# IDENTIFICATION OF PROTEINS THAT INTERACT WITH BRAIN FACTOR-1 AND CHARACTERIZATION OF THESE INTERACTIONS.

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## DECLARATION

I hereby declare that this thesis entitled:

**Identification Of Proteins That Interact With Brain Factor-1 And Characterization Of These Interactions**

is my own work, and has not been previously in its entirety or in part been submitted at any university for another degree.

**Signed:** _____ **Date:** _____

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## ABSTRACT

Brain Factor-1 (BF-1) is a winged helix transcription factor that is essential for the development of the cerebral hemispheres and olfactory neuroepithelium. BF-1 is expressed in the telencephalic neuroepithelium, olfactory placode and the nasal half of the optic stalk and retina during embryogenesis (Hatini et al 1994; Lai and Tao 1992). In these tissues, BF-1 plays multiple roles in regional patterning and regulating cell proliferation and differentiation. In order to investigate how BF-1 controls these various processes, the aim of this thesis was to identify proteins which interact with BF-1 using the Yeast Two Hybrid System (Fields and Song 1989) and to characterise these interactions.

The screening of a two-hybrid cDNA library constructed from neuroepithelial cells was successful. Surprisingly, no interactions between the BF-1 fusion bait and components of the RNA pre-initiation complex, "co-activator complexes" or cell cycle were not detected. However, four cDNA clones were shown to interact with the BF-1 fusion protein and were named $BF_1$-IP1, $BF_1$-IP2, $BF_1$-IP3 and $BF_1$-IP4. The relative strength of the interaction between the BF-1 fusion and the $BF_1$-IP1 to –IP4, were quantified and produced 86(± 21), 41 (± 13), 23 7) and 20 (± 7) units of β-galactosidase respectively. These responses totally dependent on the BF-1 fusion protein being present in yeast.

The cDNA sequences for $BF_1$-IP2, -IP3 and –IP4 were found to be 100% homologous to mouse genes encoding the mutant p53 binding protein (MBP1), acrogranin and fibulin-2 extracellular matrix proteins respectively. These extracellular matrix proteins are involved in cell growth and contain calcium binding EGF-like or granulin domains involved in mediating protein-protein interactions. Therefore if BF-1 interacts with these extracellular matrix proteins via mechanisms involving these domains, the localization of BF-1 inside and outside the cells needs to be determined if these interactions are functionally significant.

The interaction between $BF_1$-IP1 and BF-1 was robust and produced 86 (± 21) units of β-galactosidase compared to the positive control (140±18 units). The interaction was identified eight times in independent two-hybrid experiments. The $BF_1$-IP1 protein contained a coding region of 25 amino acid residues continuous with the GAL4 AD, followed immediately by one stop codon, and an untranslated region containing another two stop codons. The coding region contained a 4-disulphide core and cysteine string motif. Based on information from literature, the $BF_1$-IP protein may be able to integrate into cellular or organelle membranes via its cysteine string motif. The remainder of the $BF_1$-IP1 protein containing the WAP domain may extend into the cytoplasm, where it could interact with other proteins including BF-1. Therefore the subcellular localization of the interaction between BF-1 and $BF_1$-IP1 needs to be determined.

There are two interpretations for the results of this thesis: the first is that the interactions are an artefact of the two-hybrid system and that the observed interactions are a consequence of the artificial localization of the extracellular matrix or membrane-bound proteins and represent non-specific interactions. The second is that these interactions have a biological function and are similar to those interactions observed for mutant p53 and HIV Tat proteins (Gallagher et al 1999; Trihn et al 1999). In this case, BF-1 would be required to be present in extracellular matrix like the HIV Tat proteins or, associated with organelle membranes like the mutant p53 protein. Therefore it is important to determine the subcellular distribution pattern of BF-1 in the neuroepithelial cells. The interactions identified in this study may belong to a novel developmental pathway in which BF-1 interacts with membrane bound ($BF_1$-IP1) or extracellular matrix proteins ($BF_1$-IP2, -IP3, -IP4). Therefore the interactions between BF-1 and $BF_1$-IP1, $BF_1$-IP-2, $BF_1$-IP-3 and $BF_1$-IP–4 need to be verified using affinity chromatography and co-immunoprecipitation.

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**Abbreviations**

| | |
|---|---|
| 3-AT | 3-Aminotriazole |
| A/P | Anterior/Posterior |
| ACF | ATP-Utilising Nucleosome Assembly And Remodelling Factor |
| ACTR | Activator of TR and RAR |
| AF-2 | Activation Function 2 |
| AIB | Amplified In Breast Cancer |
| ANR | Anterior Neural Ridge |
| AP-1 | Activator Protein-1 |
| Asn | Asparagine |
| Asx | Aspartic Acid Or Asparagine |
| ATP | Adenosine Triphosphate |
| AVE | Anterior Visceral Endoderm |
| BF-1 | Brain Factor-1 |
| $BF_1$-IP | BF-1 Interacting Protein |
| BF-2 | Brain Factor-2 |
| BMP | Bone Morphogenetic Protein |
| Bp | Base Pair |
| c.f.u. | Colony Forming Unit |
| CAF-1 | Chromatin-Assembly Factor 1 |
| cCAMP | Cyclic Adenosine Mono Phosphate |
| CBP | CREB Binding Protein |
| CHD | Chromodomain-Helicase-DNA-Binding Domain |
| CHRAC | Chromatin Accessibility Complex |
| CK2 | Calcium Kinase II |
| CNS | Central Nervous System |
| CREB | Camp Response Element Binding Protein |
| CSP | Cysteine String Protein |
| CTD | C-Terminal Domain |
| D/V | Dorso-Ventral |
| DEPC | Diethylpyrocarbonate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| dNTP | Deoxy Nucleotide Triphosphate |
| DR | Vitamin D3 Receptor |
| DRIP | Vitamin D3 Receptor Interacting Proteins |
| Ds | Double Stranded |
| DSC | Discrimination of Protein Structure |
| DTT | Dithiothreitol |
| E10.5 | Embryonic Day 10.5 |
| EGF | Epithelial Growth Factor |
| FCS | Fetal Calf Serum |
| FGF | Fibroblast Growth Factors |
| GAIP | G-Alpha-Interacting-Protein |
| GAL4 AD | Gal4 Activation Domain |
| GAL4 DB | Gal4 DNA Binding Domain |
| GnRH | Gonadotropin Releasing Hormone |
| HAT | Histone Acetyltransferase |
| HDAC | Histone Deacetylase |
| HIV | Human Immunodeficiency Virus |
| ISWI | Imitation Switch |

| | |
|---|---|
| Kbp | Kilo base pairs |
| $K_d$ | Dissociation Constant |
| MBP1 | Mutant p53 Binding Protein |
| NAP-1 | Nucleosome Assembly Protein 1 |
| NcoA | Nuclear Receptor Co-Activator |
| NcoR | Nuclear Receptor Co-Repressor |
| NRs | Nuclear Hormone Receptors |
| NURF | Nucleosome Remodelling Factor |
| ONPG | o-nitrophenyl-β-D galactopyranoside |
| p/CAF | p300/CBP-Associated Factor |
| P/CIP | p300/CBP Co-Integrator Associated Protein |
| P38IP | Human p38 Interacting Protein |
| PCR | Polymerase Chain Reaction |
| PHD | Plant Homeodomain |
| PIC | Pre-Initiation Complex |
| PKC | Protein Kinase C |
| RAC3 | Receptor Associated Co-Activator 3 |
| RAR | Retinoic Acid Receptor |
| RE | Response Elements |
| RNA Pol II | RNA Polymerase II |
| RSC | Remodels-Structure-of-Chromatin |
| RTF | Regulatory Transcription Factor |
| SAGA | SPT-ADA-GCN5-Acetyltransferase |
| SD-Leu | synthetic minimal medium lacking leucine |
| SD-Leu/-Trp | synthetic minimal medium lacking leucine and tryptophan |
| SD-Leu/-Trp/-His | synthetic minimal medium lacking leucine, tryptophan and histidine |
| SD-Leu/-Trp/-His + 3AT | synthetic minimal medium lacking leucine, tryptophan and histidine and supplemented with 3-aminotriazole |
| SHH | Sonic Hedgehog |
| SMRT | Silencing Mediator Of Retinoid and Thyroid Hormone Receptor |
| SRB | Suppressor of RNA Polymerase B |
| SRC | Steroid Receptor Co-Activators |
| STAT | Signal Transducer And Activator of Transcription |
| SWI/SNF | Switching Mating Type/Sucrose Non-Fermenting |
| TAFs | TBP Associated Factors |
| TBP | TATA Box Binding Protein |
| TR | Thyroid Hormone Receptor |

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**Table (i). List of Plasmids**

| Plasmid | Description | References |
|---|---|---|
| pBF-1 | Rat Brain Factor -1 cloned *Eco*RI and *Not*I into pBluescript SK | Toa and Lai 1992 |
| PGBT9 | Two hybrid cloning vector used to generate fusion of the target protein with the GAL4 DNA binding Domain. | Bartel et al 1993a |
| PGAD10 | Two hybrid cloning vector used to generate fusions of a collection of random, unknown proteins with the GAL4 AD. | Bartel et al 1993a |
| pGalDBD/BF-$1_{(26\text{-}480aa)}$ | Two hybrid pBGT9 vector encoding BF-1 fused to the GAL4 DNA binding domain. | This study |
| PGAD10-cDNA | Two hybrid pGAD10 cloning vector encoding a collection of fusions of OP6 cDNA with the GAL4 AD. | This study |
| pCL1 | Encodes the full-length, wild-type GAL4 protein and provides a positive control for the β-galactosidase assay. | Fields and Song 1989 |
| PVA3 + pTD-1 | PVA3 encodes a fusion of murine p53 with GAL4 DNA-BD and pTD1 encodes a fusion of SV40 large T-antigen with GAL4 AD. Serves as a model for interacting proteins. | Iwabuchi et al 1993; Li and Fields 1993;Chien et al 1991 |
| PLAM5' | Encodes a fusion of human lamin C with the GAL4 DNA-BD. Serves as a control for a fortuitous interaction between an unrelated protein and the fusion protein encoded by a AD/library plasmid. | Bartel e al 1993a |

**Table (ii). List of Bacterial and Yeast Strain Genotypes**

| Host Strain | Genotype | Source |
|---|---|---|
| *E.coli* DH5α | *$F^-$ endA1 hsdR17($r_k^-$, $m_k^+$) supE44 $\lambda^-$ thi-1 recA1 gyrA96 relA1* ϕ80*lacZ*ΔM15Δ*(lacZYA-argF)*U169 | Clontech |
| *E.coli* XL-1 Blue | *RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB $lacI^q$Z*ΔM15 Tn*10*($Tet^r$) | Stratagene |
| *E.coli* HB101 | *SupE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | Stratagene |
| *S.cerevisiae* Y190 | MATa, ura3- 52, his3- 200, ade2- 101, lys2-801,HIS3, trp1- 901, leu2- 3, 112, gal4Δ, gal80Δ, $cyh^r$ 2, LYS2 :: $GAL1_{UAS}$-$HIS3_{TATA}$-HIS3, URA3 :: $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ | Clontech |
| *S.cerevisiae* SFY526 | MATa, ura3-52, his 3-200 ade 2-101, lys 2-801, trp1-901 leu2-3, 112, $can^r$, gal4-542, gal80-538, URA3:: GAL1-lacZ | Clontech |

# CHAPTER 1 INTRODUCTION

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# CHAPTER 1 INTRODUCTION

## 1.1. INTRODUCTION

The development of the mammalian brain is tightly controlled during embryogenesis by many proteins such as regulatory transcription factors, which interpret positional and temporal signals and relay them to the transcriptional machinery, resulting in the activation or repression of genes. The expression of developmental proteins can be controlled at several steps in the synthesis pathway such as, activation and initiation of transcription, translation and post-translational modification. The most common level of control occurs at transcription via multiple protein-protein interactions between various protein complexes involved. This chapter will review two fields, namely that of transcription and forebrain development. The first section will discuss our current understanding on the activation and initiation of transcription, and will focus on the large multiprotein co-activator complexes which relay the signals from the regulatory transcription factors to the transcriptional machinery assembled at the promoter. The second section will discuss development of the forebrain, summarizing (1) the various inductive signals that induce the neuroepithelium in the neural tube to develop into neural tissue, (2) the patterning mechanisms along the A/P and D/V axes of the neural tube that regionalize the forebrain and (3) the role of Brain Factor-1 in forebrain development.



## 1.2. THE ACTIVATION AND INITIATION OF TRANSCRIPTION

The most common level where gene expression is controlled is at the level of transcription where (1) the structure of the gene has to be "activated" so that the DNA, which is bound up in chromatin, becomes accessible to the transcriptional machinery and (2) where the RNA Polymerase II and the general transcription factors assemble in a pre-initiation complex (PIC) at the promoter to initiate transcription at some defined point.

### 1.2.1. RNA Polymerase II and the Basal Transcription Factors

In simple terms, the initiation and activation of transcription requires the ordered assembly of a pre-initiation complex (PIC) at the TATA box of the promoter of the target gene that is to be transcribed. The first step of PIC assembly is the binding of TFIID to the TATA-box via its TATA box binding protein (TBP). TFIID consists of TBP and TBP associated proteins (TAFs). Thereafter an ordered binding of the various basal TFs occurs: after TFIID has bound, TFIIA, TFIIB and TFIIF then bind sequentially. TFIIF is thought to recruit the RNA Pol II by providing a platform onto which the RNA Pol II can bind. TFIIF consists of RAP74 , an ATP-dependent DNA helicase possibly involved in unwinding the DNA, and RAP38 which has some homology to the prokaryotic sigma factor that contacts the core polymerase. TFIIE, TFIIH and TFIIJ then join the forming PIC, where TFIIE is needed for promoter clearance, while the ATPase, helicase and kinase activity (which phosphorylates the CTD tail of RNA Pol II) of TFIIH contributes to the initiation of transcription. After the initiation of transcription has occurred and the RNA Pol II has moved away from the promoter, most of the basal TFs are released from the PIC, except TFIIE and TFIIH, whose ATPase and helicase activities respectively are needed for RNA Pol II movement. In a similar manner, the PIC can assemble at TATA-less promoters where the initiator element, instead of the TATA- box, can act as the positioning element .



The PIC consists of RNA Pol II and a subset of proteins on which RNA Pol II is dependent for its function (Buratowski et al 1989; Orphanides et al 1996; Zawel, L. and Reinberg, D. 1995; Hampsey, M. 1998). However *in vitro* studies using the PIC alone could only initiate low levels of transcription. For high levels of transcription, additional regulatory transcription factors (RTFs) bound to response elements (RE) or enhancer sequences upstream from the promoter's initiation point, are needed. These RTFs which are responsible for controlling transcription in a temporal and spatial manner, are themselves regulated by their temporal and spatial synthesis and activation.

When bound to their RE or enhancer sequences, RTFs act in either a direct or indirect manner that increases the rate of the initiation of transcription. The RTFs can directly contact and recruit basal TFs to the growing PIC via their activation domain, which acts independently of the DNA binding domain; or the RTFs can indirectly contact the PIC via large multiprotein co-activator complexes which have multiple functions that enhance the activation of transcription (discussed later). Therefore a general overview of the activation and initiation of transcription (Figure 1.1) can be perceived as follows: the PIC assembles as depicted in Figure 1.1 over the TATA box and initiator (Inr) region. Activated sequence-specific regulatory transcription factors (R) bind to their specific RE or enhancer sequences that lie upstream of the promoter (UAS) and regulate the rate of transcription by directly affecting the rate at which the PIC assembles, or indirectly via the co-activator complex (C). The co-activator complex has multiple functions, which include interacting with the PIC and chromatin-remodelling and –modifying activity. These latter functions facilitate the activation of transcription by modifying and unravelling the chromatin and allowing (1) DNA binding proteins access to the DNA resulting in the recruitment of the basal TF and RNA Polymerase II to the promoter, (2) clearance of the promoter by RNA Polymerase II and (3) elongation through the gene.

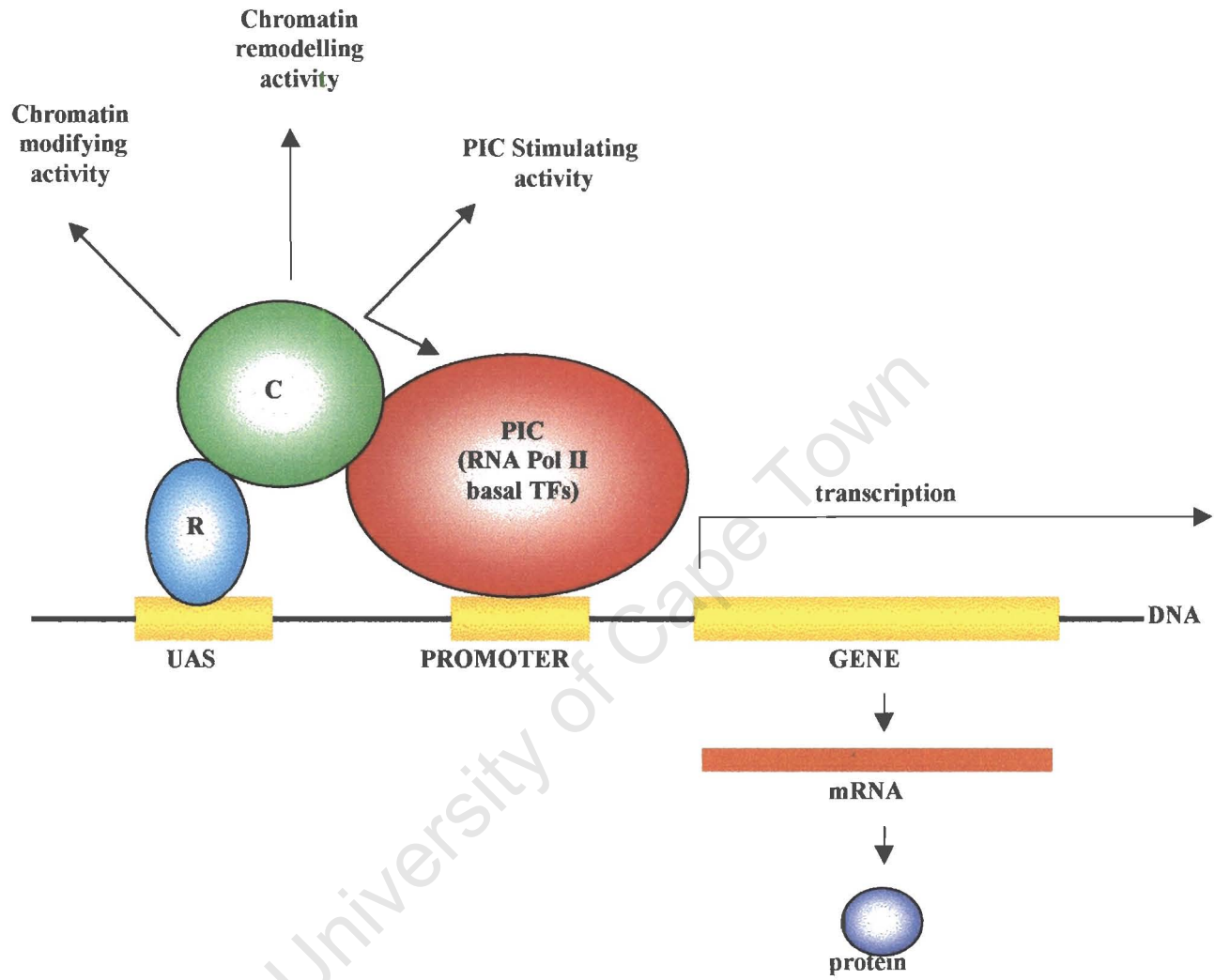




**Figure 1.1.** General overview of transcription. The PIC assembles over the TATA box and initiator (Inr) region. Sequence-specific regulatory transcription factors (R) bind to their specific RE or enhancer sequences that lie upstream of the promoter (UAS) and regulate the rate of transcription by directly affecting the rate at which the PIC assembles, or indirectly via the co-activator complex (C). The "co-activator complex" has multiple functions, which include interacting with the PIC and chromatin-remodelling and –modifying activity.

### 1.2.2. Chromatin-Modifying And –Remodelling Complexes

In eukaryotes the DNA is tightly packaged into chromatin which enables all of the DNA to fit into the small space of the nucleus. Chromatin itself consists of nucleosomes (DNA wound around histone octamers) which are organised into fibres. However, this compact packaging of the DNA inside the nucleus requires that the chromatin be rapidly and reversibly unwound allowing various enzymes access to the DNA when necessary e.g. in the case of transcription. Conversely, the repression of transcription requires that the chromatin structure be restored. (Paranjape et al 1994). The state of chromatin can be changed from an inactive to active state, and vice versa, by chromatin-modifying or -remodelling complexes (Felsenfeld et al 1996; Kingston et al 1996).



#### 1.2.2.1. Chromatin Modifying Complexes

Acetylation of histones has been correlated with gene activity whereas histone deacetylation has been correlated with gene repression (Allfrey et al 1964; Csordas 1990). Chromatin-modifying complexes act on nucleosomes by either acetylating or deacetylating the lysine residues of histone tails. Although the histone tails are not involved in maintaining the structural integrity of the nucleosome, they do play role in higher order chromatin structure interactions and in binding non-histone chromosomal proteins. The N- and C-terminal tails of core histones, in particular H3 and H4, are flexible and extend out from the histone octamer. Acetylation of these tails is postulated to neutralize the charge on the tails, leading to a reduced affinity of the histone- DNA interactions and destabilization of the chromatin structure, facilitating the binding of transcription factors and RTFs to the DNA. The mechanism by which acetylation is targeted to appropriate genes came from the discovery that the *Tetrahymena* histone acetyltransferase type A (HAT A) was a homologue of the yeast transcription factor GCN5 (Brownell et al 1996). Shortly thereafter several other proteins and the were found to have HAT activity, and were often associated with multiprotein co-activator

complexes. The chromatin modifying complexes often contained one or more subunits that have histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. The first histone modifying complex isolated from yeast, *Saccharomyces cerevisiae,* was the SPT-ADA-GCN5-acetyltransferase (SAGA) complex. The SAGA complex contains GCN5, SPT3, SPT7, SPT8, SPT20/ADA5, ADA2 and ADA3 proteins, where the GCN5 protein acted as the catalytic acetyltransferase (Grant et al 1997; Roberts and Winston 1997). However, since the yeast GCN5 was found to only acetylate free histones, it was thought that the recruitment of the ADA complex enabled yeast GCN5 to acetylate nucleosomal histones. The SPT 20 SPT3 and ADA2 proteins have been shown to interact with TBP, thereby further linking acetylation to the activation of transcription. (Roberts and Winston 1997). Several mammalian co-activator complexes, such as those that interact with nuclear receptors, have been isolated and contain several HAT proteins (discussed later). More recently, studies by Wei et al (1998) have suggested that phosphorylation of histone tails may be equally important for transcriptional regulation. They showed that antibodies generated against the N-terminal histone tails which were phosphorylated at a conserved serine 10, were highly specific for decondensed and actively transcribed chromatin.



**1.2.2.2. Chromatin- Remodelling Complexes**

Chromatin remodelling complexes remodel nucleosomal structure and facilitate transcription in an ATP-dependent manner. The remodelling of chromatin can be defined as detectable change in the structure or configuration of the chromatin or nucleosome structure. Biochemical analyses have shown that the chromatin remodelling complexes may disrupt histone –DNA interactions in an ATP-dependent manner, possibly involving the local unwinding of the DNA from the nucleosomes, which facilitate the binding of RTFs and TFs to the DNA resulting in transcription from the chromatin template. Several remodelling complexes also have the ability to regulate the spacing of the histone octamers along the DNA. The catalytic subunit of chromatin remodelling complexes are

DNA-dependent ATPases which are thought to act as ATP-driven DNA–translocating motors that disrupts histone-DNA interactions (Kornberg and Lorch 1995; Pazin and Kadonga 1997). The ATPase can be either a SWI2/SNF2 homologue, or the more distantly related ISWI protein, or a CHD protein.

The first chromatin-remodelling complex to be identified was the yeast Switching mating type/Sucrose non-fermenting (SWI/SNF) complex, which was required for the relief of chromatin repression and the activation of inducible genes. The ATPase contained in this complex is the SWI2/SNF2 protein. (Kwon et al 1994; Cote et al 1994). The SWI/SNF complex consisted of 11 subunits, many of which were encoded by genes originally identified on the basis of their requirement for normal transcription. A closely related complex also isolated from yeast, is the remodels-structure- of-chromatin (RSC) complex which possesses both remodelling and nucleosome spacing activities. (LeRoy et al 1998). The RSC consists of 15 subunits and 6 of these were ATPases encoded by the *STH1* gene, which is homologous to the *SWI/SNF* gene. Despite the similarities between the SWI/SNF and RSC complexes, they are functionally distinct complexes since RSC is much more abundant in cells than the SWI/SNF and its subunits are encoded by essential genes involved mitotic growth, unlike SWI/SNF.



Several chromatin remodelling complexes, such as nucleosome remodelling factor (NURF) (Tsukiyama et al 1995a), chromatin accessibility complex (CHRAC) (Varga-Weisz et al 1997) and ATP-utilising nucleosome assembly and remodelling factor (ACF) (Ito et al 1997) have been isolated from extracts of *Drosophila* embryos. In all these complexes the ATPase protein was the "Imitation Switch" (ISWI), which had a helicase motif but lacked the C-terminal bromodomain characteristic of the SWI2/SNF2 protein (Tsukiyama et al 1995b).

NURF is a 500kDa complex that consisted of 4 subunits. NURF has been shown to facilitate the binding of TFs to its sites in chromatin in an ATP-dependent manner and recently NURF has been shown to activate transcription from chromatin template due to its facilitated activator binding. Since one of the NURF subunits, NURF-38, possesses inorganic pyrophosphatase activity that does not appear to contribute to chromatin remodelling *in vitro*, an additional function of NURF is therefore thought to be to deliver pyrophosphatase activity to transcriptionally active chromatin, thereby removing accumulated unhydrolyzed pyrophosphate, which otherwise inhibits transcription.

CHRAC is a 670kDA chromatin remodelling complex that contains two ATPase subunits, the ISWI and topoisomerase II. CHRAC functions in both chromatin assembly and remodelling in vitro and converts irregularly spaced nucleosomes into an evenly spaced array as well as increasing the accessibility of the reconstituted chromatin to DNA binding proteins in an ATP-dependent manner. CHRAC therefore acts as histone chaperone which can either donate or accept histones during chromatin assembly or disassembly. It was shown that the ISWI subunit of CHRAC alone triggered chromatin remodelling, which therefore suggested that it was the functional core of CHRAC that recognised nucleosomes and remodelled chromatin (Langst et al 1999). The function of Topoisomerase II (Topo II), an enzyme involved in DNA topology, in CHRAC is uncertain, since its activity did not contribute to chromatin remodelling *in vitro*. One suggestion was that CHRAC-mediated chromatin remodelling facilitated the binding of Topo II to chromatin thereby enhancing its function. Another was that Topo II targeted CHRAC to specific chromosomal sites (Varga-Weisz et al 1997).

ACF is a 220kDa complex that consists of 4 subunits, one of which is the ATPase ISWI. ACF has the dual capacity to assemble regularly spaced nucleosomes in combination with a histone chaperone such as nucleosome assembly protein 1 (NAP-1) or chromatin-assembly factor 1

(CAF-1). Both the assembly and remodelling activities are ATP-dependent. ACF was also shown to mediate promoter specific chromatin remodelling by GAL4-VP16 (a sequence-specific DNA binding transcriptional activator) in an ATP-dependent manner (Ito et al 1997). Therefore ACF can regulate both chromatin assembly and the remodelling of nucleosomes that accompanies transcriptional activation. Interestingly, the core histone binding proteins NAP-1 and nucleoplasmin can stimulate the binding of RTFs to mononucleosomes by a mechanism that involves the removal of histone H2A and H2B by the histone binding proteins. (Walter et al 1995), which further enhances the initiation of transcription.

A further class of related proteins involved in ATP-dependent chromatin remodelling are the Chromodomain-Helicase-DNA-Binding Domain Proteins (CHD proteins). CHD proteins contain a helicase motif and two chromodomains (a motif involved in protein-protein interactions in heterochromatin) and two plant homeodomain (PHD) zinc finger motifs. Unlike SWI/SNF and ISWI containing complexes, complexes containing some CHD proteins, such as CHD4, are often associated with repressive chromatin remodelling (Tong et al 1998; Zhang et al 1998b).



### 1.2.3. Co-Activator Complexes

Regulatory transcription factors bound at upstream RE or enhancer sequences influence the initiation of transcription by contacting basal TFs that are assembling into the PIC. This contact may be direct or indirect and does not involve contacting the RNA Pol II. A direct interaction occurs when a RTF uses its activation domain, which acts independently of the DNA binding domain, to contact and recruit the basal TFs to the promoter. This recruitment suggests that the major function of the RTF is to increase the rate at which the PIC assembles. Indirect contact between RTFs and the PIC occurs via large multiprotein co-activator complexes which have multiple functions that facilitate the initiation of transcription.

Several mammalian "co-activator complexes" have been identified (Greenblatt, J. 1997; Myer and Young 1998) and found to contain common subunits such as subsets of basal transcription factors, SRB proteins, chromatin-modifying and -remodelling proteins and various other DNA binding proteins as well as unique subunits that interact with specific RTFs. Therefore the RTFs can target specific regulatory co-activator complexes to specific promoter or enhancer regions. The differences between the various "co-activator complexes" were due to the different methodologies used to isolate these large complexes, the different functional requirements imposed during purification, and the criteria used to define subunit composition.

Since the nuclear hormone receptors (NRs) are such well studied class of regulatory transcription factors, the discussion on co-activator complexes will focus on those that interact with the NR's. The nuclear receptors are composed of distinct functional domains involved in DNA binding, dimerization, ligand binding, and transcriptional activation or repression. In the absence of their cognate ligands, the NRs for all-trans retinoic acid (RAR) and thyroid hormone (THR) repress transcription by interacting with histone deacetylase (HDAC)-containing co-repressor complexes. However, upon binding of their cognate ligands, the NR's can activate transcription by interacting with several histone acetylase proteins such as CBP, PCAF, TIF2, SRC, and ACTR found in histone acetylase (HAT)-containing co-activator complexes. These observations led to the idea that gene activation by NRs involved targeted local histone acetylation and chromatin decondensation (Torchia et al 1998).

The activation by NRs can be viewed as a multistep process: in the first step, the binding of ligands to the NRs releases the bound co-repressor complex and induces the recruitment of HAT-containing co-activator complex which then modifies the chromatin structure leading to the activation of transcription. The NR's may then interact with "NR-specific co-activator complexes" such as TRAP and DRIP (discussed later) that then act in concert with the TAFs and basal TFs in the PIC enhance transcriptional initiation (Figure 1.2). A few of the co-activator complexes that have been shown to interact with NRs are briefly discussed below.

The CBP/p300 proteins are structurally conserved proteins which have been shown to interact and serve as a co-activator for many transcription factors (reviewed in Shikama et al 1997) such a CREB, activator protein-1 (AP-1) and signal transducer and activator of transcription (STAT) proteins as well as nuclear hormone receptors (reviewed by Kamei et al 1996). CBP was initially isolated on the basis of its association with CREB in response to cAMP signalling (Chrivia et al 1993). Its close homologue, p300, was purified as a cellular binding protein of the adenoviral protein E1A (Eckner et al 1994). The activation of CBP/p300 with nuclear receptors relies on the conserved nuclear receptor functional domain AF-2 (activation function 2). The main function of CBP/p300 appears to be to modifying chromatin, since loss of CBP/p300 HAT activity resulted in the loss of transcription for many transcription factors (Ogryzko et al 1996; Bannister and Kouzarides 1996; Naar et al 1998; Kraus et al 1998).

The p300/CBP-associated factor (P/CAF) complex was the first mammalian HAT discovered on the basis of its homology to the yeast GCN5p protein (Yang et al 1996). p/CAF contains an extended amino terminus which enables it to interact with CBP/p300, nuclear receptors (Chen et al 1997) and members of the nuclear receptor co-activator (NcoA) family (Blanco et al 1998); Korzus et al 1998).

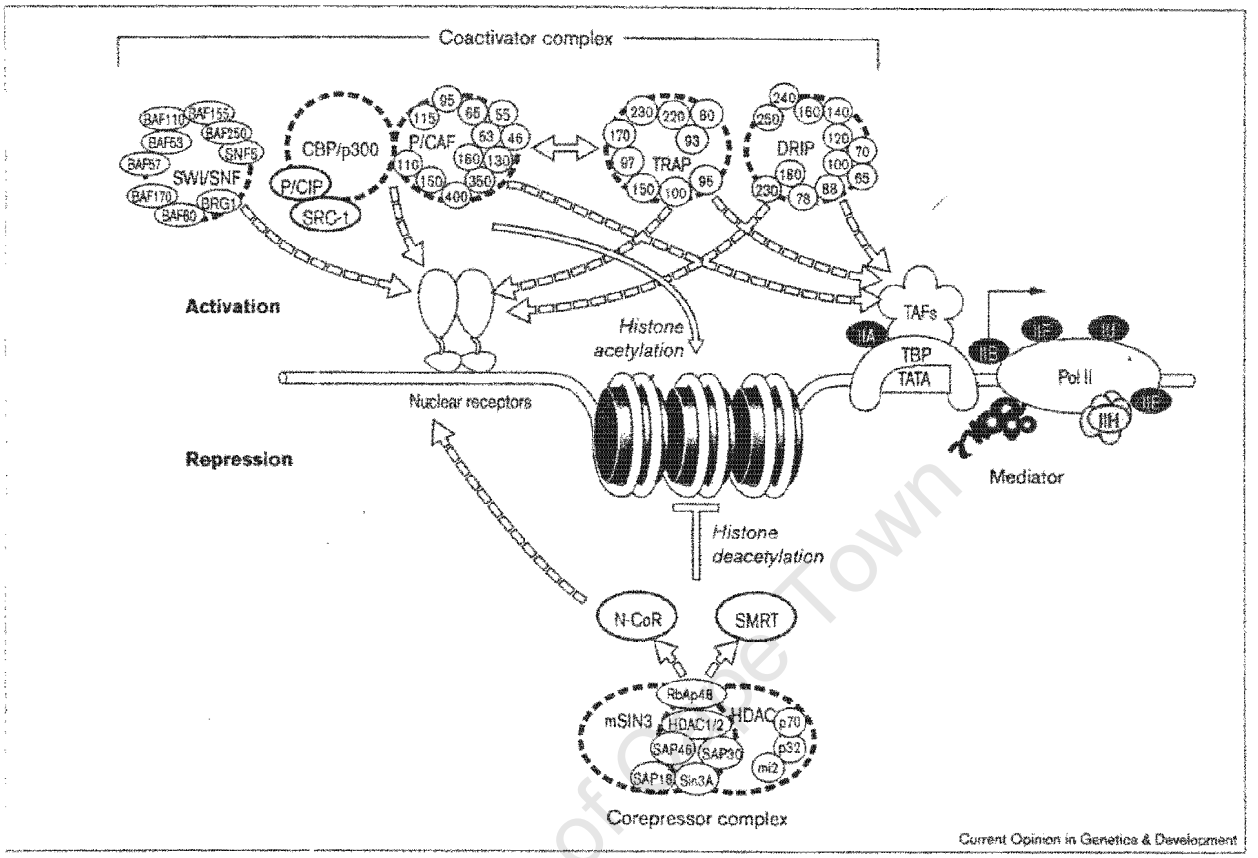


**Figure 1.2.** The functions of nuclear receptors are regulated by multiple co-activator and co-repressor complexes. Protein complexes involved in hormonal regulation of gene transcription are shown; unknown factors within the complexes are indicated by their apparent molecular sizes (in kilo Daltons). The binding of ligands to the nuclear receptors induce the recruitment of co-activator complexes, leading to the activation of transcription. The double headed arrow suggests a sequential model in which the TRAP and DRIP complexes activate transcription initiation after the chromatin has been remodelled by CBP/p/CAF or SWI/SNF complexes. The CBP/p300/pCAF complexes as well as the TRAP and DRIP complexes, may provide a link between nuclear receptors and the histone core machinery (indicated by dashed arrows). The mSin3/HDAC co-repressor complexes which contain histone deacetylases, are linked to nuclear receptors via NcoR (nuclear receptor co-repressor) or SMRT (silencing mediator of retinoid and thyroid hormone receptor) in the absence of ligands. (*This is a copy of the figure and legend taken from Current Opinion in Genetics and Development 9:141, 1999)*

Although CBP is required for functions of many transcription factors, the role of p/CAF and NcoAs was shown to be more selective, which raised the possibility that the assembly of CBP, p/CAF and NcoAs is determined by DNA bound transcription factors (Korzus et al 1998; Woolshin et al 1995).

The steroid receptor co-activators (SRC)/nuclear receptor co-activators (NcoA) were initially identified as 160kDa proteins (p160) which interacted directly with nuclear hormone receptors in an agonist and AF-2 dependent manner (Halchmi et al 1994; Cavailles et al 1994; Kurokawa et al 1995). Member of this p160 family include SRC/NcoA-1, TIF2/GRIP1/NcoS-2 and p/CIP/ACTR/AIB1/RAC3/TRAM-1. Each of these p160 proteins have a number of splice variants. SRC-1 was initially identified on the basis of a two hybrid screen in yeast using the ligand bound progesterone receptor as bait (Onate et al 1995). SRC-1 stimulated the transcriptional activities of a number of nuclear hormone receptors in response to their respective ligands (Onate et al 1995). In parallel studies, the mouse homologue of SRC-1, termed NcoA-1, was identified by its interaction with the oestrogen receptor-ligand binding domain and the C-terminal p160 interaction domain of the cAMP response element binding protein (CREB) binding protein (CBP). A related p160 protein has been cloned from humans (TIF2) and mice (GRIP1/Nco-2) and both have been shown to activate transcription for several nuclear hormone receptors (Voegel et al 1996; Hong et al 1996; Torchia et al 1997). A third related p160 protein has been identified and called the p300/CBP co-integrator associated protein( p/CIP) in mice (Torchia et al 1997), and amplified in breast cancer (AIB) 1 (Anzick et al 1997), activator of TR and RAR (ACTR) (Chen et al 1997), receptor associated co-activator 3 (RAC3) (Li et al 1997a) and TR activator molecule (TRAM-1) (Takeshita et al 1997).

Interactions between all the above SRC/NcoA family members and the carboxy terminus of CBP/p300 has been shown to occur via a different domain than the domains that interacts with the nuclear receptors. This nuclear receptor interaction domain of p/CIP, SRC-1/NCoA-1 and TIF2/GRIP1 contains three highly conserved motifs that share a consensus amino acid sequence, LXXLL, where the X is any amino acid. Analysis of these regions suggested that they represent amphipathic helical domains common in membrane proteins. Extensive mutagenesis revealed that different LXXLL motifs within the SRC-1/NcoA-1 are selectively required to support functions of different nuclear receptors. This functional specificity correlates with the difference in affinity between each LXXLL motif and different nuclear receptors.

The Thyroid Receptor Associated Proteins (TRAP) and Vitamin D3 Receptor Interacting Proteins (DRIP) co-activator complexes were isolated based on their interactions with nuclear hormone receptors. The TRAP co-activator complex was isolated on the basis of its interaction with the thyroid hormone receptor (TR) in a ligand-dependent manner (Fondell et al 1996) from HeLa cells. Similarly, the DRIP complex was identified by its ability to bind selectively to the liganded vitamin D3 receptor (DR) *in vitro* (Rachez et al 1998). The TRAP complex was shown to significantly enhance the transcriptional activation function of TR on naked DNA templates; whereas the DRIP complex could only enhance the transcriptional activation of VDR on a chromatin template (Rachez et al 1999). The components of the DRIP complex are almost identical to the activator recruited factor (ARC) complex, which is required by several other transcription factors such as SREBP, NFκB and VP16 (Rachez et al 1999; Naar et al 1999). In addition several components of the DRIP/ARC complex are also found in a complex that interacts with PIC (Li et al 1995b).

### 1.2.3.1. Functional redundancy of the co-activator complexes

Experiments have showed that CBP/p300, p/CIP, p/CAF, NcoAs, TRAP and DRIP can all associate with much larger co-activator complexes. The discovery of several co-activator complexes has suggested that these complexes may be functionally redundant. Since each of these complexes possess HAT activity, the question of functional HAT redundancy is raised. However, the presence of different HAT proteins in the complex can be explained as follows: (1) the HAT activities could be restricted to specific substrates which may include non-histone proteins such as p53, which when acetylated by p300 has increased DNA binding (Gu et al 1997) (2) different classes of RTFs may require specific acetylases for their function at a particular gene: e.g. the TRAP and DRIP complexes may have different target genes compared to those genes that need CBP/p300, NcoAs and p/CAF for their transcription. Therefore, although all the complexes are capable of being recruited by nuclear receptors, different complexes may be targeted to different hormone-inducible genes *in vivo*.



### 1.2.4. Summary

The precise regulation of gene expression is mediated by RTFs that specifically recruit co-complexes, containing remodelling, HAT and/or HDAC activities, that activate or repress genes respectively. The chromatin remodelling and HAT/HDAC mechanisms of these complexes (1) modify and remodel the structure of nucleosomes making the DNA accessible or inaccessible to the basal TFs such a TBP, resulting in transcriptional activation or repression; (2) the acetylation activity could acetylate non-histone proteins, such as p53, which enhances their DNA binding; (3) acetylation of histones or other proteins could act as a specific signal, similar to phosphorylation, promoting recognition by other factors. Therefore the rate of initiation and activation of transcription is controlled via multiple protein-protein interactions between the components of the PIC, regulatory transcription factors and various protein subunits of the co-activator complexes.

## 1.3 FOREBRAIN DEVELOPMENT

The development of the forebrain is tightly controlled during embryogenesis by many regulatory proteins, including RTFs. During gastrulation in vertebrates, signals from the "organizer" (discussed later) induce part of the ectoderm to adopt neural fates while the remainder of the ectoderm develops into epidermis. The resulting sheet of neuroepithelial cells, called the neural plate, then folds to form the neural tube. After neural tube closure, constrictions along the neural tube morphologically subdivide the future central nervous system (CNS) into the fore-, mid- and hindbrain and the spinal chord. The anterior neural tube is further subdivided into the future forebrain, into regions called prosomeres, which can be grouped into two sub-regions: the posterior diencephalon and the anterior prosencephalon (includes the telencephalon) (Puelles and Rubenstein 1993; Rubenstein et al 1994). This section of the review will discuss the role of Brain factor-1 (BF-1) in the development of the forebrain and will briefly summarize the "organizers" that secrete signals which induce the ectoderm to develop into neural tissue, and the patterning mechanisms along the A/P and D/V axes of the neural tube that regionalize the forebrain.



### 1.3.1. Induction And Patterning Of The Forebrain

A simplified overview of the induction and patterning of the forebrain is depicted in Figure 1.3. The ectoderm is initially fated to become epidermis via a Bone Morphogentic Protein (BMP)-4 signalling pathway. However, anti-BMP protein signals secreted by specialized structures called the "organizers", induce the ectoderm to become neural tissue. The regions of the ectoderm that develop into neural tissue are specified by the action of proneural genes, which specify which cells in the ectoderm will adopt neural fates. The resulting neuroectoderm then folds to form the neural tube and is further regionalized by the combined patterning mechanisms along its anterior-posterior (A/P) and dorso-ventral (D/V) axes.

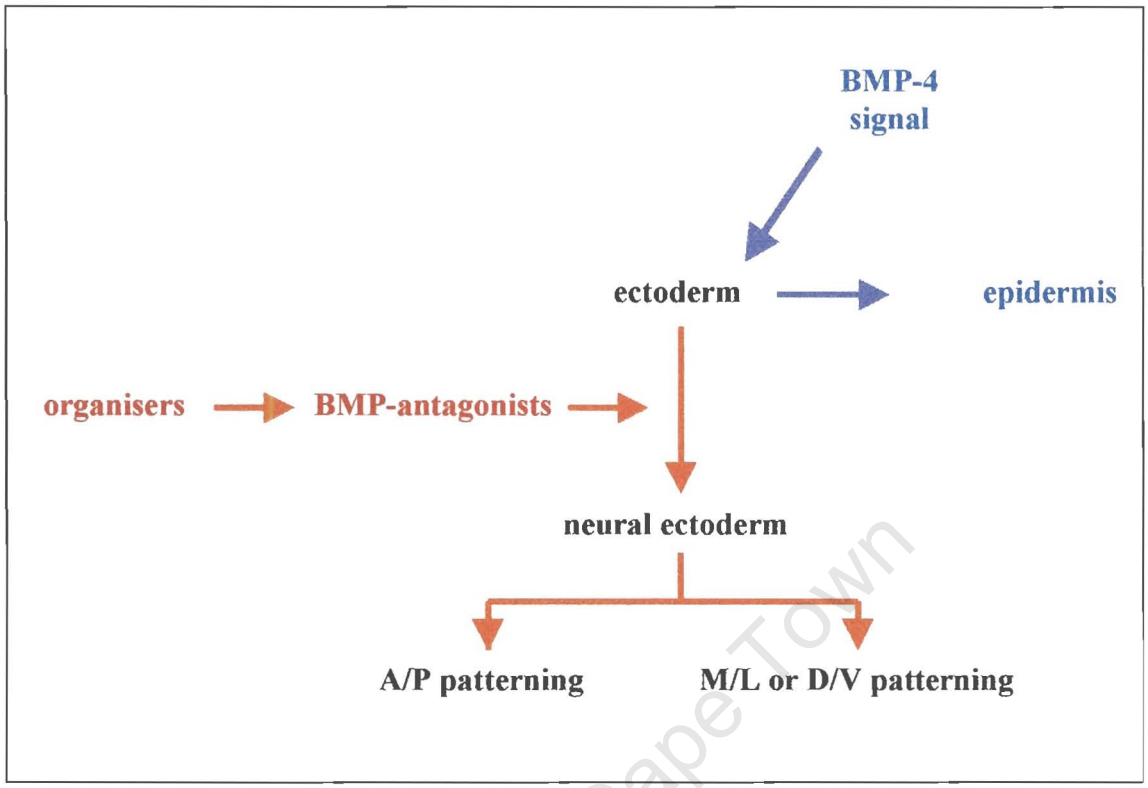


**Figure 1.3.** Flow diagram of neural induction and regional patterning of the forebrain.

This patterning occurs just after neural induction, and is also regulated via the signals secreted from the organizers, which can be spread in either a vertical or planar fashion. Vertical signals travel from prechordal mesendoderm (inductive tissue underlying the neural plate) to the target tissue (neural plate), whereas planar signals travel in the plane of the ectoderm from the ectodermal organizers (e.g. anterior neural ridge) to the target tissue (Doniach 1993; Ruis i Altaba 1994).

#### 1.3.1.1. Induction by the organizers

The concept of an "organizing centre" that controls the induction and patterning of forebrain development was introduced by Spemann and Mangold (1924). The classical organizer, called the prechordal plate (prechordal mesendoderm) or "organizer" has been found in many vertebrates such as amphibians (Spemann and Mangold 1924), chicken (Storey et al 1992; Waddington 1932), mouse (Beddington 1994), teleost (Oppenheimer 1936; Sagerstrom et al 1996; Shih and Fraser 1996) and zebrafish (Houart et al 1998) (Figure 1.4). The organizer secretes protein signals, most of which antagonise the epidermalising Bone Morphogenetic Proteins (BMPs) present in the ectoderm, resulting in the suppression of epidermal fates and promotion of neural fates. These secreted BMP antagonists include noggin (Smith and Harland 1992; Zimmerman 1996), follistatin (Hemmati-Brivanlou 1994, Xnr3 (Smith et al 1995; Hansen et al 1997) and chordin (Sasai et al 1994; Piccolo et al 1996) and they effect their antagonising function by binding BMPs directly (Piccolo et al 1996; Zimmermann et al 1996). Other organizer-secreted signals such as Cerberus (Bouwmeester et al 1996; Glinka 1997; Hsu et al 1998) and dickkopf-1 (Glinka 1998) are thought to antagonise the Wnt-signalling pathway, which is known to posteriorise neural tissue.

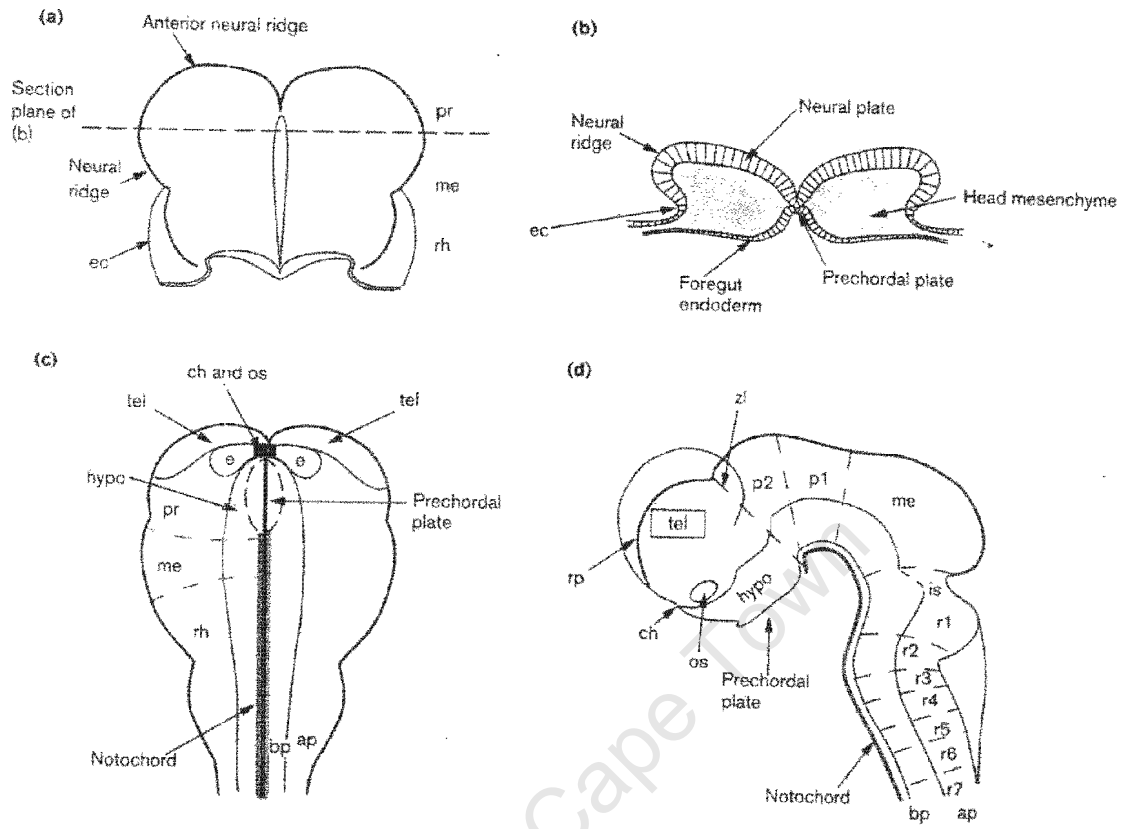


**Figure 1.4**. Various views of the morphology, tissue types and regional organization of the vertebrate neural plate and tube. **(a)** Dorsal view of the anterior neural plate showing the anterior neural ridge, neural ridge and non-neural ectoderm (ec). The approximate positions of the primordia of the prosencephalon (pr), mesencephalon (me) and rhombencephalon (rh) are shown. The dotted lined represents the plane of the section through the neural plate that generates the view in (b). **(b)** Cross-section view of the prosencephalic neural tube showing the relationship of the neuroectoderm to the non-neural ectoderm (ec), head mesenchyme, prechordal mesendoderm and foregut endoderm. **(c)** Schematic view of the neural plate showing the underlying axial tissues and the approximate location of several forebrain primordia: the hypothalamus (hypo) overlies the prechordal mesendoderm, and is just prior to the chiasmatic plate (ch) and optic stalks (os). Lateral to these structures is the primordia of the eyes (e). The location of the telencephalic vesicles (tel) map in a region similar to the expression of BF-1. **(d)** Schematic view of the neural tube showing the topographic relationships of the structures described in (a-c). Note that the neural ridge becomes the roof plate (rp) of the telencephalon. Abbreviations: Ap, alar plate; bp, basal plate; p1 and p2, prosomeres 1 and 2; r1-7, rhombomeres 1-7; zl, zona limitans. (*This is a copy of the figure and legend from Current Opinion in Neurobiology 8:19, 1998).*

More recently, other signalling centres that act later in forebrain development, have been identified. The anterior neural ridge (ANR) located at the junction between the anterior neural plate and the non-neural ectoderm (including part of the telencephalic anlagen) (Figure1.4), secretes various patterning molecules including BMPs and fibroblast growth factors (FGFs) e.g. Fgf8 (Shimamura et al 1997a,b). The Fgf8 produced by the ANR induces and maintains the expression of BF-1 in the anterior neuroectoderm, which stimulates and regulates the growth of the telencephalon (discussed later). The anterior visceral endoderm (AVE) is an extra-embryonic component that has been shown to induce and patterning in the forebrain at the onset of gastrulation (Thomas and Beddington 1996) and secretes proteins that are essential for head development such as a cerebus-like protein (Belo et al 1997) and homeobox genes such as LIM1 (Shawlot et al 1999), Hesx1 (Thomas and Beddington 1996) and Otx2 (Varlet et al 1997).



**1.3.1.2. Patterning of the Forebrain**

Patterning along the A/P axis of the neural tube is regulated by proteins secreted by several organizers such as the ANR, AVE, and the underlying prechordal plate. A/P patterning creates transverse zones of cells along the A/P axis, that have different competencies to respond patterning signals secreted from axial mesendoderm, including Sonic Hedgehog (SHH) (Grinblat et al 1998; Shimamaura 1997a,b; Shimamura et al 1995; Thomas and Beddington 1996; Mathers et al 1997). The axial mesendoderm and SHH also play a role in D/V patterning, where the role of SHH is much greater: for example, the SHH-/- mouse mutant displays both A/P and D/V defects, the latter being more severe. Ventral patterning is regulated by Sonic Hedgehog (SHH) which is expressed throughout the axial mesendoderm, including the prechordal plate (Echelard 1993; Roelink 1994; Tanabe 1995; Hynes 1995; Marti 1995; Chiang 1996; Ericson 1996) and dorsal patterning is controlled by members of the Transforming Growth Factor (TGF)β family (Basler 1993; Liem 1995; Tanabe and Jessell review 1996).

The ventral forebrain develops from the medial portion of the anterior neural plate which is specified by the prechordal mesendoderm (Sulik et al 1994; Seifert et al 1993). SHH is initially secreted from the prechordal mesendoderm, followed by expression in the medial neural plate, ventral neural tube and later from the midline of the forebrain, where it plays a role in the early bilateral subdivision of the eye field, patterning within the eye, development of the optic stalks and the hypothalamus (Shimamura et al 1995). The role of SHH in inducing ventral cell fates was confirmed by the induced expression phenotype of SHH-/- mutant mouse embryos, which lacked ventral neural fates throughout the neural axis resulting in the loss of ventral forebrain structures, failure of the eye field to subdivide and subsequent formation of a cyclopic eye. The remainder of the forebrain developed as a single vesicle and expressed molecular markers characteristic of the dorsal telencephalon (Chiang et al 1996). Since these defects are similar to those observed when the prechordal mesendoderm is removed in amphibians (Adelmann 1936a and 1936b; Li et al 1997b), it suggests that SHH constitutes or contributes to the ventral-forebrain patterning signal secreted from the prechordal mesendoderm.

The dorsal forebrain develops from the lateral neural plate and is regulated by proteins produced by cells located at the boundary region between the neuroectoderm and the non-neural ectoderm. These proteins include BMPs which are members of the TGBβ signalling pathway (BMPs) (Mehler et al 1997; Furuta et al 1997; Liem et al 1995; Dickinson et al 1995). BMPs are epidermalising proteins that are expressed in posterior neural plate at earlier developmental stages than in the anterior neural plate (Shimamura et al 1997a,b; Furuta et al 1997). In the anterior neural plate, BMPs activate the expression of dorsal midline genes, such as MsxI (Shimamura et al 1997a,b; Furuta et al 1997) and repress genes involved in regulating the growth of the telencephalon, such as Brain Factor-1 (BF-1) (Furuta et al 1997). The expression of BMP-antagonists in the forebrain primordia, e.g. noggin, potentially regulate their activity by binding them directly (Shimamura et al 1995; Knecht et al

1997). Loss of function experiments have shown that other regulatory proteins also are involved in patterning the lateral neural plate: for example mutations of the genes encoding proteins such as BF-1, Gli3, Pax6 and cNot1, result in various defects of the dorsal forebrain (Xuan et al 1995; Frantz 1994; Stoykova 1996; Masai 1997).

### 1.3.2. Role Of Brain Factor-1 In Forebrain Development

After neural induction, the conversion of ectodermal cells to neural precursors, results in the activation of regulatory genes. At the neural plate stage, most cells are actively dividing (Hartenstein 1989; Hardcastle 2000) and go through successive stages of specification that is defined by the sequential activation of different sets of proneural genes, usually encoding basic helix-loop-helix (bHLH) RTFs (Lee et al 1995; Chitnis and Kinter 1996; Ma et al 1996; Bellfroid et al 1998). This specification results in only a limited number of cells within the neural plate exiting the cell cycle and begin differentiating. The differentiation process is therefore controlled by the combined action of proneural and neurogenic genes: the proneural genes specify which regions of the ectoderm will form the neuroectoderm (Guillemot et al 1993; Zimmerman et al 1993; Ferreiro et al 1994; Turner and Weintraub 1994; Lee et al 1995; Ma et al 1996; Bellefroid et al 1996; Takebayashi et al 1997; Ravassard et al 1997; Bellefroid et al 1998; Dubois et al 1998); whereas the neurogenic genes limit the number of cells that will undergo differentiation (Figure 1.5). Neurogenic genes encode RTFs such as XNotch-1, its ligand XDelta-1 and the intracellular mediator XSu-(H), and their expression is activated by the proneural genes. A negative feed-back loop of regulatory interactions between the RTFs encoded by proneural and neurogenic genes, brings about this cell-limiting action (reviewed by Tanabe and Jessell 1996).

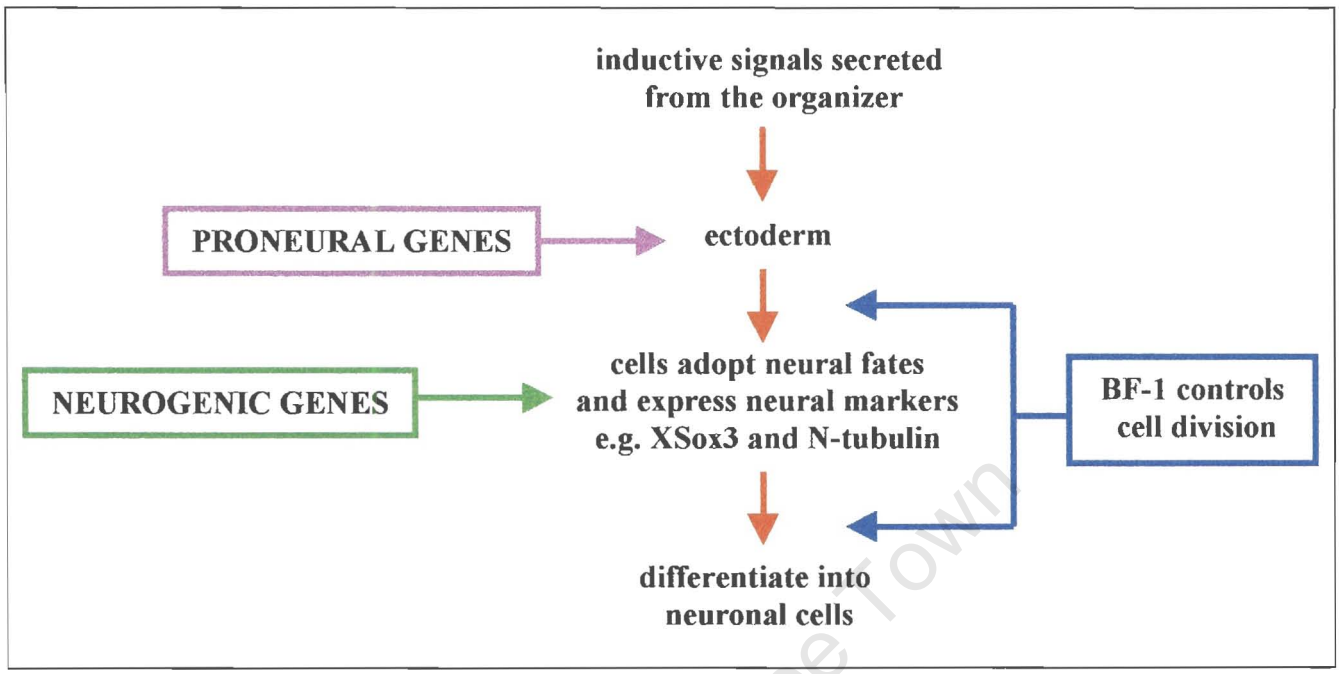


**Figure 1.5.** Flow diagram of the neural induction and differentiation pathway. After neural induction, the activity of proneural genes specify which cells in the ectoderm will adopt neural fates. Later in the pathway, the activity of neurogenic genes limit the number of cells that undergo differentiation into neurons.

Proneural and neurogenic genes have restricted expression patterns within the neural plate, which indicates that neuronal development takes place in a temporal and spatial manner: in the anterior neural plate, differentiation takes place in four regions only after the posterior neural tube has begun differentiating. These four regions are the telencephalon, ventral diencephalon, the epiphysis and olfactory bulbs (Papalopulu and Kinter 1996). These clusters gradually grow larger resulting in differentiation spreading to the rest of the brain.

The decision of the ectodermal cells to adopt a neural fate and to divide or differentiate are co-ordinated by the proneural and neurogenic RTFs during normal development. However, how are these processes mechanistically linked? One option is that there are no links and that there are RTFs that affect cell division but do not affect the cell fate, e.g. Optx2 promotes cell division without affecting cell fate in the Xenopus retina (Zuber et al 1999); or conversely, there are RTFs that mediate an epidermal to neural fate without affecting cell division. A second option, is that there are RTFs that acts as molecular links between processes, and current studies on BF-1 has made it a prime candidate to play this role, since it is involved in neural fate specification (Dou et al 1999) and cell division/differentiation (Li et al 1995a ; Chang et al 1995; Li and Vogt 1993; Xuan et al 1995; Hardcastle 2000).

BF-1 and its close relative, BF-2, are winged helix transcription factors that are essential for the development and regionalization of the forebrain. Both BF-1 and BF-1 have restricted expression patterns in the forebrain where they exert their functions. I will first briefly introduce the role of BF-2 in the forebrain, and then focus the rest of the discussion on BF-1.

BF-2 plays an important role in patterning the forebrain, optic vesicle and kidney and is expressed in these tissues. In the forebrain, BF-2 is expressed in the rostral diencephalon and temporal half of the optic stalk and is complementary to that of BF-1, creating a defined expression boundary within the developing forebrain (Hatini et al 1994). Studies in *Xenopus* showed that BF-2 was a transcriptional repressor that converted the ectoderm into neural tissue at the expense of non-neural tissue (Francesca et al 1998) and that the dorsalizing activity of BF-2 specified the dorso-lateral mesoderm by interfering with BMP4 and Xwnt8 signalling pathways in these tissues. In the dorso-lateral mesoderm, BF-2 repressed ventral mesoderm genes such as BF-1 and promoted dorso-lateral genes (e.g. XmyoD). The combined action of BMP4 antagonists and inhibition of BMP4 and its downstream genes by BF-2 creates a gradient of BMP4 activity needed for the development of the mesoderm, anterior neural plate and neural crest. BF-2 was also shown to play a role regulating the migration of neural crest cells, due to its repression of genes that are needed for the initiation of neural crest migration. (Gomez-Skarmeta et al 1999).

Similarly, BF-1 is essential for the development of the cerebral hemispheres and olfactory neuroepithelium and was also shown to act as transcriptional repressor (Li et al 1995). BF-1 was first isolated from developing rat brains by Tao and Lai (1992) and since then several homologues of BF-1 have been cloned from humans (Murphy et al 1994), chicken (Li and Vogt 1993), mouse (Li et al 1996), *Xenopus* (Gomez-Skarmeta et al 1999; Francesca et al 1998), zebrafish (Toresson et al 1998) and amphioxous (Toresson et al 1998). An alignment of the amino acid sequences of the BF-1 homologues is given in Figure1.6. The characteristic fork head DNA binding domain was observed as a "winged helix" in X-ray diffraction patterns of its crystal structure, and has since been used to classify this protein family (Clark et al 1993).

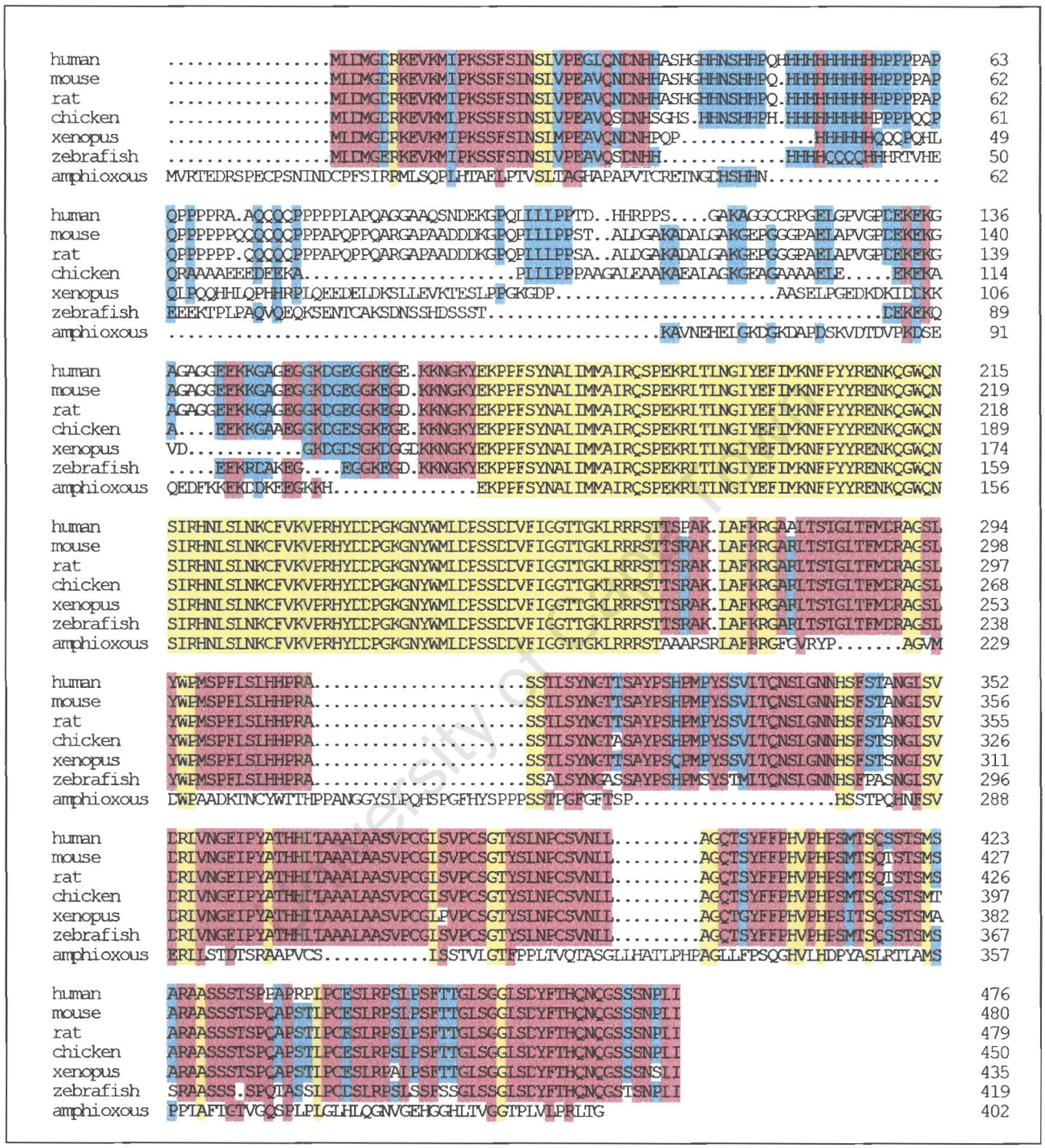


**Figure 1.6**. Alignment of the amino acid sequences of the BF-1 homologues. Shaded areas represent the following percentage homology: yellow (100%), pink (>75%) and blue (>50%).

The C-terminal end of the protein has been shown to act as a transcriptional repressor by (Li et al 1995a). At the N-terminal end of the protein there is a proline and glutamine rich region containing a striking string of ten histidine residues (noted by E. Rumbak). Although this string of ten consecutive histidines is found in many developmental RTFs, the function of this motif is unknown, but it is tempting to speculate that it may act as an activation domain. Expression studies showed that the expression pattern of BF-1 was highly restricted to telencephalic neuroepithelium and the nasal half of the optic stalk and retina and olfactory placode during embryogenesis (Hatini et al 1994; Lai and Tao 1992) and persists in the adult brain in the telencephalon derived structures including the cerebral cortex, the hippocampus, olfactory bulbs and the basal ganglia (Dou et al 1999). BF-1 has been shown to have multiple functions in forebrain development, namely (1) regional patterning (i.e. specify positional identity), (2) regulating cell proliferation and (3) regulating differentiation (Figure 1.7).



**1.3.2.1. BF-1 specifies positional identity**

BF-1 has also been shown to act earlier in the differentiation pathway than previously thought, and specifies regional identity: i.e. neural fate specification (Hatini et al 1999; Huh et al 1999; Dou et al 1999). Hatini et al (1999) showed that expression of BF-1 within the sensory cranial nerve ganglia, distinguished cells that arose from the placodes from those derived from the neural crest. BF-1 was shown to be involved in regional pattering of the eyes (Huh et al 1999). Dou et al (1999) showed that BF-1 regulated both progenitor cell proliferation and regional patterning in the forebrain by regulating the expression or activity of inductive signals which act on the telencephalic neuroepithelium. They showed that targeted disruption of the BF-1 gene in mice resulted not only in hypoplasia of the cerebral hemispheres but also dorsal-ventral patterning defects.

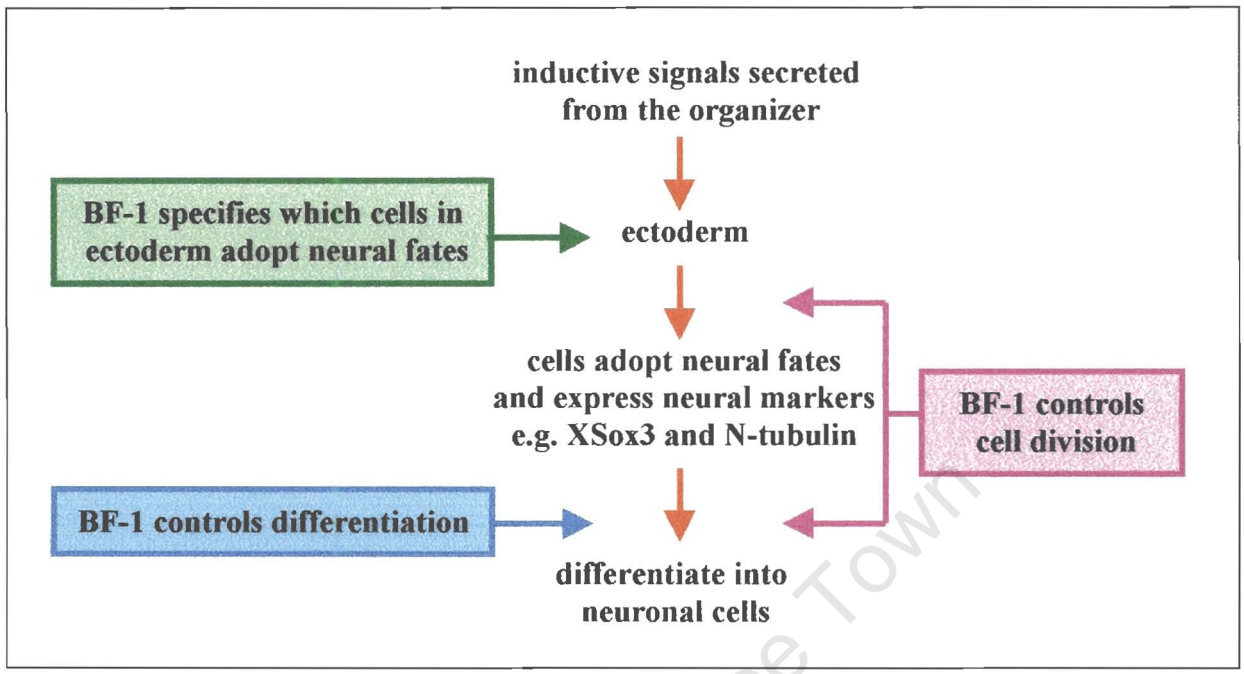


**Figure 1.7**. BF-1 can act as both a proneural and neurogenic protein in the neuronal differentiation pathway. BF-1 specifies regional identity, and controls cell proliferation and differentiation.

### 1.3.2.2. BF-1 regulates the proliferation and differentiation of cells

Studies on BF-1 null mutant mice (Xuan et al 1995) and the avian homologue of BF-1, the oncogene *qin* (Li et al 1995a ; Chang et al 1995; Li and Vogt 1993) have shown that BF-1 regulates the growth of neuroepithelial cells. Mice that had a null mutation for BF-1, failed to develop cerebral hemispheres and olfactory neurons due to a reduced rate of proliferation of the telencephalic neuroepithelium in these tissues. In these mutants the dorsal telencephalic neuroepithelial cells differentiated prematurely resulting in the early depletion of the progenitor population (Xuan et al 1995). More recent studies have showed that BF-1 regulates cell proliferation in a dose-dependent manner, where high BF-1 concentrations promote cell division and low concentrations BF-1 inhibit cell division (Bourguignon et al 1999). The molecular mechanism by which BF-1 enhances or blocks cell division was shown by Hardcastle et al (2000) to involve suppressing or activating a cell cycle inhibitor protein, $p27^{XICI}$ in a dose-dependent manner: at high BF-1 doses promoted cell division by suppressing the activity of $p27^{XICI}$ ; but at low doses, BF-1 promoted cell differentiation by inducing the activity of $p27^{XICI}$. They also provided evidence that suggested that XBF-1 regulates the activity of $p27^{XICI}$ at the transcriptional level.

BF-1 has also been shown to control the timing of differentiation in the forebrain: e.g. in BF-1 knockout mice, differentiation of the telencephalic neuroepithelium occurred prematurely (Xuan et al 1995). The activity of BF-1 in regulating differentiation was also shown to be dose-dependent (Bourguignon et al 1999), where low or high doses of BF-1 either activated or suppressed neuronal differentiation respectively (Bourguignon et al 1999; Hardcastle et al 2000). Therefore cells that express high doses of BF-1 divide and do not differentiate while cells that express low doses, stop dividing and differentiate. These dual functions of BF-1 in cell fate specification and cell division or differentiation were shown to be distinct (Hardcastle 2000).

The dose dependence of BF-1 *in vivo*, may be regulated by interactions with other factors: e.g. co-injection of Xsox3 with XBF-1 converted a low dose phenotype to a high dose one, therefore suggesting that a high-dose effect of BF-1 is determined by the presence of Xsox3, whereas a low dose effect may be determined by the absence of Xsox3 or by interactions with other factors (Hardcastle et al 2000).

### 1.2.3. Summary

Therefore the ability of BF-1 to act as both a proneural and neurogenic protein, suggests that BF-1 acts as a molecular link between neural fate specification and cell division or differentiation. Therefore the in order to investigate how BF-1 controls these various processes, the aim of this M.Sc. study was to identify proteins which interact with BF-1 in the proliferating neuroepithelium using the Yeast Two Hybrid System (Fields and Song 1989) and to characterise these interactions.

# CHAPTER 2 MATERIALS AND METHODS

## 2.1. ISOLATION AND PURIFICATION OF OP6 TOTAL RNA AND mRNA

2.1.1. Tissue Culture and Maintenance of Cell Lines

2.1.2. Total RNA Extraction from OP6 cells

2.1.3. Purification of mRNA from OP6 total RNA.

## 2.2. CONSTRUCTION OF "BF-1 BAIT" IN pGBT9

2.2.1. Cloning and Transformation into *E.coli*

2.2.2. Screening Transformants for Inserts

2.2.3. Manual Dideoxy DNA Sequencing of Positive Clones

2.2.4. Testing the BF-1 bait encoded by pGALDBD/BF-$1_{(26\text{-}480aa)}$ for self-activation of the *lacZ* reporter gene in *Saccheromyces cerevisiae* Y190.

## 2.3. CONSTRUCTION OF AN OP6 cDNA TWO HYBRID LIBRARY

2.3.1. First strand cDNA synthesis

2.3.2. Second strand cDNA synthesis

2.3.3. Adaptor Ligation

2.3.4. Phosphorylation of Adaptor-ligated ds cDNA

2.3.5. Size fractionation of the Adaptor-Ligated ds cDNA

2.3.6. Trial Ligation of ds cDNA into the pGAD10 vector

2.3.7. Transformation of the library plasmid DNA into *E.coli*.

2.3.8. Large-Scale Ligation of ds cDNA into pGAD10

2.3.9. Amplification and Titering of the Plasmid Library

## 2.4. SCREENING THE OP6 cDNA TWO-HYBRID LIBRARY FOR PROTEIN-PROTEIN INTERACTIONS

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## 2.1. ISOLATION AND PURIFICATION OF OP6 RNA

### 2.1.1. Tissue Culture and Maintenance of Cell Lines

The OP6 cells were grown under 10% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM) that was supplemented with de-complemented fetal calf serum (FCS) at a final concentration of 10%. The cells were grown at 33°C until they had reached 90-100% confluency (3.75 X $10^7$cells). For each 75cm$^2$ flask, the DMEM medium was aspirated off and the cells washed in 10ml of 1 X PBS (140mM NaCl, 2.7mM KCl, 8mM $Na_2HPO_4.7H_2O$, 0.7mM $CaCl_2$, 0.8mM $MgCl_2$ pH7.3). 3ml of Trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA, 1 X PBS pH7.8) was added and incubated for 2-3 minutes with occasional agitation to free the loosened cells. The trypsin was inactivated by the addition of 7ml of DMEM containing 10% FCS. The cells were centrifuged at 1000rpm for 3-5 minutes, the supernatant fluid aspirated off and the pellet stored at – 70°C.



### 2.1.2. Total RNA Extraction from OP6 cells

Total OP6 RNA was extracted from the OP6 cells (passage numbers 9-11 ) as described by Chomczynski et al (1987). The RNA pellet was air dried and resuspended in 50µL of DEPC water. 3µL of the RNA was kept for analysis and the remainder was stored at –70°C. The quality of the RNA was checked by determining the ratio of the absorbances at $A_{260}/A_{280}$ and by running 1µL of RNA on a 1% agarose gel that contained 2.2M formaldehyde. The concentration of the RNA was determined by measuring the $A_{260}$. All solutions, except Tris-containing buffers, used for the RNA work, were treated with DEPC to destroy any endogenous RNAses. To each solution, DEPC was added to a final concentration of 0.1% and gently shaken overnight at 37°C. The solutions were then autoclaved for 30 minutes to remove any traces of DEPC. For Tris-containing buffers, solutions were prepared using DEPC-treated water and an ultra-pure Tris stock designated for RNA work only.

### 2.1.3. Purification of mRNA from OP6 total RNA.

Messenger RNA was purified from OP6 total RNA using the PolyATtract® mRNA Isolation System (Promega) as per the manufacturer's instructions. The eluted RNA fractions were pooled and stored at –70°C. The concentration and purity of the mRNA was determined by measuring the absorbance at $A_{260}$ and $A_{280}$. To estimate the mRNA concentration, it was assumed that a 40µg/ml mRNA solution will have an absorbance of 1 at 260nm. The purity of the mRNA was estimated by determining the $A_{260}/A_{280}$ absorbance ratio, which for pure mRNA is >=2.0.The quality of the isolated mRNA was checked by running 1µl of the mRNA on a formaldehyde- denaturing 1% agarose gel.

## 2.2. CONSTRUCTION OF "BF-1 BAIT" IN PGBT9

The BF-1 cDNA (nucleotides 515-2338; amino acid residues 26-480) was subcloned from the rat pBF-1 cDNA clone (Tao and Lai 1992) in frame in the pGBT9 plasmid, containing the GAL4 DB. The N-terminal domain of BF-1 was not subcloned, since we were interested to see what proteins interacted with reported C-terminal transcriptional repressor domain (Li et al 1995a) and the proline, glutamine and histidine-rich region, which may putatively act as an activation domain. The cloning strategy is outlined in Figure 2.1.

### 2.2.1. Cloning of pGalDBD/BF-1$_{(26\text{-}480aa)}$ and Transformation into *E.coli*

5µg of pBF-1 was digested with 10U each of *Eco*81I and *Stu*I in 1 X Buffer M (10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 1mM DTT, 50mM NaCl) overnight at 37°C. 1µL of the digest was run on a 1% agarose-TAE gel to check that the 1723bp insert had been excised. Similarly, the pGBT9 Two Hybrid plasmid was digested with 10U of *Sma*I in 1X Buffer T (33mM Tris-HCl pH7.9, 10mM Mg acetate, 0.5mM DTT, 66mM K-acetate containing with 0.1% BSA) overnight at 25°C. 1µL of the digest was run alongside 1µg of uncut plasmid DNA on a 1% agarose-TAE gel to check that the digestion was complete. The pGBT9 plasmid was then digested with 10U of *Eco*RI in 1X Buffer T (33mM Tris-HCl pH7.9, 10mM Mg acetate, 0.5mM DTT, 66mM K-acetate, 0.1% BSA) overnight at 37°C. Both the BF-1 insert (1723bp) and pGBT9 plasmid were run on a 1% low melting point gel (Nusieve) and purified using the GeneClean Kit as per the manufacturer's specifications (BIO101). The *Sma*I blunt end of the PGBT9 plasmid was ligated to the *Stu*I blunt end of the BF-1 insert. 4.0µL of each of the purified pGBT9 plasmid and BF-1 insert was ligated overnight at 16°C in a total volume of 20µL in 1X Ligation Buffer (30mM Tris-HCl pH7.8, 10mM $MgCl_2$, 10mM DTT, 0.5mM ATP) containing 10U of T4 DNA Ligase. The *Eco*81I 5′ overhang of the BF-1 insert and the *Eco*RI 5′ overhang of the pGBT9 plasmid were filled-in to generate blunt ends. 10µL of above

ligation reaction was incubated for 30 minutes at 30°C in 1X T4 Polymerase Buffer (25mM Tris-HCl, 15mM $(NH_2)_2SO4$, 7mM $MgCl_2$, 0.1mM EDTA, 10mM 2-mercaptoethanol, 0.02mg/ml BSA; pH8.8) containing 2U of T4 DNA Polymerase and 0.5mM of each dNTP.

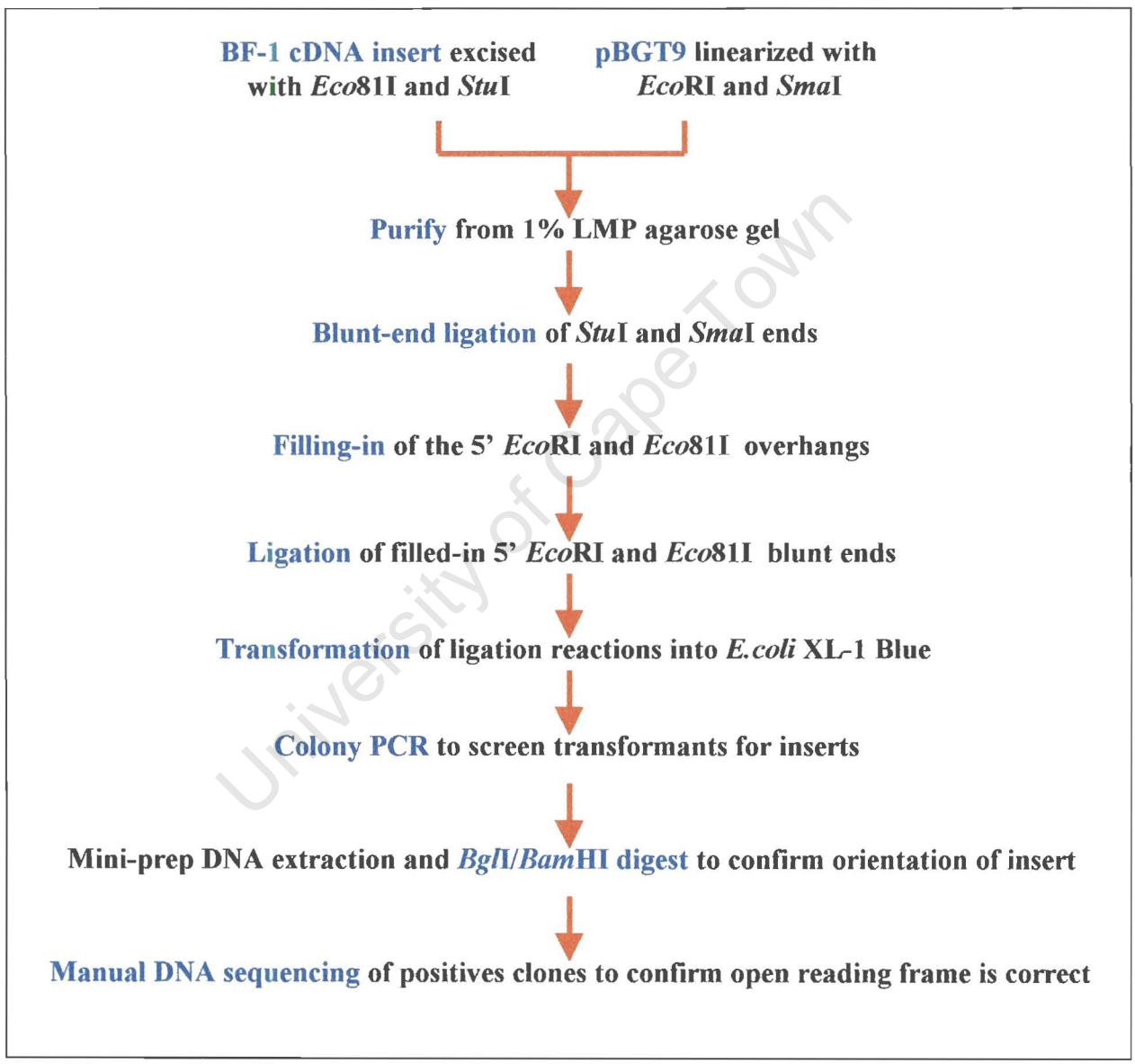




**Figure 2.1.** Outline of the strategy to subclone BF-1 into pGBT9.

The reaction was then incubated at 75°C for 15 minutes to heat denature the T4 DNA Polymerase. The blunted *Eco*81I and *Eco*RI 5'-ends were ligated by incubating 20μL of the above blunt-ending reaction at 16°C overnight in 1X T4 DNA Ligase Buffer (30mM Tris-HCl pH7.8, 10mM $MgCl_2$, 10mM DTT, 0.5mM ATP) containing 1 unit of T4 DNA Ligase. The entire ligation reaction was transformed via heat shock into *E.coli* XL-1 Blue cells prepared by the method of Inoue et al (1990). Transformation controls included checking the background of self ligated plasmid, competency of the cells and contamination of the competent cells (minus DNA control). All reactions were plated out on LB plates containing 100μg/ml ampicillin and incubated at 37°C overnight, and the colonies counted the next day. The competency controls were plated out at $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions.



**2.2.2. Screening Transformants for Inserts**

The transformants were picked from the agar plate and resuspended in 20μL of sterile water. 5μL of the resuspended colony was added to 15μL of PCR cocktail (1X Gibco PCR Buffer, 50mM $MgCl_2$, 0.2mM of each dNTP, 10μM of both the forward (5'-GCGACATCATCATCGGAA-3') and reverse (5'- GCATGCCGGTAGAGGTGT-3') primers, and 0.125U of recombinant Taq. The PCR reactions were amplified under the following conditions: 94°C for 2 minutes for 1 cycle; 94°C for 45 seconds, 54°C for 45 seconds, 72°C for 45 seconds for 30 cycles. The final extension was at 72°C for 7 minutes for cycle. 10μL of each PCR reaction was loaded with 2μL of 6X DNA loading dye and run on a 1% agarose TAE gel along with a λDNA *Pst*1 size marker. The water control substituted PCR water for cell suspension. The negative control contained 100μg of empty pGBT9 plasmid and the positive control contained 100μg of pVA3. All the controls were amplified under the same conditions as the experimental samples. For those clones which contained inserts, 1ml of 2 X YT containing 100μg/ml ampicillin was added to the remaining 15μL of resuspended cells and

grown at 37°C overnight. Plasmid DNA was extracted from the 1ml cultures using the standard mini-prep method of Sambrook et al (1989). Since the cloning strategy used resulted in 50% of the inserts ligating in either orientation to the vector, a diagnostic *Bgl*I / *BamH*I digest was used to identify those clones which contained the BF-1 insert in the correct orientation 5.0μL of the mini-prep DNA was added to 15μL of *Bgl*I / *BamH*I digestion cocktail (33mM Tris-HCl pH7.9, 10mM Mg acetate, 0.5mM DTT, 66mM K-acetate containing with 0.1% BSA containing 10U each of *Bam*HI and *Bgl*I). The reactions were incubated at 37°C overnight and run on a 1% agarose-TAE gel. Clones that displayed the correct digest pattern were selected for DNA sequencing. Glycerol stocks for positive clones were made by adding 250μL of 50% glycerol to 500μL of culture, and were stored at –70°C.



### 2.2.3. Manual Dideoxy DNA Sequencing of Positive Clones

The positive clones were sequenced using the dideoxy chain termination method of Sanger et al (1977) to confirm that no rearrangements had occurred during the cloning process. The Sequenase ™ version 2.0 DNA Sequencing Kit (United States Biochemical Corporation) was used as per the manufacturer's instructions. The sequencing reactions were run on a 6% polyacrylamide DNA sequencing gel. The gel was incubated in fixer (5%MeOH, 5% acetic acid) and dried. The dried gel was placed in an X-ray cassette with sensitive X-ray film for 48 hours and the autoradiograph developed, fixed and the sequences read and recorded.

### 2.2.4. Testing the BF-1 bait encoded by pGALDBD/BF-1$_{(26\text{-}480aa)}$ for self-activation of the *lacZ* reporter gene in *Saccheromyces cerevisiae* Y190.

Plasmid pGalDBD/BF-1$_{(26\text{-}480aa)}$ was transformed into the *S. cerevisiae* Y190 strain and checked that it did not self-activate the reporter gene. The bait plasmid, pGALDBD/BF-1$_{(26\text{-}480aa)}$, was transformed into the yeast host strain, *S. cerevisiae* Y190, by electroporation (Becker and Guarente 1991) and the transformants grown at 30°C for 3-4 days on SD-Leu

medium plates containing 1M sorbitol. In order to confirm the presence of pGALDBD/BF-$1_{(26-480aa)}$ plasmid in the yeast, a single yeast colony transformed with pGALDBD/BF-$1_{(26-480aa)}$ was picked and resuspended in 50μL of sterile water, and vortexed for 2 minutes with 0.3g of acid washed glass beads. The reaction was incubated at 95°C for 10 minutes and vortexed for a further 10 minutes to lyse the yeast cells. 25μL of the lysed yeast cells was added to 25μL of PCR cocktail (1X Gibco PCR Buffer, 0.2mM of each dNTP, 2.5mM $MgCl_2$, 0.5μM of the forward primer (5'-CGCGTTTGGAATCACTACAGG -3'), 0.5μM reverse primer (5'-GCATGCCGGTAGAGGTGT-3') and 0.5 Units of recombinant Taq). The PCR reaction was amplified under the following conditions: 94°C for 2 minutes for 1 cycle; 94°C for 45 seconds, 54°C for 45 seconds, 72°C for 45 seconds for 30 cycles; 94°C for 45seconds, 55°C for 45 seconds, 72°C for 45 seconds for 30 cycles. The final extension was at 72°C for 7 minutes for cycle. 10μL of each PCR reaction was run on a 1% agarose-TAE gel. PCR controls included a water control, yeast negative control and a positive control. For the water control, 25μL of yeast lysate was substituted with PCR water. For the yeast negative control, 25μl of colony lysate from an untransformed Y190 yeast colony was added to 25μL of PCR cocktail. For the positive control, 50ng of pGALDBD/BF-$1_{(26-480aa)}$ plasmid DNA was added to 1 X PCR cocktail in a final reaction volume of 50μL. All the controls were amplified under the same conditions as the experimental samples. The transformants were then tested for β-galactosidase activity using the colony lift filter method of Breeden and Nasmyth (1985). Positive controls for the β-galactosidase assay included screening yeast colonies transformed with the pCL1 plasmid encoding the wild-type GAL transcriptional activator or with plasmids pVA3 and pTD1, which encode interacting proteins.

## 2.3. CONSTRUCTION OF AN OP6 cDNA TWO HYBRID LIBRARY

A control cDNA library was constructed in parallel to the OP6 cDNA library using mRNA supplied in the Clontech Matchmaker Two Hybrid cDNA Construction kit. The control reactions followed the same methods up to and including the transformation into *E.coli* DH5α.

### 2.3.1. First strand cDNA synthesis

5μg of purified OP6 mRNA was added to two separate tubes which contained 5μl of either Random $p(dN)_6$ or Oligo $(dT)_{25}$ primers. Sterile DEPC-treated water was added to a final volume of 12.5μl and the contents gently mixed. The reactions were incubated at 70°C for 3 minutes in a water bath to heat denature the mRNA to get rid of any secondary structure. The reactions were then incubated on ice for 2 minutes to allow the primers to anneal to the mRNA. The contents of the tubes were briefly spun down in a bench top centrifuge at maximum speed for 30 seconds. To each tube the following components were added: 2.2μl sterile DEPC-water, 5.0μl 5X First Strand Buffer (250mM Tris pH8.3, 30mM $MgCl_2$, 375mM KCl) 0.5μl DTT, 1.3μl dNTP mix (20mMdATP, 20mM dCTP, 20mM dGTP, 20mM dTTP), 1.0μl of [α-$^{32}$P]dCTP (800Ci/mmol) and 2.5μl (500 Units) of MMLV Reverse Transcriptase (MMLV-RT). The contents of the tubes were gently mixed and the reactions incubated at 42°C for 1.5 hours. The reactions were then placed on ice while the components for second strand synthesis were added.



### 2.3.2. Second strand cDNA synthesis

While the reactions were kept on ice, the following was added: 123.5μl of sterile DEPC-water, 40μl 5X Second-strand buffer (500mM KCl, 50mM Ammonium sulphate, 25mM $MgCl_2$, 0.75mM β-NAD, 100mM Tris pH7.5, 0.25μg/ml Bovine serum Albumin (BSA), 1.5μl dNTP mix (20mMdATP, 20mM dCTP, 20mM dGTP, 20mM dTTP) and 10μl Second-strand

enzyme cocktail (0.25 units/µl RNase H, 6 units/µl *E.coli* DNA Polymerase I, 1.2 units/µl *E.coli* DNA Ligase). The contents of each tube was gently mixed and the reactions incubated at 16°C for 2 hours. 3µl (15 units) of T4 DNA Polymerase was added and the reactions incubated at 16°C for a further 30 minutes to blunt-end the cDNA transcripts. The second strand synthesis was terminated by adding 10µl of 0.2M EDTA. 200µl of phenol:chlorofom:isoamyl alcohol (25:24:1 pH7.5) was added to each tube and mixed by gentle continuous inversion for 2 minutes. The reactions were centrifuged at 12000 rpm for 2 minutes and the top aqueous layer from each tube transferred and pooled into a clean sterile tube. An equal volume (400µl) of chloroform:isoamyl alcohol (24:1) was added and gently mixed by continuous inversion for 2 minutes. The reactions were centrifuged at 12000rpm for 2 minutes and the top aqueous layer transferred to a clean sterile tube. The reaction was divided by transferring half (200µl) to a separate tube. To each tube 200µl of 4M ammonium acetate and 1ml of ice-cold 95% ethanol was added and gently mixed. The reactions were incubated at –20°C overnight. The reactions were centrifuged at 13000rpm for 35 minutes and the supernatant carefully aspirated off without disturbing the pellet. The cDNA pellet was air dried for 15 minutes and resuspended in 15µl of DEPC water.



### 2.3.3. Adaptor Ligation

To each 15µl of resuspended cDNA the following was added: 3µl 10 X Adaptor buffer (500mM Tris pH7.8, 100mM $MgCl_2$, 100mM DTT), 3µl 10mM ATP, 7µl 50µM *Eco*RI adaptors and 2µl T4 DNA Ligase (100U/µl). The components were gently mixed and the reaction incubated at 16°C overnight. The reaction was stopped by adding 3µl 0.2M EDTA. The volume of each reaction was increased to 100µl by adding 70µl of sterile DEPC-water. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1;pH8.0) was added and gently mixed for 2 minutes by inversion. The reactions were centrifuged at 13000rpm for 2 minutes

and the top aqueous layers of both reactions transferred to the same clean tube. An equal volume (200µl) of chloroform:isoamyl alcohol (24:1) was added and gently mixed by continuous inversion for 2 minutes. The reactions were centrifuged at 13000rpm for 2 minutes and the top aqueous layer transferred to a clean tube. The adaptor-ligated cDNA was precipitated by adding 1µl of Microcarrier (Life Technologies), 20µl 3M NaAc and 500µl of ice-cold 95% ethanol and incubated overnight at –20°C. The adaptor-ligated cDNA was pelleted by centrifuging at 13000rpm for 35 minutes. The supernatant was carefully aspirated off and the pellet air dried for 15 minutes.

### 2.3.4. Phosphorylation of Adaptor-ligated ds cDNA

The pellet was resuspended in 14µl of sterile DEPC-water and the following components added: 2µl 10 X adaptor buffer (500mM Tris pH7.8, 100mM $MgCl_2$, 100mM DTT)2µl 10mM ATP and 2µl of T4 Polynucleotide Kinase (30 units/µl). The reactions were incubated at 37°C for 30 minutes. The phosphorylation reaction was terminated by adding 2µl of 0.2M EDTA and incubation at 70°C for 15 minutes. The reactions were stored on ice while the cDNA fractionation columns were equilibrated.



### 2.3.5. Size fractionation of the Adaptor-Ligated ds cDNA

The adaptor-ligated ds cDNA was size fractionated so that 80% of the cDNA fragments smaller than 300bp and 100% of the nucleotides and unligated adaptors were removed. The top and bottom caps were removed from the column and the storage buffer allowed to drain out completely. Care was taken to remove any air bubbles trapped in the column. The cDNA fractionation columns (Clontech) were equilibrated by adding 750µl of STE buffer (30mM NaCl, 10mM Tris pH7.8, 0.5mM EDTA). The buffer was allowed to completely drain from the column. An additional 750µl of STE buffer was added and allowed to drain until the buffer

was just above the resin bed. 1µl of 1% xylene cyanol was added to the adaptor-ligated ds cDNA and gently mixed. The adaptor-ligated ds cDNA was loaded onto the top and middle of the resin bed and the first fraction immediately collected. The tube that contained the cDNA was rinsed with 75µl of STE buffer and was loaded onto the column. Only once the sample had been completely absorbed into the resin was a further 100µl of STE buffer was added to the column. Collection of the eluate (fraction 1) was continued until a final volume of 200µl was collected. Thereafter single-drop fractions were collected for a total of 20 fraction. After 10 fractions the column stopped flowing and a further 500µl of STE buffer was added and single-drop fractions collected. The cpm of each fraction was measure using automatic Cerenkov counting scintillation counter. The fraction that contained the highest cpm was pooled with all the preceding fractions that contained more than 400cpm. 1µl of the pooled fractions were run on a 1% agarose gel which was blotted on Whatmann 3MM paper and sealed in plastic. The gel was placed in a cassette with x-ray film and exposed overnight at –70°C. The following day the autoradiograph was developed and the quality and size of the size fractionated cDNA examined. The size-fractionated adaptor-ligated ds cDNA was precipitated by adding 1.5µl of glycogen, 1/10 the volume of 3M NaAc pH4.8 and 2.5 volumes ice-cold 95% ethanol. The cDNA was precipitated overnight at –20°C and centrifuged at 13000rpm for 35 minutes at 4°C. The cDNA pellet was resuspended in 8µl of sterile DEPC-water. The concentration of the ds cDNA was estimated by spotting 1µl of the resuspended cDNA onto an ethidium bromide agarose gel together with standard concentrations on DNA.

### 2.3.6. Trial Ligation of ds cDNA into the pGAD10 vector

Three different ligation reactions were carried out using different amounts of cDNA in order to determine what the optimal ratio of cDNA to vector DNA. Both positive and negative control ligation reactions were carried out. The positive control reaction included 100ng of intact pUC19 DNA whereas the negative control did not include any DNA. For all the ligation reactions 1.0µl of *Eco*RI-digested and dephosphorylated pGAD10 vector (0.15µg/µl), 10.0µl Plasmid ligation buffer (Clontech patent), 1.0µl 10mM ATP and 1.0µl T4 DNA Ligase (100units/µl) was added. For Ligation reactions 1, 2 and 3, 0.5µl, 1.0µl and 1.5µl of cDNA was added respectively. The unused cDNA was stored at 4°C for later use. The ligation reactions were gently mixed and incubated at 16°C for exactly 30 minutes. To each reaction 80µl of sterile DEPC-water and 1.5µl glycogen was added and mixed well. 280µl of ice-cold 95% ethanol was added and mixed gently. The reactions were incubated at –70°C overnight. The DNA was pelleted by centrifuging at 13000 rpm for 35 minutes. The supernatant was carefully aspirated off and the pellet air dried for 15 minutes. The DNA pellet was resuspended in 5µl of DEPC-water.



### 2.3.7. Transformation of the library plasmid DNA into *E.coli.*

*E.coli* DH5α electrocompetent cells were purchased from Clontech (C2022-1) and stored at -70°C. The cells were thawed on ice and used immediately. To each ligation reaction and positive and negative controls, 40µl of electrocompetent cells was added and mixed gently. The DNA/cell mixture was transferred to a pre-chilled electroporation cuvette (Biorad 0.2cm gap) and the sample electroporated using a GenePulserII Electroporator (Biorad) set at 2.5kV, 200Ω and 25µF for 4.5-5.0msec. Immediately after pulsing, 500ml of SOC medium (0.5% yeast extract, 2% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$, 10mM $MgCl_2$, 20mM glucose) was added to the cuvette and the cells suspension transferred to a sterile 1.5ml tube.

The cuvette was rinsed with 500µl of SOC medium and the wash pooled with the cell suspension collected previously. The cells were incubated at 37°C for 1 hour with gentle shaking. Both the positive and negative control reactions were plated LB agar plates that contained 100mg/ml ampicillin and incubated overnight at 37°C. For the negative (no DNA) control 100µl of the reaction was plated directly; whereas for the positive control (pUC19) reactions 100µl of $10^{0}$,$10^{-1}$, $10^{-2}$and $10^{-3}$ dilutions in SOC medium were plated. For each of the OP6 sample reactions, 10µl of the cell suspension was added to 40µl of SOC medium and mixed well. The remainder of the cell suspension was stored at 4°C. 1µl of the diluted cells was plated on LB agar plates that contained 100mg/ml ampicillin and incubated overnight at 37°C. Similarly, the remaining 49µl of cells was plated onto LB agar plates that contained 100mg/ml ampicillin and incubated overnight at 37°C. The next day the plates were examined and the number of transformants counted. The transformation efficiency and ligation efficiencies for each ligation reaction was determined. The quality of the unamplified library was tested by determining the percentage of recombinant clones for each ligation reaction. 15 independent colonies from each trial ligation reaction was picked and grown overnight at 37°C in 1ml of LB broth containing 100mg/ml ampicillin. The plasmid DNA was extracted (Sambrook et al 1989) and digested with *Eco*RI to release the cloned inserts from the pGAD10 plasmid. The restriction digests were run on a 1% agarose-TAE gel and the number and size of the cDNA inserts determined.



### 2.3.8. Large-Scale Ligation of ds cDNA into pGAD10

The remainder of the cDNA was ligation reactions were ligated into the pGAD10 vector in two separate reactions using the optimal conditions determined in the trial above. For each reaction 1.5µl of cDNA, 1.0µl of *Eco*RI digested and dephosphorylated pGAD10 vector (0.15µg/µl), 10.0µl Plasmid ligation buffer (Clontech patent), 1.0µl 10mM ATP and 1.0 T4

DNA Ligase (100units/µl) was added and incubated at 16°C for exactly 30 minutes. To each reaction 80µl of sterile DEPC-water and 1.5µl glycogen was added and mixed well. 280µl of ice-cold 95% ethanol was added and mixed gently. The reactions were incubated at –70°C overnight. The DNA was pelleted by centrifuging at 13000 rpm for 35 minutes. The supernatant was carefully aspirated off and the pellet air dried for 15 minutes. The DNA pellet was resuspended in 5µl of DEPC-water and electroporated into *E.coli* DH5α as described above.

### 2.3.9. Amplification and Titering of the Plasmid Library

The unamplified cDNA library was plated onto 150mm LB agar plates that contained 100mg/ml ampicillin and incubated overnight at 37°C. The cells were plated at a density of 10000-15000 colonies/plate. The next day, 8ml of LB broth was added to each plate and the colonies scraped off the agar using a glass spreader. The cell suspension was transferred to a sterile pre-chilled JA14 centrifuge tube and kept on ice while the remaining plates were harvested. Once all the plates had been scraped and the cells pooled and gently mixed, 2µl of the cell suspension was added to 999µl of LB broth. The remainder of the amplified library cell suspension was kept at 4°C. The diluted cells were plated at $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions onto LB agar plates that contained 100mg/ml ampicillin and incubated overnight at 37°C. The next day the plates were examined and the number of colonies/ml determined. To the amplified library cell suspension, sterile 100%glycerol was added to a final concentration of 25%. The glycerol and cell suspension was thoroughly mixed with gentle stirring. The amplified library was aliquoted into sterile 2ml cryovials, immediately frozen on dry ice for 5 minutes and stored at –70°C. Plasmid DNA was extracted from 4ml of the amplified library cell suspension using the Wizard DNA Midi Prep kit (Promega) as per the manufacturer's instructions.

## 2.4. SCREENING THE OP6 cDNA TWO-HYBRID LIBRARY FOR PROTEIN-PROTEIN INTERACTIONS

### 2.4.1. Transformation of Saccheromyces cerevisiae host strain

The phenotype of the *Saccharomyces cerevisiae* strains, SFY526 and Y190, provided in the Matchmaker Two Hybrid System (Clontech) were verified as outlined in the Clontech Matchmaker Two-Hybrid System manual (PT1265-1). Both the SFY526 and Y190 yeast strains were maintained on YPD medium and stored as frozen glycerol stocks. The SFY526 strain carried the *lacZ* reporter gene under the control of the GAL1 UAS and was used for quantitative β-galactosidase assays. Strain Y190 carried two reporter genes, the *lacZ* and *HIS3*, under the control of the GAL1 UAS and was used for library screening. Yeast transformants were grown on synthetic minimal media lacking either leucine and/or tryptophan and/or histidine. For Y190 transformants, the minimal media lacking leucine, tryptophan and histidine was supplemented with 50mM 3-aminotriazole. Electrocompetent yeast cells were prepared from Y190 transformants that contained the pGALDBD/BF-$1_{(26\text{-}480aa)}$ plasmid and ≤100μg of the OP6 library plasmid DNA was electroporated into the yeast cells (Becker and Guarente 1991). The negative (cells only) and positive (pCL1; pVA3 and pTD1) controls were plated on SD-Leu, SD-Leu/-Trp and SD-Leu/-Trp/-His medium plates containing 1M sorbitol. The reactions were plated in on large (150mm) SD-Leu/-Trp/-His minimal medium plates containing 1M sorbitol and 50mM 3-aminotriazole (Sigma #A-8056). The plates were incubated at 30°C for 7-10 days until colonies had grown >2mm in diameter.

### 2.4.2. Screening yeast transformants for Protein-Protein Interactions

Colonies in which an interaction between BF-1 fusion protein, encoded by plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$, and a library fusion protein, turned blue when assayed for β-galactosidase due to the hydrolysis of X-gal (5-bromo-4-chloro-3-indoyl-β-galatopyranoside).

The yeast transformants were screened for β-galactosidase activity using the colony lift filter method of Breeden and Nasmyth (1985). The assay was preformed over a 16 hour period during which the filters were periodically checked for the appearance of blue colonies. The β-galactosidase assay was checked, by screening yeast colonies containing the wild type full-length GAL4 protein (pCL1) or both the pVA3 and pTD1 plasmids, which encoded interacting proteins. Colonies which turned blue, were picked from the original master plate and restreaked onto a SD-Leu/-Trp/-His medium plate containing 1M sorbitol and 50mM 3-aminotriazole and incubated at 30°C for 4-5 days. These colonies were rescreened again for β-galactosidase activity, and positives from the secondary screen regrown (1) on a SD-Leu/-Trp/-His medium plate containing 1M sorbitol and 50mM 3-aminotriazole and (2) in 5ml of SD-Leu medium at 30°C. Plasmid DNA was extracted from the 5ml culture using the method of Hoffman and Winston (1987) and the plasmid DNA purified Wizard Miniprep DNA Isolation kit (Promega).



**2.4.3. Sorting the positive clones into groups**

The positive clones from the secondary screen were sorted into six groups based on the *Hap*II digest pattern of their cDNA inserts amplified by PCR. 5μL of purified yeast plasmid DNA was added to 45μL of yeast PCR cocktail (1X Gibco PCR Buffer, 0.2mM of each dNTP, 2.5mM $MgCl_2$, 0.5μM of the forward primer (5'-CGCGTTTGGAATCACTACAGG -3') and reverse primer(5'- GCATGCCGGTAGAGGTGT-3') and 0.5Units of recombinant Taq) and amplified under the following conditions: 94°C for 2 minutes for 1 cycle; 94°C for 45 seconds, 54°C for 45 seconds, 72°C for 45 seconds for 30 cycles; 94°C for 45seconds, 55°C for 45 seconds, 72°C for 45 seconds for 30 cycles. The final extension was at 72°C for 7 minutes for 1 cycle. PCR controls included a water control, negative (empty pGAD10 plasmid) and positive (pTD1) control. For the water control, 25μL of yeast lysate was substituted with PCR water.

For the negative and positive controls, 50ng of empty pGAD10 and pTD1 plasmid DNA was added respectively. All the controls were amplified under the same conditions as the experimental samples. 10μL of each PCR reaction was run on a 1% agarose-TAE gel along. For the *Hap*II digest, 10μL of each PCR reaction was digested with 3 units *Hap*II at 37°C overnight in 1 X Buffer L (10mM Tris pH 7.5, 10mM $MgCl_2$, 1mMDithiothreitol). The digests were analyzed by 1% agarose gel electrophoresis. The clones were sorted into 6 groups and a representative clone for each chosen for further analysis. These clones were named BF-1 interacting protein (BF1-IP) –1, 2, 3, 4,5 and 6 ($BF_1$-IP1, $BF_1$-IP2, $BF_1$-IP3, $BF_1$-IP4, $BF_1$-IP5 and $BF_1$-IP6).

### 2.4.4.Transformation into *E.coli* HB101 to select for the Library plasmid

Although the library plasmid DNA was selected for by growing the yeast in minimal media lacking leucine only, the plasmid DNA prepared from the yeast contained contaminating pGalDBD/BF-$1_{(26\text{-}480aa)}$ plasmid. In order to isolate the library plasmid, the yeast plasmid DNA was electroporated into HB101. 1-2μl of the purified yeast plasmid DNA was electroporated into the HB101 cells prepared using the method of Dower et al (1988). The reactions were plated on M9 minimal medium agar plates containing 100μg/ml ampicillin, 40μg/ml proline, 1nM thiamine-HCl and incubated at 37°C for 2 days. The HB101 transformants were screened for inserts using colony PCR as described previously. Plasmid DNA was extracted from transformants containing plasmid DNA for each clone using the High-Pure Plasmid Isolation kit (Boehringer Mannhein #1754785) as per the manufacturer's instructions.

### 2.5.5. Control Transformations and β-galactosidase Assays

In order to verify the interactions between BF-1 and the putative BF-1 interacting proteins, control transformations and β-galactosidase assays reactions were performed. The BF-1-

interacting plasmids were transformed either alone or together with plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$ or with one of the control plasmids described in the list of plasmids in the used on page (ix). A total 50ng of each plasmid was electroporated into freshly prepared electrocompetent yeast cells as described previously, and each transformation plated onto the appropriate SD selection medium and incubated at 30°C until colonies had grown to >2mm in size. The transformants were screened for activation of the *lacZ* reporter gene by assaying for β-galactosidase activity. A colony lift filter assay was performed for each reaction as described previously.

## 2.5. CHARATERIZATION OF THE CLONES

### 2.5.1. Liquid β-Galactosidase Assays in *S. cerevisiae* SFY526

Plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$ and plasmids for $BF_1$-IP1, -IP2, -IP3, -IP4, -IP5 and -IP6 were transformed either alone or together into a different yeast strain, *S.cerevisiae* SFY526. 50ng of each plasmid was electroporated into freshly prepared electrocompetent *S. cerevisiae* SFY526 cells as described previously, and each transformation plated onto the appropriate SD selection medium and incubated at 30°C until colonies had grown to >2mm in size. Five individual *S.cerevisiae* SFY526 colonies for each reaction was assayed for β-galactosidase activity in triplicate using the method of Miller (1972). The units of β-galactosidase produced for each reaction was calculated using the equation below derived by Miller (1972):

$$\beta\text{-galactosidase units} = (1000 \times OD_{420}) / (t \times V \times OD_{600})$$

t = time of incubation in minutes

V= 0.1ml X concentration factor

$OD_{600}$ = $A_{600}$ of 1ml of culture

### 2.5.2. Automatic DNA Sequencing of the BF1-Interacting Protein cDNA Inserts

For each BF-1 interacting clone, the plasmid DNA isolated from *E.coli* HB101 was transformed into *E.coli* DH5α via the heat shock method of Inoue (1990) and plasmid DNA for each reaction extracted from 3ml cultures using the High-Pure Plasmid Isolation kit (Boehringer Mannheim #1754785) as per the manufacturer's instructions. The cDNA inserts for each of the BF1-IP plasmids were sequenced using Automatic Dye Terminator Automatic DNA Sequencing. The clones were sequenced in both the forward and reverse directions using the appropriate primers (forward: 5'- TACCACTACAATGGATG-3'; reverse 5'-GCATGCCGGTAGAGGTGT-3'). The sequencing results were analyzed using the software package DNAMAN Version 4.0 (Lynnon BioSoft). The amino acid sequences of the clones were searched for protein motifs using the "PPSEARCH" programme of the European Bioinformatics Institute at http://www.ebi.ac.uk/ppsearch/ web page.

# CHAPTER 3 RESULTS

## 3.1. INTRODUCTION

## 3.2. CONSTRUCTION OF VECTORS FOR THE YEAST TWO HYBRID SCREENING ASSAY

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3.2.2.1. Purification of OP6 mRNA

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3.2.2.3. Adaptor Ligation, Phosphorylation and Size-fractionation

3.2.2.4. Ligation of ds cDNA into the pGAD10 vector and Transformation into *E.coli*

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## 3.1 INTRODUCTION

The aim of this study is to identify proteins that interact with BF-1 and to characterize these protein-protein interactions. Protein-protein interactions have been previously studied using conventional *in vitro* methods such as affinity chromatography and co-immunoprecipitation. The yeast two-hybrid system was chosen over these *in vitro* methods for a number of reasons including (1) it did not require highly purified antibodies and target protein fractions; (2) it was an *in vivo* system that detected protein-protein interactions and therefore promoted the proteins to be in their native conformation; (3) it is a sensitive assay which can detect weak and transient interactions that are common in transcriptional complexes and (4) since it is a genetic based system, the cDNA encoding the interacting protein(s) can be used directly to search genetic databases for the identities of the candidate protein partners.



The Two Hybrid System is based on the fact that many eukaryotic regulatory transcription factors consist of two physically separable domains: one which acts as a DNA binding domain and the other which acts as an activation domain. The DNA binding domain localizes the transcription factor to specific DNA sequences upstream of the genes that it regulates. The activation domain contacts other proteins which may directly or indirectly regulate transcription. In the GAL4 Two Hybrid system used in this study (Figure 3.1), the sequences encoding these two GAL4 functional domains have been cloned into separate vectors. The gene for BF-1 was cloned in frame to the GAL4 DNA binding domain in the pGBT9 vector (Figure 3.2) to generate a fusion protein when expressed in the yeast host strain. Similarly, cDNA representing a library of proteins is cloned in frame to the GAL4 activation domain in the pGAD10 vector (Figure 3.2). Both the BF-1 "bait" plasmid and library plasmids were transformed into and co-expressed in the yeast reporter strain, *Saccheromyces cerevisiae* Y190. Interactions between the BF-1 fusion protein and a library fusion protein, resulted in transcription of the *HIS3* and *lacZ* reporter genes,

because this interaction brought the GAL4 DNA binding domain and activation domains together at the promoter (Figure 3.1). Activation of the *HIS3* and *lacZ* reporter genes was selected for by growing the transformed colonies on minimal media and assaying for blue colonies.

The embryonic neuroepithelial OP6 cell line was used as the source of mRNA for construction of the two-hybrid cDNA library. The OP6 cell line was generated by infecting primary cultures of olfactory neuroepithelium from E10.5 mouse embryos with a retrovirus carrying temperature-sensitive alleles of the SV40 large T antigen (Boolay 1999; Ph. D thesis). When grown at 33°C the OP6 cells proliferated and expressed BF-1, Olf-1 and nestin; whereas at 39°C, the SV40 large T antigen was inactivated resulting in the initiation of differentiation and termination of cell proliferation (Boolay, S. Ph.D. Thesis). An outline of the methodology used in this study is given in Figure 3.3.

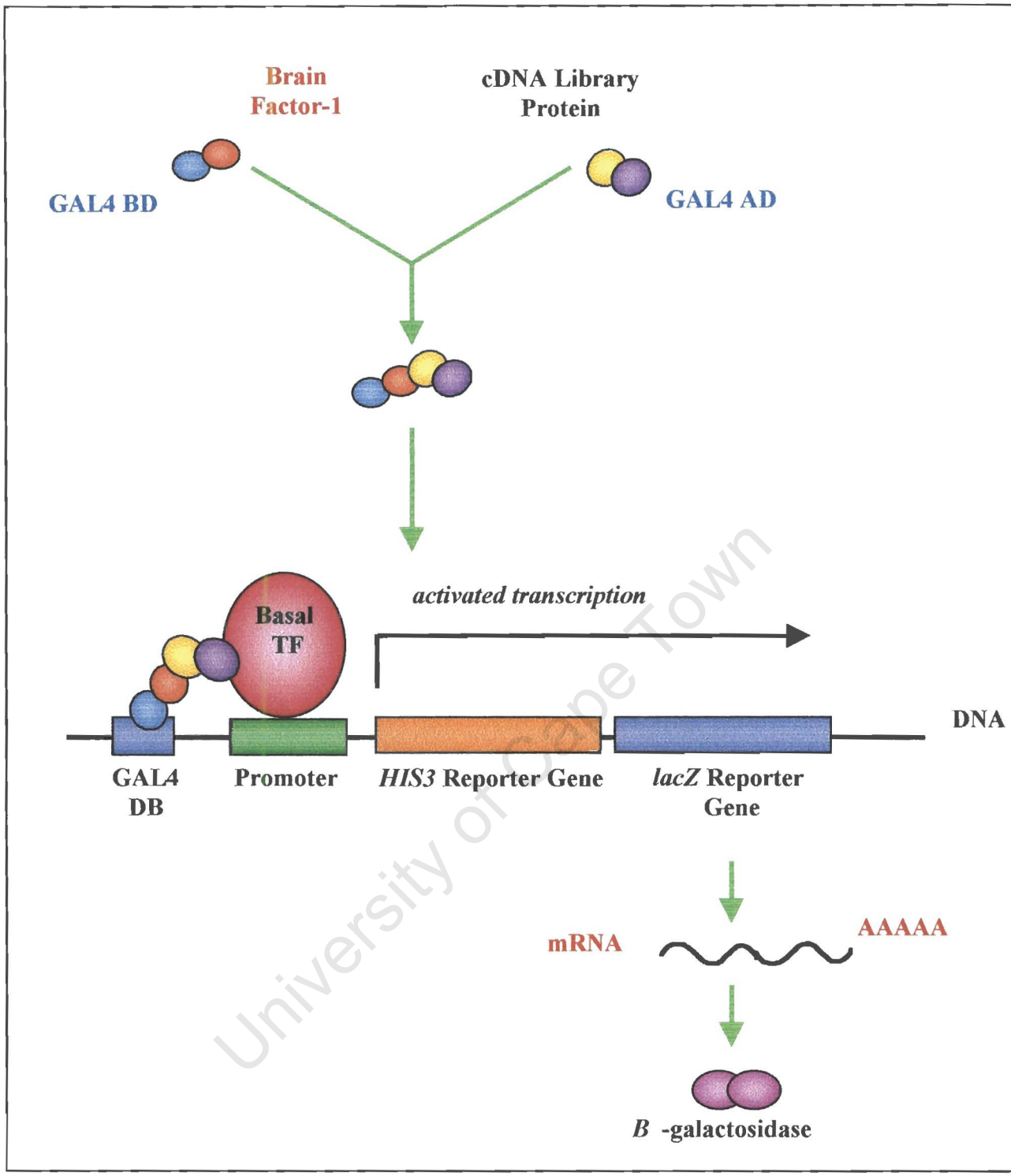





**Figure 3.1.** The Yeast Two hybrid system was used to search for proteins that interacted with BF-1. The BF-1 "bait" protein was cloned in frame to the GAL4 DB, thereby creating a fusion protein; whereas a cDNA library was cloned in frame to the GAL4 AD, creating a library of fusion proteins. An interaction between the BF-1 "bait" and a cDNA library fusion protein resulted in the GAL4 DNA Binding Domain (DB) and Activation domain (AD) being brought together at the promoter, resulting in the activation and expression of the *HIS3* and *lacZ* reporter genes. The interaction is detected by assaying for β-galactosidase activity.

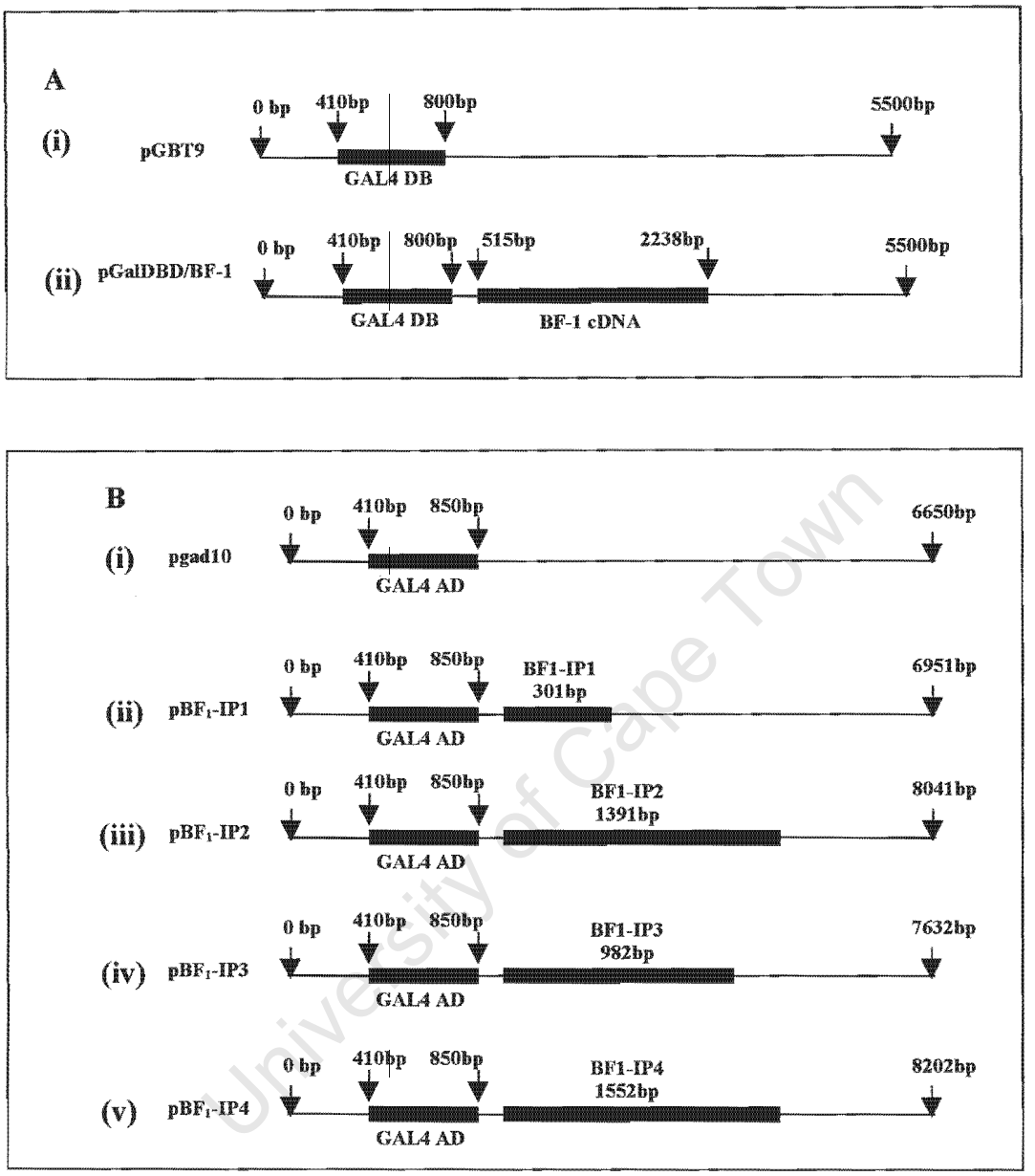





**Figure 3.2.** Schematic representation of plasmids used in the Two Hybrid Assay. **(A)** BF-1 cDNA was cloned in frame to the GAL4 DB on the pGBT9 plasmid (i), which generated a fusion protein encoded by plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$ (ii). **(B)** An OP6 cDNA library was cloned in frame to the GAL4 AD on the pGAD10 plasmid (i) to generate a library of fusion proteins. Plasmids (ii)-(v) encoded fusion proteins which interacted with the BF-1 bait encoded by pGalDBD/BF-$1_{(26\text{-}480aa)}$.

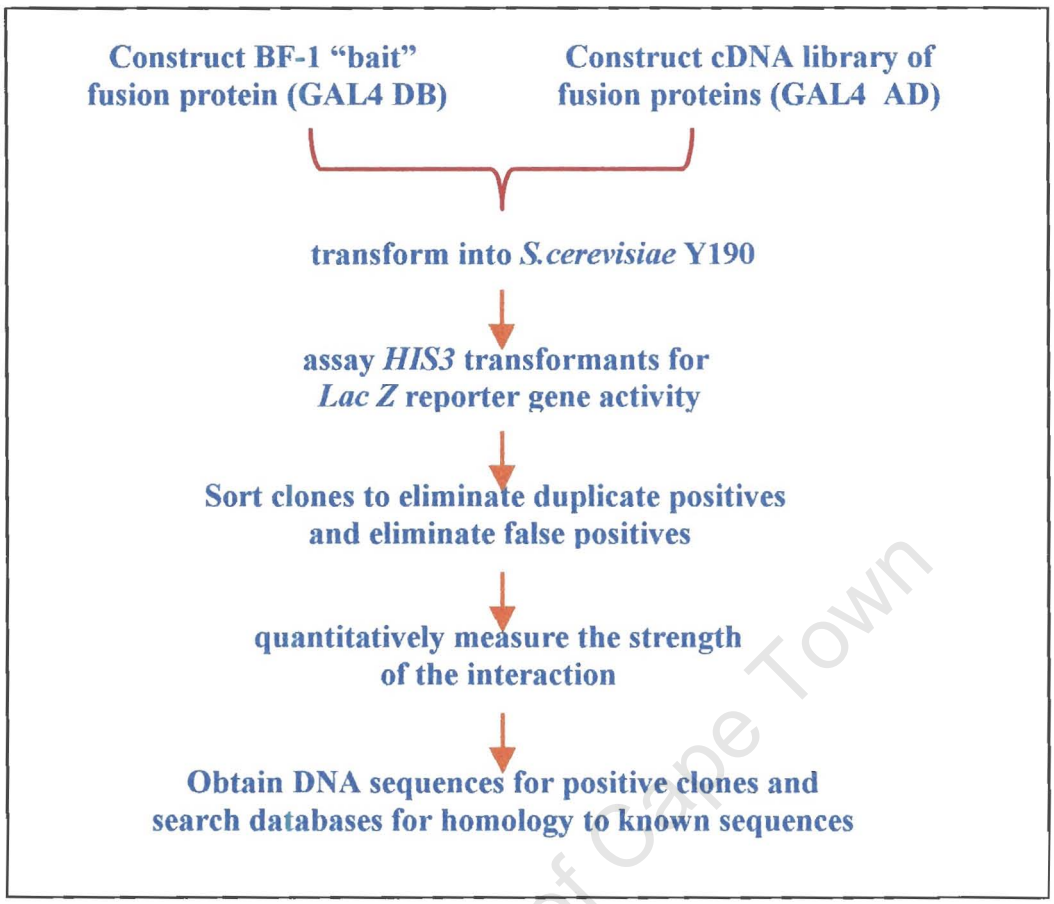



**Figure 3.3.** Outline of the Yeast Two Hybrid screening assay.

## 3.2. CONSTRUCTION OF VECTORS FOR THE YEAST TWO HYBRID SCREENING ASSAY

The first part of my thesis involved the construction of the bait plasmid encoding the BF-1 GAL4 DB fusion protein and the fusion plasmids encoding the OP6 cDNA library GAL4 AD fusion proteins for the Yeast Two Hybrid Screen.

### 3.2.1. Construction of "BF-1 bait" in pGBT9

The BF-1 cDNA (nucleotides 515-2238; amino acid residues 26-480) was successfully subcloned from the rat pBF-1 cDNA clone (Tao and Lai 1992) in frame with the GAL4 DB in the pGBT9 plasmid. DNA sequencing of the pGALDBD/BF-$1_{(26\text{-}480aa)}$ plasmid confirmed that there had been no rearrangements during the cloning process and that the BF-1 insert was cloned in-frame to the open reading frame of the pGBT9 vector (Figure 3.4). The "bait" fusion protein encoded by the pGALDBD/BF-$1_{(26\text{-}480aa)}$ plasmid, was transformed into *S.cerevisiae* Y190 and SFY526 and was negative for β-galactosidase activity, thereby indicating that it did not self-activate the *lacZ* reporter gene in these reporter yeast strains.



### 3.2.2. Construction of the OP6 cDNA Library in pGAD10

#### 3.2.2.1. Purification of OP6 mRNA

A total of 1.379mg of total RNA was isolated from OP6 cells and was analyzed by denaturing 1% agarose gel electrophoresis (Figure 3.5 A). The ratio of 28S to 18S eukaryotic ribosomal RNA bands was visually estimated to be approximately 2:1, indicating that the RNA was intact and of high integrity. 12.01μg of mRNA was isolated from the total RNA and a fraction was analyzed by denaturing 1% agarose gel electrophoresis to check its quality before preceding with the construction of the cDNA library (Figure 3.5 B). The mRNA appeared as a smear extending from about the 28S rRNA band to just above the 5S rRNA band. No ribosomal RNA bands were seen.

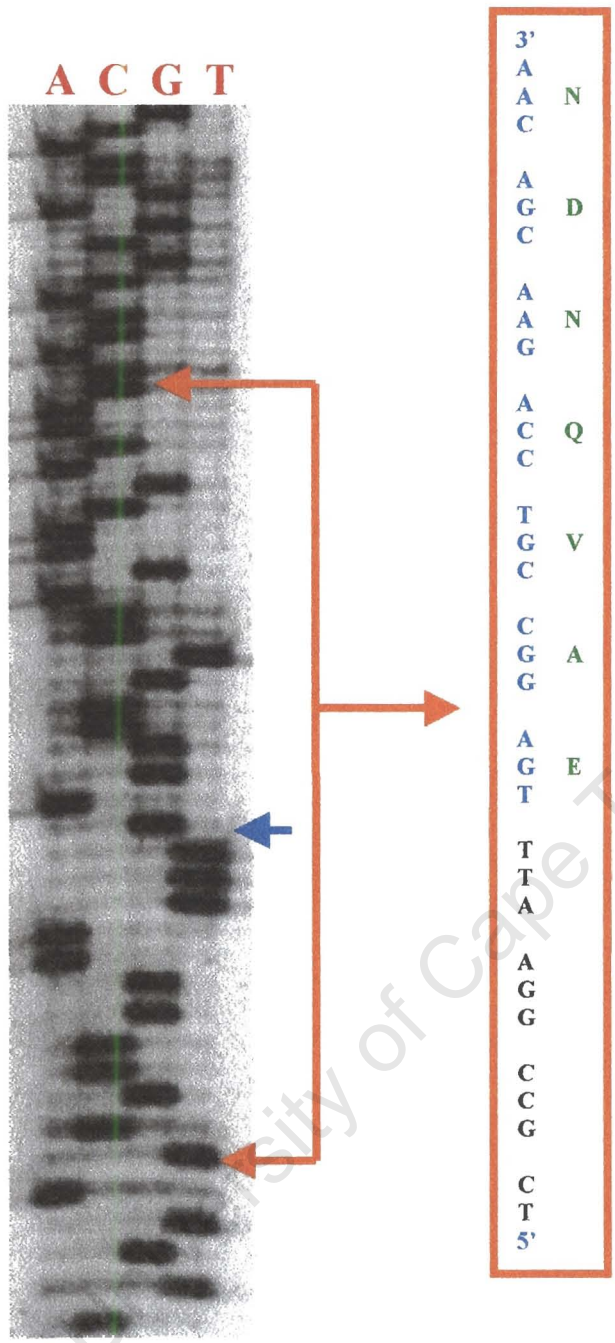





**Figure 3.4.** DNA sequencing autoradiograph of plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$. The DNA sequence of pGalDBD/BF-$1_{(26\text{-}480aa)}$ over the cloning site at the junction where the vector (black) and BF-1 (blue) sequences are joined. The blue arrow shows the point of insertion of the BF-1 cDNA sequence. The amino acid residues (green) encoded by the BF-1 sequence are shown

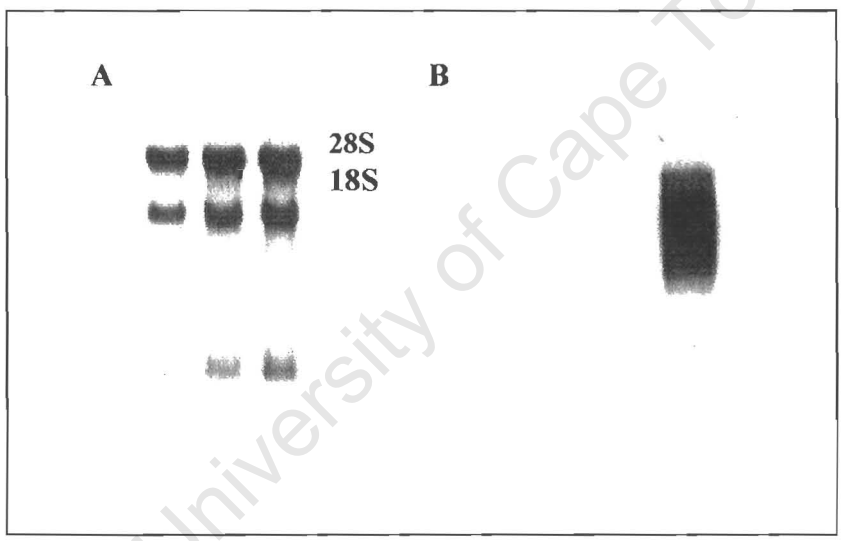





**Figure 3.5.** Denaturing 1% agarose gel electrophoresis of (A) total RNA and (B) mRNA extracted from OP6 cells.

### 3.2.2.2. First and second strand cDNA synthesis

The quality of the first and second strand cDNA synthesis was assayed by measuring the percentage incorporation of [α-32P]dCTP and running the products on a 1% agarose gel. The percentage incorporation was used as a measure of the efficiency of cDNA synthesis. Although the control reaction had a higher incorporation with the random primers, the amount of incorporation with oligo dT primers was comparable with the OP6 cDNA synthesis (Table 3.1). The gels reflected the results from the percentage incorporation (Figure 3.6 A and B) and showed that synthesis of cDNA with the oligo(dT) primers was much better. For the OP6 samples, the first strand cDNA appeared as a smear from about 300bp to 1.7kbp (Figure 3.6 A, lanes 1 and 2), whereas the second strand cDNA appeared as a smear from about 300bp to 4.5kbp (Figure 3.6 B, lanes 1 and 2). For the control samples, the first and second strand cDNA both appeared as smears from 200bp to greater than 11.5kbp (Figure 3.6 A, lanes 3 and 4) (Figure 3.6 B, lanes 3 and 4).



**Table 3.1. Percentage Incorporation of [α-$^{32}$P]-dCTP during First and Second Strand cDNA Synthesis.**

| Sample | Primer | Total CPM | Incorporated CPM | Percentage Incorporation |
|---|---|---|---|---|
| OP6 First Strand | Random p(dN)$_6$ | 105065 | 117.5 | 1.12 |
| OP6 First Strand | oligo (dT)$_{25}$(dN) | 975333 | 3395 | 3.48 |
| Control First Strand | Random p(dN)$_6$ | 91593 | 13980 | 15.26 |
| Control First Strand | oligo (dT)$_{25}$(dN) | 107150 | 4537.5 | 4.23 |
| OP6 Second Strand | Random p(dN)$_6$ | 14555 | 152.5 | 1.04 |
| OP6 Second Strand | oligo (dT)$_{25}$(dN) | 11797.5 | 560 | 4.746 |
| Control Second Strand | Random p(dN)$_6$ | 14259 | 3057.5 | 21.456 |
| Control Second Strand | oligo (dT)$_{25}$(dN) | 10027.5 | 532.5 | 5.31 |

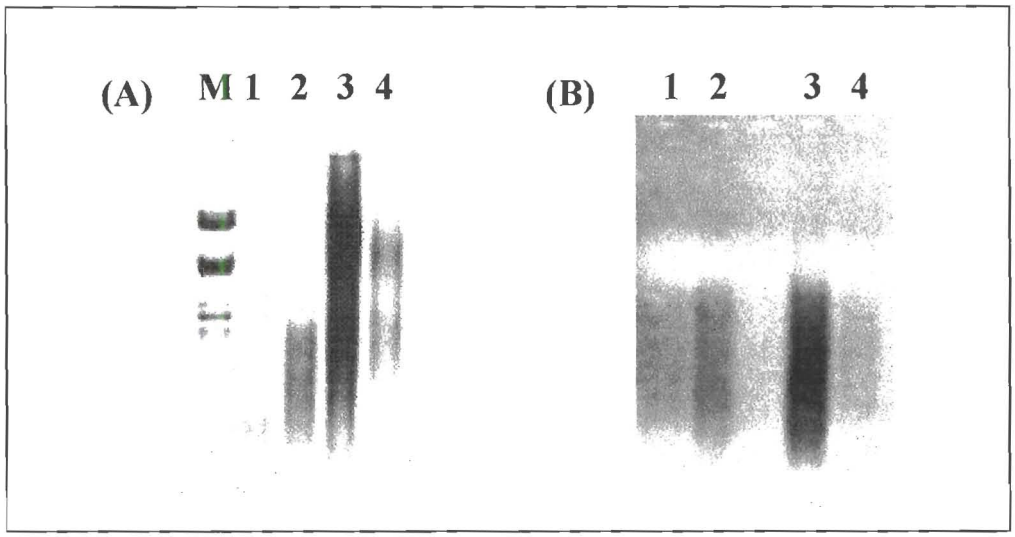



**Figure 3.6.** 1% agarose gel electrophoresis of first and second strand cDNA synthesis. **(A)** Lane M contains λ *Pst*I marker; lane 1 contains OP6 random primed 1st strand cDNA; lane 2 contains OP6 oligo dT primed 1st strand cDNA; lane 3 contains control random primed 1st strand cDNA ; and lane 4 contains control oligo dT primed 1st strand cDNA. **(B)** lane 1 contains OP6 random primed 2nd strand cDNA; lane 2 contains OP6 oligo dT primed 2nd strand cDNA; lane 3 contains control random primed 2nd strand cDNA ; and lane 4 contains control oligo dT primed 2nd strand cDNA.



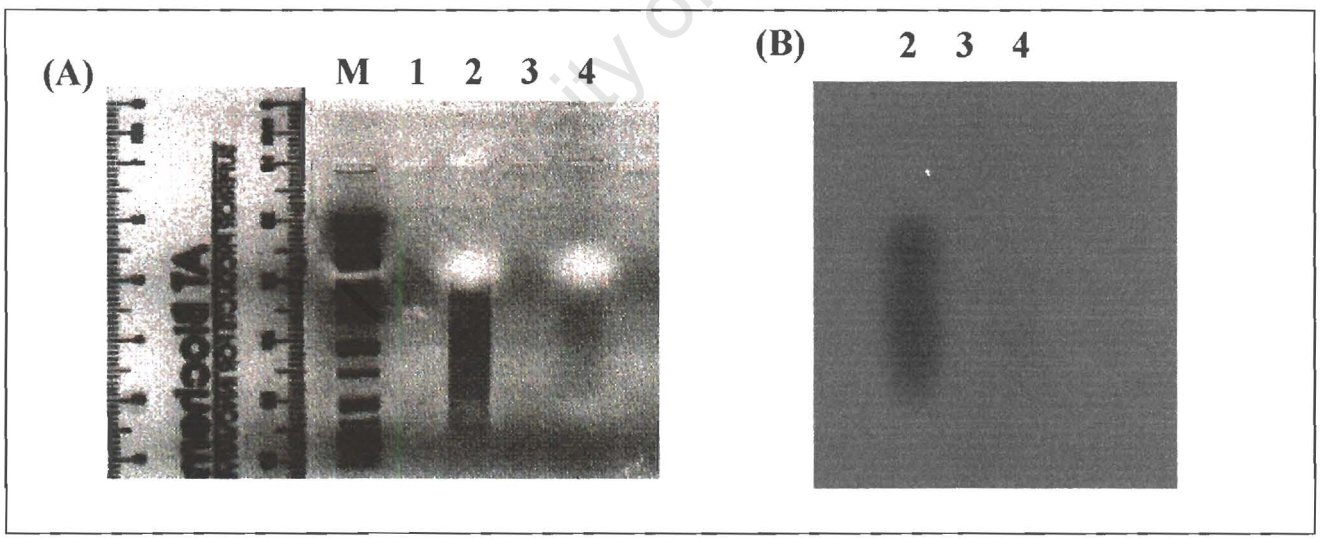



**Figure 3.7.** 1% agarose gel electrophoresis of size fractionated OP6 and control cDNA. **(A)** Fractions 1-10 were pooled and analyzed by gel electrophoresis. **(B)** autoradiograph of the gel. For both (A) and (B), lane M contains λ *Pst*I marker; lanes 1 and 3 are blank; lane 2 contains control cDNA ; and lane 4 contains OP6 cDNA.

### 3.2.2.3.Adaptor Ligation, Phosphorylation and Size-fractionation

The oligo(dT)- and random-primed cDNA reactions were pooled and the cDNA ends were successfully ligated with adaptors. The adaptor-ligated ds cDNA was phosphorylated and size fractionated, and fractions 1-10 were pooled for both the OP6 and control reactions. 3µl of the pooled fractions were analyzed by 1% agarose gel electrophoresis and autoradiography (Figure 3.7). The control cDNA synthesis gave a much higher yield than the OP6 cDNA as expected. The OP6 cDNA appeared as a faint smear from about 300bp to 4.5kbp (Figure 3.7A). The autoradiograph (Figure 3.7 B) showed that the OP6 cDNA had a reasonable range of sizes, comparable with that of the control cDNA. The concentration of the precipitated size-fractionated adaptor-ligated ds cDNA was estimated to be approximately 300ng/µl (Figure3.8) and the yield of the OP6 ds cDNA was approximated to be 2.4µg.



### 3.2.2.4. Ligation of ds cDNA into the pGAD10 vector and Transformation of the library plasmid DNA into E.coli.

A series of vector to insert ratios were ligated to determine the optimum for construction of the cDNA library. For vector to insert ratios of (i)1 to 1, (ii) 1 to 2 and (iii) 1 to 1.5, gave 1.66 X $10^4$, 7.3 X $10^3$ and 1.7 X $10^4$ c.f.u. per ml respectively. The remaining cDNA was ligated into the pGAD10 vector, using the vector to insert ratio of 1 to 1.5.

### 3.2.2.5. Amplification, Titering and Quality control of the Plasmid Library

The library contained 0.5 X $10^6$ original clones which were amplified and harvested. The titer of the frozen amplified cDNA library was calculated to be 4.6 X $10^{13}$ c.f.u./ml. The percentage of recombinant clones for the unamplified library was calculated to be approximately 40% (33 of the 87 colonies had inserts) and the size of the cDNA inserts ranged from 300bp to 1200bp.

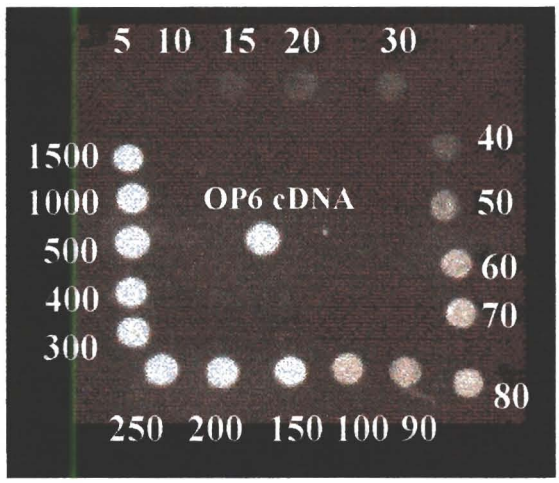



**Figure 3.8.** Concentration of the size-fractionated OP6 ds cDNA. The cDNA was estimated to be approximately 300ng/μl as estimated by comparison to DNA standards spotted onto an ethidium bromide agarose gel. The numbers in the figure refer to concentrations of the DNA standards in μg/μl.

## 3.3. SCREENING THE OP6 cDNA TWO-HYBRID LIBRARY FOR PROTEIN-PROTEIN INTERACTIONS

Transformation of the pGALDBD/BF-$1_{(26\text{-}480aa)}$ and BF-1 interacting plasmids into the yeast cells was performed sequentially since simultaneous transformation gave ten fold lower transformation efficiencies ($10^3$ –$10^4$ c.f.u./μg) than sequential transformation ($10^5$-$10^6$ c.f.u./μg). After 3-5 days incubation a background of small white colonies appeared that never grew greater than 2mm in diameter. These small white colonies did not turn pink as they aged, which strongly suggested that they were yeast mutants that had reverted to the wild type phenotype. The pink colour of the reporter yeast strain is due to the *ade2-101* mutation. After 10-12 days of incubation at 30°C, a few large pink colonies appeared, which were screened for β-galactosidase activity using the colony-lift filter assay of Breeden & Nasmyth (1985). A total of 1.32 X$10^6$ c.f.u. of the OP6 cDNA Two Hybrid library were screened for interactions between the BF-1 bait and library fusion proteins. Forty-four colonies turned blue in the original library screening (Figure 3.9 A) and were re-streaked from the master plate onto another SD-Leu/-Trp/-His/+3AT plate. In the secondary screening, 20 of the 44 colonies turned blue (Figure 3.9 B). Nine colonies turned blue within 60-100 minutes, whereas the remaining colonies turned blue within a 16 hour period (the reaction was run overnight). Yeast transformed with the positive control plasmids turned blue after approximately 30 minutes (pCL1) and 60 minutes (pVA3 + pTD1) as expected. The plasmid pCL1 encoded the wild type full-length GAL4 protein and plasmids pVA3 and pTD1 served as a model of interacting proteins.

### 3.3.1. Sorting positive clones into groups to Eliminate Duplicate Positive Clones

In order to eliminate duplicate plasmids bearing the same insert, the 20 clones were sorted into 6 groups based on the *Hap*II digestion pattern of their inserts (Figure 3.10). A representative clone from each group chosen for further analysis (Figure 3.10). These clones were named BF-1 interacting protein ($BF_1$-IP) $BF_1$-IP1, -IP2, -IP3, -IP4, -IP5 and - IP6. The cDNA inserts for $BF_1$-IP1 –6 ranged from 100bp to 1300bp (Figure 3.11).

### 3.3.2. Control Transformations

The control transformations set out in Table 3.2. served to (1) test the original interaction between the pGALDBD/BF-$1_{(26\text{-}480aa)}$ bait fusion protein and $BF_1$-IP1, -IP2, -IP3, –IP4, -IP5 and –IP6, (2) test whether the BF1 interacting proteins self-activated the reporter genes and (3) to eliminate false positives. In the yeast Two-Hybrid System, "true positives" are defined as those yeast colonies in which the *lacZ* and *HIS3* reporter genes are activated and expressed, *only* when the hybrid fusion proteins encoded by the GAL4 DB and AD hybrid plasmids, directly interact with each other. "False positives" are defined as transformants that display $His^+$ and/or $LacZ^+$ phenotypes, even though the fusion proteins encoded by both the hybrid, do *not directly interact* with each other (Bartel et al 1993a). The various types of controls checking for false positives are summarized in Table 3.2.

Even though the library plasmid was selected for by growing the positive yeast colonies in minimal media lacking only leucine, contaminating pGalDBD/BF-$1_{(26\text{-}480aa)}$ was isolated in the plasmid preparation. In order to purify only the library plasmids, the yeast plasmid DNA was electroporated into the *E.coli* HB101 strain, which carried a *leuB* mutation that was complemented by the *LEU2* gene on the library plasmid.

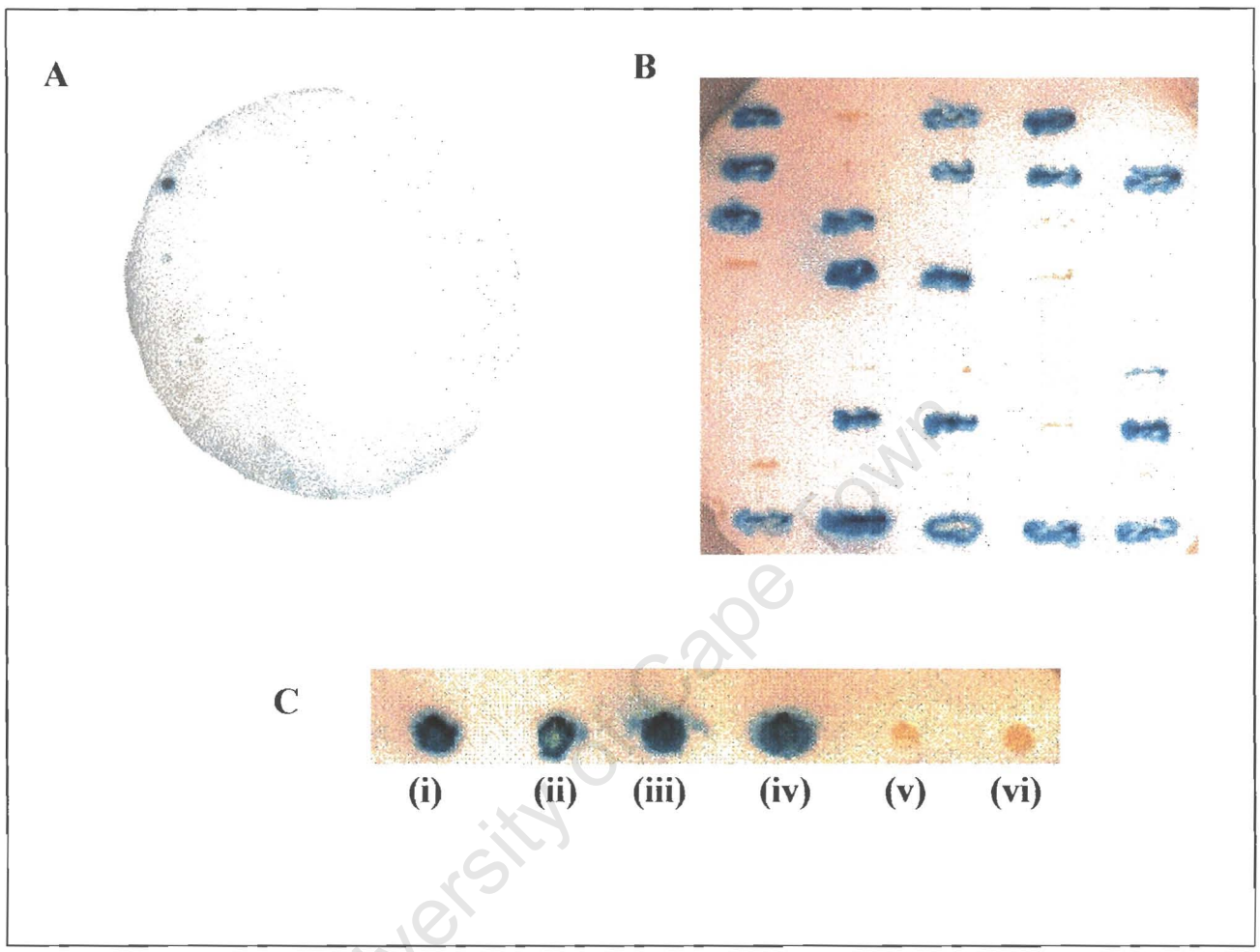





**Figure 3.9.** Colony lift β-galactosidase Assays. (A) An example of a blue colony from the first two-hybrid screen. (B) 20 out of the 44 colonies turned blue in the secondary screen. (C) Control reactions of yeast transformed with pCL1 (i and ii), pVA3 + pTD1 (iii and iv), pGALDBD/BF-$1_{(26\text{-}480aa)}$ only (v and vi).

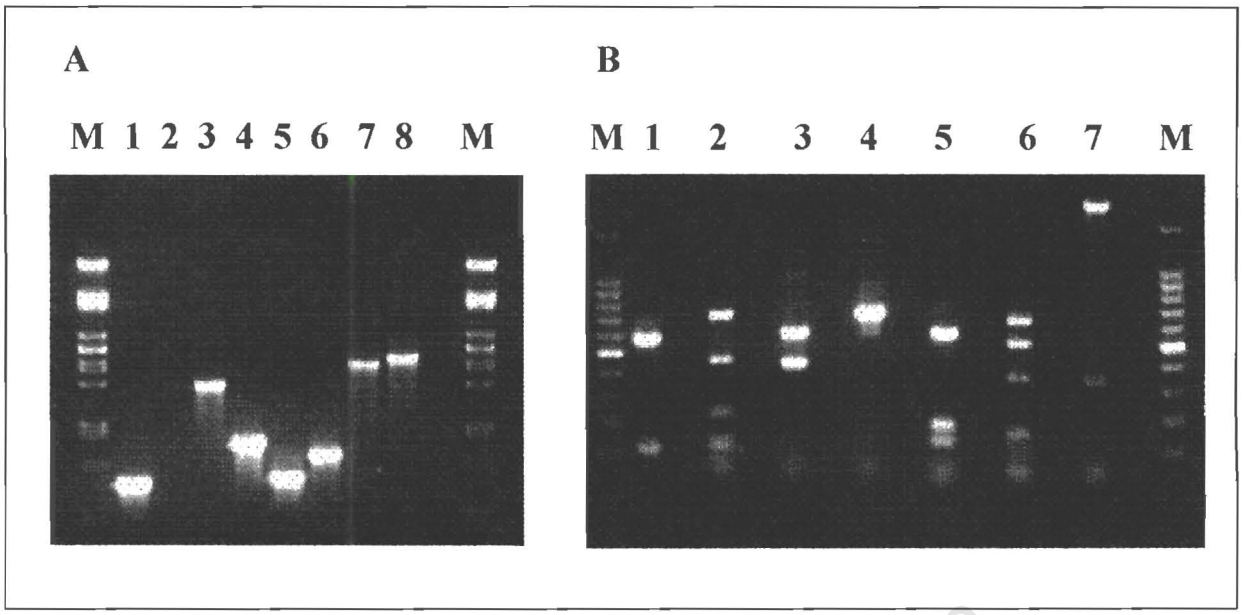



**Figure 3.10.** 1% agarose gel electrophoresis of the **(A)** PCR amplified cDNA inserts of the BF1-interacting proteins. Lanes M contain λ *Pst*I marker; lane 1 contains PCR product for the empty pGAD10 vector; lane 2 contains the PCR water control; lanes 3-8 contain the amplified cDNA inserts for $BF_1$-IP3 (lane 3); $BF_1$-IP1 (lane 4); $BF_1$-IP5 (lane 5); $BF_1$-IP6 (lane 6); $BF_1$-IP2 (lane 7); and $BF_1$-IP4 (lane 8). **(B)** *Hap*II digest of the PCR amplified inserts. Lanes M contain a 100bp ladder (Promega); lanes 1-7 contain the *Hap*II digest pattern for pGAD10 (lane 1); $BF_1$-IP3 (lane 2); $BF_1$-IP1 (lane 3); $BF_1$-IP5 (lane 4); $BF_1$-IP6 (lane 5); $BF_1$-IP2 (lane 6); and $BF_1$-IP4 (lane 7).



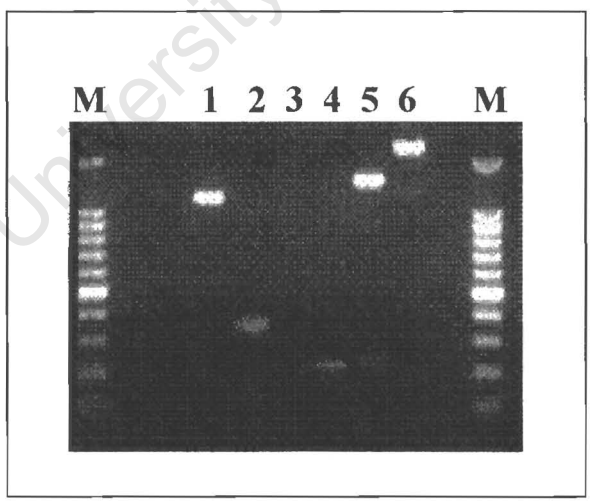



**Figure 3.11.** Determination of the cDNA insert Sizes. 1% agarose gel electrophoresis of the *Eco*RI digests of the BF-1 interacting clones. Lanes M contain a 100bp ladder (Promega); lanes 1-6 contain the *Hap*II digest pattern for $BF_1$-IP3 (lane 1); $BF_1$-IP1 (lane 2); $BF_1$-IP5 (lane 3); $BF_1$-IP6 (lane 4); $BF_1$-IP2 (lane 5); and $BF_1$-IP4 (lane 6).

Plasmid DNA for $BF_1$-IP1, -IP2, -IP3 and –IP4 was extracted from the HB101 transformants and were re-transformed in *S.cerevisiae* Y190 either alone or together with plasmid pGalDBD/BF-$1_{(26\text{-}480aa)}$ or with one of the control plasmids as in Table 3.2. The yeast transformants were screened for β-galactosidase activity. These results of the tests are given in Table 3.2.

Negative control tests (2,3 ,4 ,6-12) checked that the various components of the yeast two hybrid assay did not interact with each other. Control tests 1 and 5 were positive controls for the β-galactosidase assay. The pCL1 plasmid (control test 1) encoded the wild type GAL4 protein. The pTD1 and pVA3 plasmids (control test 5) served as a model of interacting fusion proteins. pVA3 encoded murine p53 and pTD1 encoded large T antigen fusion proteins.

The fusion protein encoded by plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$ did not self-activate the *lacZ* reporter gene by itself (control 14) nor in the presence of a GAL4 AD (control 13). The original interaction between the pGalDBD/BF-$1_{(26\text{-}480aa)}$ bait fusion protein and each BF1-interacting proteins was confirmed (control 17). None of the BF-1-interacting proteins self-activated the *lacZ* reporter gene (control 15), or fortuitously interacted with the GAL4 DNA-BD encoded by pGBT9 (control 16). The control 18 checked whether the interacting library protein interacted non-specifically with other GAL4 DB fusion proteins e.g. human lamin C, encoded by the pLAM5 plasmid used in this control. Clones $BF_1$-IP1, -IP2, -IP3 and –IP4 were negative and therefore encoded proteins that directly interacted with the BF-1 bait fusion protein; whereas $BF_1$-IP5 and –IP6 were positive for this control and were discarded.

**Table 3.2. Control Transformations to Eliminate False Positives**

| Control | GAL4 DNA Binding Plasmid | GAL4 Activation Domain Plasmid | Description of Control | Expected result | Obtained Results |
|---|---|---|---|---|---|
| 1 | PCL1 | - | positive control that tested the β-galactosidase assay | Blue | Blue |
| 2 | PGBT9 | - | negative control that confirmed that a GAL4 DNA BD did not activate the reporter genes by itself. | White | White |
| 3 | - | PGAD424 | negative control that confirmed that a GAL4 AD did not activate the reporter genes by itself. | White | White |
| 4 | PGBT9 | PGAD424 | negative control that confirmed that the GAL4 DNA BD and AD did not interact by themselves to activate the reporter genes. | White | White |
| 5 | PVA3 | PTD1 | positive control that served as a model of interacting fusion proteins. pVA3 encoded murine p53 and pTD1 encoded large T antigen fusion proteins. | Blue | Blue |
| 6 | PVA3 | - | negative control that confirmed that a GAL4 DNA BD fusion protein did not activate the reporter genes by itself. | White | White |
| 7 | PVA3 | PGAD424 | negative control that confirmed that a GAL4 DB fusion protein and a AD protein did not interact to activate the reporter genes. | White | White |
| 8 | - | PTD1 | negative control that confirmed that a GAL4 AD fusion protein did not activate the reporter genes by itself. | White | White |
| 9 | PGBT9 | PTD1 | negative control that confirmed that a GAL4 DNA BD and a AD fusion protein did not interact to activate the reporter genes. | White | White |
| 10 | PLAM5' | - | negative control that confirmed that a GAL4 DNA BD fusion protein did not activate the reporter genes by itself. | White | White |
| 11 | PLAM5' | PTD1 | negative control that confirmed that an unrelated GAL4 DNA BD fusion protein did not interact with the GAL4 AD fusion protein to activate the reporter genes. | White | White |
| 12 | PLAM5' | PCL1 | positive control that confirmed that the GAL4 DNA BD –lamin C fusion protein did not inhibit the wild-type-GAL4 protein from activating the reporter genes. | Blue | Blue |
| 13 | pGALDBD/BF-$1_{(26\text{-}480aa)}$ | pGAD10 | checked if the GAL4 DNA BD of the BF-1 fusion protein interacted with the GAL4 AD. | White | White |
| 14 | pGALDBD/BF-$1_{(26\text{-}480aa)}$ | - | checked if the BF-1 GAL4 DB fusion self activated the reporter genes. | White | White |

**Table 3.2. Control Transformations (continued)**

| Control | GAL4 DNA Binding Plasmid | GAL4 Activation Domain Plasmid | Description of Control | Expected result | Obtained Results |
|---|---|---|---|---|---|
| 15 | - | $BF_1$-IP1 | Checked if the $BF_1$-IP's self-activated the *lacZ* and *HIS3* reporter genes. | White | White |
| | | $BF_1$-IP2 | | White | White |
| | | $BF_1$-IP3 | | White | White |
| | | $BF_1$-IP4 | | White | White |
| | | $BF_1$-IP5 | | White | White |
| | | $BF_1$-IP6 | | White | White |
| 16 | PGBT9 | $BF_1$-IP1 | checked if there was a fortuitous interaction between the $BF_1$-IP's and GAL4 DNA-BD. | White | White |
| | | $BF_1$-IP2 | | White | White |
| | | $BF_1$-IP3 | | White | White |
| | | $BF_1$-IP4 | | White | White |
| | | $BF_1$-IP5 | | White | White |
| | | $BF_1$-IP6 | | White | White |
| 17 | PGALDBD/BF-$1_{(26\text{-}480aa)}$ | $BF_1$-IP1 | checked the initial observation of the interaction between the pGALDBD/BF-$1_{(26\text{-}480aa)}$ bait fusion protein and the BF1-IP's. | Blue | Blue |
| | | $BF_1$-IP2 | | Blue | Blue |
| | | $BF_1$-IP3 | | Blue | Blue |
| | | $BF_1$-IP4 | | Blue | Blue |
| | | $BF_1$-IP5 | | Blue | Blue |
| | | $BF_1$-IP6 | | Blue | Blue |
| 18 | PLAM5' | $BF_1$-IP1 | Checked if the interacting protein interacted non-specifically with other GAL4DB fusion proteins. | White | White |
| | | $BF_1$-IP2 | | White | White |
| | | $BF_1$-IP3 | | White | White |
| | | $BF_1$-IP4 | | White | White |
| | | $BF_1$-IP5 | | White | Blue |
| | | $BF_1$-IP6 | | White | Blue |

## 3.4. CHARACTERIZATION OF CLONES

### 3.4.1. Liquid β-Galactosidase Assays in *S. cerevisiae* SFY526

Liquid culture β-Galactosidase assays were carried out in order to rank the BF-1 interacting proteins, $BF_1$-IP1, -IP2, -IP3 and -IP4, according to the relative strength of their interactions with the BF-1 bait encoded by plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$. Although these liquid assays were quantitative, *no direct correlation* between β-galactosidase activity and the $K_d$ of an interaction exists (Estojak et al., 1995).

Plasmid pGALDBD/BF-$1_{(26\text{-}480aa)}$ and plasmids for $BF_1$-IP1, -IP2, -IP3 and -IP4 were transformed either alone or together into *S.cerevisiae* SFY526. To reduce variability of the results due to differences in plasmid copy number, five separately transformed colonies for each $BF_1$-IP clone was assayed in triplicate. The results represented as a bar graph in Figure 3.12. The quantitative data between different interactions were compared using the same yeast host strain, since different host strains had different *lacZ* reporter constructs and therefore different promoter strengths. The pCL1 wild-type control strongly activated the *lacZ* reporter gene as observed by the appearance of an intense yellow colour after 6.5 minutes, thereby generating 12006 units of β-galactosidase. The pVA3 + pTD1 positive control which served as a model of interacting proteins, produced 140 (±18) units of β-galactosidase. The interactions between the BF-1 fusion protein encoded by pGALDBD/BF-$1_{(26\text{-}480aa)}$ and $BF_1$-IP1, -IP2, -IP3 and –IP4 produced 86 (± 21), 41 (± 13), 23 (± 7) and 20 (± 7) units β-galactosidase respectively. Neither the BF-1 bait nor the BF-1-interacting proteins self-activated the *lacZ* reporter gene.

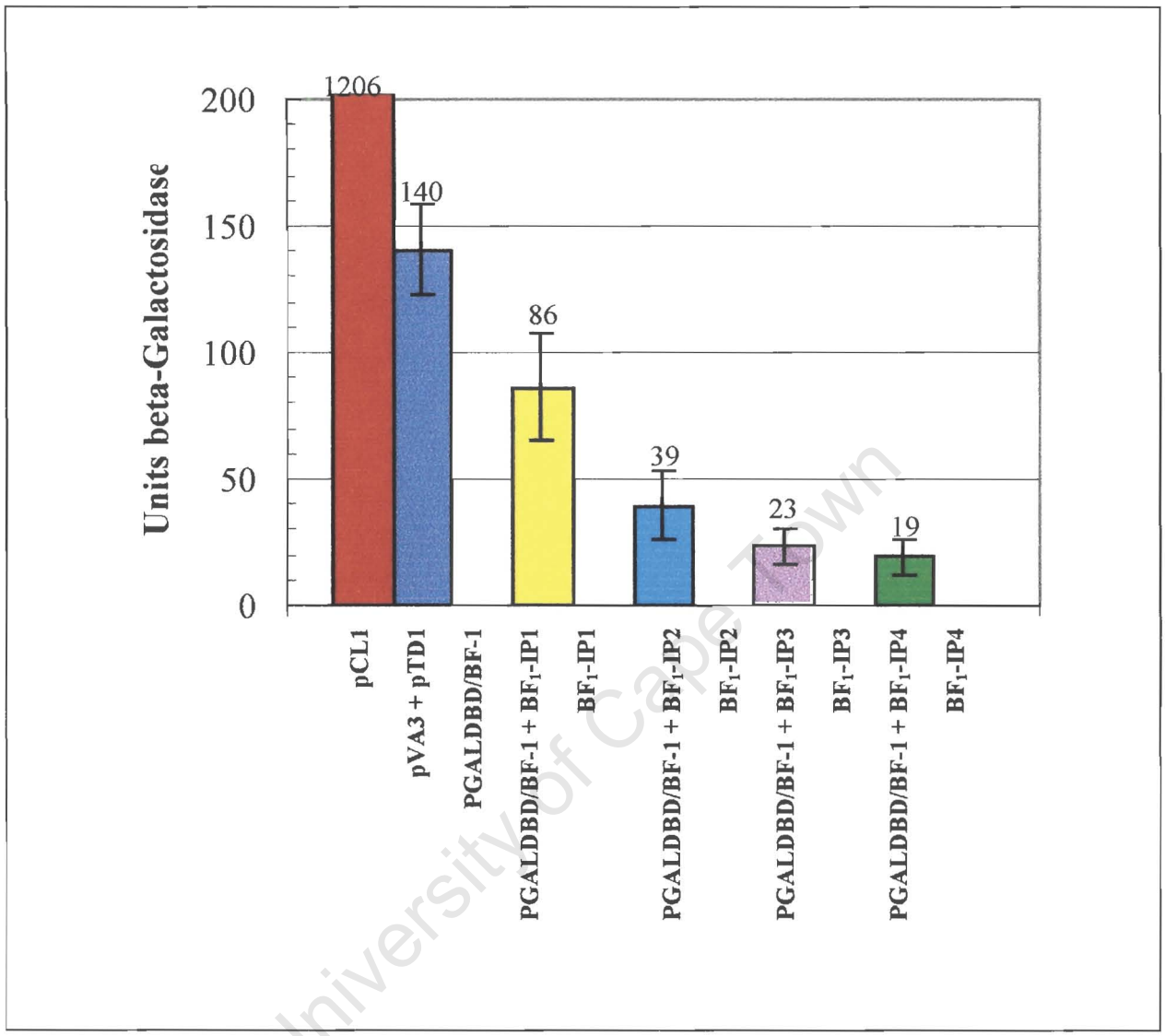





**Figure 3.12.** Quantitative liquid β-galactosidase assays to measure the relative strengths of the Two-Hybrid interactions. The plasmids used for each reaction is given as a legend below each bar in the graph.

### 3.4.2. Analysis of DNA and Amino Acid Sequences

The cDNA inserts for $BF_1$-IP1, -IP2, -IP3 and –IP4 were automatically sequenced in both the forward and reverse directions. The forward and reverse sequences of each clone were merged and analyzed using DNAMAN version 4.0 (Lynnon BioSoft) software. Analysis of the DNA and amino acid sequences is discussed in Chapter 4.

# CHAPTER 4 ANALYSIS AND DISCUSSION

## 4.1. INTRODUCTION

## 4.2. ANALYSIS OF BF-1 INTERACTING PROTEINS

4.2.1. BF-1 Interacting Protein-2

4.2.2. BF-1 Interacting Protein-3

4.2.3. BF-1 Interacting Protein-4

4.2.4. BF-1 Interacting Protein-1

## 4.3. CONCLUSION

## 4.1. INTRODUCTION

The screening of the OP6 cDNA two-hybrid library was successful, but surprisingly no interactions between the BF-1 fusion bait and components of the RNA PIC, "co-activator complexes" or cell cycle, specifically the mouse homologue $p27^{XICI}$, was detected. However, four cDNA clones were shown to interact with the BF-1 fusion protein and were named $BF_1$-IP1, $BF_1$-IP2, $BF_1$-IP3 and $BF_1$-IP4. The merged DNA sequences for $BF_1$-IP1, -IP2, -IP3 and –IP4 were used to search the GenBank database for homology to known genes. A summary of the results is given in Table 4.1. $BF_1$-IP2, -IP3 and –IP4 were found to be 100% homologous to mouse genes encoding extracellular matrix proteins involved in cell growth; whereas $BF_1$-IP1 appeared to encode a novel protein.

The amino acid sequences of the $BF_1$-IP1, $BF_1$-IP2, $BF_1$-IP3 and $BF_1$-IP4 proteins were searched for known protein motifs to elucidate the function these proteins, as well as to determine if there were any shared common motifs that might mediate the protein-protein interactions with BF-1. A summary of these motifs is given in Table 4.2. The "PPSEARCH" programme of the European Bioinformatics Institute at the Htt://www.ebi.ac.uk/ppsearch/ website was used to search for protein motifs in the PROSITE database. The analysis of $BF_1$-IP2, $BF_1$-IP3 and $BF_1$-IP4 will be discussed first, and the analysis of $BF_1$-IP1 will be discussed last.

**Table 4.1. DNA Sequencing and Analysis of BF-1 Interacting Proteins.**

| Group | Number of times isolated | Size of Insert (bp) | Clone | Units β-galacto-sidase | Homology (nucleotide) |
|---|---|---|---|---|---|
| 1 | 8 | 982bp | $BF_1$-IP3 | 23.4 | Acrogranin (100%) |
| 2 | 8 | 301 bp | $BF_1$-IP1 | 86.4 | Unknown |
| 3 | 1 | 100 bp | $BF_1$-IP5 | ND | ND |
| 4 | 1 | 200 bp | $BF_1$-IP6 | ND | ND |
| 5 | 1 | 1391 bp | $BF_1$-IP2 | 41.5 | MBP1 (100%) |
| 6 | 1 | 1552 bp | $BF_1$-IP4 | 20.3 | Fibulin-2 (100%) |

ND = not determined



**Table 4.2. Protein Motifs Identified in the BF-1 Interacting Proteins**

| NAME | DOMAINS | | | | | PHOSPHORYLATION SITES | | | OTHER POST-TRANSLATIONAL MODIFICTIONS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | WAP | Cysteine String | EGF-like | $Ca^{2+}$ - EGF-like | Granulin | PKC | CKC | CAMP | Myristolation | Asx hydroxylation | Asn glycosylation |
| $BF_1$-IP1 | 1 | 1 | - | - | - | - | - | - | - | - | - |
| $BF_1$-IP2 | - | - | 4 | 6 | - | 4 | 9 | - | 10 | 4 | 2 |
| $BF_1$-IP3 | - | - | - | - | 2 | 2 | - | - | 5 | - | 1 |
| $BF_1$-IP4 | - | - | 3 | 5 | - | 7 | 6 | 1 | 11 | 4 | 1 |

## 4.2. ANALYSIS OF BF-1 INTERACTING PROTEINS

### 4.2.1. BF-1 Interacting Protein-2

The DNA sequence obtained for $BF_1$-IP2 was 1391bp long and was 100% homologous to the region from 122bp–1512bp in the mutant p53 binding protein (MBP1) (accession #: gb/AF104223.1/AF104223) (Figures 4.1A and 4.2). Therefore it was concluded that $BF_1$-IP2 encoded MBP1. The translated amino acid sequence of $BF_1$-IP2 (Figure 4.3) corresponding to amino acids 26–443 in the MBP1 protein sequence (accession #: gb/AAD45219.1) (Figures 4.1B and 4.4) contained motifs that have been previously identified in MBP1 (Gallagher et al 1999). These include two asparagine glycosylation (Asn) sites, four asparagine (Asx) hydroxylation sites and six calcium binding epithelial growth factor (EGF)-like domains. In addition, the $BF_1$-IP2 amino acid sequence also contains four non-calcium-binding EGF-like domains, ten myristolation sites and phosphorylation sites for Protein Kinase C (PKC) and Calcium kinase II (CK2). The results are summarized in Table 4.2 and represented in Figure 4.5.

MBP1 was isolated in a two-hybrid screen using a common tumour derived mutant p53 protein as the bait (Gallagher et al 1999) and is the mouse homologue of the hamster H411 protein. MBP1 is a member of the fibulin family of extracellular matrix proteins and contains several EGF-like and calcium binding EGF-like domains. The EGF-like domain contains 40-50 residues including six conserved cysteine residues that form three disulphide bonds. EGF-like domains have been shown to mediate protein-protein interactions by binding calcium which helps stabilize the interactions (Selander et al 1992; Rao et al 1995; Kuroda and Tanizwa 1999). Several proteins involved in blood coagulation and fibrinolysis, activation of complement, cell adhesion, cell growth and inhibition, cell signalling and determination of embryonic cell fates, contain EGF-like domains (Campbell and Bork 1993).

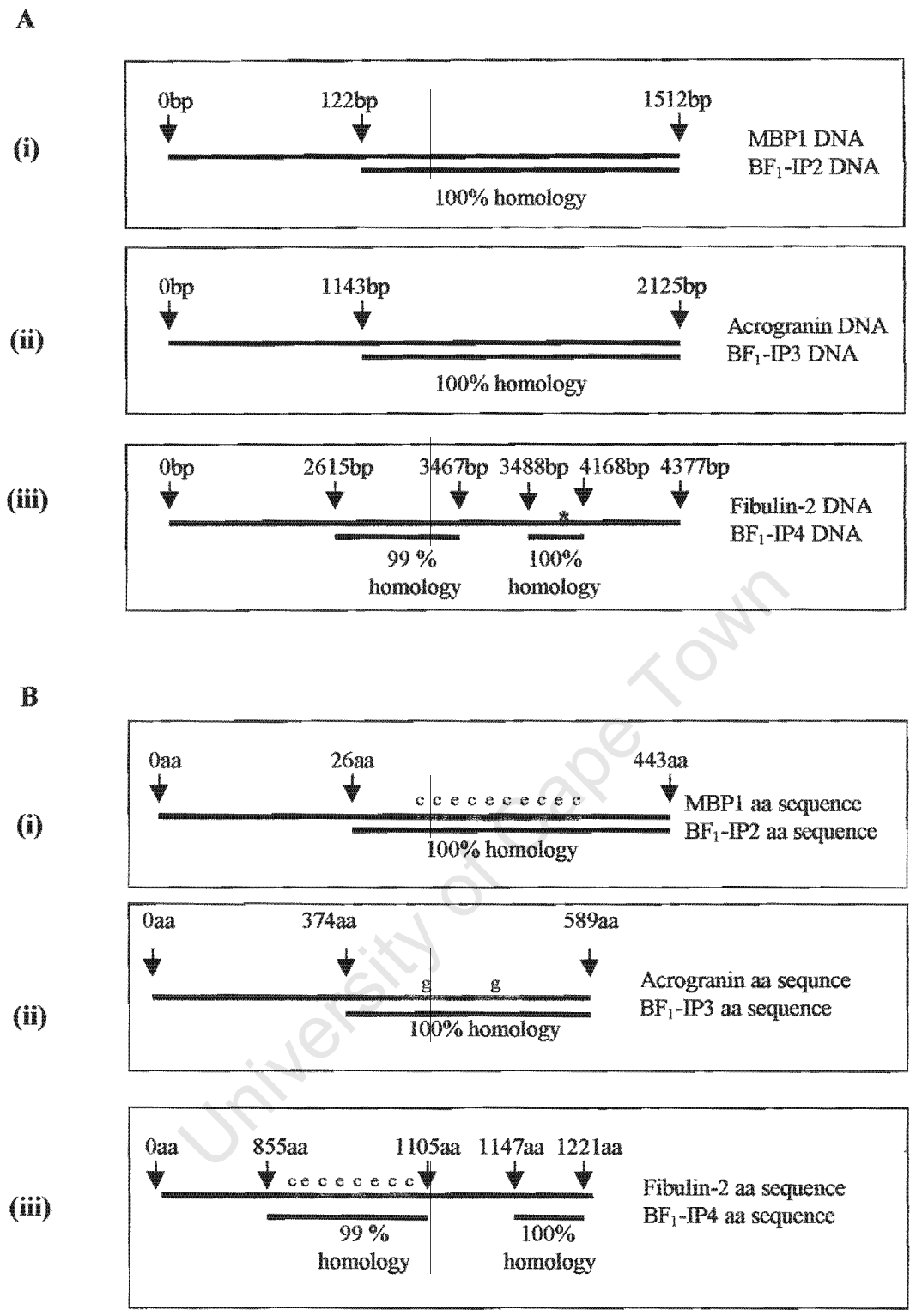





**Figure 4.1.** Schematic drawing representing the alignments of (A) DNA sequences and (B) amino acid sequences (aa) for (i) $BF_1$-IP2 and MBP1, (ii) $BF_1$-IP3 and acrogranin, and (iii) $BF_1$-IP4 and fibulin-2. The nucleotide or amino acid residue number is given above the arrows. The percentage homology between sequences is given. The stop codon in the fibulin-2 DNA sequence at 3715bp, is denoted by a red asterix. The EGF-like (e), calcium binding EGF-like (c) and granulin (g) domains in the amino acid sequences are depicted as purple, teal blue and green coloured lines respectively.

Most of these EGF-like domain containing proteins are secreted extracellular matrix or membrane associated proteins. However some intracellular proteins also contain EGF-like domains e.g. NELL proteins which act as intracellular signalling molecules in the cytoplasm of neuronal cells by interacting with Protein Kinase C through their EGF-like domains (Kuroda and Tanizwa 1999).

Although the interaction between BF-1 and MBP1 was checked using a variety of yeast controls, the biological significance of the interaction between BF-1, a transcription factor, and MBP1, a membrane bound or secreted protein, is under question and needs to be verified. MBP1 was originally isolated in a yeast two hybrid screen to look for proteins that interacted with a mutant form of the p53 transcription factor, a tumour suppressor (Gallagher et al 1999). In their studies, Gallagher et al (1999) showed that MBP1 was expressed in a variety of tissues, including the brain. Since the MBP1 protein sequence contains a putative topogenic sequence which defines protein trafficking events, they speculated that MBP1 could be either a membrane-bound type 1 protein which faces the cytoplasm or a secreted protein. Therefore as a membrane protein, MBP1 would be available for interaction with the mutant p53 protein due to the exposure of its C-terminus into the cytosol; or as a secreted protein, MBP1 could interact with mutant p53 in the cytoplasm before it is secreted from the cell. If BF-1 interacts with MBP1 by similar mechanisms, it would need to be in the cytoplasm or in the extracellular matrix. Therefore, the subcellular distribution pattern of BF-1 during forebrain development needs to be determined in order to decide whether the interaction between BF-1 and MBP1 is biologically relevant or not.

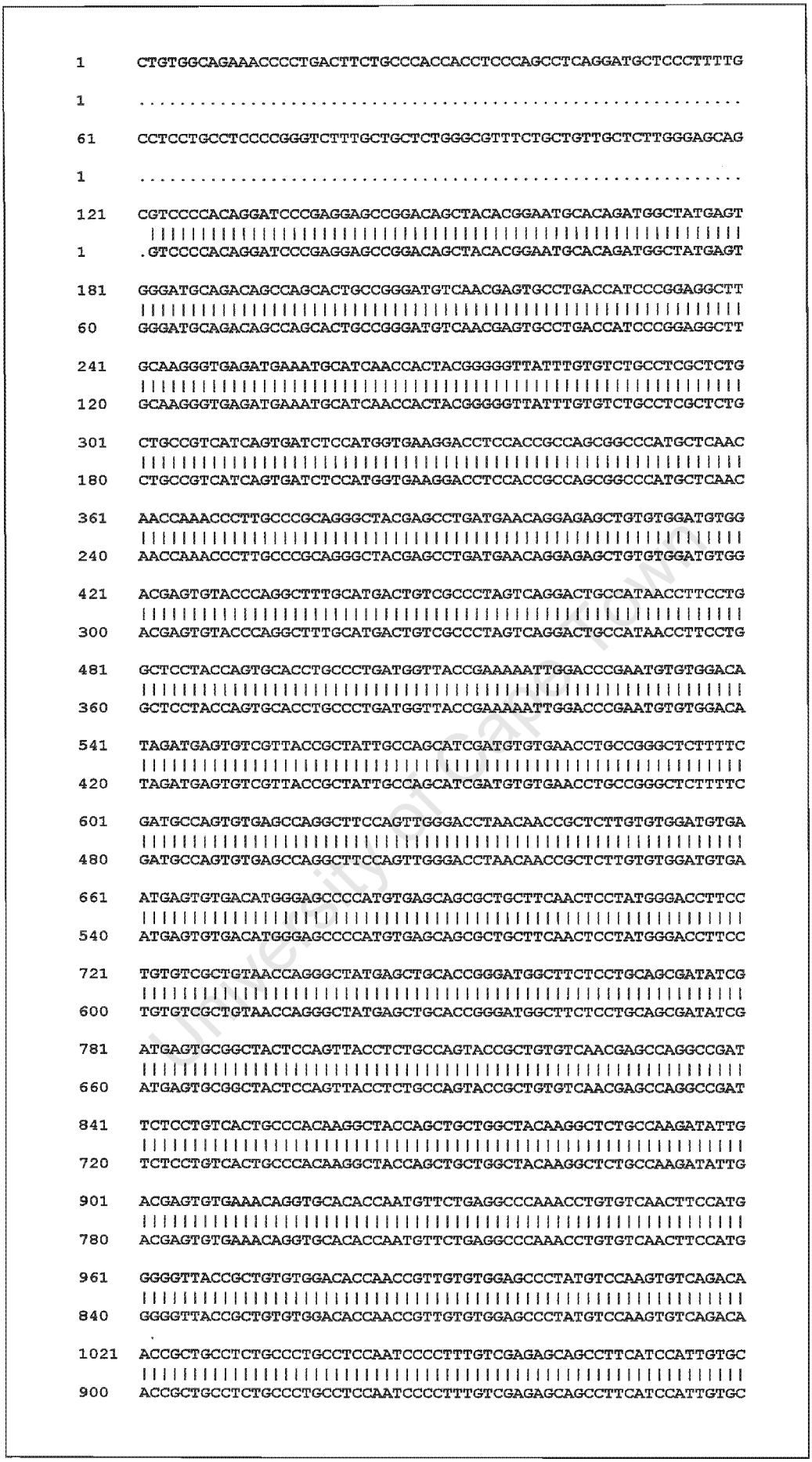



**Figure 4.2.** Alignment DNA Sequences of MBP1 (upper line) and $BF_1$-IP2 (lower line). Base pair values for each sequence is given on the left.

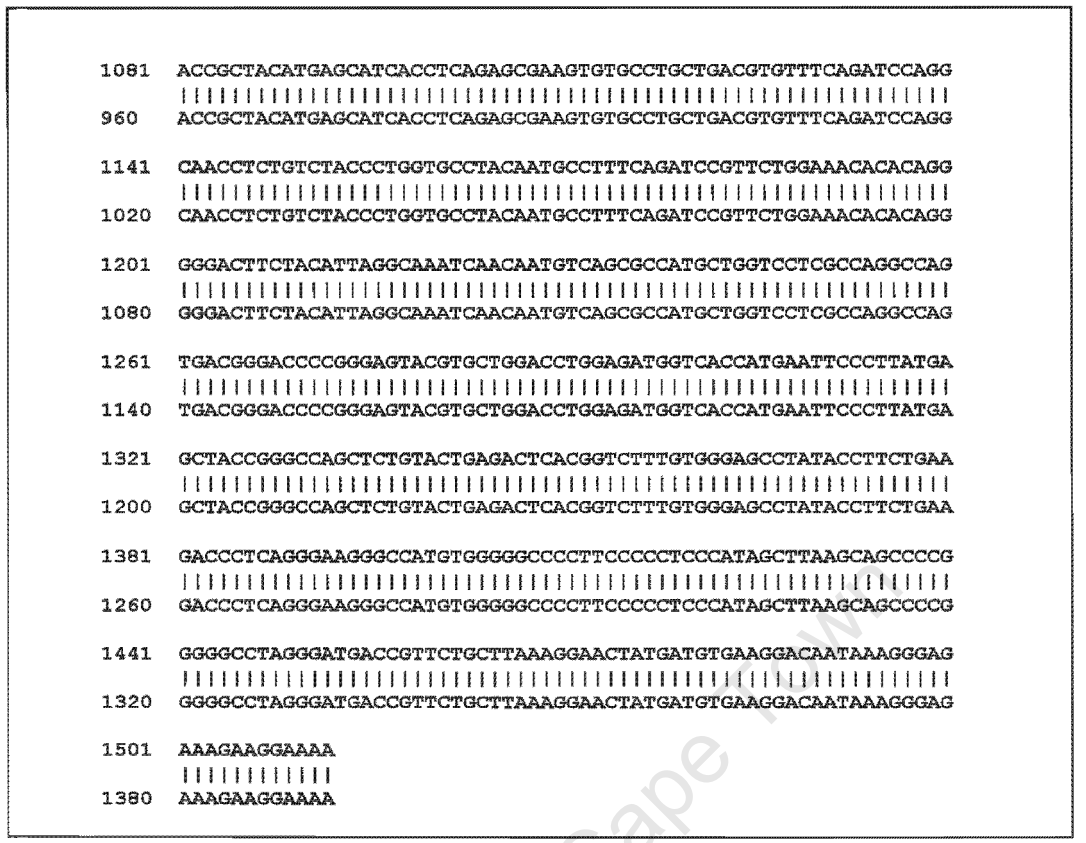



**Figure 4.2.** Alignment DNA Sequences continued: MBP1 (upper line) and $BF_1$-IP2 (lower line). Base pair values for each sequence is given on the left.

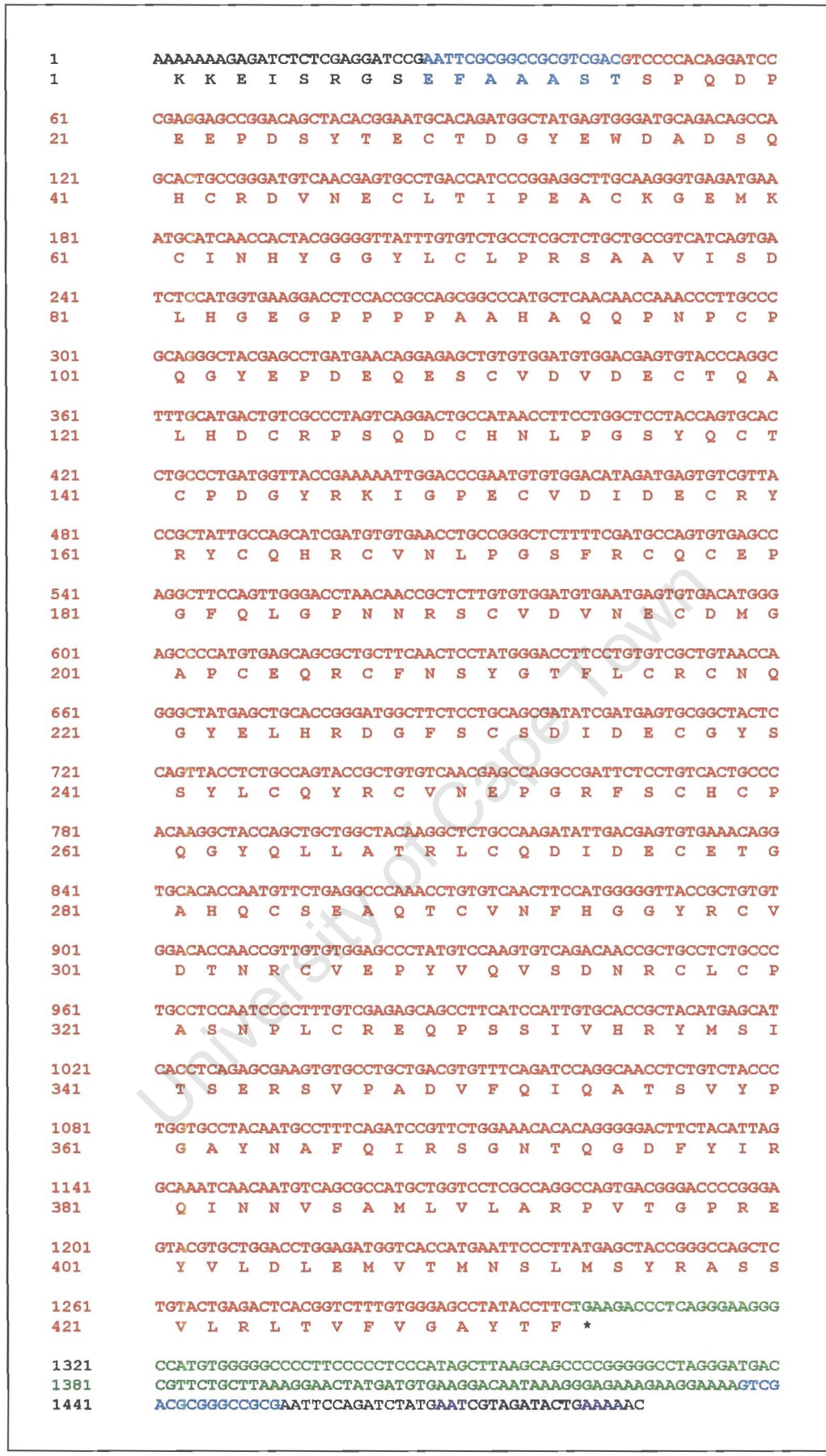





**Figure 4.3.** DNA sequence (upper line) and translation (lower line) of $BF_1$-IP2. The amino acid sequence of $BF_1$-IP2 is continuous with the open reading frame of the GAL4 Activation Domain. The end of the GAL4 Activation domain sequence is in black, the vector sequence is in dark blue, the adaptor sequence is in blue, the coding region of the $BF_1$-IP2 insert is in red, and the untranslated 3' end of the insert is in green.

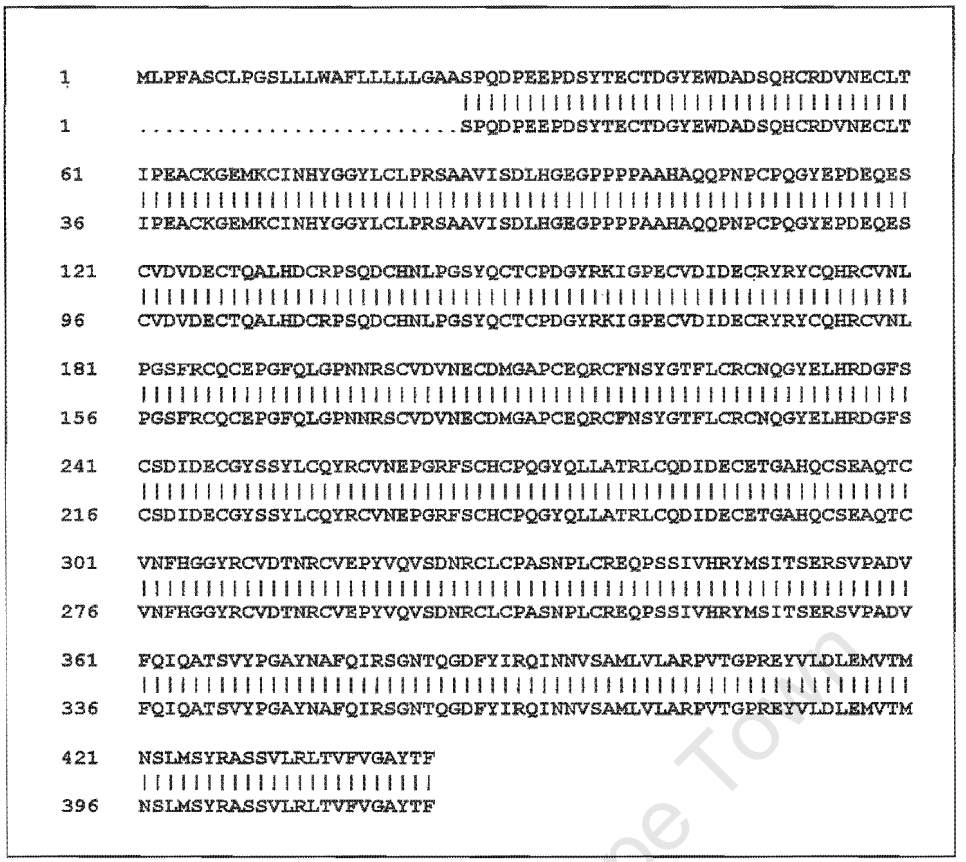



**Figure 4.4.** Alignment of amino acid sequences of MBP1 (upper line) and $BF_1$-IP2 (lower line). Amino acid residue values for each sequence is given on the left.

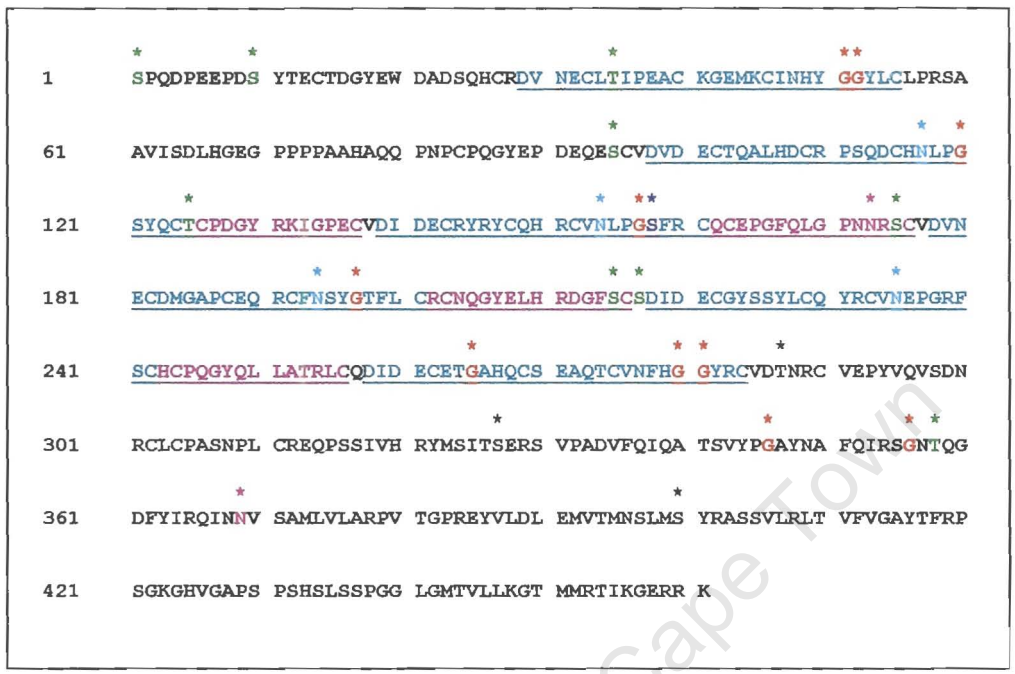



**Figure 4.5.** Protein motifs within the $BF_1$-IP2 amino acid sequence. A coloured asterix above a coloured residue denotes the following modification sites: Asparagine glycosylation (pink), Protein Kinase C (dark blue) and Calcium kinase II (green) phosphorylation, myristolation (red), aspartic acid and asparagine hydroxylation (turquoise). The Epithelial Growth Factor- like domains (purple text) and calcium binding EGF-like domains (teal blue text) are underlined

### 4.2.2. BF-1 Interacting Protein-3

The DNA sequence for $BF_1$-IP3 was 100% homologous to the region from 1143bp –2125bp in the acrogranin gene (accession #: emb/X62321/MMEPIT1) (Figures 4.1A and 4.6). Therefore it was concluded that $BF_1$-IP3 encoded the mouse acrogranin gene. The translated $BF_1$-IP3 amino acid sequence (Figure 4.7) corresponding to amino acids 374–589 in the acrogranin protein sequence (accession #:gi/191767) (Figures 4.1B and 4.8) contained a single asparagine (Asn) glycosylation site, two Protein kinase C (PKC) phosphorylation sites, five myristolation sites and two granulin domains (Table 4.2 and Figure 4.9).

Acrogranin is the precursor molecule for the granulin and epithelin growth factors which were first isolated by Bateman et al (1990) and Shoyab et al (1990) respectively. Acrogranin is expressed throughout the body, but is predominant in epithelial and haematopoietic cells, and can be processed in different ways: for example, intact acrogranin can be secreted (Zhou et al. 1993) or stored in a vesicular organelles such as the sperm acrosome (Baba et al. 1993). It can also be processed into small 6-kDa peptides, which in neutrophils can be stored in vesicles (Bateman et al. 1990, Couto et al. 1992). All these various forms of acrogranin regulate the growth of epithelial and mesenchymal cells. Recently, Diaz-Cueto et al (2000) showed that acrogranin is secreted by pre-implantation mammalian embryos into the surrounding medium and regulates the appearance of the epithelium in the developing mouse blastocyst, the growth of the trophectoderm, and/or the function of the embryonic epithelium.



Acrogranin contains several granulin domains (grnA - grnG) which contain twelve highly conserved cysteine residues. The granulin domain is found in several proteins that are regulators of cell growth and is implicated in mediating protein-protein interactions in a similar manner as EGF-like domains. There are several parallels between the granulin and EGF-like containing proteins: (1) both are cysteine-rich peptides of approximately 6 kDa that can modify cell growth;

(2) they have similar, but not identical, biological activities; (3) the three-dimensional folds of the granulin and EGF-like domains are partially superimposible and (4) the precursors proteins for granulin/epithelin proteins and EGF-like domain containing proteins all contain multiple repeats of conserved cysteine modules.

Acrogranin has been previously shown to interact with the HIV Tat transcription factor, which is required for HIV replication and transcription. Trinh et al (1999) used a yeast two hybrid screen to identify proteins which interacted with the Tat protein encoded by exon 1. This exon contains an activation domain and nascent LTR-leader RNA binding domain. Mutagenesis and deletion studies showed that the grnA and grnB di-repeat in acrogranin interacted with a highly conserved cysteine rich region in the activation domain in exon 1 of the Tat protein. In this thesis, the BF-1 transcription factor has also been shown to interact with acrogranin. Once again, the localization of BF-1 inside and outside the cells needs to be investigated to show whether this result has functional significance.

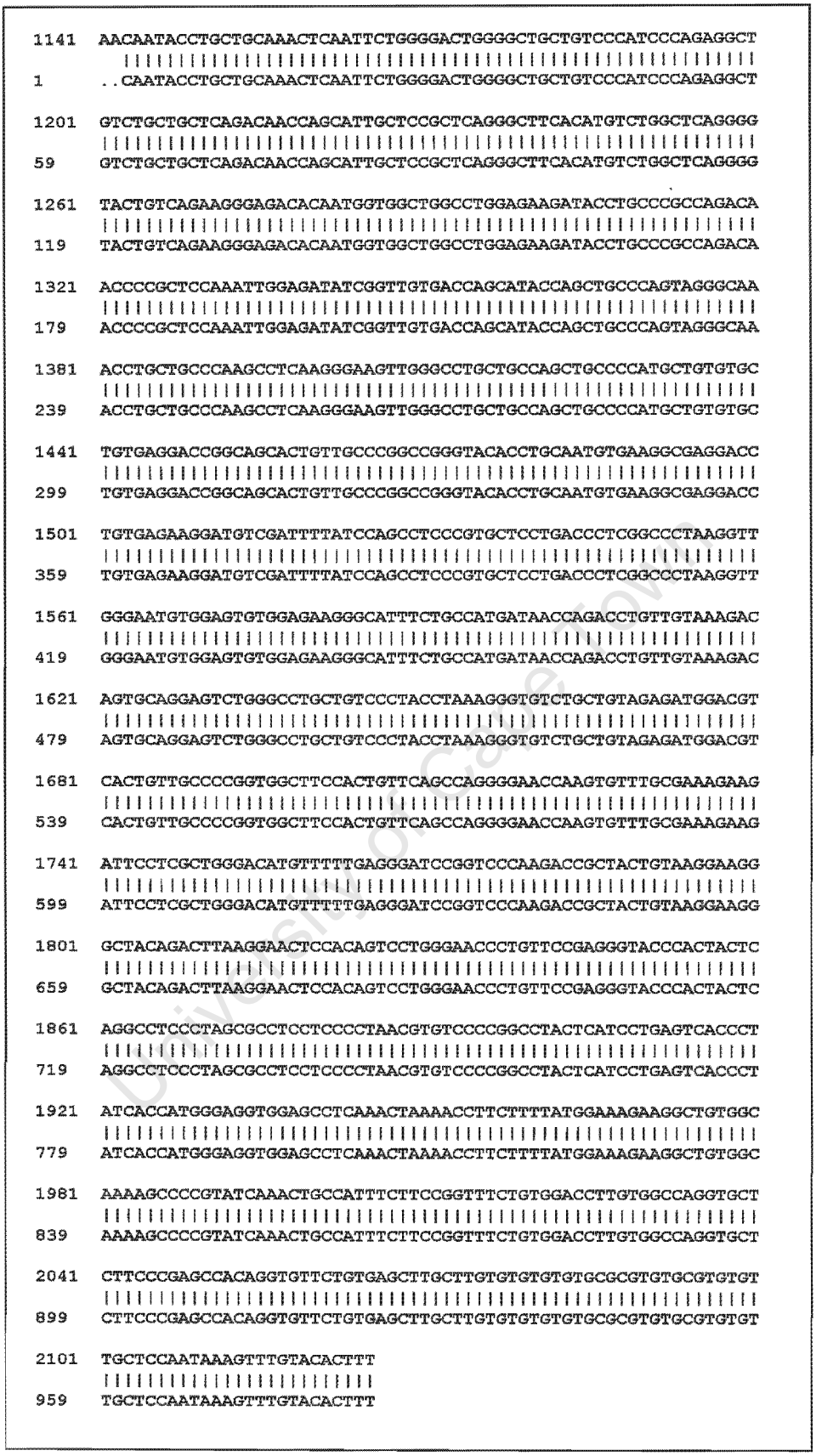



**Figure 4.6.** Alignment of DNA Sequences: Acrogranin (upper line) and $BF_1$-IP3 (lower line). Base pair values for each sequence is given on the left.

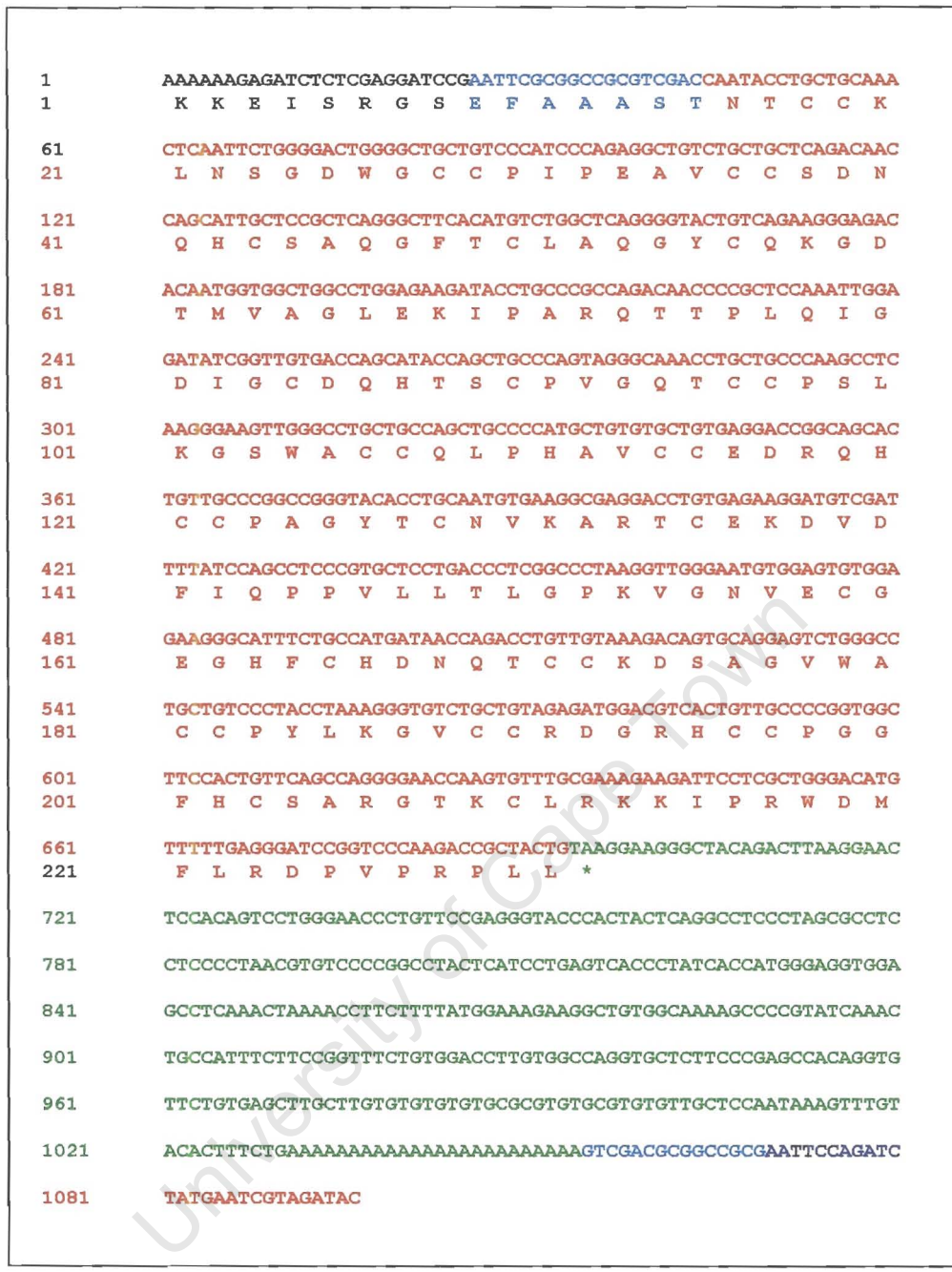



**Figure 4.7.** DNA sequence (upper line) and translation (lower line) of $BF_1$-IP3. The amino acid sequence of $BF_1$-IP3 is continuous with the open reading frame of the GAL4 Activation Domain. The end of the GAL4 activation domain sequence is in black, the vector sequence is in dark blue, the adaptor sequence is in blue, the coding region of the $BF_1$-IP3 insert is in red, and the untranslated 3' end of the insert is in green.

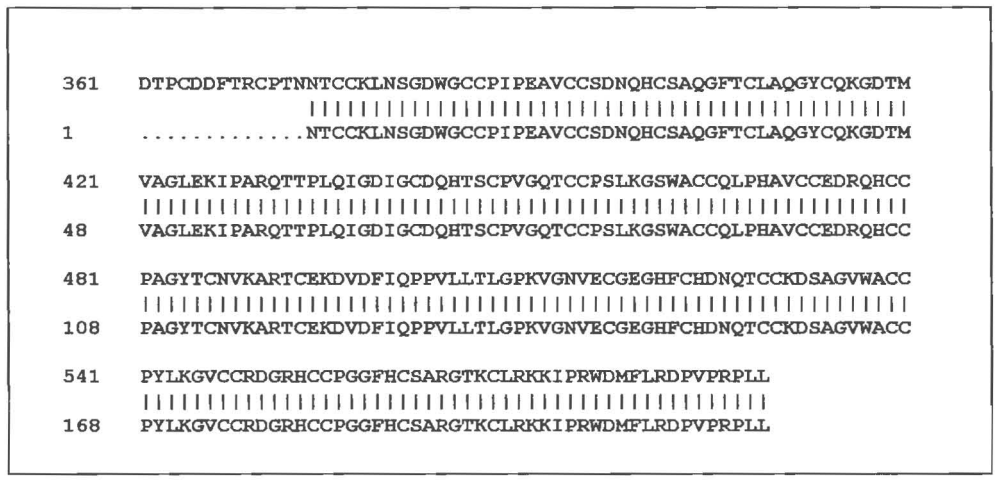



**Figure 4.8.** Alignment of amino acid sequences of acrogranin (upper line) and $BF_1$-IP3 (lower line). Amino acid values for each sequence is given on the left.

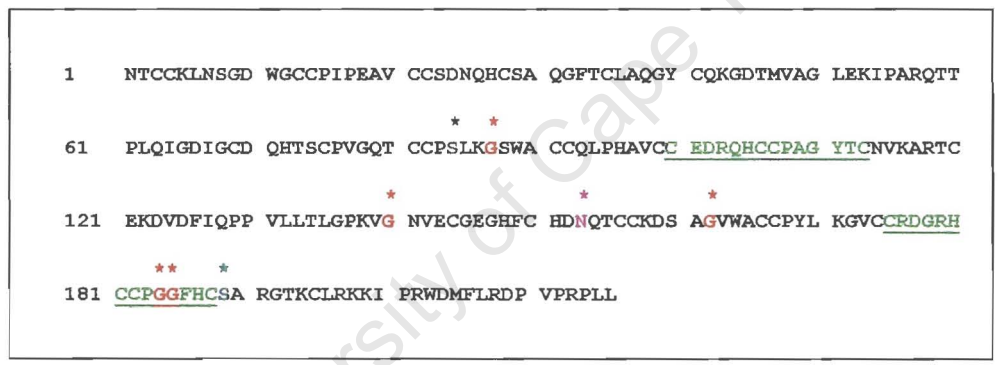



**Figure 4.9.** Protein motifs within the $BF_1$-IP3 amino acid sequence. A coloured asterix above a coloured residue denotes the following modification sites: asparagine glycosylation (pink), Protein Kinase C (dark blue) phosphorylation and myristolation (red). The granulin domains (green text) is underlined.

### 4.2.3. BF-1 Interacting Protein-4

The forward and reverse DNA sequences for $BF_1$-IP4 covered 1431bp of the cDNA insert (1552bp). Both the forward and reverse sequences were 100% homologous to region from 2616 to 3467bp and 3488bp to 4168bp, in the fibulin-2 gene (emb/X75285/MMRFFIB2) (Figures 4.1A and 4.10). Therefore it was concluded that BF1-IP4 encoded the mouse fibluin-2 gene. The translated amino acid sequence of $BF_1$-IP4 (Figure 4.11) corresponding to the regions (amino acids 855–1105 and 1147-1221) in the fibulin-2 protein sequence (accession #: gb/AAD34456.1) (Figures 4.1B and 4.12) contained motifs characteristic of fibulin-2.

The mouse fibulin-2 gene has been previously shown to consist of four domains. The N-terminal domain which contains a glycosylation site, consists of two separate subdomains, a cysteine-rich segment of 150 residues (Na subdomain) and a cysteine-free segment with a stretch of acidic amino acids (Nb subdomain). Domain I contains three anaphylatoxin-like modules and a glycosylation site. Domain II contains and two glycosylation sites and tandem arrays of eleven EGF-like domains, ten of which bound calcium. Domain III is globular and is at the C-terminus of the protein (Pan et al 1993; Zhang et al 1994). The amino acid sequence for $BF_1$-IP4 starts half way in domain II, and contains five calcium binding EGF-like domains interspersed with three EGF-like domains. Analysis of the $BF_1$-IP4 protein showed that it contained a single asparagine glycosylation site and several myristolation sites, as well as phosphorylation sites for cAMP-and cGMP-dependent protein kinase (CAMP), Protein Kinase C and Calcium Kinase II (Table 4.2 and Figure 4.13).

Fibulin-2 is an extracellular matrix protein that belongs to the fibulin family of proteins. Sasaki et al 1997 showed that fibulin-2 exists as a disulphide-linked homodimer of 175 kDa which has a rod-like shape of 80nm length based on an anti-parallel association of two subunits through their central globular domains I. The fibulin proteins have various isoforms that occur due to alternative splicing of the cDNA transcripts: e.g. in the mouse fibulin-2 gene, exon 9 was shown to be subjected to alternative splicing (Grassel et al 1999). Fibulin-2 is expressed in the basement membrane and stroma of many tissues and its expression pattern suggests that it plays an essential role in organogenesis, particularly in embryonic development of the heart and blood vessels as well as, skeletal and neuronal structures ( Pan et al 1993; Zhang et al 1994, 1995, 1996; Miosge 1996). Specifically, Zhang et al (1996) showed that fibulin-2 was located at sites where polarized cells convert into mesenchyme in the endocardial cushion tissue and in neural crest cells. In addition, expression studies by Miosge et al (1996) showed that fibulin-2 was detected primarily within the neuroepithelium, spinal ganglia and peripheral nerves in human embryos of gestational weeks 4-10.

Fibulin-2 has been shown to bind to many extracellular matrix ligands via the calcium binding EGF-like domains found in domain II. These extracellular matrix ligands include the following proteins such as nidogen, perlecan and fibulin-1 (Sasaki et al 1995), Integrins alpha IIb beta 3 and alpha V beta 3 (Pfaff et al 1995), Fibronectin (Sasaki et al 1996), Fibrillin-1 (Reinhardt et al 1996), Fibulin-1 (Miosge et al 1998), Tropoelastin (Sasaki et al 1999) and endostatin (Miosge et al 1999).

Therefore if BF-1 interacts with fibulin-2 via a similar mechanisms involving the EGF-like domains, the localization of BF-1 inside and outside the cells needs to be determined if the interaction is biologically significant.

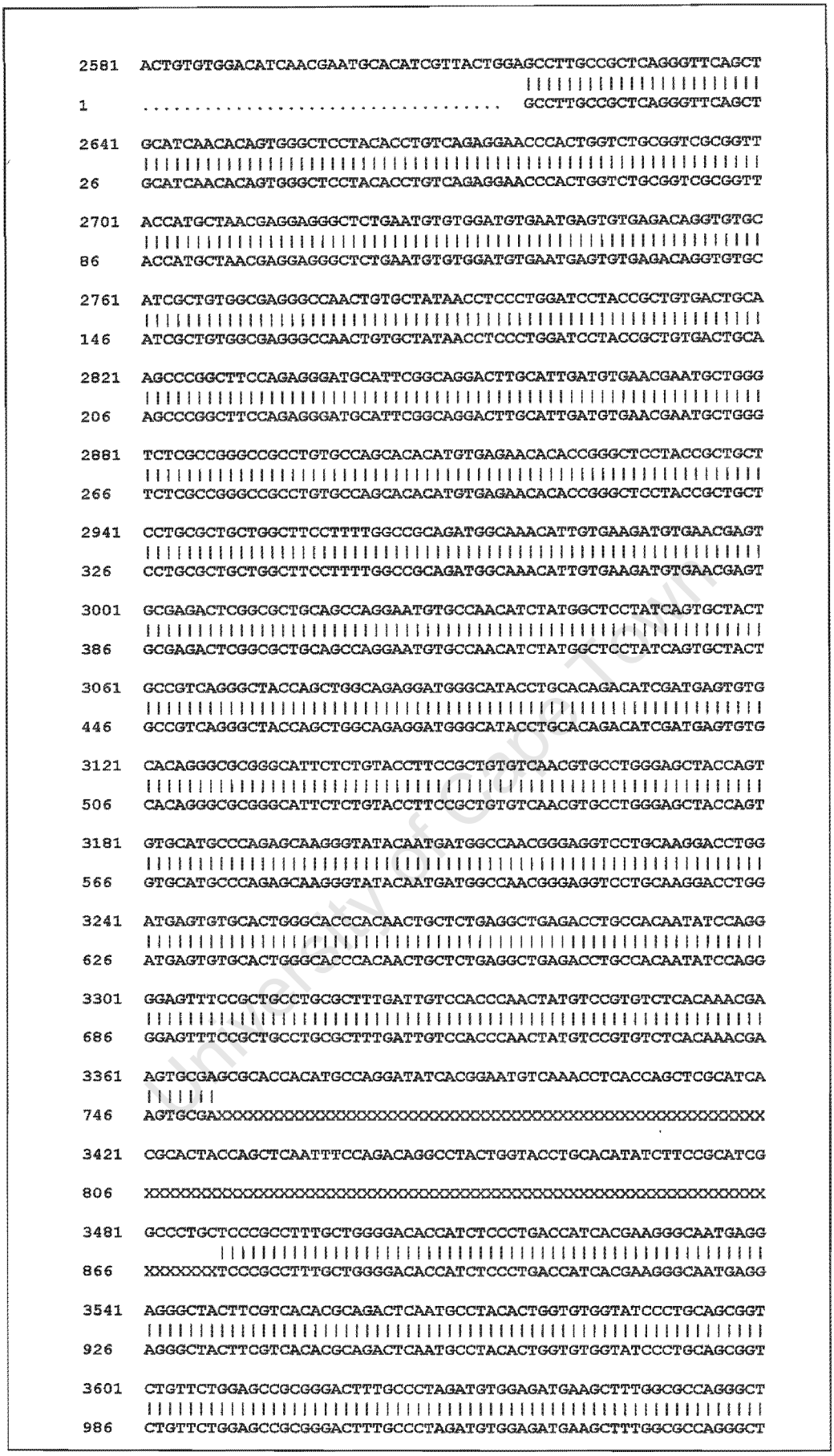



**Figure 4.10.** Alignment DNA sequences of Fibulin-2 (upper line) and $BF_1$-IP4 (lower line). The base pair value of each sequence is given on the left. The "X" denotes outstanding DNA sequence.

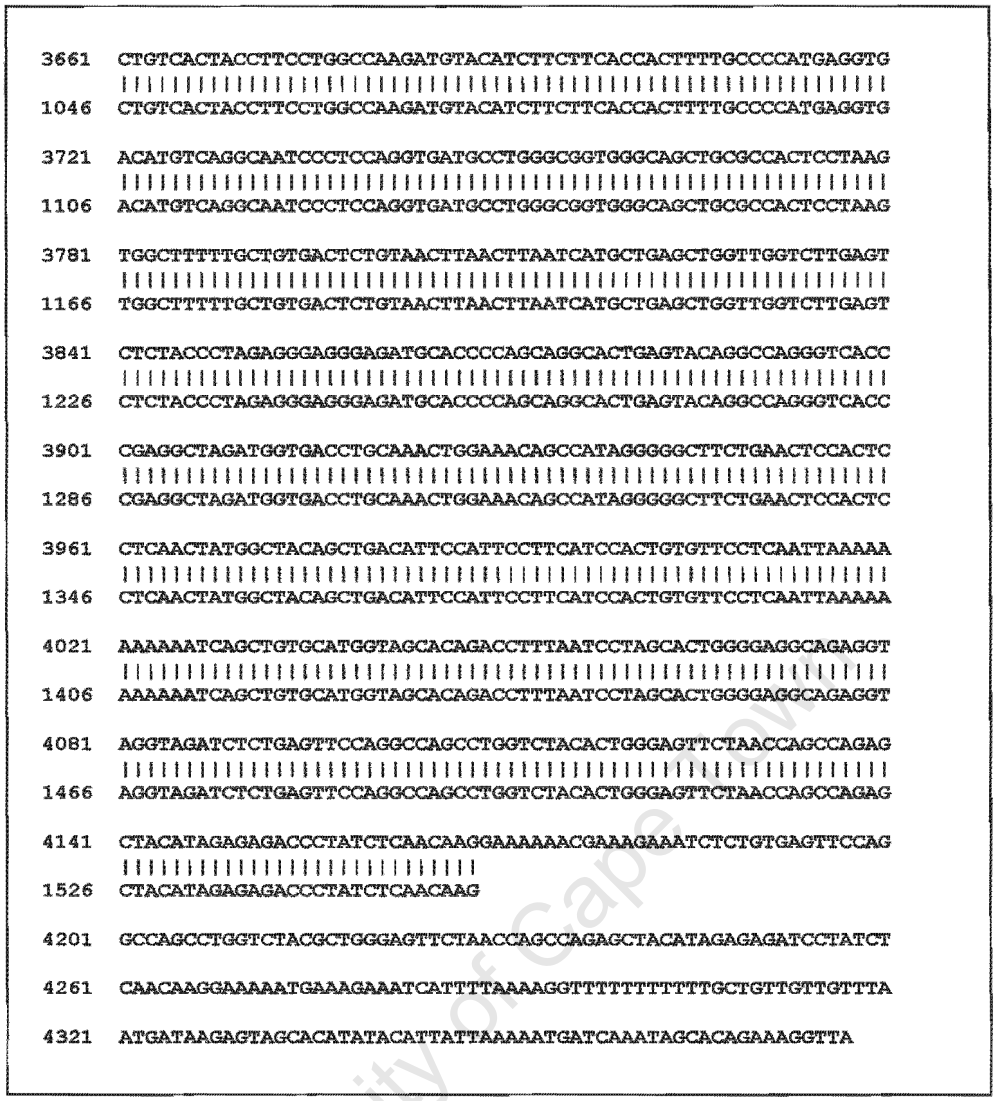



**Figure 4.10.** Alignment DNA sequences (continued): Fibulin-2 (upper line) and $BF_1$-IP4 (lower line). The stop codon is shown in red. The base pair value of each sequence is given on the left. The "X" denotes outstanding DNA sequence.

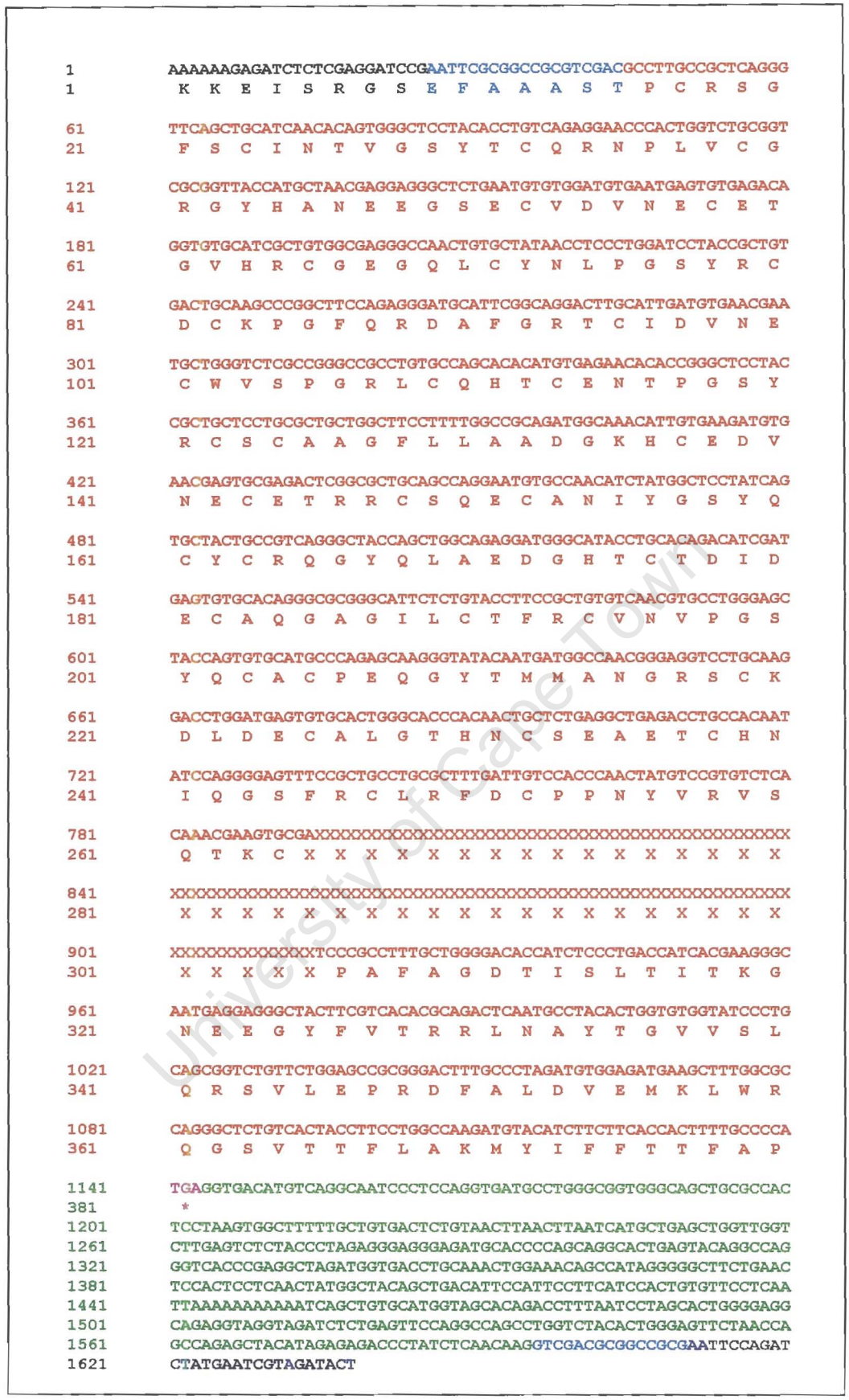





**Figure 4.11.** $BF_1$-IP4 DNA sequence and translation. The amino acid sequence of $BF_1$-IP4 is continuous with the open reading frame of the GAL4 Activation Domain. The end of GAL4 activation domain sequence is in black, the vector sequence is in dark blue, the adaptor sequence is in blue, the coding region of the $BF_1$-IP4 insert is in red, and the untranslated 3' end of the insert is in green.


**Figure 4.12.** Alignment of amino acid sequences of Fibulin-2 (upper line) and $BF_1$-IP4 (lower line). The amino acid residue values for each sequence is given on the right.

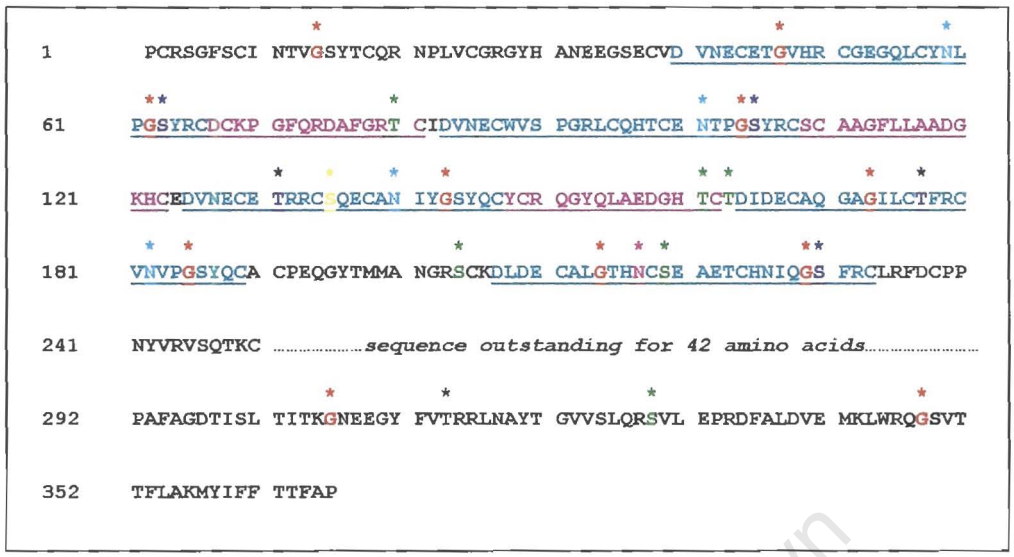



**Figure 4.13.** Protein motifs within the $BF_1$-IP4 amino acid sequence. A coloured asterix above a coloured residue denotes the following modification sites: asparagine glycosylation (pink), Protein Kinase C (dark blue) and Calcium kinase II (green) phosphorylation, myristolation (red), aspartic acid and asparagine hydroxylation (turquoise). The Epithelial Growth Factor- like domains (purple text) and calcium binding EGF-like domains (teal blue text) are underlined.

### 4.2.4. BF–1 Interacting Protein –1

The DNA sequence for $BF_1$-IP1 was 301 bp long and was sequenced in both the forward and reverse directions to double check the sequence information obtained (Figure 4.14A). The amino acid sequence of $BF_1$-IP1 contained a coding region of 25 amino acid residues continuous with the GAL4 AD, followed immediately by one stop codon and later by another two stop codons (Figure 4.14). The $BF_1$-IP1 clone therefore could encode the end of the coding region of a gene, since it includes the three stop codons and an untranslated 3' region.

The amino acid sequence did not have homology to any known protein sequence in the non-redundant GenBank database, thereby strongly suggesting that $BF_1$-IP1 encodes an unknown protein. The amino acid sequence also contained a potential 4-disulfide core domain, as well as an unusual motif of ten consecutive cysteine residues at the C-terminal end (Figure 4.14B). Analysis of the amino acid composition of $BF_1$-IP1 (Figure 4.15) showed that the protein is rich in cysteine (70% mol/mol) and that the N-terminal domain is hydrophilic while the C-terminal domain is predominantly hydrophobic (Figure 4.16). The secondary structure of the $BF_1$-IP1 protein was predicted using the DNAMAN software, which used the DSC (Discrimination of protein Structure Classes) method developed by King and Sternberg (1996). The plot (Figure 3.17) showed that $BF_1$-IP1 had no significant structure and had a high probability of forming coils.

Interestingly, nucleotide residues 2 – 18 on the plus strand of the $BF_1$-IP1 DNA sequence was found to be 100% homologous to a 17bp region on the minus DNA strand of human genes encoding a p38 Interacting Protein (Accession # gb/AF093250.1/AF093250) and a transcription factor (accession # emb/AJ130894.1/HSA130894) (Figure 4.18). However, since the homology was to the minus strand of these genes, the match was not considered significant and $BF_1$-IP1 therefore considered to encode an unknown protein.

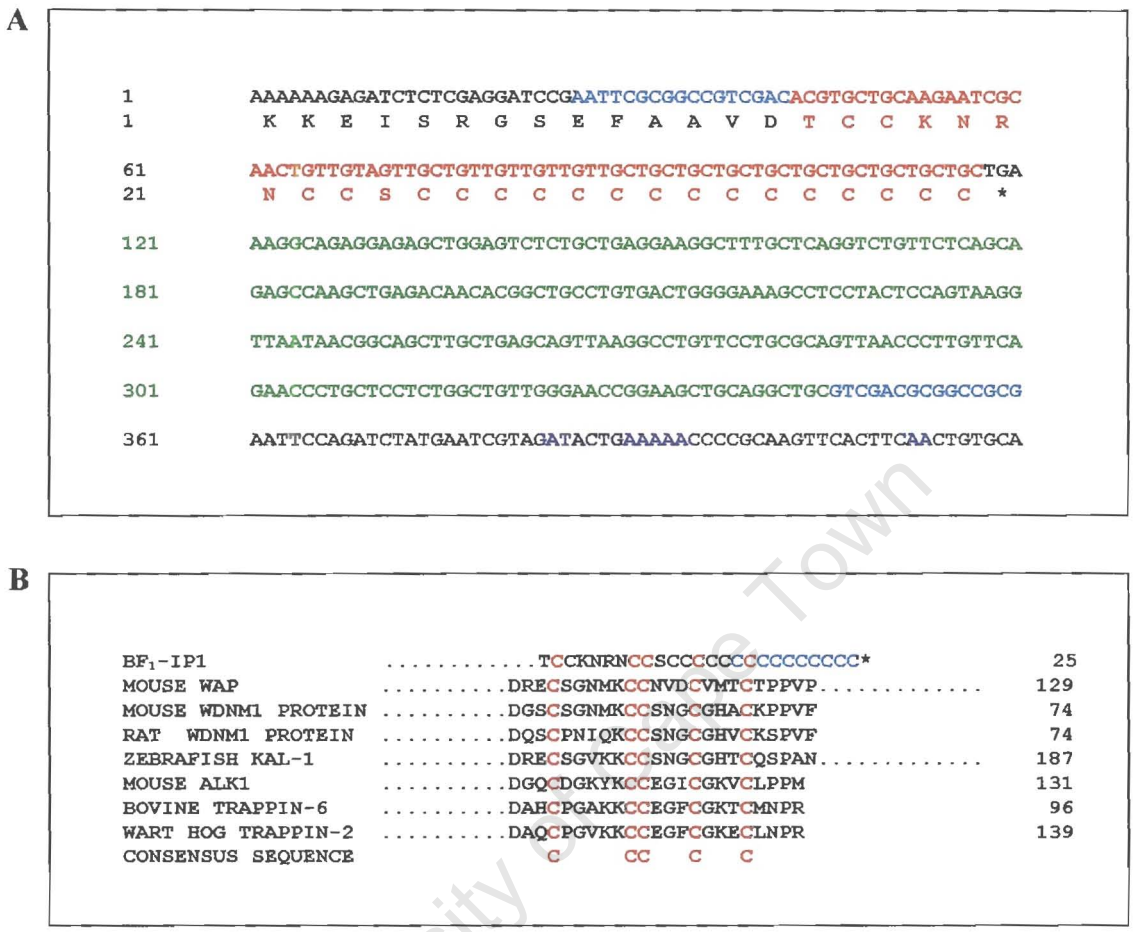





**Figure 4.14.** **(A)** Translation of $BF_1$-IP1. The amino acid sequence of $BF_1$-IP1 is continuous with the open reading frame of the GAL4 Activation Domain. The GAL4 Activation domain sequence is in black, the vector sequence is in dark blue, the adaptor sequence used to clone the insert is in blue, the coding region of the $BF_1$-IP1 insert is in red, and the untranslated 3' end of the insert is in green. **(B)** The amino acid sequence contains a potential 4-disulpihide core domain (red) and a cysteine string (blue) domain. Alignment of the $BF_1$-IP1 and other proteins containing a 4-disulpihide core domain. The conserved cysteine residues of the consensus sequence are highlighted in red.

**A**

| Amino acid | | Num | %mol/mol | %w/w |
|---|---|---|---|---|
| A | Ala | 0 | 0.00 | 0.00 |
| C | Cys | 19 | 70.37 | 68.48 |
| D | Asp | 1 | 3.70 | 3.96 |
| E | Glu | 0 | 0.00 | 0.00 |
| F | Phe | 0 | 0.00 | 0.00 |
| G | Gly | 0 | 0.00 | 0.00 |
| H | His | 0 | 0.00 | 0.00 |
| I | Ile | 0 | 0.00 | 0.00 |
| K | Lys | 1 | 3.70 | 4.35 |
| L | Leu | 0 | 0.00 | 0.00 |
| M | MET | 0 | 0.00 | 0.00 |
| N | Asn | 2 | 7.41 | 7.86 |
| P | Pro | 0 | 0.00 | 0.00 |
| Q | Gln | 0 | 0.00 | 0.00 |
| R | Arg | 1 | 3.70 | 5.18 |
| S | Ser | 1 | 3.70 | 3.13 |
| T | Thr | 1 | 3.70 | 3.54 |
| V | Val | 1 | 3.70 | 3.49 |
| W | Trp | 0 | 0.00 | 0.00 |
| Y | Tyr | 0 | 0.00 | 0.00 |

**B**

1 EFAAVDTCCK NRNCCSCCCC CCCCCCCCC C

**Figure 4.15**. **(A)** Amino Acid Composition of the $BF_1$-IP1 Amino Acid Sequence. **(B)** The amino acids highlighted in blue were analysed.

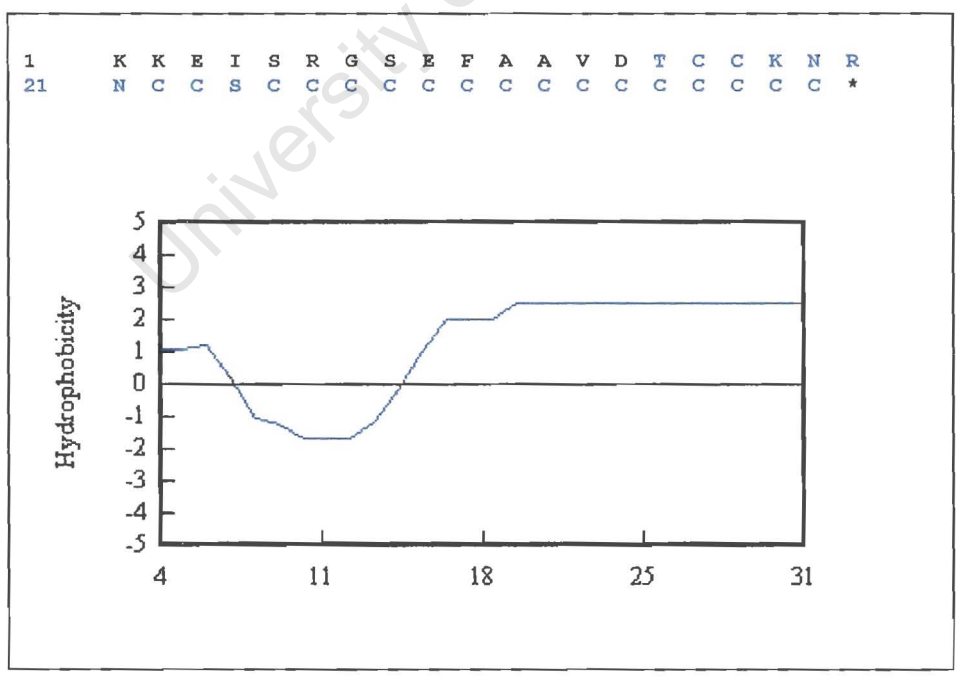
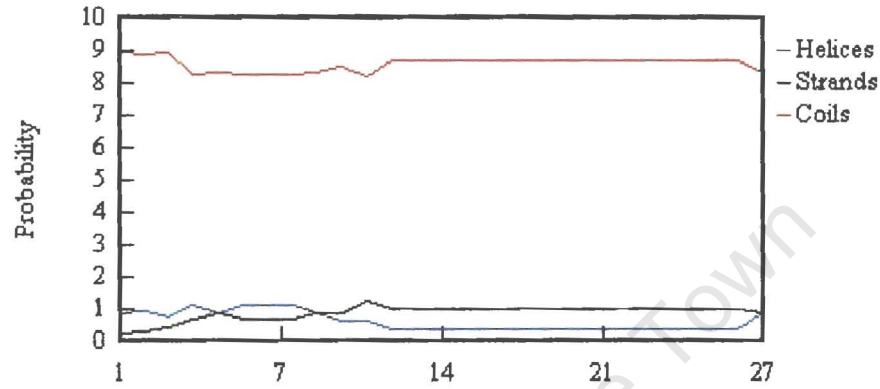



**Figure 4.16.** The hydrophobicity plot of $BF_1$-IP1 based on the method Kyte and Doolittle (1982). The amino acids highlighted in blue were analysed.

```
1     K K E I S R G S E F A A V D T C C K N R
21    N C C S C C C C C C C C C C C C C C C *
```




Prediction accuracy= 83.32%

Structure Probability Table

| Pos | aa | STR | HELIX | STRAND | COIL |
|---|---|---|---|---|---|
| 1 | V | Coil | 0.09 | 0.02 | 0.89 |
| 2 | D | Coil | 0.09 | 0.03 | 0.88 |
| 3 | T | Coil | 0.07 | 0.04 | 0.89 |
| 4 | C | Coil | 0.11 | 0.07 | 0.82 |
| 5 | C | Coil | 0.09 | 0.09 | 0.83 |
| 6 | K | Coil | 0.11 | 0.07 | 0.82 |
| 7 | N | Coil | 0.11 | 0.07 | 0.82 |
| 8 | R | Coil | 0.11 | 0.07 | 0.82 |
| 9 | N | Coil | 0.09 | 0.09 | 0.83 |
| 10 | C | Coil | 0.06 | 0.09 | 0.85 |
| 11 | C | Coil | 0.06 | 0.12 | 0.82 |
| 12 | S | Coil | 0.03 | 0.10 | 0.87 |
| 13 | C | Coil | 0.03 | 0.10 | 0.87 |
| 14 | C | Coil | 0.03 | 0.10 | 0.87 |
| 15 | C | Coil | 0.03 | 0.10 | 0.87 |
| 16 | C | Coil | 0.03 | 0.10 | 0.87 |
| 17 | C | Coil | 0.03 | 0.10 | 0.87 |
| 18 | C | Coil | 0.03 | 0.10 | 0.87 |
| 19 | C | Coil | 0.03 | 0.10 | 0.87 |
| 20 | C | Coil | 0.03 | 0.10 | 0.87 |
| 21 | C | Coil | 0.03 | 0.10 | 0.87 |
| 22 | C | Coil | 0.03 | 0.10 | 0.87 |
| 23 | C | Coil | 0.03 | 0.10 | 0.87 |
| 24 | C | Coil | 0.03 | 0.10 | 0.87 |
| 25 | C | Coil | 0.03 | 0.10 | 0.87 |
| 26 | C | Coil | 0.03 | 0.10 | 0.87 |
| 27 | C | Coil | 0.09 | 0.09 | 0.83 |



**Figure 4.17.** The predicted secondary structure of the amino acid sequence (highlighted in blue) for $BF_1$-IP1was based on the DSC method of King and Sterngberg (1996).

**A**

```
1       ACGTGCTGCA AGAATCGCAA CTGTTGTAGT TGCTGTTGTT GTTGTTGCTG CTGCTGCTGC
61      TGCTGCTGCT GCTGCTGAAA GGCAGAGGAG AGCTGGAGTC TCTGCTGAGG AAGGCTTTGC
121     TCAGGTCTGT TCTCAGCAGA GCCAAGCTGA GACAACACGG CTGCCTGTGA CTGGGGAAAG
181     CCTCCTACTC CAGTAAGGTT AATAACGGCA GCTTGCTGAG CAGTTAAGGC CTGTTCCTGC
241     GCAGTTAACC CTTGTTCAGA ACCCTGCTCC TCTGGCTGTT GGGAACCGGA AGCTGCAGGC
301     TGC
```

**B**

```
BF1-IP1:    2    tgctgcaagaatcgcaa 18
                 ||||||||||||||||||
p38IP:    2314   tgctgcaagaatcgcaa 2298

BF1-IP1:    2    tgctgcaagaatcgcaa 18
                 ||||||||||||||||||
HumanTF:  2404   tgctgcaagaatcgcaa 2388
```



**Figure 4.18. (A)** DNA Sequence of $BF_1$-IP1 and **(B)** BLAST Results. The DNA stop codon is highlighted in red and the untranslated region is in green. The sequence that matched 100% to a region in p38IP and a human transcription factor is highlighted in blue.

As mentioned previously, the $BF_1$-IP1 protein contains a 4-disulphide core domain and cysteine string motif. The 4-disulphide core domain was first discovered in whey acidic proteins and is also called whey acidic protein (WAP) domain. This domain consists of eight conserved cysteine residues that fold to form four disulphide bonds which stabilizes the domain. The biochemical properties of the WAP domain in various proteins is largely unknown, but they have been implicated in playing a role in protein-protein interactions (Schalkwijk et al 1999). Studies on protease inhibitor proteins called trappins (TRransglutaminase substrate and wAP domain containing ProteINs) revealed that the WAP domain of trappin-2 inhibited the activity of serine proteases by binding the proteases (Schalkwijk et al 1999). The cysteine string motif, consisting of 10 consecutive cysteine residues, is found in cysteine string proteins (CSPs) as well as other proteins. Cysteine-string proteins (CSPs) are highly conserved components of secretory organelles such as synaptic vesicles, where they are responsible for the regulated secretion of neurotransmitters and peptide exocytosis. (Graham and Burgoyne 2000; Umbach et al 1998; Ranjan et al 1998; Leveque et al 1998; Zhang et al 1998a; Chamberlain and Burgoyne 1998b; Morales et al 1999). CSPs have also been shown to play a role outside of neurotransmission, based on their expression in the synaptic vesicles of a variety of non-neural tissues (Eberle et al 1998; Kohan et al 1995; Jacobsson and Meister 1996; Chamberlain and Burgoyne 1996a).



Both WAP domain-containing proteins and cysteine-string-containing proteins have been shown to play a role in neuronal development. The KAL-1 gene, which encodes a "WAP" domain extracellular matrix glycoprotein called Anosmin-1 (Dandekar et al 1982), was shown by Ardouin et al (2000) to be important for development of the olfactory tract and bulb and the migration of GnRH neurons and their release of GnRH. Studies by Nie et al 1999 implicated cysteine string proteins in embryonic development. They showed that the individual over-expression of various CSP isoforms in wild-type *Drosophila* resulted consistently in impaired viability and interference with wing and eye development.

However, the role of CSP's in wing and eye development was unknown, but it is intriguing that BF-1, which also plays a role in eye development, interacts with a protein which also contains a cysteine string motif ($BF_1$-IP1). The cysteine-string motif has been shown to create a hydrophobic region that targets and integrates CSPs into membranes of secretory vesicles (Mastrogiacomo et al 1998; Chamberlain and Burgoyne 1998c). Moreover the human RGSZ1 protein, which has no membrane-spanning region, was shown to be membrane bound via its cysteine string motif (Wang et al 1998).

On the basis of its homology to the cysteine string motif, we can speculate that the protein encoded by $BF_1$-IP may be able to integrate into cellular or organelle membranes via its cysteine string motif. The remainder of the $BF_1$-IP1 protein containing the WAP domain may extend into the cytoplasm, similar to the G-Alpha-Interacting-Protein (GAIP) which is membrane bound via its cysteine string and faces the cytoplasm (De Vries et al 1996). The WAP domain extended in the cytoplasm could interact with other proteins, as speculated by Schalkwijk et al (1999). As mentioned previously, proteins containing WAP domains have been have implicated in playing a role in the development of the olfactory tract and bulb (Ardouin et al 2000), which are also areas regulated by BF-1. Therefore, the WAP domain of $BF_1$-IP1 may interact with BF-1 or with other regulatory proteins in the olfactory neuroepithelium during development.



Consistent with this theory is the hydrophobic profile of the $BF_1$-IP1 (Figure 4.16), in which the small part at the N-terminal of the protein is hydrophilic and the remainder of the protein containing the cysteine string is hydrophobic. The $BF_1$-IP1 protein therefore has a high probability of integrating into the hydrophobic environment of a membrane. In addition, the predicted secondary structure of the $BF_1$-IP1 protein (Figure 4.17 ) showed that $BF_1$-IP1 has high probability of forming coils, which would allow it to integrate into membranes.

## 4.3. CONCLUSION

Four proteins have been identified in a yeast two hybrid screen to interact with BF-1. Three of these proteins are extracellular matrix proteins and the fourth is a novel protein. There are two interpretations to the results of this thesis: the first is that the interactions are an artefact of the two-hybrid system, and the second is that they are biologically relevant.

The yeast two hybrid system is an *in vivo* screen in which the interaction between the bait fusion (GAL4 DB) protein and a library (GAL4 AD) fusion protein are selected for in the nucleus of the yeast cell. The fusion proteins are expressed within the yeast cell and are specifically targeted to the nucleus via a nuclear targeting sequence. Therefore extracellular matrix proteins encoded by cDNAs fused to the GAD4 AD in the two hybrid system, could therefore be targeted to the nucleus. It is possible that the observed interactions are a consequence of the artificial localization of the extracellular matrix proteins and represent non-specific interactions. However, a number of tests were conducted to try and eliminate false positives. Yeast colonies containing the $BF_1$-IP1, $BF_1$-IP2, $BF_1$-IP3 and $BF_1$-IP4 plasmids only turned blue in the presence of pGalDBD/BF-$1_{(26\text{-}480aa)}$, encoding BF-1 fused to the GAL4 DNA binding domain, and not PGBT9 or pLAM5, encoding the GAL4 DB and GAL4DB-laminC fusion proteins respectively.

The alternative explanation is that these interactions have a biological function and are similar to those interactions observed for mutant p53 and HIV Tat proteins (Gallagher et al 1999; Trihn et al 1999). An important question is how BF-1, a transcription factor, interacts with these extracellular matrix proteins *in vivo*. A first step in answering this question is to determine the subcellular distribution pattern of BF-1 in the neuroepithelial cells. The amino acid sequence of BF-1 does not contain an obvious signal sequence. We do not know if BF-1 is secreted into the extracellular matrix like the HIV tat proteins or, whether it associates with organelle membranes like the mutant p53 protein.

In addition, $BF_1$-IP2 and $BF_1$-IP4 share common motifs such as calcium binding EGF-like domains (Figure 4.19) that have been shown to mediate protein-protein interactions. Similarly, $BF_1$-IP3 contains granulin domains which have also been implicated in protein-protein interactions. Therefore the interaction between BF-1 and these extracellular matrix proteins may be mediated by mechanisms involving these domains.

Analysis of the $BF_1$-IP1 DNA and amino acid sequence suggested that it is a novel protein. The interaction between $BF_1$-IP1 and BF-1 was robust and produced 86.4 ($\pm$ 21.09) units of β-galactosidase compared to the pVA3 and pTD1 control (140.5$\pm$18 units). In addition the BF1-IP1 clone was isolated eight times in independent two-hybrid experiments. Since the $BF_1$-IP1 clone is so small and little functional information available, the full length gene therefore needs to be cloned and characterized in order to analyse the novel protein more completely with respect to its function and expression. The expression and distribution of the $BF_1$-IP1 protein within the cells, and the site of its interaction with BF-1 needs to be determined.

The pathway whereby BF-1 elicits it proliferative activity and specifies regional identity on the developing neuroepithelial cells is unknown. The interactions identified in this study may belong to a novel developmental pathway in which BF-1 interacts with membrane bound ($BF_1$-IP1) or extracellular matrix proteins ($BF_1$-IP2, -IP3, -IP4). It is therefore important that the interactions between BF-1 and $BF_1$-IP1, $BF_1$-IP-2, $BF_1$-IP-3 and $BF_1$-IP–4 are verified using affinity chromatography and co-immunoprecipitation. Of further interest, would be to map the domains of BF-1 involved in the protein-protein interactions. The C-terminal end of the BF-1 has been shown to act as a transcriptional repressor, and at the N-terminus of BF-1 there is an striking 10 histidine repeat of unknown function that is found in many developmental transcription factors (noted by E.Rumbak). Therefore it would be interesting to see if either of these two regions are involved in mediating protein-protein interactions.


**Figure 4.19.** Alignment of the Amino Acid sequences for $BF_1$-IP2 and $BF_1$-IP4. The Calcium binding EGF-like domains are indicated by bold teal text. The EGF like domains are shown in bold purple text.

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