliminate false positives.

Interaction of putative positives with the bait pDB62capB1 in yeast strain HF7c

GROUP	CLONE NUMBER	GROWTH ON SD (-H-L-W)[a]	β-GAL FILTER ASSAY
A	302	+	+
B	101	+	+
D	308	-	-
E	305	+	+
G	316	+	+

[a] Synthetic dropout medium lacking histidine, leucine and tryptophan.

The sequence of clone 316 from group G was obtained and data-base searches did not reveal similarity with any known sequences. For this reason, this clone was not included in any further tests.

For the second test clones 101, 305 and 302 were independently co-transformed with three different plasmids in strain HF7c. Co-transformation with pDB62capB1 was designed to confirm the bait-prey interactions and as a positive control for the assay. Co-transformations with pGBT9/rat somatostatin and with pDB62 were performed to test the clones for non-specific interactions. Also, each of the putative positives was singly transformed into HF7c to show that they did not activate the reporter gene on their own. Transformants were assayed for β-galactosidase expression as before and the results of these assays are summarized in Table VIII. The three clones tested did not interact with the DNA-binding domain (DB) vector alone or with the DB/rat somatostatin hybrid. Also there was no activation of the reporter gene when the cDNA clones were transformed individually into the strain. However, all cDNA constructs tested showed interaction with the p34 hybrid. These results imply that the three hybrid proteins encoded by the cDNAs tested in this assay interacted specifically with the bait protein p34.

The last round of transformations to detect false positives was performed in Y153, the original strain used for library screening. The genotype of Y153 shows that it harbours two copies of the GAL1-*lacZ* reporter gene (Table I). Expression of β-galactosidase from two copies of the reporter results in the development of a more intense blue color when filter assays are performed. The heightened color intensity of Y153 colonies expressing *lacZ* enhances the difference between positive and negative interactions.

Clones 101, 302, and 305 were individually co-expressed in strain Y153 with seven different DNA-binding domain hybrids as well as with the DNA-binding domain vector. This last test was designed to confirm the results of previous assays and to obtain more interaction data between the cDNA clones and a variety of DNA-binding domain hybrids. Two of the DNA-binding domain hybrids were the bait proteins and served as positive

Table VIII: Second test to eliminate false positives.

β-galactosidase filter assays of *S. cerevisiae* HF7c colonies cotransformed with putative positives and DNA-binding domain hybrids

DNA-BINDING DOMAIN HYBRIDS	PUTATIVE POSITIVES IN pGAD424		
	101	302	305
pDB62capB1	+	+	+
pGBT9/rat somatostatin	-	-	-
pDB62	-	-	n/d
None	-	-	-

n/d - not done

controls for the assay. The hybrid carrying the rat somatostatin gene and plasmid pDB62 were included as controls for non-specific interactions. The other four DNA-binding domain constructs carried the proteins CABP1A, CABP1B, annexin VII, and p24. In addition, the three cDNA constructs were singly transformed into the strain.

The results of the last test for false positives are summarized in Table IX. Clone 101 was found to interact with CABP1A and CABP1B in addition to its interactions with the p34 and p31 bait constructs. These interactions were also confirmed in HF7c (data not shown).

As expected from previous data [J. Gisser, personal communication] clone 305 (CABP1B) interacted with itself as well as with CABP1A, the larger gene product of *capA*. Clone 302 interacted with p34 and p31 but not with CABP1A or CABP1B suggesting that the interactions are highly specific.

3.5 DNA sequencing of cDNA clone 316

Clones 316, 323, 326, and 327 were identical copies of the same extension product. Both strands of cDNA clone 316 were sequenced in full. Its 303-nucleotide sequence is reported in Figure 6. A distinguishing feature of this sequence is that 79.9% of its nucleotides are A or T. As mentioned in the previous section a search of protein data banks based on the three reading frames of both strands did not reveal similarity with known sequences.

3.6 Analysis of cDNA clone 101

3.6.1 DNA sequencing of clone 101

The product of clone 101 was shown above to interact with both the products of *capA* and *capB*. Clones 101, 303, and 321 were isolated independently and their sequences are identical except that clone 101 is longer in the 5' end than 303 and 321 by 33 and 66 nucleotides respectively. Clone 101 was sequenced in its full length although not in both strands. Figure 7 illustrates the sequencing strategy used and the restriction map of these clones.

Table IX: Third test to eliminate false positives.

β-galactosidase filter assays of *S. cerevisiae* Y153 colonies cotransformed with putative positives and DNA-binding domain hybrids

DNA-BINDING DOMAIN HYBRIDS	PUTATIVE POSITIVES IN pGAD 424		
	101	302	305
pDB62capB1	+	+	+
pDB62capB2	+	+	n/d
pGBT9/rat somatostatin	-	-	-
pDB62capA1	+	-	+
PDB62capA2	+	-	+
PDB62nxnA	-	-	-
PDB62/p24	-	-	-
pDB62	-	-	-
None	-	-	-

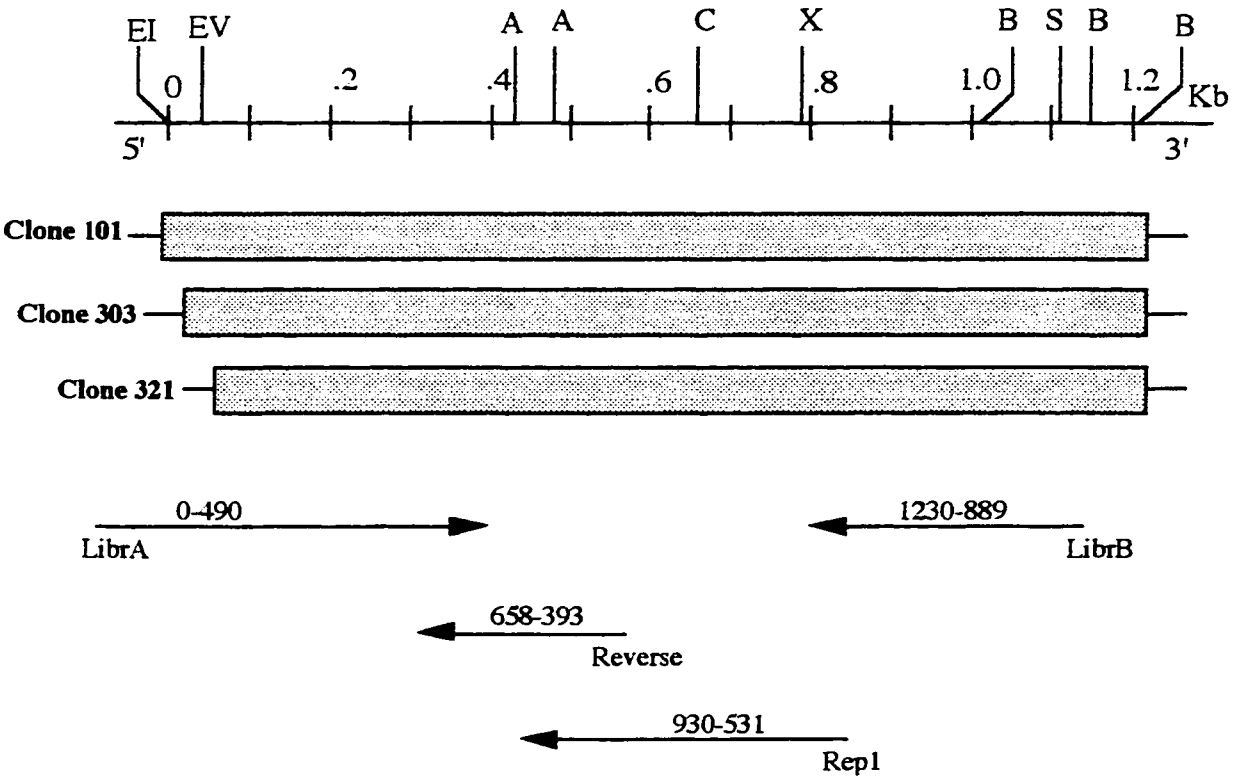
n/d - not done

Figure 6. Nucleotide sequence of cDNA clone 316. Nucleotide numbers are indicated on the left. The sequence composition is as follows: 136 A (44.9%); 34 C (11.2%); 27 G (8.9%); 106 T (35%).

```
  1 TGATTTTGAAAGTGCAGAGATATCAATCCAACAGTTACAATCTTATTTGG
 51 TAAATCAATACAACCAATATCATAATTTTTATAGTCAAAATCAACAAATG
101 ATTCAATTGCAACAACTATATGAAATTTCAAAAAAAAATTAATTTATTAAA
151 AGTTTATATCGAGTTAAGAAAAGGAAATACAGAAAATGCATTAAATATAA
201 TTTTAAGTGGAAGCCTTCTCCAAATTAATTAATTGGTTTTATAAATTTTT
251 TTAAAAAAATAAAATAATTCTTAATTCCTTTTTTTTTCCTTACAAAAAAA
301 AAA
```

Figure 7. Restriction map and sequencing strategy for cDNA clone 101.

Each arrow in the figure represents sequencing done using a particular primer. The name of the primer used is indicated below the arrow, the bases sequenced with the primer are indicated above the arrow. Sequencing of the 5' and 3' ends was done using the primers LibrA and LibrB respectively. These primers were designed to sequence or to PCR-amplify inserts cloned into the two hybrid vector pGAD424. The restriction fragment *Eco*RI/*Cla*I was subcloned into the plasmid vector Bluescript KS+® (Stratagene). Sequencing of the 3' end of the subcloned fragment was performed using the Reverse primer (Stratagene). Primer Rep1 was designed from sequences specific for this clone as determined by DNA sequencing. Clones 101, 321, and 303 are depicted as shaded horizontal bars. The restriction enzymes indicated in the map are: EI, *Eco*RI; EV, *Eco*RV; A, *Apa*LI; C, *Cla*I; X, *Xba*I; B, *Bgl*II; S, *Sac*I. The 5' end of the cDNA clone was joined to the GAL4 transcriptional activation domain of pGAD424 at the *Eco*RI cloning site. The *Bgl*II site is located six base pairs downstream from the 3' cloning site (*Xho*I/*Sal*I).


EI
EV
A
A
C
X
B
S
B
B
0
.2
.4
.6
.8
1.0
1.2
Kb
5'
3'
Clone 101
Clone 303
Clone 321
0-490
LibrA
1230-889
LibrB
658-393
Reverse
930-531
Rep1

Translation of the 5'-half of clone 101 revealed that its predicted amino acid sequence coded for a region rich in the amino acids glycine, tyrosine, proline, and glutamine. In addition, we found five different amino acid sequences repeated throughout this region. There are two repeats of each of the sequences QAGQYGQPPP and QGYPPQ, three repeats of APPQA, four repeats of QYGQPPYG, and five repeats of QPGQYGAP. The nucleotide and deduced amino acid sequences of clone 101 are illustrated in Figure 8.

The repeated sequences in the 5' portion of this cDNA prevented the design of a specific primer within this region. To facilitate sequencing of the middle portion of the clone an *Eco*RI/*Cla*I restriction fragment was sub-cloned into the corresponding sites of plasmid Bluescript KS+® (Stratagene). The Reverse primer (Stratagene) was used to sequence 265 bases upstream from the *Cla*I restriction site. Primer Rep1 was designed to complete sequencing of the region between the *Cla*I site and the sequence read from the primer LibrB (Figure 7).

A protein data bank search using the deduced amino acid sequence of the 3'-half of cDNA 101 revealed similarity with the carboxy end of a potential protein-coding region from the genome of a cyanobacterium [Kaneko, *et al.*, 1996]. Alignment of these two sequences, presented in Figure 9, shows that the last 91 residues of the carboxy end of cDNA 101 shares a 50% identity in amino acid composition with the bacterial open reading frame (ORF). If conservative amino acid changes are taken into account the similarity of the two sequences increases to 68%.

3.6.2 RNA blot analysis of clone 101

RNA blot analysis was performed to determine the approximate size and the pattern of expression of the mRNA transcript that produced clones 101, 303, and 321. Total RNA was extracted from *D. discoideum* cells at 0, 4, 8, 12, 16, and 20 hours of development. A 4-μg aliquot of each sample was resolved by denaturing agarose gel electrophoresis, and transferred to a nylon membrane. The probe was prepared by the random priming labelling

Figure 8. Nucleotide and deduced amino acid sequences of cDNA clone 101. Nucleotide numbers are indicated on the left while amino acid numbers are indicated on the right. Repeated amino acid sequences are underlined.

```
   1 AAT ATA AAT ATG AAA CCA GGC GAA TAT AAC ATA CCA AAA GGA TAT CCT
     N   I   N   M   K   P   G   E   Y   N   I   P   K   G   Y   P  16

  49 CAA AAT TCT CAA CCT CCT TTA GGT CCT CCT CAA CCC GGC CAA TAT GGA
     Q   N   S   Q   P   P   L   G   P   P   Q   P   G   Q   Y   G  32

  97 GCA CCA CAA CAA GGA TAC CCA CCA CAA CAA GGT TAC CCA CCA CAA TAT
     A   P   Q   Q   G   Y   P   P   Q   Q   G   Y   P   P   Q   Y  48

 145 TCT CAA CAA CCT TTA GGC CCA CCA CAA CCT GGA CAA TAT GGA GCA CCA
     S   Q   Q   P   L   G   P   P   Q   P   G   Q   Y   G   A   P  64

 193 CAA CCA GGT CAA TAT GGA GCA CCA CAA CCA GGT CAA TAT GGA GCA CCA
     Q   P   G   Q   Y   G   A   P   Q   P   G   Q   Y   G   A   P  80

 240 CAA CCA GGT CAA TAT GGA GCA CCA CCA CCA CAA GCA GGA CAA TAT GGC
     Q   P   G   Q   Y   G   A   P   P   P   Q   A   G   Q   Y   G  96

 288 CAA CCA CCA CCA CAA GCA GGT CAA TAT GGT CAA CCA CCA CCA CAA CAA
     Q   P   P   P   Q   A   G   Q   Y   G   Q   P   P   P   Q   Q 112

 336 TAT AAA CCA CAA GCA GGT CAA TAT GGT CAA CCA CCA TAT GGC CAA CCA
     Y   K   P   Q   A   G   Q   Y   G   Q   P   P   Y   G   Q   P 128

 384 CCA CAA GCA GCA GGT CAA TAT GGT CAA CCA CCA TAC GGT CAA CCA CCT
     P   Q   A   A   G   Q   Y   G   Q   P   P   Y   G   Q   P   P 144

 432 TAT GGT GCA CCA CCA CAA GCA GCA CAA TAT GGC CAA CCA CCT TAT GGT
     Y   G   A   P   P   Q   A   A   Q   Y   G   Q   P   P   Y   G 160

 480 GCA CCA CCA CAA GCA GGT CAA TAC GGT CAA CCA CCA TAT GGC GCA CCA
     A   P   P   Q   A   G   Q   Y   G   Q   P   P   Y   G   A   P 176

 528 CCA CAA GCA GGT CAA TAC GGT CAA CCA CAA CCA ATG GGA TAT GGT AAA
     P   Q   A   G   Q   Y   G   Q   P   Q   P   M   G   Y   G   K 192

 576 CCA GCA CCA ACT ATG CCA GGT GTA TCA TTA GTT AAA TTC TTT GAT CAT
     P   A   P   T   M   P   G   V   S   L   V   K   F   F   D   H 208

 624 GTT AAA CAT CGT TAT GCA AGA GAT TAT ACA CTT ATT ATC GAT AAG AGT
     V   K   H   R   Y   A   R   D   Y   T   L   I   I   D   K   S 224

 672 GGT AGT ATG AGT GGT GGT TTA TGG AAA CAA TGT GAA GCA GCA GTA GCA
     G   S   M   S   G   G   L   W   K   Q   C   E   A   A   V   A 240

 720 AAA ATT GCA CCA TTT GCA TGT GGA GCA GAT CCA GAT GGT ATT AAT ATT
     K   I   A   P   F   A   C   G   A   D   P   D   G   I   N   I 256

 768 TAT TTC TTT GGA AGT CCA AGT TCT AGA CAT CCA AAA TAT GAA AAT ATT
     Y   F   F   G   S   P   S   S   R   H   P   K   Y   E   N   I 272

 816 AGA GAT GCA CAA ACA GTA ATG GGA TTA TTT GCA AGA GAA AAA CCA AGT
     R   D   A   Q   T   V   M   G   L   F   A   R   E   K   P   S 288

 864 GGT ACT ACA GAT TTA TAT GGT GTT TTA AAC CAA GCT ATT AAT GAT CAC
     G   T   T   D   L   Y   G   V   L   N   Q   A   I   N   D   H 304

 912 TTT GTA AAA GGT AAT AAA CCA GAA ACT ATT TTG GTT ATT ACT GAC GGT
     F   V   K   G   N   K   P   E   T   I   L   V   I   T   D   G 320

 960 ATT CCA AAC TCT GAA TCC GAT GTT AAA AAA TTA ATT ATA TCA ACA ACT
     I   P   N   S   E   S   D   V   K   K   L   I   I   S   T   T 336

1008 AAA AAA CTT AAT AGA GAT GAA GAT CTT TCA ATT TCA TTC ATT CAA ATT
     K   K   L   N   R   D   E   D   L   S   I   S   F   I   Q   I 352

1056 GGT AAT GAT AGA TCA GCT TCT AAA TTT TTA AAG AAA TTA GAT GAT AAT
     G   N   D   R   S   A   S   K   F   L   K   K   L   D   D   N 368

1104 TTA AAG AAG GAA GGA GCT CGT TTT GAT ATT GTC GAT GTG CAA ACA TTT
     L   K   K   E   G   A   R   F   D   I   V   D   V   Q   T   F 384

1152 GAA GAT CTT AAA CAT TTA ACA TTT GAA CAA TTG ATT GAT TTG TCA ATT
     E   D   L   K   H   L   T   F   E   Q   L   I   D   L   S   I 400

1200 GAC GAT TAG AAGAAAAAAAAAAAAAAAAA
     D   D   -
```

Figure 9. Comparison of the deduced amino acid sequence of clone 101 with a hypothetical protein encoded by a bacterial open reading frame (ORF). The ORF corresponds to a hypothetical protein from the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803.II [Kaneko, *et al.*, 1996]. This figure is based on the alignment produced by the TFASTA program [Pearson and Lipman, 1988]. Double dots indicate identical amino acids (:), single dots (.) represent conservative amino acid substitutions, and dashes (-) indicate gaps introduced by the program to achieve maximum alignment.

```
Clone 101   NINMKPGEYNIPKGYPQNSQPPLGPPQPGQYGAPQQGYPPQQGY
            PPQYSQQPLGPPQPGQYGAPQPGQYGAPQPGQYGAPQPGQYGAP
            PPQAGQYGQPPPQAGQYGQPPPQQYKPQAGQYGQPPYGQPPQAA
            GQYGQPPYGQPPYGAPPQAAQYGQPPYGAPPQAGQYGQPPYGAP
            PQAGQYGQPQPMGYGKPAPTMPGVSLVKFFDHVK

Clone 101   HRYARDYTLIIDKSGSMS-------GGLWKQCEAAVAKIAPFAC
                :::::::::::::::          . :.  ....  .:.  :
ORF         MSNQRDYTLIIDKSGSMSIIEPKFQKSRWELVQESTLALAR-KC

Clone 101   GA-DPDGINIYFFGSPSSRHPKYENIRDAQTVMGLFAREKPSGT
            .. :..::..: :   :.. ..:.:. .: .:  .:. ..: :.
ORF         DQLDANGITVYTF---SGKFRRYDNV-NASKVEQIFQENEPVGG

Clone 101   TDLYGVLNQAINDHFVK-----GNKPETILVITDGIPNSESDVK
            :.: .::..:.:. . .     . . ::::::::: ::. ..:
ORF         TNLTAVLQDALNNFLQRKKSNQAPTGETILVITDGEPNDRRSVF

Clone 101   KLIISTTKKLNRDEDLSISFIQIGNDRSASKFLKKLDDNLKKEG
            ..:: ..: :. ::.:.:::.:::::.::.:::. :::.:.. :
ORF         EIIIQASKCLDADEELAISFMQIGNDPSATKFLQALDDQLMEVG

Clone 101   ARFDIVDVQTFEDLKHLTFEQLIDLSIDD
            :.::::::. ::.....:.....  .:.:
ORF         AKFDIVDTVTFDEMEDLTLTEVLLNAIED
```

method using clone 321 as a template. The blot was hybridized and washed under low stringency conditions.

As shown in Figure 10 the probe detected a transcript approximately 1.7 Kb in length present at all times of development in variable amounts, as well as two smaller transcripts. Based on the size of the cDNA we believe that the 1.7 Kb bands correspond to the transcript for clone 101. The other bands detected by the probe are smaller than the cDNA, and most likely they are the product of cross-hybridization to the repeated sequences found in clone 101 as well as in a number of other *Dictyostelium* genes. Non-translated sequences constitute approximately 100-200 bases of *D. discoideum* transcripts. Hence, the coding sequence of the gene corresponding to clones 101, 303, and 321 was estimated to have an approximate size of 1.5-1.6 Kb.

3.7 Analysis of cDNA clone 302

3.7.1 DNA sequencing of clone 302

Clones 302 and 403, with an approximate size of 1.6 Kb, were the longest cDNAs isolated. Clone 302 was isolated using the p34 hybrid as a bait while the identical clone 403 was isolated with the p31 hybrid as a bait (Table IV). As shown in Table IX they interacted specifically with both *capB* gene products, but not with the gene products of *capA* a gene which shares extensive similarity with *capB*.

Sequencing of clone 302 from the 5' and the 3' end was performed using the primers LibrA and LibrB respectively. Restriction fragments *Eco*RI/*Cla*I, *Cla*I/*Cla*I, and *Cla*I/*Bgl*II were subcloned into the appropriate sites of plasmid Bluescript KS+® (Stratagene). The subcloned fragments were sequenced using the primers Reverse, Universal, T3, and T7 (all from Stratagene). Sequencing of the cDNA was completed using six primers designed for this purpose and described in detail in Table III. The sequencing strategy and restriction map for clone 302 are shown in Figure 11.

Protein data-base searches showed that this cDNA encoded an amino acid sequence with extensive similarity to the carboxyl end of eukaryotic DNA topoisomerase II.

Figure 10. RNA blot analysis of transcripts complementary to clone 321. A radiolabelled probe prepared by random primer extension using cDNA clone 321 as a template. Total RNA was extracted from *D. discoideum* cells at 0, 4, 8, 12, 16, and 20 hours of development and 4-μg aliquots were size-fractionated under denaturing conditions by agarose gel electrophoresis. The blot was hybridized at 37°C for 20h, washed with 2 x SSC/0.1% SDS, and visualized by the Phosphoimaging Analysis System (Bio-Rad Laboratories) The positions of molecular size markers (in Kb) are shown on the right side of the figure.

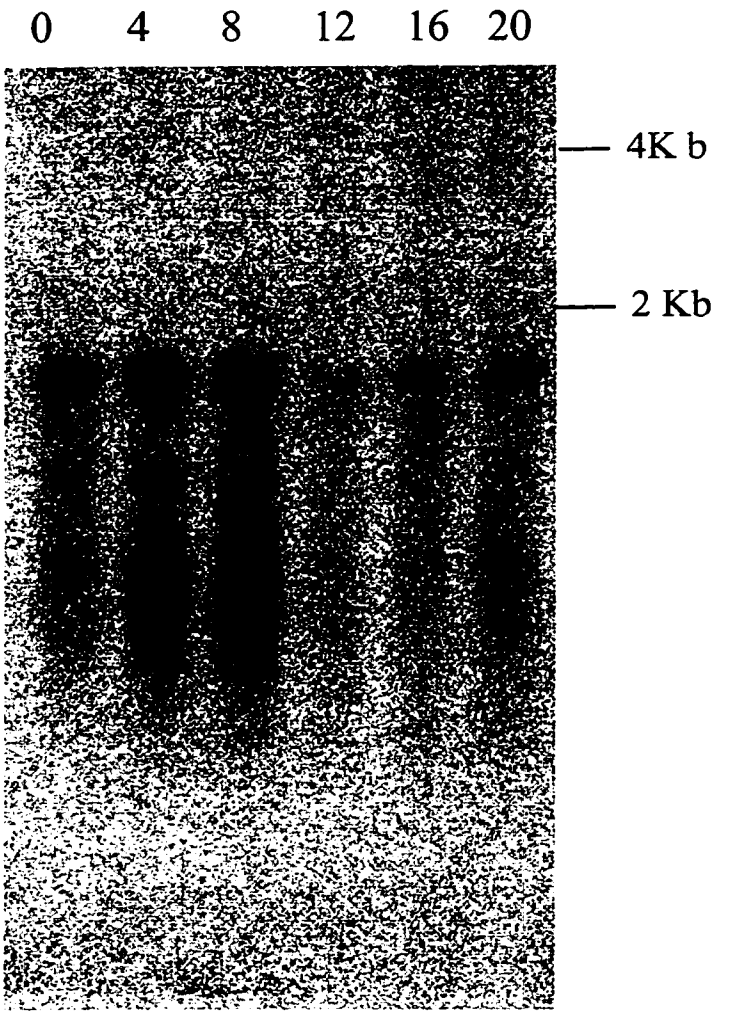
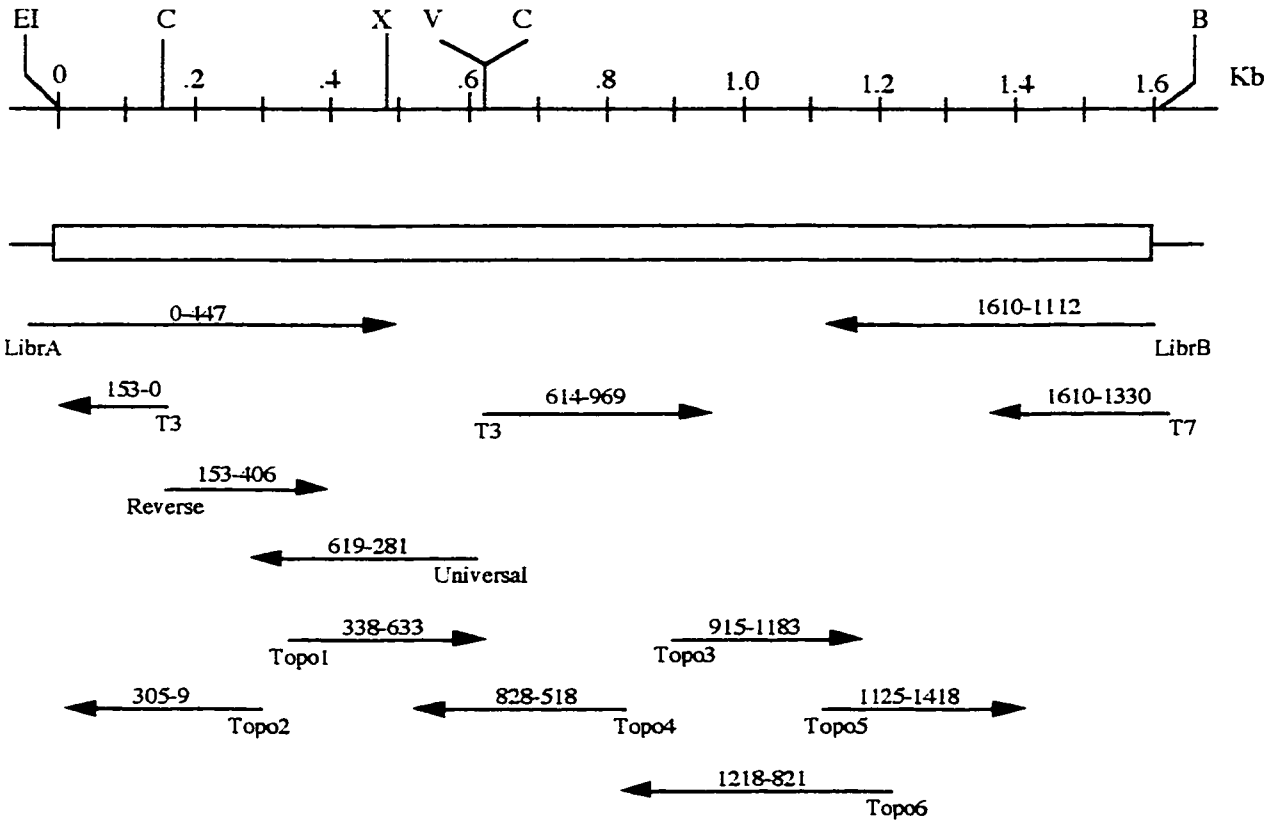

0
4
8
12
16
20
4K b
2 Kb

Figure 11. Restriction map and sequencing strategy for cDNA clone 302.

Each arrow in the figure represents sequencing done using a particular primer. The names of the primers used are indicated below the arrows, the bases sequenced with the primers are indicated above the arrows. Sequencing of the 5' and 3' ends was performed using the primers LibrA and LibrB respectively. These primers were designed to sequence or to PCR–amplify inserts cloned into the two hybrid vector pGAD424. Restrictions fragments *Eco*RI/*Cla*I, *Cla*I, and *Cla*I/*Bgl*II were subcloned respectively into the sites *Eco*RI/*Cla*I, *Cla*I and *Cla*I/*Bam*HI of the plasmid vector Bluescript KS+® (Stratagene). Sequencing of the subcloned fragments was done using the primers T3, T7, Universal and Reverse (Stratagene). Primers Topo1-6 were designed from sequences specific for this clone determined by DNA sequencing. The restriction enzymes indicated in the map are: EI, *Eco*RI; C, *Cla*I; X, *Xba*I; V, *Eco*RV; B, *Bgl*II. *Eco*RI is the 5' cloning site where the cDNA clone was joined to the GAL4 transcriptional activation domain of pGAD424. The *Bgl*II site is six base pairs downstream from the 3' cloning site (*Xho*I/*Sal*I).


EI
C
X
V
C
B
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
Kb
0-447
LibrA
1610-1112
LibrB
153-0
T3
614-969
T3
1610-1330
T7
153-406
Reverse
619-281
Universal
338-633
Topo1
915-1183
Topo3
305-9
Topo2
828-518
Topo4
1125-1418
Topo5
1218-821
Topo6

3.7.2 DNA blot analysis of clone 302

To characterize clone 302 further we performed genomic DNA blot analysis. One microgram of genomic DNA from strain AX2 was digested with various restrictions enzymes and transferred to a nylon membrane. The probe was prepared by random primer labelling using clone 302 as a template. The blot was hybridized and washed under low stringency conditions.

Figure 12A shows the pattern of hybridization for probe 302 when genomic DNA was digested with *Cla*I, *Eco*RI and *Eco*RV. The *Cla*I digest revealed four hybridization bands with approximate sizes of 4.0 Kb, 0.9 Kb, 0.8 Kb, and 0.5 Kb. The results of probing the *Eco*RI digest showed three hybridization fragments with approximate sizes of 6.6 Kb, 4.5 Kb, and 4.0 Kb. In the *Eco*RV digest, the probe detected two bands with approximate sizes of 4.0 Kb and 1.8 Kb respectively.

The January 25, 1997 posting of the electronic edition of CSM News (http://dicty.cmb.nwu.edu/dicty/newsletter/) made us aware that this gene had been isolated and characterized by a group in Japan [Komori *et al.*, 1997a]. The gene, called *topA*, encodes a type II DNA topoisomerase that was localized to the mitochondria. Figure 12B illustrates the restriction map of *top*A published by Komori *et al.* (1997a). The portion of TopA encoded by clone 302 is represented in the same figure as a solid line.

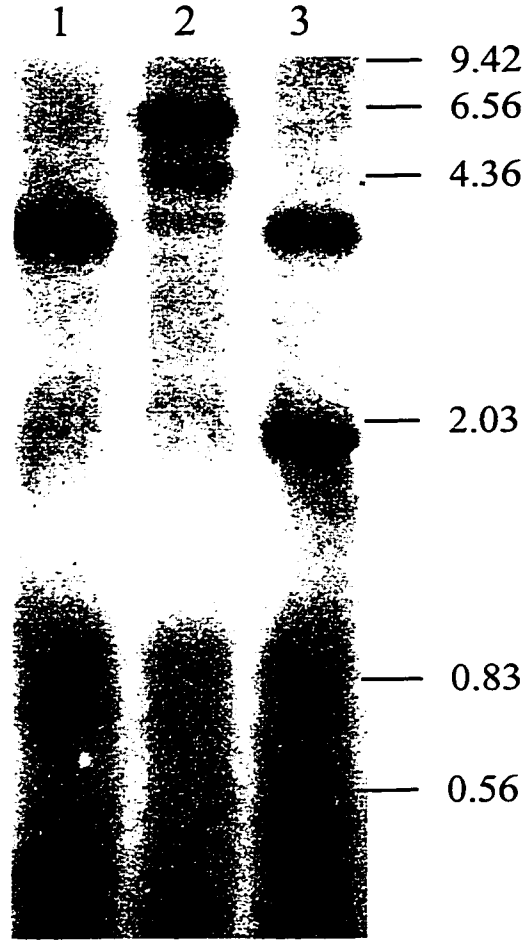
Figure 12B shows that there are two *Eco*RV sites present in the coding sequence of *topA* spaced approximately 1.8 Kb apart. The two hybridization bands detected by probe 302 in the *Eco*RV digest (lane 3 of Figure 12A) are consistent with the restriction map of *topA*.

A comparison of the *Cla*I digest probed with clone 302 (lane 1, Figure 12A) and the restriction map of *topA* in Figure 12B shows that the hybridization bands with sizes 4.0 Kb, 0.8 Kb and 0.5 Kb detected in lane 1 correspond to the restriction pattern expected for *Cla*I. The detection of a fourth hybridization band 0.9 Kb in size is likely to be the product of nonspecific hybridization due to the low stringency of the washes.

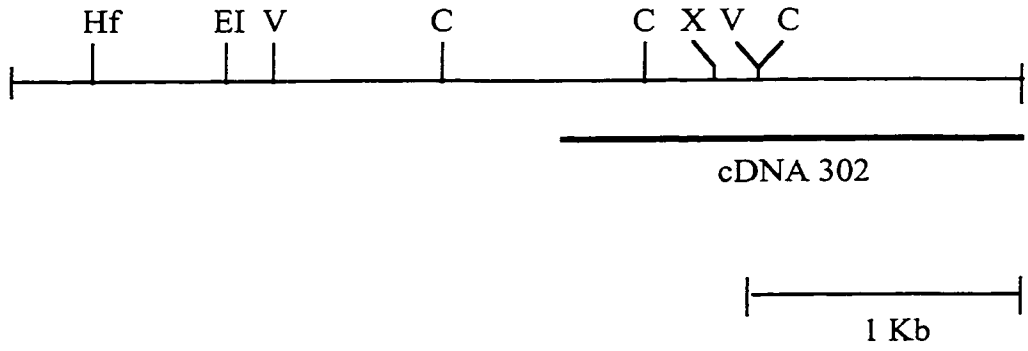
Figure 12A. DNA-blot analysis of cDNA clone 302. Genomic DNA from strain AX2 of *D. discoideum* was digested with a variety of enzymes, size-fractionated on a 0.9% agarose gel, transferred to a nylon membrane, and hybridized with a random priming labelled probe generated using clone 302 as a template. The membrane was hybridized in a buffer containing 50% formamide for 20h, and washed in 2 x SSC/0.1% SDS twice at room temperature and twice at 65°C. Restriction enzymes were: lane 1, *Cla*I; lane 2, *Eco*RI; lane 3, *Eco*RV. The positions of molecular size markers (in Kb) are shown on the right side of the figure.

Figure 12B. The restriction map of *topA*. Clone 302 corresponds to the 3' end of the gene *topA* which encodes a DNA topoisomerase II localized in the mitochondria of *D. discoideum* cells [Komori, *et al.*, 1997a]. The portion of TopA encoded by cDNA 302 is represented by a solid line. The restriction enzymes indicated in the map are: EI, *Eco*RI; V, *Eco*RV; C, *Cla*I; X, *Xba*I.

A



B



The restriction map of *topA* shows that there is an *Eco*RI site approximately 1 Kb downstream from the 5' end of the gene. In their paper Komori, *et al.* presented a DNA blot probed with a labelled oligo. An examination of their experimental data revealed that the oligo probe designed by the Komori group and cDNA probe 302 (Figure 12B) should detect the same hybridization fragment in an *Eco*RI digest. However the oligo probe [Komori *et al.*, 1997a] hybridized to a single 9.0 Kb band in an *Eco*RI digest whereas probe 302 (Figure 12A, lane 2) detected a strong 6.6 Kb band and two smaller, relatively weaker bands. The genomic DNA used in the blot presented by Komori *et al.* was extracted from a different *Dictyostelium* strain (AX3) than the one we used in our experiments (AX2). It is likely that the 6.6 Kb band detected with probe 302 corresponds to the 9.0 Kb band detected by Komori *et al.*, the size difference between the two fragments is probably due to a difference in the location of the EcoRI site in the sequence flanking the 3' end of the *top*A gene. We think that the 4.5 Kb and the 4.0 Kb bands are the products of non-specific hybridization due to the low stringency of the washes.

The hybridization and washing conditions used for our DNA blot cannot be compared with those reported by Komori *et al.* due to the difference in the type of probes used. The labelled oligo used to probe the blot presented by Komori, *et al.* annealed to a specific 21-nucleotide region around the middle of the gene whereas the labelled probe generated from clone 302 by random primer extension consisted of fragments with an average size of 200 nucleotides that annealed to random regions throughout the length of the template. It is possible that the non-specific bands detected by probe 302 in the *Cla*I and *Eco*RI digests were the result of cross-hybridization to homologous regions of a nuclear topoisomerase. Detection of non-specific hybridization fragments might be avoided by increasing the stringency of the washes.

CHAPTER 4: DISCUSSION

As reported in Chapter 1 efforts to determine the function of *capB* through genetic and immunological studies resulted in limited information. Protein-protein interactions form the basis of many essential cell processes such as signal transduction, cell growth, cell differentiation, and gene regulation. The identification of proteins that interact with a protein of interest can often shed light on the regulation and function of that protein. For these reasons we chose the two-hybrid system to screen a *D. discoideum* cDNA library to identify proteins that interacted with the gene products of *capB*.

A major advantage of using this screening method is that the genes or gene-fragments encoding the polypeptides isolated are immediately available. Furthermore the two-hybrid system is highly sensitive to weak or transient interactions that cannot be detected by other methods [Van Aelst *et al.*, 1993]. However the system is not without shortcomings. The proteins tested must be able to retain their function and fold to their native conformation in the nucleus of the yeast cell. Expression of the proteins as hybrids with the GAL4 domains may interfere with the interactions. Also, the hybrid proteins may be toxic to the yeast cell.

When screening a library false positives may arise from activation of the reporter gene due to spurious interactions between the transcriptional activation hybrid and the promoter region of the reporter gene. False positives can be identified by subjecting the cDNA clones to a series of tests such as those described in Section 3.4. Furthermore, once we have identified a protein or proteins that interact specifically with the bait hybrid the significance of these interactions should be demonstrated by showing that the proteins associate inside the host organism. Immunoprecipitation is a commonly used method of testing protein-protein interactions detected in the two-hybrid system.

I performed two-hybrid system screenings of more than 3×10^7 transformants with the bait plasmids pDB62capB1 and pDB62capB2 and isolated 36 clones expressing *lacZ*

which were classified in 7 groups representing 6 different genes. Table VI shows that analysis of the putative positives identified clones in groups E and F as *capA*2, clones in group C as *capB*, and clones in group A as *topA*. The clones in group B encoded a polypeptide that has a high degree of similarity with a bacterial open reading frame (Figure 9). In addition its amino terminus was found to be very rich in the amino acids glycine, tyrosine, proline, and glutamine. The clones in group D were scored as false positives after appropriate testing (Section 3.4 and Table VII), those in group G were believed to be an artifact caused by imperfect transcriptional control (Section 4.6).

The proteins p34/31, CABP1B, and TopA were isolated by interactions with both p34 and p31, whereas the three cDNAs corresponding to group B (Section 3.6) were isolated by interaction with p34. Subsequent tests showed that p31 also interacted with the hybrids encoded by group B clones (Table IX). The two groups of clones (D and G in table VI) identified as false positives were isolated using the p34 bait.

Table IV reveals that, although I screened an equivalent number of transformants with each of the baits, I isolated 33 clones with p34 and three clones with p31. Moreover the three clones isolated with p31 were copies of cDNAs isolated with p34. We can only speculate on the causes of this 10-fold difference in the ability of each bait to isolate interacting proteins. The sequences of p34 and p31 are identical except that amino acids 12-45 in the p34 sequence are absent from p31, hence the GYPQ region of p31 is 34 amino acids shorter than that of p34. The high proportion of residues capable of forming hydrogen-bonds in the GYPQ region makes it a possible site for protein-protein interactions. If we assume that the interactions of p34 and p31 with certain proteins are mediated through this region it may be possible that the bulk of the GAL4 DNA-binding domain fused to the N-terminus of the bait proteins could destabilize the interactions. The N-terminal GYPQ region of p31 is 37% smaller than the equivalent region of p34, hence it is possible that the steric interference of the GAL4 domain could have a more pronounced

effect on the smaller putative interacting domain. This effect could account for the striking difference in the number of clones isolated using p34 versus p31.

4.1 Thirteen interacting clones were identified as the gene products of *capB*

As mentioned in section 3.3.2 we expected to isolate clones coding for *capB* when using its gene products as bait. Of the five clones sequenced two were identified as p34, the remaining three fell short of the region spliced from the *capB* transcript to generate p31 and this prevented their identification as either *capB* product. Isolation of the *capB* clones served to increase our confidence in the results of the library screenings.

4.2 Ten interacting clones were identified as CABP1B

The gene products of *capB* were isolated by co-purification with the two subunits of CABP1 [Tsang and Tasaka 1986]. In consequence p34, p31, CABP1A and CABP1B were systematically tested for interactions in the two-hybrid system [J. Gisser, personal communication], these tests failed to reveal any interactions between the proteins encoded by *capA* and those encoded by *capB*. Moreover immunoprecipitation assays also failed to detect an interaction between these proteins [C. Bonfils, personal communication]. For these reasons the isolation of CABP1B and confirmation of its interactions with both p34 and p31 were unexpected findings. In addition, given the high degree of similarity between the *capA* subunits, it was surprising to isolate CABP1B and not CABP1A in the library screenings.

The data presently available is not sufficient to explain these contradictory results. The two-hybrid system has been known to detect protein-protein interactions that could not be detected by other techniques [Van Aelst *et al.*, 1993], hence we could use this argument to explain the lack of interaction between the products of *capA* and *capB* in immunoprecipitation assays. The conflicting results of the assays performed by J. Gisser and those presented in Section 3.4 cannot be easily explained. Furthermore a direct comparison of the two set of results might not be a valid approach. The strains used to screen the library (Y153) and to test the putative positives (HF7c) were different from the

strain used by J. Gisser in his assays (PCY3). Estojak *et al.* (1995) have shown that different two-hybrid system reporters had different thresholds for the minimum affinity required to detect interactions between a given pair of proteins. It is plausible to assume that the interactions between the gene products of *capA* and *capB* were not detected in the assays performed by J. Gisser because the affinity between these proteins is below the detection threshold of the strain used in those assays. Therefore a comparison of the results reported in Section 3.4 with those obtained by J. Gisser would require repeating all possible co-transformations between p31, p34, CABP1A, CABP1B, and the CABP1B library clone isolated in PCY3, Y153 and HF7c. In addition, the CABP1B cDNA would have to be cloned into the DNA-binding domain plasmid in order to test the interactions in both orientations.

4.3 Clone 101 codes for a novel polypeptide

Clone 101 encodes a 402-residue polypeptide that will be called p101 for the purposes of this discussion. Its sequence can be separated into two regions based on amino acid composition. More than 81% of the N-terminal-half of p101 is made up of glycine, tyrosine, proline, and glutamine (GYPQ), the remaining 19% is made up of 9% alanine, and 10% other amino acids. The nature of its N-terminal places this polypeptide in the same group as the *Dictyostelium* proteins described in section 1.1 which encode a similar GYPQ repetitive region, these proteins include the products of *capB*, *capA*, *nxnA*, and *p24*.

The GYPQ region of p101 contains two repeated sequences identical to repeats found in annexin VII [Greenwood and Tsang, 1991] and p24 [Noegel *et al.*, 1990]. Furthermore two other repeated sequences in p101 are degenerate copies of repeats found in the proteins encoded by *capA* [Grant and Tsang, 1990]. The repeats in the sequence of p101 are shown underlined in Figure 8.

The hybrid protein encoded by clone 101 interacted with the products of *capA* and *capB* in the two-hybrid system (Table IX). As we mentioned before the GYPQ region might mediate protein-protein interactions. However the data currently available is not

sufficient to determine whether the product of clone 101 and the gene products of *capA* and *capB* interact through this region. Table IX shows that p101 did not interact with the products of *nxnA* or *p24* despite the fact that all three proteins bear identical GYPQ repeats. These results suggest that if the interactions are mediated by the GYPQ region those interactions are specific to particular pairs of proteins, and that the presence of such a region in polypeptides co-expressed in two-hybrid assays is not sufficient to mediate the interactions.

We also found that the C-terminus of the polypeptide shared a 50% identity (68% including conserved amino acids) with the C-terminus of a hypothetical 200-residue protein from the genome of a cyanobacterium [Kaneko *et al.*, 1996]. Unfortunately this information did not provide us with any clues about the identity of the protein encoded by clone 101.

Based on the data presented in Section 3.6 we believe that clone 101 encodes a novel protein, its estimated length based on the size of its RNA transcript (Figure 10) is approximately 500 amino acids. Two-hybrid system assays showed that p101 interacts with the products of *capA* and *capB*. As mentioned in section 4.2 we have obtained ambiguous data regarding the interactions between the products of *capA* and *capB*.

The interactions between the gene products of *capA* and *capB* and clone 101 remain to be confirmed using a quantitative method. Further studies are required to characterize the gene encoding p101 in order to determine the function of this protein, and to understand the physiological and biochemical significance of its interactions with the products of *capA* and *capB*.

4.4 The sequence of clone 302 codes for the carboxyl-end of a mitochondrial type II topoisomerase

4.4.1 The structure and function of DNA topoisomerases

DNA topoisomerases are enzymes that catalyze changes in DNA topology, they are classified according to their mechanism of action. Type I topoisomerases introduce breaks

in a single strand of DNA, pass the intact strand through the break, and religate the broken strand. Type II topoisomerases introduce double stranded breaks and pass an intact part of the DNA duplex through the break before resealing it. The catalytic activity of DNA topoisomerases is ATP dependent. There are three type of reactions catalyzed by DNA topoisomerases: relaxation/supercoiling, knotting/unknotting, and catenation/decatenation. Type I and type II topoisomerases can catalyze all three reactions, however type I topoisomerases can perform knotting/unknotting and catenation/decatenation only if there is a nick or a gap in one of the strands of the DNA duplex [reviewed in Bates and Maxwell, 1993].

The most extensively studied eukaryotic topoisomerases belong to human and yeast. In eukaryotes the function of most type I topoisomerases seems to be relaxation of supercoiling during transcription and replication [Watt and Hickson, 1994]. On the other hand studies in *S. cerevisiae* indicate that the main function of eukaryotic type II topoisomerases is to untangle daughter strands of chromosomes during mitosis and mieosis to allow proper segregation. Type II topoisomerases are also involved in condensation and decondensation of chromatin, and the suppression of abnormal recombination [reviewed in Watt and Hickson, 1994].

4.4.2 The structure of type II DNA topoisomerases

Structurally DNA topoisomerase II enzymes can be divided into three major domains. The N-terminal third has ATPase activity, it is homologous to the ATP-binding subunit of bacterial gyrase. The central part of the enzyme, homologous to the catalytic subunit of gyrase, is the site of binding and cleaving DNA. These two domains are conserved across species. The C-terminal does not have an equivalent in bacteria, it is hydrophilic and highly divergent [reviewed in Watt and Hickson, 1994].

Jensen *et al.* (1996) performed a detailed study of the functional organization of DNA topoisomerase II from human and *S. cerevisiae*. Alignment of topoisomerase II

sequences from various organisms revealed a pattern of 13 conserved regions throughout the N-terminal and central parts of protein separated by unconserved sequences.

Complementation studies in *S. cerevisiae* with both the human and the yeast enzymes showed that the conserved domains are essential for topoisomerase II function. Deletion of any of the conserved domains abolished activity and generated uncomplementing proteins. Truncation of the C-terminal did not lead to loss of catalytic activity, however some of the truncated proteins did not complement yeast *top2* mutants. It was determined that the non-complementing enzymes were no longer targeted to the nucleus. Jensen *et al.* reported that these data correlated with the existence of potential nuclear localization sequences identified in the C-terminal domains of the human and yeast enzymes. In the same study Jensen *et al.* suggest that the C-terminal domain might be involved in topoisomerase II dimerization, interactions with other proteins, and the regulation of enzymatic activity by phosphorylation.

4.4.3 TopA was identified as a *D. discoideum* mitochondrial topoisomerase II

As reported in Section 3.7 independent screenings using *capB*1 and *capB*2 as baits isolated two identical cDNA clones that coded for the carboxyl-end of TopA. This protein is a *D. discoideum* mitochondrial topoisomerase II isolated and characterized by Komori *et al.* (1997a). Structurally TopA can be divided into the three domains characteristic of all type II topoisomerases: an N-terminal ATPase domain, a central catalytic domain, and an unconserved C-terminal. However they also found several characteristics that distinguished TopA from other topoisomerase II enzymes: an extended N-terminal domain 70 residues long that contained a mitochondrial localization sequence, an insert of 34 hydrophilic amino acids near the ATP-binding site that had no similarity to other protein sequences, and a C-terminal 100-300 amino acids shorter than other known type II topoisomerases.

RNA blot analysis identified a 4.5 Kb transcript present at all stages of development, the levels of the transcript decreased gradually as development progressed. Immunoblot analysis with polyclonal antibodies recognized a 135 KDa polypeptide, the developmental

profile of the protein was similar to that of the mRNA transcript [Komori *et al.*, 1997a]. Intracellular localization studies demonstrated that TopA resided in the mitochondria. Furthermore immunodepletion studies determined that TopA was not associated with topoisomerase activity in the nucleus of *Dictyostelium* cells [Komori *et al.*, 1997b].

4.4.4 The C-terminal of TopA interacts specifically with p34 and p31 in the two-hybrid system

Clone 302 encodes the last 520 residues of TopA. Three series of tests designed to eliminate false positives determined that the interactions detected between the carboxyl end of TopA and p34 or p31 were specific (Tables VII-IX). These interactions remain to be confirmed through a quantitative method.

As reported in section 1.3 immunofluorescence analysis of *Dictyostelium* cells overexpressing *capB* showed a punctuate pattern of fluorescence throughout the cell [Bain, 1990]. Moreover preliminary studies with a *capB*/GFP fusion showed a similar pattern of fluorescence [A. Tsang, personal communication]. The results of these microscopy studies support the possibility that the gene products of *capB* might be associated with mitochondria. Immunoblot analysis had previously localized p34/31 in the nucleus of developing cells [Kay *et al.*, 1987]. However the method used for sample preparation was not designed to separate mitochondria from nuclei. Precipitation of mitochondria with the nuclear fraction could have accounted for the detection of the *capB* products in the nucleus of *D. discoideum*.

It must be mentioned that analysis of the amino acid sequence of p34 and p31 has not identified any putative targeting peptides. Nevertheless, the isolation of TopA by interaction with p34/31 in the two-hybrid system together with the results of immunofluorescence and GFP-fusion microscopy studies suggest the *capB* products might be localized in mitochondria.

In contrast to the high degree of conservation present in the N-terminal and central regions of eukaryotic type II topoisomerases the C-terminal region of these enzymes is very

divergent between species. This region has been implicated in dimerization, nuclear localization, and regulation of enzymatic activity [reviewed in Watt and Hickson, 1994]. The interaction of p34/31 with the C-terminal half of TopA leads us to speculate that these polypeptides might be involved in the modulation of topoisomerase activity. The involvement of p34/31 in mitochondrial function could explain the growth and developmental delay exhibited by *capB* disruption mutants [Bain *et al.*, 1991]

4.5 Clone 316 might be the product of imperfect transcriptional control

An examination of the sequence of clone 316 revealed that 80% of its nucleotides are A or T. This clone may belong to a class of cDNA inserts described in an article submitted by Bill Loomis to CSM News [Fuller *et al.*, 1997]. Their analysis of 42 cDNA clones isolated randomly from a lambda ZAP cDNA library turned up ten inserts with no significant reading frame. Many of these inserts were very A/T rich. They proposed that these clones may be the result of imperfect transcriptional control and that, as long as they are not translated, their presence would not be detrimental to the cell.

4.6 Conclusion

The results presented in this thesis combined with previously obtained data have uncovered new directions for the study of the intracellular role of p34/31.

The interaction of p34/31 with TopA taken together with the impaired growth phenotype of *capB* mutants and the cytoplasmic pattern of localization visualized through microscopy studies [Bain, 1990; A. Tsang, personal communication] suggest an *in vivo* role for these polypeptides in mitochondrial function.

On the other hand the isolation of CABP1B in independent screenings with p34 and p31 and the isolation of p34/31 by co-purification with CABP1A/CABP1B [Tsang and Tasaka, 1986] supports the possible existence of an *in vivo* protein complex involving these polypeptides. In addition, the interaction of p101 with all four gene products of *capA* and *capB* leads us to speculate that it may also be a member of the putative complex involving p34/31 and the subunits of CABP1.

The possibilities presented above raise many questions regarding the intracellular localization and function of these polypeptides. What is the intracellular localization of p34/31? Is their interaction with TopA, CABP1, and p101 biologically significant? Do p34/31 have more than one intracellular function or do they perform the same function in different cellular compartment? A lot of work remains to be done in order to begin addressing some of these questions. First, we must start by corroborating the two-hybrid interactions using a quantitative method. Second, we must determine the intracellular localization of p34/31 during various developmental stages. Proteinase K experiments using mitochondrial extracts could be used to determine whether p34/31 it is found inside mitochondria. Finally, cloning and characterization of the gene encoding p101, the generation of mutants for this gene, and determination of the intracellular localization of the protein might provide some answers regarding its function as well as its relationship with the gene products of *capA* and *capB*.

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