Functional characterisation of Mss11p, a transcriptional regulator of pseudohyphal development, starch degradation and flocculation in *Saccharomyces cerevisiae*

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

The yeast *Saccharomyces cerevisiae* is able to sense and respond to changes in its immediate environment. Information regarding the nutritional status of the extracellular environment is sensed by membrane receptor systems and relayed through signalling pathways to the nuclear interior, affecting the transcription of specific genes., Transcription factors, which function downstream of these signal transduction pathways, have to be transported into the nucleus after synthesis in the cytoplasm in order to regulate transcriptional events. Transport into the nucleus occurs in a tightly regulated manner at the nuclear pore complex, which is located in the nuclear membrane, and requires the recognition of transport signal sequences, which are present in the proteins that are to be transported. Signalling pathways control the nuclear accessibility of transcriptional regulators by modifying their respective signal sequences.

In response to a limited availability of carbon or nitrogen, cells are able to change their morphology from a unicellular ovoid form to elongated cells attached to each other. This morphological change is associated with daughter cells that remain attached to their respective mother cells following unipolar budding, thus forming filamentous structures referred to as pseudohyphae. The regulation of the development of pseudohyphae is correlated with other physiological processes, such as starch degradation and the invasion of agar-containing media. Mss11p performs a central role in the regulation of the genes required for these processes and it has been shown to specifically regulate the expression of *FLO11*, which encodes a cell surface protein critical for pseudohyphal development, and *STA2*, which encodes an extracellular glucoamylase functioning in the degradation of starch.

The aim of this study was to characterise the functioning of Mss11p. Overexpression analysis indicates that Mss11p functions as an inducer of invasive growth, cell elongation and flocculation. Furthermore, *MSS11* deletion improves biomass formation and suppresses the growth defect of yeast from a ∑1278b genetic background transformed with the *RAS2*^{val19} allele on non-fermentable carbon sources. Biochemical analysis shows that Mss11p is a nuclear protein of approximately 97 kDa in apparent size that is maintained at relatively low levels in yeast. Finally, the data suggest a model in which Mss11p functions as a mediator of the transcriptional regulation of various genes.

OPSOMMING

Die gis *Saccharomyces cerevisiae* is in staat om veranderinge in sy onmiddelike omgewing waar te neem en daarop te reageer. Inligting betreffende die beskikbaarheid van voedingstowwe in die omgewing word vanaf membraan reseptorsisteme deur middel van seintransduksiekaskades na die nukleus herlei, waar die transkripsie van spesifieke gene beïnvloed word. Transkripsie faktore wat stroom af van hierdie seintransduksie funksioneer, moet na die nukleus vervoer word na vervaardiging in die sitoplasma, om sodoende transkripsionele gebeurtenisse te reguleer. Die vervoer van faktore na die binnekant van die nukleus vind onder streng regulering plaas by die nukleêre porie kompleks, wat in die nukleêre membraan gesitueer is. Vervoer vind plaas deur middel van die herkenning van nukleêre lokaliseringsekwense wat in die proteïene wat vervoer word, teenwoordig is. Seintransduksiekaskades beheer die beskikbaarheid van proteïene tot die nukleus deur hul onderskeidelike nukleêre lokaliseringsekwense te modifiseer.

Selle is in staat om hul morfologie te verander van 'n eensellige eliptiese vorm tot verlengde selle wat aan mekaar geheg bly in reaksie op die beperkende beskikbaarheid van koolstof of stikstof bronne. Hierdie morfologiese verandering word geassosieer met dogterselle wat ná monopolêre botselvorming aan hul moederselle geheg bly, en dus filamentagtige strukture vorm wat pseudohifes genoem word. Die regulering van die ontwikkeling van pseudohifes word gekorreleer met ander fisiologiese prosesse, soos styselafbraak en die penetrerende groei van selle op agar-bevattende media. Mss11p vervul 'n sentrale rol in die regulering van gene wat vir hierdie prosesse benodig word en reguleer die uitdrukking van *FLO11,* wat kodeer vir 'n selwandproteïen wat krities is vir die ontwikkeling van pseudohifes, en *STA2*, wat kodeer vir 'n ekstrasellulêre glukoamilase wat vir die afbraak van stysel benodig word.

Die doel van hierdie studie was om Mss11p-funksie te karakteriseer. Deur middel van oorproduksie is Mss11p as die induseerder van penetrerende groei, selverlenging en flokkulasie geïdentifiseer. Verder is bevind dat *MSS11*-delesie lei tot verhoogde biomassa formasie, en dat dieselfde delesie lei tot 'n oorkoming van 'n groeidefek van gis van die ∑1278b genetiese agtergrond wat met die $RAS2^{val19}$ aleel op nie-fermenteerbare koolstofbronne getransformeer is. Biochemiese analise dui daarop dat Mss11p 'n nukluêre proteïen is van ongeveer 97 kDa in oënskynlike grootte, wat teen lae vlakke in gis onderhou word. Die data stel 'n model voor waarin Mss11p as bemiddelaar optree vir die transkripsionele regulering van verskeie gene.

BIOGRAPHICAL SKETCH

Michael Bester was born in Port Elizabeth, South Africa on 9 October 1976. He matriculated at the DF Malan High School, Bellville in 1994.

In 1995, he enrolled at Stellenbosch University and obtained a BSc degree in Biochemistry and Microbiology in 1998. The following year, he completed a BSc Hons degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University.

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PREFACE

This thesis is presented as a compilation of four chapters. Additional information can be found in the appendix.

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CHAPTER 1

INTRODUCTION AND PROJECT AIMS

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Fungi, which lack chlorophyll and belong to the eukaryote kingdom eumycota, generally exist as multicellular organisms that absorb nutrients from their immediate environment. Typically, fungal organisms are able to form elongated filaments or hyphae, often in response to environmental changes. Fungi that live as unicellular organisms and that have generally lost the ability to form hyphae are collectively referred to as "yeasts". A considerable number of yeasts, however, are still able to grow filamentous structures called pseudohyphae, which consist of elongated cells that remain attached to each other. This ability to change cell morphology can be correlated to the virulence of pathogenic fungi (for recent reviews see Gow *et al.*, 2002; Rooney and Klein, 2002). The pathogen *Candida albicans*, which is a yeast that forms pseudohyphae, is even able to form true hyphae (for a review see Gow, 1997).

Saccharomyces cerevisiae is the yeast that has been studied the best to date. Under conditions of sufficient nutrient availability, it grows as ovoid-shaped cells and reproduces vegetatively by means of bud formation. Bud formation occurs in a bipolar fashion in diploid cells, whereas daughter cells emerge in an axial pattern from cells with a haploid genotype (Casamayor and Snyder, 2002). Cells with the above-mentioned behaviour and morphology are referred to as the "yeast" form. Under conditions of limited nutrient availability, especially with regard to carbon and/or nitrogen sources, *S. cerevisiae* is able to change its cell morphology to the pseudohyphal form (Gimeno *et al.*, 1992). Pseudohyphal cells of both haploid and diploid genotypes produce buds in a polar fashion, are more elongated in shape and the daughter cells remain attached to their respective mother cells after the completion of budding (Kron *et al.*, 1994). Pseudohyphal cells of the haploid genotype have been associated with invasive growth into agar, and those of a diploid genotype with pseudohyphal development. Pseudohyphae, however, invade agar (Gimeno *et al.*, 1992) and haploid cells can form pseudohyphae consisting of less elongated cells, indicating that the two processes are closely related. The "yeast" form is predominantly observed under laboratory conditions of excess nutrients. Specific media with limited amounts of carbon and/or nitrogen sources are used in yeast cultivation to induce pseudohyphal morphologies. It appears that the morphological switching from "yeast" to pseudohyphal form enables yeast to grow directionally to regions of higher nutrient content (for a review see Vivier *et al.*, 1997). This ability to alter its morphology also makes *S. cerevisiae* an important model system for the study of pathogen invasion (Goldstein and McCusker, 2001).

In *S. cerevisiae,* the sensing of extracellular nutrients occurs through membrane receptor systems. Information from the receptors is relayed to intracellular signal transduction pathways that control the expression of the genes needed for morphological change (reviewed in Bauer and Pretorius, 2001; Gancedo, 2001;

Palecek *et al.*, 2002; Pan *et al.*, 2000). Two signal transduction pathways have been characterised to date, one involving a mitogen-activated protein kinase (MAPK) pathway, and the other the cyclic adenosine mono phosphate (cAMP) pathway (see **Figure 1.1**). Upstream, these two pathways both receive signals from the GTP-binding protein Ras2p (Gimeno *et al.*, 1992; Mösch *et al.*, 1996; Mösch *et al.*, 1999), while, at the output level, both pathways affect the transcription of *FLO11* (Gagiano *et al.*, 1999a; Rupp *et al.*, 1999), a gene encoding for a cell surface flocculin (Lo and Dranginis, 1996) that is required for both diploid pseudohyphal growth and haploid invasive growth (Lambrechts *et al.*, 1996a; Lo and Dranginis, 1998). The MAPK pathway consists of elements of the pheromone response pathway, but contains a MAPK kinase kinase, Kss11p, which is specific for invasive growth and pseudohyphal development (Cook *et al.*, 1996, 1997). *FLO11* is also able to induce flocculation, a process in which cells clump together when overexpressed (Guo *et al.*, 2000). The MAPK pathway affects the transcription of genes whose promoters contain Filamentation and invasive Response Elements (FREs) (Madhani and Fink, 1997). Factors regulating pseudohyphal development and invasive growth that do not fit into clear signalling pathways include Ash1p (Chandarlapaty and Errede, 1998), Elm1p (Edgington *et al.*, 1999), Msn1p (Gagiano *et al.*, 1999a,b; Lambrechts *et al.*, 1996b; Lorenz and Heitman, 1998), Mss11p (Gagiano *et al*, 1999a,b; Lorenz and Heitman, 1998), Phd1p (Gimeno and Fink, 1994; Lorenz and Heitman, 1998), Sok2p (Pan and Heitman, 2000; Ward *et al.*, 1995) and Spt3p (Laprade *et al.*, 2002).

Components or modules are often shared between signalling pathways, such as the MAPK kinase kinase Ste11p, which regulates the pheromone response, invasive growth, pseudohyphal development and cell wall integrity (Lee and Elion, 1999; Lorenz and Heitman, 1998). In *S. cerevisiae* strains carrying the *STA1-3* genes, which encode for glucoamylases that enable yeast to grow on starch as sole carbon source (for a review see Pretorius *et al.*, 1991), the signalling processes that control starch utilisation and pseudohyphal/invasive growth also share various signalling components. The three transcriptional regulators Flo8p, Msn1p, and Mss11p were shown to positively affect the transcription of both *FLO11* and *STA2* when overexpressed (Gagiano *et al.*, 1999a,b; Lambrechts *et al.*, 1996a,b, Webber *et al.*, 1997). All three of these proteins were shown to act through the promoters of *FLO11* and *STA2* in order to perform their regulatory functions. The *FLO11* and *STA2* promoters have 99% similarity over a region of ~3000 base pairs, but are divergently regulated because of critical sequence differences (Gagiano *et al.*, 1999a,b).

Flo8p was originally identified as a dominant activator of flocculation (Kobayashi *et al.*, 1996; Yamashita and Fukui, 1983), a process in which cells clump together and thus sediment from liquid media (Stratford, 1993). A *FLO8* nonsense mutation (Liu *et al.*, 1996) and disruption (Kobayashi *et al.*, 1996) disable the ability of yeast to flocculate. Flo8p acts as a transcription factor downstream of the cAMP signalling pathway (Pan and Heitman, 1999; Rupp *et al.*, 1999) and positively

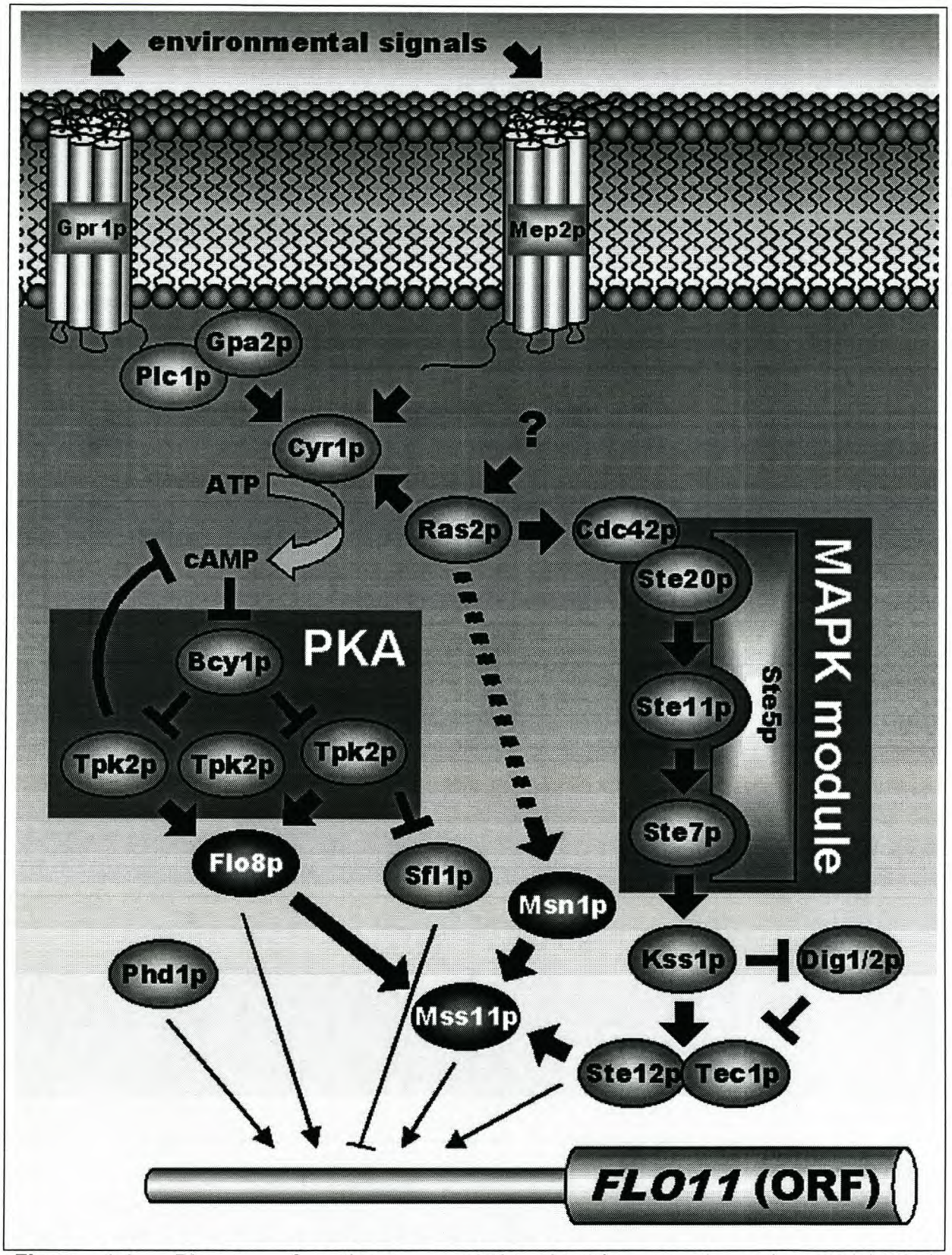

Figure 1.1 Diagram of pathways regulating invasive growth and pseudohyphal development. The cAMP-PKA and MAPK pathways are indicated, as well as other factors not fitting into the first two signalling pathways. Flo8p, Msn1p and Mss11p, which co-regulate *FLO11* and *STA2* expression, are indicated in white with a black background.

regulates the transcription of *FLO1* (Kobayashi *et al.*, 1996; Kobayashi *et al.*, 1999) and *FLO11* (Gagiano *et al.*, 1999a; Kobayashi *et al.*, 1999; Pan and Heitman, 1999;

Pan and Heitman, 2002; Rupp *et al.*, 1999). Msn1p has previously been implicated in the ability to overcome temperature sensitive *snf1* (Estruch and Carlson, 1990) and *swi6* mutants (Sidorova and Breeden, 1999). It is furthermore able to enhance yeast growth in iron-limited media (Eide and Guarente, 1992), and is required for proper osmotic stress-induced transcription (Rep *et al.*, 1999). Msn1p was also found to be able to suppress a nonsense mutation in *FLO8* when overexpressed (Lambrechts *et al.*, 1996b), and to regulate *FLO11* transcription (Lambrechts *et al.*, 1996a). Mss11p was initially identified as a regulator of starch metabolism (Webber *et al.*, 1997) and has additionally been implicated in cell cycle control (Stevenson *et al.*, 2001). Evidence suggests that Mss11p performs a central role in the transcriptional regulation of *STA2* and *FLO11* (Gagiano *et al.*, 1999a,b). Recently, it was shown that specific domains from Mss11p that have been fused to the DNA-binding domain of the Gal4p transcription factor are able to activate the transcription of a reporter gene *in vivo* (Gagiano *et al.*, 2002).

As has already been mentioned, Flo8p, Msn1p and Mss11p are involved in various processes, but all act on the similar promoter regions of *FLO11* and *STA2*. Furthermore Mss11p was found to perform a central role in the transcriptional regulation of these genes. Thus, the effects of *MSS11* differential expression on yeast physiology were further investigated in this study. Apart from previously established phenotypes, such as invasive growth and starch degradation, Mss11p was identified as a strong activator of flocculation when present in high numbers. *MSS11* deletion improved yeast biomass production and suppressed the growth defect of yeast from the ∑1278b genetic background transformed with the hyperactive $RAS2^{val19}$ allele on non-fermentable carbon sources. Mss11p was further characterised by size determination, subcellular location and protein levels under certain physiological conditions. To determine the intracellular location of Flo8p, Msn1p and Mss11p, the proteins were fluorescently tagged. The nuclear location of Flo8p (Liu *et al.*, 1996) and Msn1p (Estruch and Carlson, 1990) has previously been established, thus providing this study with an internal control for the method employed in localising proteins.

During the course of this study, all the proteins were localised to the nucleus. It was therefore decided to select as topic for Chapter 2 the nuclear import and export of proteins. The transport process itself, the regulation thereof and the manner in which it functionally connects with intracellular signalling pathways are discussed in greater detail.

For the sake of clarity, additional information is included in the form of an appendix at the end of this thesis. Simplified diagrams, illustrating the cloning strategies followed, as well as additional sequence alignments, can be found in the appendix.

1.2 PROJECT AIMS

The three transcriptional regulators Flo8p, Msn1p and Mss11p co-regulate starch metabolism, pseudohyphal development and invasive growth. Mss11p performs a central role in the regulation of these processes (Gagiano *et al.*, 1999a,b). Because of these findings, it was decided to pursue the following aims:

(i) to assess the effects of Mss11p overproduction on cell physiology
(ii) to study the effects of *MSS11* deletion and the differential expression of Mss11p on cell growth
(iii) to investigate possible relations between *MSS11* function and intracellular cAMP levels
(iv) to raise antibodies against Mss11p for determination of protein levels and size
(v) to determine the intracellular location of Mss11p
(vi) to follow the intracellular location of Flo8p, Msn1p and Mss11p during various physiological stages

1.3 LITERATURE CITED

Bauer, F. F. and Pretorius, I. S. (2001). Pseudohyphal and invasive growth in *Saccharomyces cerevisiae*. *Focus on Biotechnology – Applied Microbiology* **2**, 109-133.

Casamayor, A. and Snyder, M. (2002). Bud-site selection and cell polarity in budding yeast. *Current Opinion in Microbiology* **5**, 179-186.

Chandarlapaty, S. and Errede, B. (1998). Ash1, a daughter cell-specific protein, is required for pseudohyphal growth of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **18,** 2884-2891.

Cook J. G., Bardwell, L., Kron, S. J. and Thorner, J. (1996). Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast *Saccharomyces cerevisiae*. *Genes and Development* **10**, 2831-2848.

Cook J. G., Bardwell, L. and Thorner, J. (1997). Inhibitory and activating functions for MAPK Kss1 in the *S. cerevisiae* filamentious growth signalling pathway. *Nature* **390**, 85-88.

Edgington, N. P., Blacketer, M. J., Bierwagen, T. A. and Myers, A. M. (1999). Control of *Saccharomyces cerevisiae* filamentous growth by cyclin-dependent kinase Cdc28. *Molecular and Cellular Biology* **19,** 1369-1380.

Eide, D. and Guarente, L. (1992). Increased dosage of a transcriptional activator gene enhances iron-limited growth of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **138,** 347-354.

Estruch, F. and Carlson, M. (1990). Increased dosage of the MSN1 gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase. *Nucleic Acids Research* **18,** 6959-6964.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999a). Divergent regulation of the evolutionarily closely related promoters of the *Saccharomyces cerevisiae* STA2 and MUC1 genes. *Journal of Bacteriology* **181,** 6497-6508.

Gagiano, M., Van-Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999b). Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Molecular Microbiology* **31,** 103-116.

Gagiano, M., Bester, M., Van Dyk, D., Franken, J., Bauer, F. F. and Pretorius, I. S. (2003). Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability. *Molecular Microbiology* **47**, 119-134.

Gancedo, J. M. (2001). Control of pseudohyphae formation in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **25,** 107-123.

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. and Fink, G. R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68,** 1077-1090.

Gimeno, C. J. and Fink, G. R. (1994). Induction of pseudohyphal growth by overexpression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Molecular and cellular biology* **14,** 2100-2112.

Goldstein, A. L. and McCusker, J. H. (2001). Development of *Saccharomyces cerevisiae* as a model pathogen: a system for the genetic identification of gene products required for survival in the mammalian host environment. *Genetics* **159**, 499-513.

Gow, N. A. (1997). Germ tube growth of *Candida albicans*. *Current Topics in Medical Mycology* **8**, 43-55.

Gow, N., Brown, A. and Odds, F. (2002). Fungal morphogenesis and host invasion. *Current Opinion in Microbiology* **5,** 366.

Guo, B., Styles, C. A., Feng, Q. and Fink, G. R. (2000). A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 12158-12163.

Kobayashi, O., Suda, H., Ohtani, T. and Sone, H. (1996). Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Molecular and General Genetics* **251**, 707-715.

Kobayashi, O., Yishimoto, H. and Sone, H. (1999). Analysis of the genes activated by the *FLO8* gene in *Saccharomyces cerevisiae*. *Current Genetics* **36**, 256-261.

Kron, S. J., Styles, C. A. and Fink, G. R. (1994). Symmetric cell division in pseudohyphae of the yeast *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **5**, 1003-1022.

Lambrechts, M. G., Bauer, F. F., Marmur, J. and Pretorius, I. S. (1996a). Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 8419-8424.

Lambrechts, M. G., Sollitti, P., Marmur, J. and Pretorius, I. S. (1996b). A multicopy suppressor gene, MSS10, restores STA2 expression in *Saccharomyces cerevisiae* strains containing the STA10 repressor gene. *Current Genetics* **29,** 523-529.

Laprade, L., Boyartchuk, V. L., Dietrich, W. F. and Winston, F. (2002). Spt3 plays opposite roles in filamentous growth in *Saccharomyces cerevisiae* and *Candida albicans* and is required for *C. albicans* virulence. *Genetics* **161**, 509-519.

Lee, B. N. and Elion, E. A. (1999). The MAPKKK Ste11 regulates vegetative growth through a kinase cascade of shared signalling components. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 12679-12684.

Liu, H., Styles, C. A. and Fink, G. R. (1996). *Saccharomyces cerevisiae* S288C has a mutation in FLO8, a gene required for filamentous growth. *Genetics* **144,** 967-978.

Lo, W. S. and Dranginis, A. M. (1996). FLO11, a yeast gene related to the STA genes, encodes a novel cell surface flocculin. *Journal of Bacteriology* **178,** 7144-7151.

Lo, W. S. and Dranginis, A. M. (1998). The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **9,** 161-171.

Lorenz, M. C. and Heitman, J. (1998). Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. *Genetics* **150,** 1443-1457.

Madhani, H. D. and Fink, G. R. (1997). Combinatorial control required for the specificity of yeast MAPK signaling. *Science* **275,** 1314-1317.

Mösch, H. U., Kubler, E., Krappmann, S., Fink, G. R. and Braus, G. H. (1999). Crosstalk between the Ras2p-controlled mitogen-activated protein kinase and cAMP pathways during invasive growth of *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **10,** 1325-1335.

Mösch, H. U., Roberts, R. L. and Fink, G. R. (1996). Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 5352-5356.

Palecek, S. P., Parikh, A. S. and Kron, S. J. (2002). Sensing, signalling and integrating physical processes during *Saccharomyces cerevisiae* invasive and filamentous growth. *Microbiology Reading, UK* **148,** 893-907.

Pan, X. and Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **19**, 4874-4887.

Pan, X., Harashima, T. and Heitman, J. (2000). Signal transduction cascades regulating pseudohyphal differentiation of *Saccharomyces cerevisiae*. *Current Opinion in Microbiology* **3,** 567-572.

Pan, X. and Heitman, J. (2000). Sok2 regulates yeast pseudohyphal differentiation via a transcription factor cascade that regulates cell-cell adhesion. *Molecular and Cellular Biology* **20,** 8364-8372.

Pan, X. and Heitman, J. (2002). Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation. *Molecular and Cellular Biology* **22**, 3981-3993.

Pretorius, I. S., Lambrechts, M. G. and Marmur, J. (1991). The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus*: an overview. *Critical Reviews in Biochemistry and Molecular Biology* **26,** 53-76.

Rep, M., Reiser, V., Gartner, U., Thevelein, J. M., Hohmann, S., Ammerer, G. and Ruis, H. (1999). Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. *Molecular and Cellular Biology* **19,** 5474-5485.

Rooney, P. J. and Klein, B. S. (2002). Linking fungal morphogenesis with virulence. *Cellular Microbiology* **4,** 127-137.

Rupp, S., Summers, E., Lo, H. J., Madhani, H. and Fink, G. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. *The EMBO journal* **18,** 1257-1269.

Sidorova, J. and Breeden, L. (1999). The MSN1 and NHP6A genes suppress SWI6 defects in *Saccharomyces cerevisiae*. *Genetics* **151,** 45-55.

Stevenson, L. F., Kennedy, B. K. and Harlow, Ed. (2001). A large-scale overexpression screen in *Saccharomyces cerevisiae* identifies previously uncharacterized cell cycle genes. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3946-3951.

Stratford, M. (1993). Yeast flocculation: flocculation onset and receptor availability. *Yeast* **9**, 85-94.

Vivier, M. A., Lambrechts, M. G. and Pretorius, I. S. (1997). Co-regulation of starch degradation and dimorphism in the yeast *Saccharomyces cerevisiae*. *Critical Reviews in Biochemistry and Molecular Biology* **32**, 405-435.

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

Webber, A. L., Lambrechts, M. G. and Pretorius, I. S. (1997). MSS11, a novel yeast gene involved in the regulation of starch metabolism. *Current Genetics* **32,** 260-266.

Yamashita, I. and Fukui, S. (1983). Mating signals control expression of both starch fermentation genes and a novel flocculation gene FL08 in the yeast Saccharomyces. *Agric Biol Chem* **47,** 2889-2896.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

NUCLEOCYTOPLASMIC COMMUNICATION AND SIGNALLING IN *Saccharomyces cerevisiae*

2.1 INTRODUCTION

The living cell adjusts its intracellular processes in response to external or internal stimuli. Adjusting the internal machinery, or maintaining its current status, is a tightly regulated process. This regulation is an energy "expensive" process for the cell. Soluble components of the cell's internal machinery tend to display "unwanted" intracellular location and activity, due to biophysical factors such as diffusion, intrinsic activity and stoichiometric ratios (McAdams and Arkin, 1999; Swain *et al.*, 2002). This "background" activity thus occurs independently of any regulating mechanisms. Limiting the extent of background activity therefore should prove to be energetically favourable for the cell, as it will have to spend less energy on processes that are not performing desired functions (for a review see Bird, 1995). The control of such background activity might prove to be problematic in a singular aqueous compartment in which all the water-soluble components are able to interact with one another.

A solution for this problem is provided by the compartmentalisation of intracellular processes by means of the physical separation of intracellular areas by lipid membrane barriers (for reviews see Bird, 1995; Buiatti and Buiatti, 2001). Specific processes thus can be controlled more efficiently in separate compartments. By means of fluorescent indicators, the messenger, cyclic AMP, has been shown to localise in specific intracellular compartments. Considering the diversity of events that this messenger is involved in, compartmentalisation should result in an increase in specificity regarding signalling events (for a review see Zaccolo *et al.*, 2002). These specialised compartments, which usually are referred to as organelles, are present in cells belonging to eukaryotic organisms, but not in those of prokaryotes. Considering the great amount of differences between eukaryotes and prokaryotes, the ability to compartmentalise intracellular contents clearly plays a key role in allowing cells to acquire higher complexity through the process of evolution (Bird, 1995). Although certain prokaryotes contain intracellular structures such as inclusion bodies, vacuoles and mesosomes, it has not been specifically shown that these structures are involved in compartmentalising-specific processes. In eukaryotic organisms, processes such as protein processing and respiration are limited to specific organelles, namely the Golgi apparatus and mitochondria respectively. This compartmentalisation of processes contributes to the ability of eukaryote organisms to form complex and highly differentiated multicellular structures.

Limiting processes to designated organelles would serve little purpose if the coordinated exchange of components between organelles, or between organelles and the general intracellular environment, was not possible. If such an exchange would happen in an uncontrolled manner, it would possibly cancel out the advantage of intracellular compartmentalisation. Eukaryotes have thus developed highly

regulated mechanisms for the efficient and selective exchange of components between organelles and the cytoplasm (for a review see Huijbregts *et al.*, 2000).

Eukaryotes derive their name from the presence of a predominant organelle in the cytoplasm, referred to as the nucleus (Eu-karyon: "true nucleus"; pro-karyon: "before the nucleus"). The nucleus consists of a double-layered membrane, the nuclear envelope (NE) (reviewed in Dingwall and Laskey, 1992), which encloses an aqueous phase called the nucleoplasm. The outer lipid membrane of the NE is continuous with another cytoplasmic membranous assembly, the endoplasmic reticulum (ER). Located in the nucleus is most of the genetic material of the cell, organised in the form of chromosomes. A small structure, the nucleolus, also resides in the nucleus and appears denser than the rest of the nucleoplasm. The nucleolus is essential for the assembly of ribosomal subunits and is also implicated in less clearly defined functions, including control over certain stages of mitosis and meiosis (reviewed in Garcia and Pillus, 1999; Olson *et al.*, 2000). Evidence of further subnuclear compartmentalisation of different processes (reviewed in Strouboulis and Wolffe, 1996), clear subnuclear "compartments" (Baxter *et al.*, 2002; Leger-Silvestre *et al.*, 1999; Trumtel *et al.*, 2000), and the existence of an internal nucleoskeleton (reviewed in Hozak, 1996, using evidence from HeLa cells), indicates further intranuclear organisation not based on membrane barriers.

The NE lipid membrane, which is impregnable to most molecules in the cytoplasm and nucleoplasm, is perforated, allowing the exchange of water soluble content between the two compartments. The great majority of this nucleocytoplasmic exchange does not, however, occur through random free diffusion, but rather through a highly regulated, selective and energy-dependent transport process. This process is facilitated by large proteinaceous assemblies located at the site of the pores in the NE, referred to as Nuclear Pore Complexes (NPCs) (for recent reviews see Adam, 2001; Fahrenkrog *et al.*, 2001; Rout and Aitchison, 2001). Active energy-dependent transport of substrates, in and out of the nucleus, is facilitated by physical translocation through the central region of the NPC. Translocation depends greatly on the initial specific and differential recognition of substrates at the NPC. Nuclear translocation at the NPC is thus a specific and regulated process, assigning a "gatekeeping" function to the NPC. Although ATP is required for nucleocytoplasmic transport, no ATP is hydrolysed during any stage of translocation. It is rather a member of the small ras family of guanine triphosphate (GTP)-binding proteins, Ran, which provides the energy for transport by GTP or guanine diphosphate (GDP) binding, and also for GTP hydrolysis. A cytosolic GTPase-activating protein (GAP) maintains high levels of cytosolic GDP-bound Ran, while a GDP-GTP exchange factor (GEF) keeps nuclear Ran in a GTP-bound state. This gradient of Ran across the NE and Ran GTP hydrolysis are seemingly the only energy-providing role players in nucleocytoplasmic transport (for recent reviews see Clarke and Zhang, 2001; Moore, 2001).

Substrates destined for nuclear import and/or export include proteins, messenger RNAs (mRNAs), heterogeneous nuclear RNA-binding proteins (hnRNPs), transfer RNA (tRNA), and ribosomal proteins and subunits. All these substrates must first be recognised at the cytoplasmic or nucleoplasmic side of the NPC by import or export receptors via direct interaction, or via adapter proteins. These receptors all belong to a superfamily of similar proteins, referred to as importins (Strom and Weis, 2001; Ullman *et al.*, 1997, Wozniak *et al.*, 1998), and interact directly with proteins of the NPC (for an example, see Allen *et al.*, 2001). Receptors recognise their substrates via specific transport sequences, referred to as nuclear localisation signals (NLSs) for nuclear import or nuclear export signals (NESs) for nuclear export (for a review see Jans *et al.*, 2000). Different importins recognise different and/or similar cargo with different binding affinities and, together with Ran and its interacting proteins, thus play a central regulatory role in NPC translocation.

Following receptor recognition, the receptor/substrate complex undergoes a rapid docking process at the NPC, after which the complex is translocated through the NPC's central transport region at a relatively slower rate compared to the docking process. The complexes all follow the same multiple steps independently of the nature of the recognition process.

Another factor greatly affecting the nucleocytoplasmic distribution of transport cargo is its availability for transport and the modifications, or absence thereof, it may undergo. Many proteins are kept physically in the nucleus or cytoplasm, preventing them from being recognised by import or export receptors. The activation or inhibition of nuclear transport can also occur by means of the covalent modification of substrates. Phosphorylation or dephosphorylation of NLSs/NESs by the respective kinases and phosphatases, usually acting downstream of the signalling pathways, may activate or inhibit nuclear transport, generally at the level of receptor recognition. This is also true for the formation or destruction of disulphate bridges, revealing or masking signal sequences. Lastly, competition for binding sites may inhibit cargo recognition, for example in the case of overlapping nuclear receptor and DNA binding sites (for reviews see Jans and Hubner, 1996; Jans *et al.*, 2000; Nigg, 1997).

In the following sections of this review, the structure of the NPC, the transport process itself, and the effect of nucleocytoplasmic transport on general intracellular signalling are discussed.

2.2 THE NUCLEAR PORE COMPLEX (NPC)

2.2.1 ARCHITECTURE OF THE NPC

The NPC consists of an assembly of proteins and roughly has the shape of a symmetrical conical structure (**Figure 2.1**). Viewed in the plane of the NE, the complex exhibits an eight-fold cylindrical symmetry surrounding a central structure,

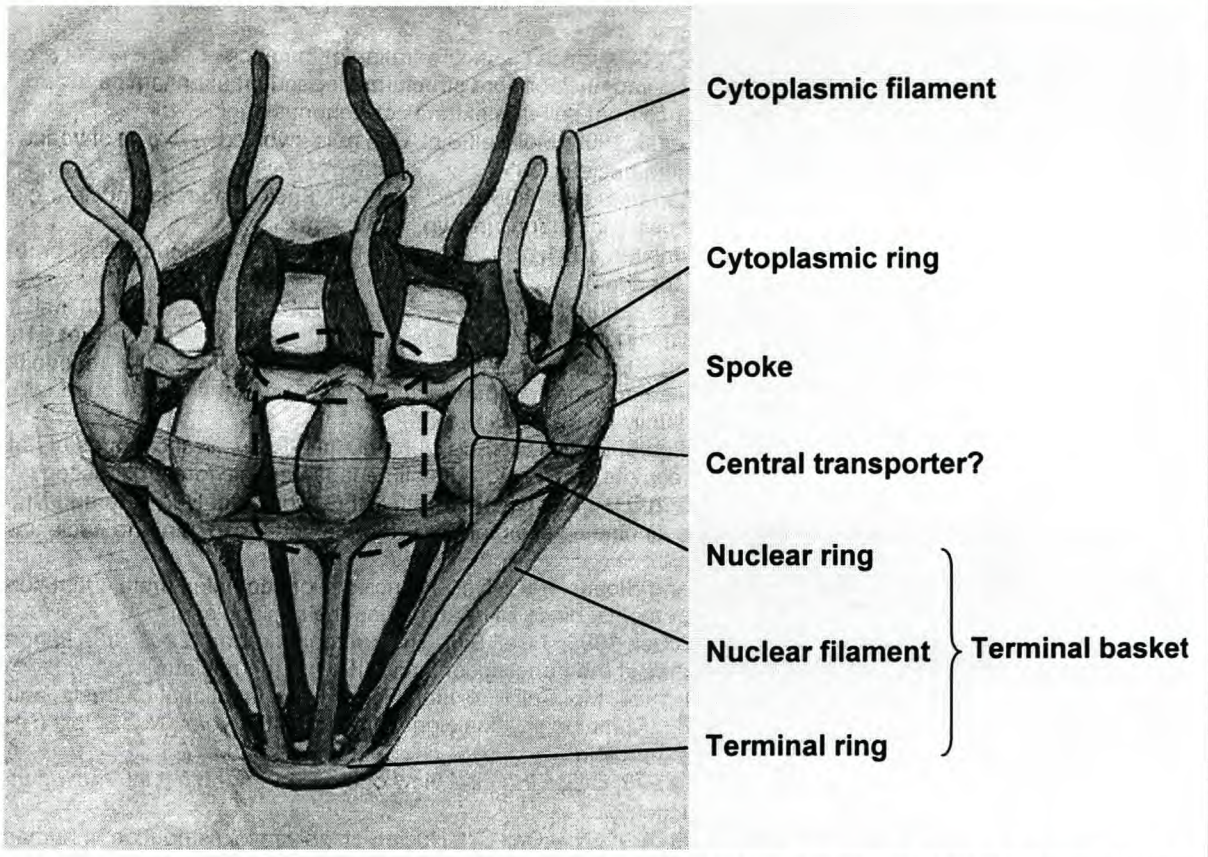

Figure 2.1 A schematic representation of the ultrastructural morphology of the NPC as viewed at an elevated level to the horizontal plane of the NE from the cytoplasmic side of the NPC (Drawing derived from schematic information obtained from Adam, 2001; Dingwall and Laskey, 1992; Rout and Aitchison, 2001; Ohno *et al.*, 1998).

which is referred to as the central transporter (previously "nuclear plug"), as observed by electron microscopy (EM) and atomic force microscopy (AFM) (Allen *et al.*, 1998, Danker & Oberleithner, 2000; Stoffler *et al.*, 1999a) (**Figure 2.2 A, C and D**). Perpendicular to the axis of the central structure, eight spoke-like structures connect a cytoplasmic and nucleoplasmic ring. Filaments that are connected to the ring structures stretch into the cytoplasm and nucleoplasm, forming a "nuclear basket" in the latter case (Fahrenkrog *et al.*, 1998) (**Figure 2.2 B, E and F**). In higher eukaryotes, the "nuclear basket" is anchored to an intranuclear filamentous network, referred to as the nucleoskeleton, which stretches throughout the nucleus (Fontoura *et al.*, 2001; Philimonenko *et al.*, 2001; Smythe *et al.*, 2000). Recently, it was shown that the interaction of Tpr, a component of the intranuclear filaments, with the nuclear basket was dependent on alpha helical coiled-coil secondary structures forming heptad repeats (Hase *et al.*, 2001). In yeast, it was shown that two related genes, MLP1 and MLP2, encode the nucleoskeleton components and that Mlp2p interacts *in vitro* with a component of the nuclear basket (Kosova *et al.*, 2000; Strambio-de-Castillia *et al.*, 1999).

The yeast NPC is roughly 50 MDa in size, consisting of ~30 different proteins (Rout *et al.*, 2000, listed in **Table 2.1**). Compared to other macromolecular assemblies like ribosomal sub-units, it could be expected that, with consideration of

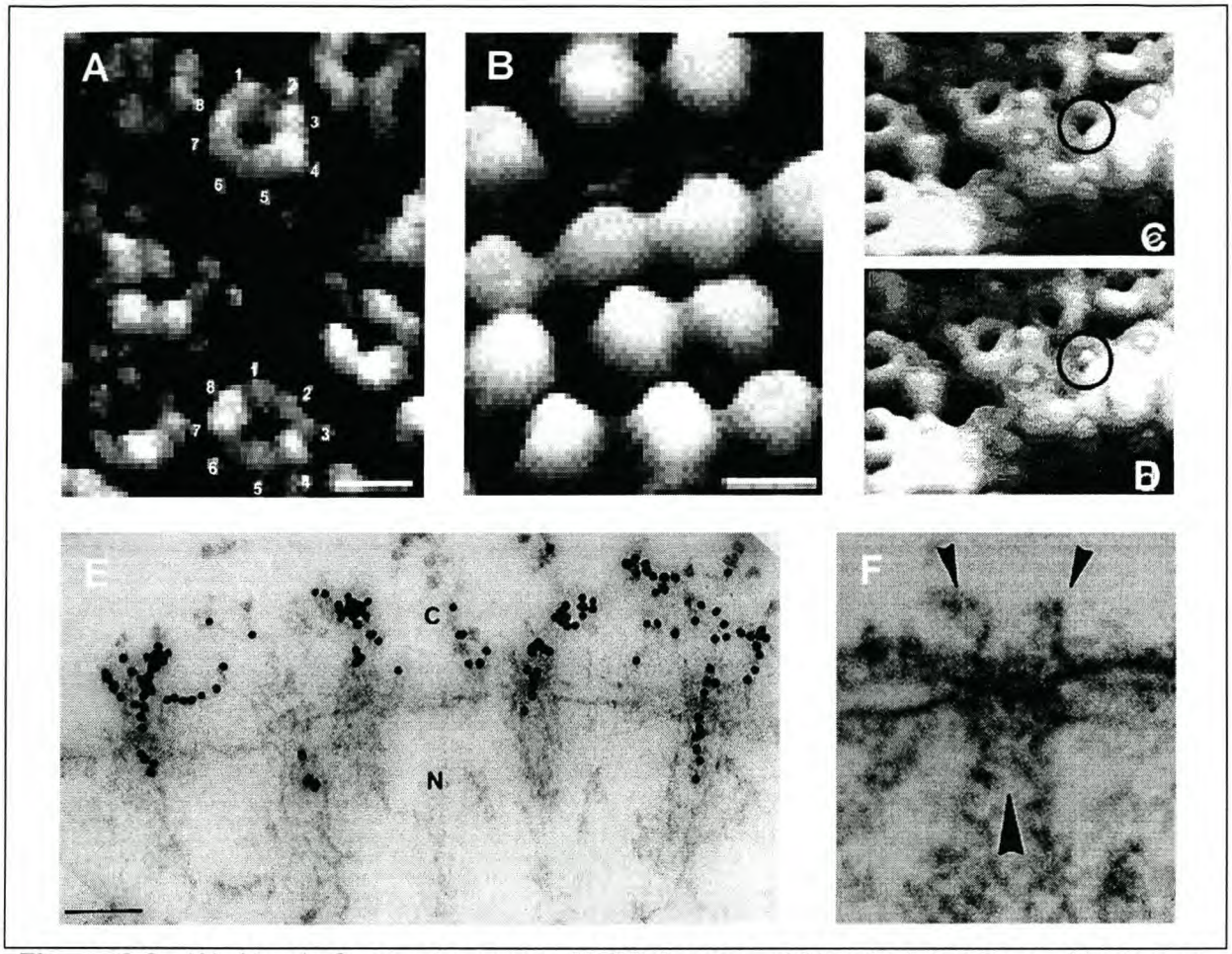

Figure 2.2 (A) Atomic force microscopy (AFM) images of NEs isolated from aldosterone-sensitive Madin-Darby canine kidney (MDCK) cells. Viewed from the cytoplasmic side, the eight-fold symmetry can be seen (individual spokes numbered), while the nuclear basket can be seen from the nucleoplasmic side. (B) (Danker and Oberleithner, 2000). Using stringent AFM scanning, macromolecular structures (encircled, (C)), possibly cargo in transit, can be removed from NE-embedded NPCs (D) isolated from *Xenopus laevis* cells (Stoffler *et al.*, 1999b). (E) Nucleoplasmin-coated gold particles localise to the cytoplasmic fibrils and central transport region of NPC's, as seen with the aid of electron microscopy (EM) in a cross section of *Xenopus laevis* oocyte NE. The bar represents 0,2 μm, "C" the cytoplasm side, and "N" the nucleoplasmic side of the NE. (F) A sectioned *S. cerevisiae* NPC showing cytoplasmic fibrils (small arrowheads) and the nuclear basket (large arrow), using EM (Fahrenkrog *et al.*, 1998).

the size of the NPC, that a much higher amount of different protein constituents should be required to fulfill the respective size requirement (4 Mda ~80 different proteins for ribosomal sub-units). It can be speculated, however, that the symmetrical nature of the NPC requires only a limited amount of different components, due to the eight-fold symmetry in the plane of the NE and the perpendicular symmetry to the NE. In support of this hypothesis, many components have been shown to localise both to the cytoplasmic and nucleoplasmic sides of the NPC in single or multiple copies (Allen *et al.*, 2001; Fahrenkrog *et al.*, 1998; Rout *et al.*, 2000; Stoffler *et al.*, 1999a).

Table 2.1 Nucleoporins present in the NPC

NPC protein	Molecular weight (kDa)	Latest reference for NPC location
Gle1p	62	Rout *et al.*, 2000
Gle2p	41	Rout *et al.*, 2000
Ndc1p	74	Chial *et al.*, 1998
Nic96p	96	Rout *et al.*, 2000
Nsp1p	87	Strambio-de-Castillia *et al.*, 1995
Nup1p	114	Strambio-de-Castillia *et al.*, 1995
Nup2p	78	Rout *et al.*, 2000
Nup42p	43	Rout *et al.*, 2000
Nup49p	49	Strambio-de-Castillia *et al.*, 1995
Nup53p	53	Rout *et al.*, 2000
Nup57p	58	Strambio-de-Castillia *et al.*, 1995
Nup59p	59	Rout *et al.*, 2000
Nup60p	59	Rout *et al.*, 2000
Nup82p	82	Rout *et al.*, 2000
Nup84p	84	Rout *et al.*, 2000
Nup85p	85	Rout *et al.*, 2000
Nup100p	100	Strambio-de-Castillia *et al.*, 1995
Nup116p	116	Strambio-de-Castillia *et al.*, 1995
Nup120p	120	Rout *et al.*, 2000
Nup133p	133	Pemberton *et al.*, 1995
Nup145p	146	Strambio-de-Castillia *et al.*, 1995
Nup157p	157	Rout *et al.*, 2000
Nup159p	159	Rout *et al.*, 2000
Nup170p	169	Rout *et al.*, 2000
Nup188p	189	Nehrbass *et al.*, 1996
Nup192p	191	Rout *et al.*, 2000
Pom34p	34	Rout *et al.*, 2000
Pom152p	152	Rout *et al.*, 2000
Seh1p	39	Rout *et al.*, 2000

2.2.2 COMPONENTS OF THE NPC

The proteins identified to date that perform structural functions in the yeast NPC are listed in **Table 2.1**. By convention, genes encoding for structural proteins of the NPC

are designated as *NUP* (nuclear pore), followed by the molecular mass in kDa of the gene product, with historical exceptions (*NUP1, NUP2, NIC96* and *NSP1*). A *NUP* gene product is in general referred to as a nucleoporin. Likewise, genes encoding for membrane-spanning components are designated *POM* (pore membrane). In a comprehensive study analysing the components of the yeast NPC and associated factors, additional non-*NUP* or *POM* gene products were found to perform structural roles (Rout *et al.*, 2000).

2.2.2.1 Motifs present in NPC components

The majority of nucleoporins contain structural motifs consisting of FG, FXFG or GLFG repeats (single amino acid code, X – any amino acid) (Starr and Hanover, 1991). These repeats can be present in any number and/or in different combinations in a NPC component (for comparative motif organisations of FG repeats containing nucleoporins, see **Figure 2.3**). These repeats are found exclusively in nucleoporins and are highly conserved in eukaryotes (Aris and Blobel, 1989).

Classes of FG repeats seem to play different roles. FSFG repeats are found predominantly in nucleoporins located on the cytoplasmic fibrils, FXFG repeats reside more in components of the nuclear basket structure, and GLFG and XXFG repeats are more commonly found in the central transport region (Allen *et al.*, 2001). When the GLFG repeat region of Nup116p was replaced with either the FXFG repeats of Nsp1p or the GLFG repetitive sequence of Nup100p, Nup116p function was abolished (Iovine *et al.*, 1995), emphasising the importance and specificity of FG repeats, as they cannot be replaced by repeats belonging to a different or even same class of repeats. Importantly in the same group it was shown that the GLFG repeat regions of Nup100p and Nup116p directly bind two receptors belonging to the importin receptor family, importin α and Mex67p, *in vitro* (Strawn *et al.*, 2001). Numerous *in vitro* (Belanger *et al.*, 1994; Fahrenkrog *et al.*, 1998; Hood *et al.*, 2000; Marelli *et al.*, 1998; Rexach and Blobel, 1995; Seedorf *et al.*, 1999) and *in vivo* (Damelin and Silver, 2000) data suggest physical interaction between nucleoporins containing FG repeats and soluble transport factors, emphasising the importance of FG repeats in the process of transporting cargo across the NPC.

Nucleoporins with or without FG (including FG, FXFG and GLFG) repeats may, in addition, contain other structural motifs. For instance, Nsp1p contains a coiled-coil domain (Grandi *et al.*, 1993), whereas Nup107p and Nup153p contain a leucine zipper domain and zinc finger domains respectively (Radu *et al.*, 1994 and Sukegawa and Blobel, 1993 respectively).

2.2.2.2 Function and location of NPC structural components

Because proteins are part of the structure of the NPC, they must perform functions favouring the structural stability of the NPC, or play a more functional role in the transport process, or both. Similarities and differences in physical size,

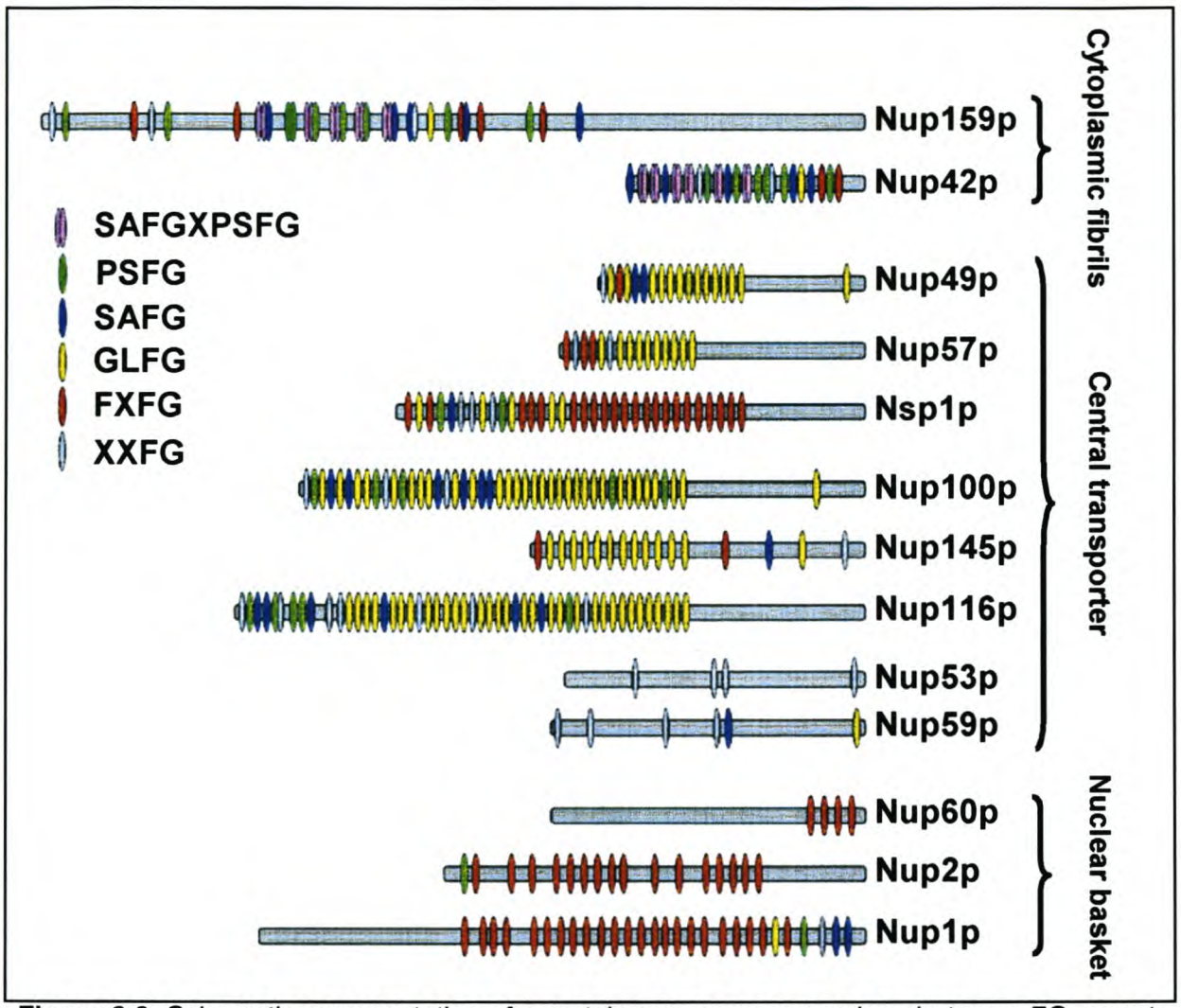

Figure 2.3 Schematic representation of a protein sequence comparison between FG-repeat containing nucleoporins. The frequencies of occurrence, and locations of the FG-like motifs are indicated, using specific colour-coding. Also indicated are the predominant locations of these nucleoporins in the NPC (Allen *et al.*, 2001).

hydrophobicity, sub-NPC location and primary structure suggest specialised and partially redundant roles for individual components.

Pore membrane proteins containing transmembrane domain(s) are localised in the NE and anchor the NPC structure to the NE. This is supported by the finding that two previously identified pore membrane proteins, Ndc1p (Chial *et al.*, 1998) and Pom152p (Strambio-de-Castillia *et al.*, 1995; Wozniak *et al.*, 1994), together with a novel pore membrane protein, Pom34p, co-sediment with NE membranes isolated from yeast (Rout *et al.*, 2000). Interestingly, an essential integral NE protein, Brr6p, was found to be required for the export of mRNA and a NES-reporter, but was not identified in the previously mentioned study. The authors speculate that Brr6p might be located in a novel membrane domain adjacent to the NPC (De Bruyn-Kops and Guthrie, 2001).

Coiled-coil, leucine zipper and zinc finger domains are probably essential for the stability of the NPC core, nucleoplasmic basket and cytoplasmic fibril structure. FG repeats in contrast, being unique to the NPC, are required for substrate recognition

and docking at the cytoplasmic fibrils and nuclear basket. Differences in FG repeat content and distribution in the nucleoporins probably regulate their ability to associate with transport cargo and/or transport complexes. Allen *et al.* (2001) performed *in vitro* binding assays using various nucleoporins (Nup1p, Nup2p, Nup42p, Nup49p, Nup57p, Nup60p, Nup100p, and Nup116p) bound to resin and yeast protein extract. 1 M NaCl and SDS (very tightly bound proteins) eluates were resolved on an SDS-PAGE gel, after which individual proteins were identified with MALDI-TOF mass spectrometry and, where required, by LC mass spectrometry. The above-mentioned nucleoporins were found to bind a wide array of nucleoporins and soluble transport factors. Certain bound proteins could not even be recovered with 1 M NaCl, due to the degree of association with the respective nucleoporins. Strikingly, specific binding of different nucleoporins to unique substrates was shown, as well as the binding of groups of nucleoporins to similar factors (Allen *et al.*, 2001).

Considering that the translocation of macromolecules across the NPC is an energy-dependent process, it was surprising that no ATPase or GTPase activity, or even sequence homology to proteins possessing such activity, was found in NPC components. The only possible energy-providing process is the gradient of GTP- or GDP-bound Ran across the NE (for a review see Mattaj and Englmeier, 1998).

As proposed by Rout *et al.* (2000), FG repeats can serve as docking sites for substrates on the cytoplasmic and nucleoplasmic fibrils, as well as for improving the facilitated diffusion of cargo through the central channel. This is most probably achieved by covering the inside channel and the cytoplasmic/nucleoplasmic fibrils with FG repeats, creating a continuous area with which transport factors and substrates can interact. Such a model would account for the essential role that FG repeats play in gating transport cargo through the NPC (Allen *et al.*, 2001; Rout *et al.*, 2000).

Nucleoporins associate physically with other NPC constituents to form NPC subcomplexes. A heterotrimeric complex consisting of Nsp1p, Nup49p and Nup57p can be reconstituted *in vitro* using purified proteins (Schlaich *et al.*, 1997). Alpha-helical coiled-coil domains, which are present in the C-terminal domain each of the components, were shown to be responsible for keeping this complex intact. Nic96p associates with this complex through direct interaction by an N-terminal coiled-coil domain (Grandi *et al.*, 1995b). In a similar fashion Nsp1p bind to Nup82p through alpha-helical coiled-coil interactions (Grandi *et al.*, 1995a). Using immuno-gold labelling, distinct subcomplexes containing Nsp1p were localised to specific regions of the NPC, as observed by EM. The subcomplex consisting of Nsp1p, Nup49p, Nup57p and Nic96p localised to the cytoplasmic and nucleoplasmic periphery of the NPC, while the Nsp1p-Nup82p and Nsp1p-Nic96p subcomplexes localised exclusively to the cytoplasmic periphery and to the terminal ring of the nuclear basket respectively (Fahrenkrog *et al.*, 1998). Nic96p was also shown to interact with Nup53p by two-hybrid analysis and *in vitro* binding assays, and co-localised with Nup53p to both the cytoplasmic and nucleoplasmic sides of the central

transporter and the terminal ring of the nuclear basket, as observed by immuno-gold EM (Fahrenkrog *et al.*, 2000). In addition Nup53p was found to form a subcomplex with Nup59p and Nup170p, which was observed with the use of immuno-gold EM to be localised to the cytoplasmic and nucleoplasmic sides of the NPC (Marelli *et al.*, 1998). Two soluble transport factors essential for the general import and export of various substrates, Kap95p/importin α and Kap121p/Pse1p, were also shown to interact *in vitro* with Nsp1p and Nup53p (Fahrenkrog *et al.*, 2000; Marelli *et al.*, 1998; Seedorf *et al.*, 1999).

By localising to distinct areas of the NPC, subcomplexes can perform specific functions in the docking and subsequent translocation of substrates. Different subcomplexes contain similar components, indicating that the function of an individual protein can be extended greatly by association with different components. Additionally, certain nucleoporins directly bind soluble factors associated with the transport process.

2.3 SOLUBLE COMPONENTS INTERACTING WITH THE NPC

Apart from components that form part of the NPC structure, a large array of other soluble components participate in the process of nuclear import and export (for an extensive analysis, see Allen *et al.*, 2001). Besides the transport substrates that undergo translocation across the NPC (mRNA, ribosomal subunits and proteins), these soluble components include the proteins that recognise these import/export substrates (importin-α, importin-β receptors), the proteins that regulate the transport process, as well as the small G-protein Ran, which provides the energy for the translocation process. All these components have specific characteristic motifs or properties that allow them to participate in this process.

2.3.1 CARGO DESTINED FOR NUCLEAR IMPORT/EXPORT

Except for peptides that are small enough to enter or exit the nucleus by passive diffusion through the NPC, proteins have to be recognised by the transport apparatus before translocation. For this purpose, proteins contain signal sequences for nuclear import and export, called nuclear localisation signals (NLSs) and nuclear export signals (NESs) respectively (**Table 2.2**).

In general, NLS motifs consist of short stretches (5-20 amino acids) of positively charged amino acids, mainly arginine and lysine residues. The first NLS to be identified, from the SV40 large T-antigen (Kalderon *et al.*, 1984a), was able, when fused to the normally cytoplasmic heterologous proteins β-galactosidase and pyruvate kinase, to confer nuclear localisation to these proteins (Kalderon *et al.*, 1984b), and displayed the same localisation behaviour in living cells when fused to green fluorescent protein (GFP) (Shulga *et al.*, 1996). The SV40 NLS consists of a positively charged group of lysine and arginine, and is also known as the "classical" NLS. Later, an NLS that contained two clusters of positively charged residues

Table 2.2 Sequences required for nuclear import/export. Residues in bold are essential for translocation. Underlined residues indicate consensus when the *Saccharomyces cerevisiae* Ssb1p sequence is compared to homologues of *Candida albicans*, *Schizosaccharomyces pombe* and *Kluyveromyces marxianus*. The double underlined glycine, when mutated to alanine, abolishes both the nuclear import and export of human RNP A1. Residues underlined with a dashed line indicate yeast Nab2p homology with higher eukaryote M9-like sequences (Kalderon *et al.*, 1984a; Robbins *et al.*, 1991; Shulga *et al.*, 1999; Siomi *et al.*, 1998).

Transport signal sequence	Origin
P**KKKRK**V	SV40 large T-antigen
KRPAATKKAGQA**KKKK**	Human nucleoplasmin
KKRRTSNAQRVKEFRKHSTSLDNDHNNA**RKR**QHSSCKAE**KKKK**	Yeast Lys14p
IEAALSDALAALQI	Yeast Hsp70 Ssb1p
NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY	Human RNP A1
APVDNSQRFTQRGGGAVGKNRRGGRGGNRGGRNNNSTRFNPLAKALG	Yeast Nab2p

separated by a 10 amino acid spacer region was identified in nucleoplasmin (Robbins *et al.*, 1991). In accordance with the primary structure of the motif, NLS sequences from the SV40 large T-antigen and nucleoplasmin are referred to as monopartite and bipartite NLSs respectively. Interestingly, a tripartite NLS that contained three positive clusters necessary for proper nuclear localisation was identified in Lys14p (El-Alami *et al.*, 2000). Many NLS sequences have since been identified that, although they show low sequence homology, generally contain clusters of arginine and lysine residues flanked by uncharged residues (a comprehensive database of experimentally determined NLSs is available at http://maple.bioc.columbia.edu/predictNLS/data/exptnlsdb.html; Cokol *et al.*, 2000).

No real consensus sequence exists for the nuclear export sequence. In general, it consists of a short hydrophobic motif containing a number of leucine residues. Replacing single individual leucine residues with alanine does not abolish NES activity. Rather, it was shown that spaced leucine residues, which generally are surrounded by hydrophobic residues, are essential as a unit for efficient export (Wen *et al.*, 1995). The heat shock protein Ssb1p contains such a sequence, which is essential for relocation to the cytoplasm following nuclear import (see **Table 2.2**; Shulga *et al.*, 1999).

In contrast to the general consensus for NLS and NES signals, a 38 amino acid motif, termed M9, was identified in human pre-mRNA/mRNA-binding heterogeneous nuclear ribonucleoprotein (hnRNP) A1, containing no significant amount of arginine, leucine or lysine residues. Strikingly, the M9 sequence was shown to be essential for both hnRNP A1 nuclear import and nuclear export (Michael *et al.*, 1995; Siomi and

Dreyfuss, 1995; Weighardt *et al.*, 1995). In yeast, the hnRNP-like protein Nab2p contains a 55 amino acid sequence, termed NAB35, which is homologous to the M9 sequence. The NAB35 motif was shown to be functional for the import process in yeast, as well as in mammalian systems (Siomi *et al.*, 1998; Truant *et al.*, 1998). The M9 motif is evolutionarily conserved and other M9-like motifs are present in proteins of *Drosophila melanogaster* and *Xenopus laevis* (**Table 2.2**, Siomi *et al.*, 1998).

2.3.2 RECEPTORS FOR NLS/NES-BEARING PROTEINS

The recognition of transport substrates by the transport apparatus is mediated by import and/or export receptors, collectively referred to as importins (also called karyopherins and transportins). Phylogenetic analysis subdivides importins into two subclasses of importin-α-, and importin-β-like receptors (Malik *et al.*, 1997). Proteins containing NLSs are recognised by an importin-α/importin-β heterodimer (discussed in more detail later). Fifteen receptors have been identified in yeast so far. Ten are involved in import, four in export, and one functions in both processes.

2.3.2.1 Importin-α

Only one 60 kDa importin-α-like homologue has been identified in yeast so far, and is referred to here as importin-α. Encoded by *SRP1/KAP60*, it was first identified as a suppressor of temperature-sensitive RNA polymerase I mutations (Yano *et al.*, 1992). Able to bind SV40 large T-antigen NLS fused to GST, importin-α was shown to play an essential role in certain nuclear import pathways (Gorlich *et al.*, 1994; Rexach and Blobel, 1995).

Structurally, importin-α consists of ten tandem armadillo (arm) repeats, flanked by an N-terminal basic domain and a C-terminal acidic domain (Herold *et al.*, 1998). Arm repeats are found in functionally unrelated proteins in *Drosophila melanogaster* (Peifer *et al.*, 1994) and typically consist of roughly 40 amino acids, forming three alpha helices. X-ray crystallographic analysis reveals that importin-α bound or unbound to a monopartite NLS takes the secondary form of a right-handed superhelix (**Figure 2.4A**) (Conti *et al.*, 1998). Two binding sites for arginine and lysine clusters are present in the helical surface groove. Monopartite signals bind to only one of the sites and bipartite motifs occupy both binding sites (Conti and Kuriyan, 2000). The 41 basic residues at the N-terminal of importin-α are essential for importin-β1 binding and NLS cargo import and therefore are referred to as the importin-β-binding (IBB) domain (Gorlich *et al.*, 1996; Weis *et al.*, 1996). Thus, the IBB domain can be viewed as the NLS of importin-α. Crystallographic evidence of the IBB domain complexed with the NLS binding domain suggests an auto-inhibitory role for the IBB domain (Kobe, 1999).

2.3.2.2 Importin-β receptors

Up to 14 importin-β family members have been identified in yeast, which is in contrast with having only one importin-α family member. Importin-β receptors are

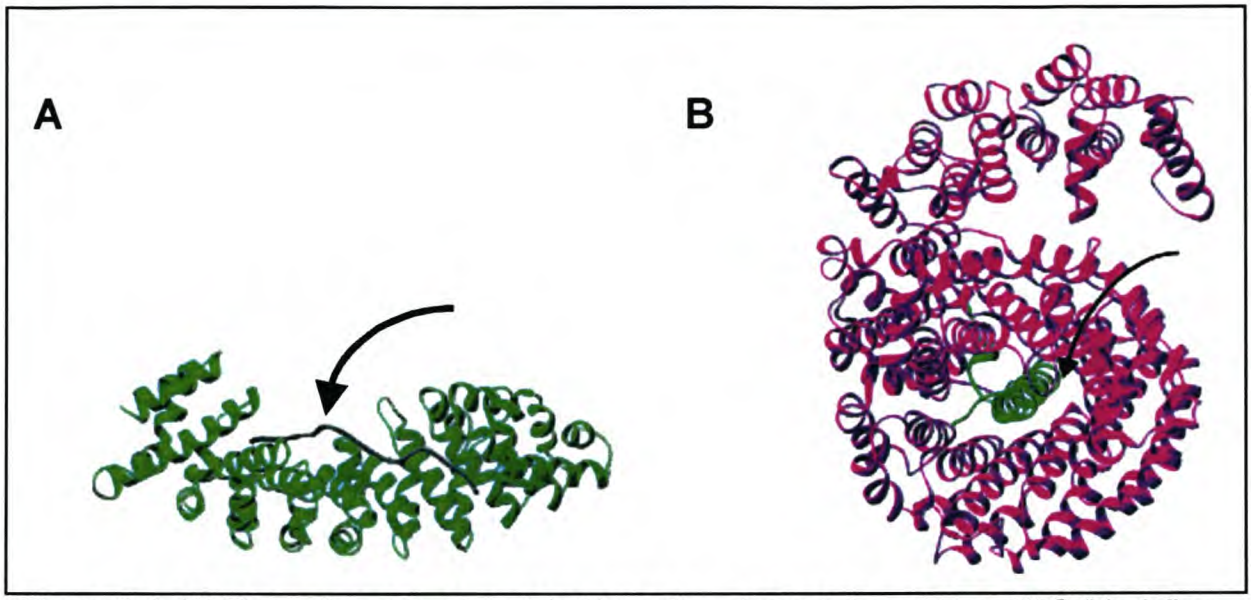

Figure 2.4 (A) Three-dimensional model of importin-α (green), bound to an NLS (black line, arrow), and (B) importin-β (purple), bound to the IBB domain of importin-α (green, arrow), based on crystallographic data obtained from the respective complex crystals (Conti and Izaurralde, 2001).

characterised by tandem repeats of HEAT motifs (the HEAT acronym is based on the proteins in which the repeats were first identified). HEAT sequences are between 38 to 45 amino acids in length (for a review see Andrade *et al.*, 2001) and form two alpha helices, as first determined from the crystal structure of phosphatase 2A (Groves *et al.*, 1999). Similar to importin-α, importin-β is an all-helical structure, but is arranged in a right-handed, snail-like superhelix. Importin-β1, belonging to the importin-β receptor family, acts as the receptor for the importin-α - NLS-bearing protein complex (Gorlich *et al.*, 1995). The crystal structure of human importin-β bound to the IBB domain of human importin-α and of a 462 a.a. fragment bound to Ran-GTP (non-hydrolysable) were resolved by Cingolani *et al.* (1999) and Vetter *et al.* (1999) respectively (**Figure 2.4 B**). Generally, binding occurs in the alpha-helical groove, where conserved importin-β residues interact with positive groups of the importin-β binding domains of Ran and importin-α. Importin-β undergoes various substrate-dependent conformational changes, but its twisted structural nature remains essential for function (Lee *et al.*, 2000).

As has already been mentioned, many receptors similar in structure to importin-β have been identified in yeast. The genes encoding these proteins are designated *KAP*, for karyopherin, followed by the molecular mass in kDa (e.g. *KAP95* encodes for importin-β1). In addition to any number of tandemly arranged HEAT repeats, all display an acidic isoelectric point and recognise basic motifs such as NLSs. The highest sequence similarity between importin-β members occurs at the N-terminus, which has been shown to be the Ran binding site in importin-β1. In contrast, sequence similarity in HEAT repeats between importin-β receptors was found to be low. It can be suggested that all importin-β members bind Ran through a conserved motif, but recognise a wide array of different substrates due to the variation in the HEAT repeats (Cingolani *et al.*, 1999; Kutay *et al.*, 1997; Vetter *et al.*, 1999).

The importin-β members, importin-β/Kap95p, Kap104p, Kap108p/Sxm1p, Kap111p/Mtr10p, Kap114p, Kap119/Nmd5p, Kap121p/Pse1p, Kap122/Pdr6p and Kap123p/Yrb4p, have been shown to function exclusively as nuclear import receptors. Crm1p/Xpo1p, Kap109p/Cse1p, Los1p and Kap120p/Lph2p, on the other hand, are only implicated in nuclear export. Kap142p/Msn5p functions as both a nuclear import and export receptor (for details of importin-β receptors and references, see **Table 2.3**).

Table 2.3 The importin-β-like family of transport receptors, excluding importin-β1. References: 1. Aitchison *et al.*, 1996; 2. Rosenblum *et al.*, 1997; 3. Senger *et al.*, 1998; 4. Morehouse *et al.*, 1999; 5. Pemberton *et al.*, 1999; 6. Albertini *et al.*, 1998; 7. Ferrigno *et al.*, 1998; 8. Polizotto and Cyert, 2001; 9. Kaffman *et al.*, 1998a, 10. Chaves and Blobel, 2001; 11. Delahodde *et al.*, 2001, 12. Leslie *et al.*, 2002, 13. Titov and Blobel, 1999; 14. Grosshans *et al.*, 2001; 15. Rout *et al.*, 1997; 16. Schlenstedt *et al.*, 1997; 17. Jensen *et al.*, 2000; 18. Kunzler *et al.*, 2000; 19. Maurer *et al.*, 2001; 20. Moy and Silver, 1999; 21. Hood and Silver, 1998; 22. Kunzler and Hurt, 1998; 23. Solsbacher *et al.*, 1998; 24. Hellmuth *et al.*, 1998; 25. Stage-Zimmermann *et al.*, 2000; 26. Yoshida and Blobel, 2001; 27. Blondel *et al.*, 1999; 28. Kaffman *et al.*, 1998b; 29. Komeili *et al.*, 2000; 30. Mahanty *et al.*, 1999.

Receptor	Other name	Import or export	Substrates	Reference
Kap104p		import	Nab2p. Nab4p	1
Kap108p	Sxm1p	import	Lph1p	2
Kap111p	Mtr10p	import	Npl3p	3
Kap114p		import	TBP	4, 5
Kap119p	Nmd5p	import	TFIIS, Hog1p, Crz1p	6 – 8
Kap121p	Pse1p	import	Pho4p, Spo12p Pdr1p. Ste12p	9 – 12
Kap122p	Pdr6p	import	Toa1p, Toa2p, SRP	13, 14
Kap123p	Yrb4p	import	L25, SRP	14 – 16
Crm1p	Xpo1p	export	SRP, Ace2p, Hog1p, Yrb1p, 20S	8, 14, 17 – 20
Kap109p	Cse1p	export	importin-α	21 – 23
Los1p		export		24
Kap120p	Lph2p	export	Rpl11p	25
Kap142p	Msn5p	import & export	RPA, Far1p, Pho4p, Rtg1p, Rtg3p, Ste5p	26 – 30

Importin-β receptors are important for the import and export of a diverse selection of factors, ranging from mRNA-binding proteins and general transcription factors to specific transcription factors and ribosomal proteins. Kap142p/Msn5p, which has been implicated in various cellular processes such as carbon source utilisation, calcium tolerance, mating, cyclin-specific functions and pseudohyphal differentiation (Alepuz *et al.*, 1999; Lorenz and Heitman, 1998), mediates the import and export of

various substrates. It plays an essential role in the import of the yeast trimeric replication protein A (RPA) (implicated in DNA replication, repair, and recombination), because *KAP142* null mutants display marked sensitivity to bleomycin (causes DNA double strand brakes) (Yoshida and Blobel, 2001). Kap142/Msn5p-mediated export includes that of Far1p, which is required for cell cycle arrest and establishing cell polarity during mating (Blondel *et al.*, 1999), Pho4p (transcriptional regulator of the phosphate system, Kaffman *et al.*, 1998a), the Rtg1p/Rtg3p dimer (targets of the rapamycin-sensitive (TOR) pathway, Komeili *et al.*, 2000), and Ste5p (scaffold protein for MAP kinases, Mahanty *et al.*, 1999).

2.3.2.3 The central transport apparatus

The core transport apparatus of soluble components includes Ran, Rna1p, Prp20p, NTFII and Yrb1p. Unlike the discussed transport receptors and mediators, which regulate nucleocytoplasmic transport by means of differential specificity for each other, the central transport mechanism is responsible for the non-discriminatory import or export of docked transport complexes.

Yeast Ran, encoded by two homologous genes, *GSP1* and *GSP2,* belongs to the ras family of small GTP-binding proteins. *GSP1* has been shown to be a highly expressed essential gene, whereas the non-essential *GSP2* is expressed only under specific conditions (Belhumeur *et al.*, 1993). Although the vast majority of Ran is encoded by *GSP1*, Ran is not generally referred to as Gsp1p. The relatively low intrinsic GTPase activity of Ran is increased greatly by the mainly cytosolic GTPase activating protein (GAP), Rna1p (Becker *et al.*, 1995; Hopper *et al.*, 1990). Located in the nucleus, the GDP-GTP exchange factor (GEF), Prp20p, exchanges GDP for GTP-bound Ran (Amberg *et al.*, 1993). This results in a cytoplasmic pool of GDP-bound Ran and a GTP-bound pool of Ran in the nucleus. The switching of Ran between GTP- and GDP-bound forms is referred to as the Ran cycle. This process ensures that a Ran gradient exists across the NPC, which is the essential energy-providing system for active directional nuclear translocation in and out of the nucleus (**Figure 2.5**) (Izaurralde *et al.*, 1997). Using *in vitro* transport assays, Nachury and Weis (1999) were able to invert the direction of nucleocytoplasmic transport by creating high levels of GTP-bound Ran in the cytoplasm.

Together with Ran, the homodimeric nuclear transport factor 2 (NTFII) is essential for nuclear import. NTFII binds exclusively to GDP-bound Ran (Stewart *et al.*, 1998), interacts with certain nucleoporins (Clarkson *et al.*, 1996) and importin-β (Bayliss *et al.*, 2000a), and is essential for the *in vivo* nuclear import of Ran-GDP (Ribbeck *et al.*, 1998; Smith *et al.*, 1998; Steggerda *et al.*, 2000). Two models have been proposed for NTFII-mediated Ran import, according to which the Ran-GDP/NTFII complex can either enter the nucleus as part of an import cargo complex, or as an independent complex (Quimby *et al.*, 2000).

Yrb1p, which is found predominantly in the cytosol, has been shown to activate the GAP activity of Rna1p, as shown by *in vitro* enzyme assays (Bischoff *et al.*,

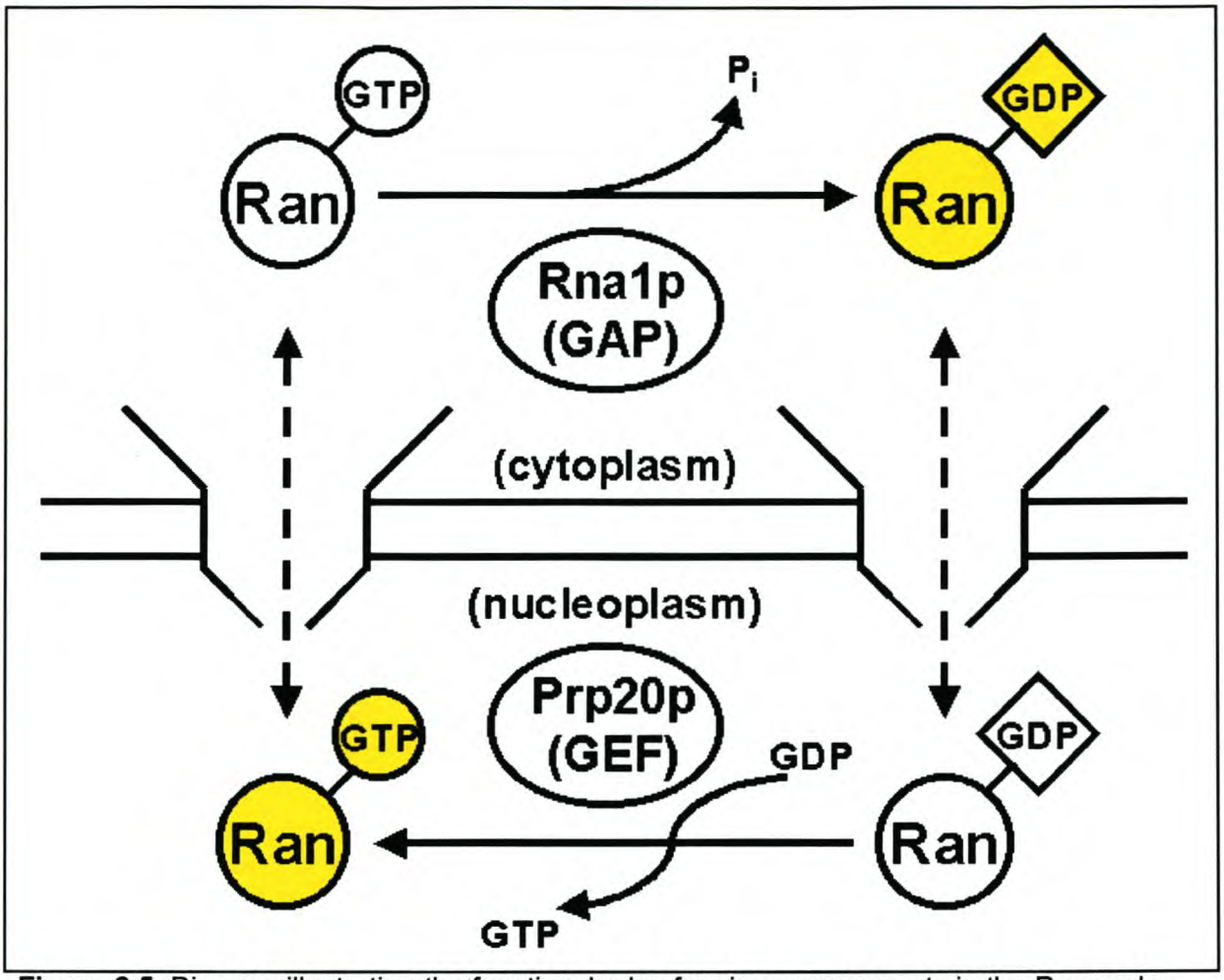

Figure 2.5 Diagram illustrating the functional role of various components in the Ran cycle

1995). In addition, *YRB1* mutants display a reduction in transport efficiency (Kunzler *et al.*, 2001). Yrb1p most likely plays a role in the release of cargo complexed to Ran, following the translocation step into the cytoplasm.

2.4 GENERAL MECHANISM OF TRANSLOCATION, WITH IMPORTIN-α NUCLEOCYTOPLASMIC TRANSPORT AS EXAMPLE

Mechanistically, the transport process can be subdivided into multiple steps (Akey and Goldfarb, 1989). Complexes consisting of cargo, transport receptors and mediators are first recognised by the cytoplasmic fibrils at the cytoplasmic side of the NPC, or by the nuclear basket at the opposite side, followed by a docking process, after which the cargo complexes are directed to the periphery of the NPC central transport channel. This is supported by the observation that the transport substrate still locates at the NPC periphery, even when transport is blocked (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). In addition, when nucleoplasmin (importin-α homologue)-coated gold particles were injected into *Xenopus* oocytes, the particles accumulated at the cytoplasmic fibrils and the central transporter (Richardson *et al.*, 1988) (**Figure 2.2 E**). The same authors also showed that, following the relatively quick docking process, the translocation process

comparatively was much slower, as well as being dependent on the presence of ATP. Interestingly, no ATPase activity has been attributed to any NPC component. The transport kinetics of the nuclear translocation process have been found to be very fast, using permeabilised HeLa cells (~10^3 translocation events per second) (Ribbeck and Gorlich, 2001). It has been proposed that efficient translocation is dependent on the ability of the NPC to facilitate diffusion of recognised cargo complexes in an affinity-dependent manner, while still remaining impermeable to other molecules (Ribbeck and Gorlich, 2001; Rout *et al.*, 2000).

All proteins bearing transport signals are actively transported through the NPC by the same general mechanism of docking and subsequent translocation. This transport mechanism appears to be conserved between higher and lower eukaryotes. When colloidal gold coated with BSA-bipartite NLS conjugates or a reporter protein containing the human hnRNP A1 M9 shuttling signal were micro-injected into amoebae, which are simple eukaryotes, the substrates were excluded from the nucleus. When vertebrate importin-α and importin-β were co-injected with these substrates, nuclear accumulation of the substrates could be detected, indicating that a higher eukaryote receptor-cargo complex still could be recognised by the core transport apparatus of a simple eukaryote (Feldherr *et al.*, 2002). In the following section, the import and export processes involving importin-α, importin-β1 and Kap109p/Cse1p, are discussed briefly.

NLS-bearing proteins are recognised in the cytoplasm by the heterodimer formed by importin-α and importin-β1 (Enenkel *et al.*, 1995). Importin-α serves as an adapter between the NLS-bearing protein and importin-β. In addition, importin-α associated with importin-β has a higher affinity for NLS motifs than importin-α alone (Gilchrist *et al.*, 2002; Rexach and Blobel, 1995). Interacting with the FXFG repeats of cytoplasmic nucleoporins, importin-β1 directs the trimeric importin-α/importin-β/NLS protein complex to the central transporter (Bayliss *et al.*, 2000a,b). The complex is transported through the central channel in a process dependent on ATP, Ran and NTFII (Quimby *et al.*, 2000). Once in the nucleoplasm, GTP-bound Ran dissociates the transported complex by interacting with importin-β, and the hydrolysis of GTP occurs. Importin-β1 remains associated with the NPC, and importin-α, as well as the imported cargo, resides in the nucleoplasm after dissociation (Gorlich *et al.*, 1995; Rexach and Blobel, 1995). Using the components of the "classical" import pathway in yeast, Nup1p, Nup2p, Cse1p and Gsp1p were shown to greatly accelerate NLS-cargo/importin-α/importin-β1 disassembly in the nucleoplasm. These factors may well serve as release factors in the nuclear basket following nuclear import (Gilchrist *et al.*, 2002).

Kap109p/Cse1p, functioning as the export receptor for importin-α, forms a trimeric complex with importin-α and GTP-bound Ran in the nucleus (Richards *et al.*, 1997). This complex is readily exported to the cytoplasm, effectively recycling importin-α. NLS-bound importin-α is unable to bind Kap109p/Cse1p and prevents the export of the imported cargo with importin-α to the cytoplasm. After export out of the

nucleus, the complex is disassembled by GTP hydrolysis, which is induced by Yrb1p (Hood and Silver, 1998; Kunzler and Hurt, 1998; Solsbacher *et al.*, 1998) (**Figure 2.6**).

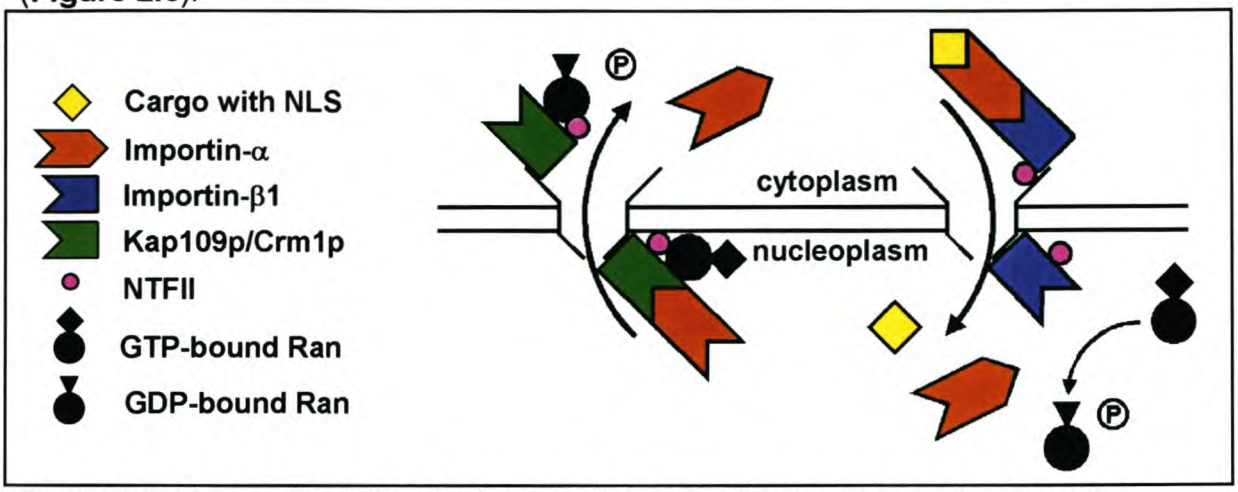

Figure 2.6 Diagram illustrating the "classical" import pathway and importin-α recycling

2.5 REGULATION OF NUCLEOCYTOPLASMIC TRANSPORT

2.5.1 NLS RECOGNITION

NLS recognition directly affects the nuclear import process, controlling the accessibility of factors to the nuclear import machinery. Apart from NLS composition, the accessibility of importin-α in the cytoplasm additionally regulates the process. *In vitro* and *in vivo* evidence show that NLS recognition releases importin-α from cytosolic aggregates in Xenopus and HeLa cell extracts (Percipalle *et al.*, 1999). Crystallographic data from mouse importin-α indicates that the IBB domain binds to the armadillo repeats in the absence of an NLS, suggesting monomeric autoinhibition of importin-α (Kobe, 1999). Using various biophysical techniques, Catimel *et al.* (2001) confirmed importin-α autoinhibition. The binding of importin-α to importin-β1 greatly increased importin-α affinity for the NLS (KD = 1.1×10^{-8}). Importin-α/importin-β complex affinity for mono- and bipartite NLSs was found to be similar, comparable to the affinity of an IBB domain lacking importin-α for the same respective NLSs (Catimel *et al.*, 2001).

Another factor that was found to greatly affect NLS recognition is the presence of phosphorylation sites located in, or adjacent to, the NLS. Depending on the location of phosphorylation sites, they can, upon phosphorylation, enhance or inhibit nuclear import. Global cytoplasmic phosphorylation inhibits nuclear import, but not CRM1-mediated export, as shown with permeabilised HeLa cells and phosphatase inhibitors (Kehlenbach and Gerace, 2000). The best-studied NLS that is affected by phosphorylation events is the SV40 large T-antigen NLS, which will be discussed as a model.

2.5.1.1 SV40 large T-antigen NLS phosphorylation

Rihs and Peters (1989) identified a short region of phosphorylation motifs adjacent to the SV40 large T-antigen NLS core region that greatly enhanced nuclear import (**Figure 2.7**). Residues 126-132, containing the NLS alone, and residues 111-135, containing the NLS with adjacent phosphorylation sites, were all fused to beta-galactosidase and injected into the cytoplasm of rodent hepatoma cells. Beta-galactosidase fused only to the region corresponding to the basic NLS was taken up in the nucleus, but the process took several hours to reach a steady state. In contrast, the fusion protein containing both the NLS and N-terminal phosphorylation sites accumulated in the nucleus with a half time of 8-10 minutes, reaching a steady state nuclear-to-cytoplasmic ratio of 15/1 (Rihs and Peters, 1989). Exhibiting the reverse effect, the exclusive phosphorylation of threonine at position 124 was shown to inhibit NLS-mediated nuclear import (Jans *et al.*, 1991). Further *in vitro* and *in vivo* evidence showed that serine residues 111/112 and threonine residue 124 are phosphorylated by casein kinase II (CKII) and cyclin-dependant kinase Cdc2 respectively. When replacing the CKII motif with motifs responsive to either cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC), the same enhanced protein import was observed *in vivo* (Xiao *et al.*, 1996, Xiao and Jans, 1998). In addition, a serine residue was identified at position 120 that enhanced protein import when phosphorylated and was responsive to the double-stranded DNA-dependent protein kinase (dsDNA-PK) (Xiao *et al.*, 1997). Negatively charged phosphorylated residues near the NLS may act positively or negatively on NLS recognition. Possibly, phosphorylated $Ser^{111/112}$, located at a relative distance, acts additively to NLS recognition, and closely located negative phosphate residues, such as Thr^{124}, may cancel out positive charges in the NLS through the formation of salt bridges. Although the precise mechanism is unclear, phosphorylation events therefore dramatically affect the rate-limiting step of NLS recognition (Hubner *et al.*, 1997; Jans and Jans, 1994; Rihs *et al.*, 1991; Xiao *et al.*, 1998).

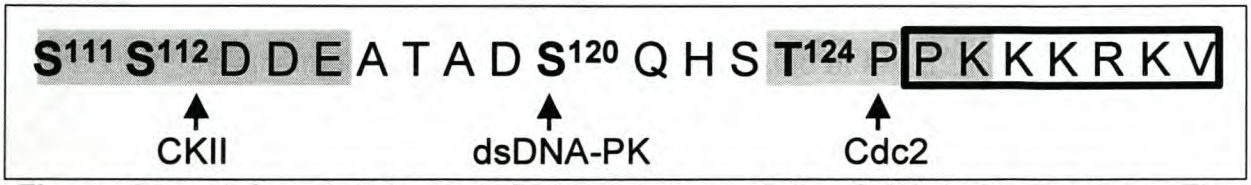

Figure 2.7 NLS and N-terminal flanking regions of the SV40 large T-antigen. The monopartite NLS is boxed, the phosphorylation motifs are shaded in gray, and the target residues for phosphorylation appear in bold (Jans and Hubner, 1996).

2.5.1.2 Phosphorylation regulating yeast nucleocytoplasmic transport

Experimental data for SV40 T-Ag NLS regulation was obtained by using higher eukaryotic systems. In yeast, numerous proteins have been identified whose nuclear location is affected by phosphorylation. Similar to the SV40 T-Ag, some of these yeast proteins are phosphorylated near or inside the NLS motifs.

Yeast calcineurin, which is responsive to intracellular Ca^{2+} levels, dephosphorylates the transcription factor Crz1p, resulting in the translocation of Crz1p into the nucleus (Stathopoulos-Gerontides *et al.*, 1999). Crz1p interaction with Kap119p, which mediates Crz1p import, has recently been shown to be dependent on this dephosphorylation (Polizotto and Cyert, 2001). Another transcription factor, Gln3p, is hyperphosphorylated in a TOR (target of rapamycin)-dependent manner under high nitrogen conditions, causing it to be inaccessible to the nucleus. Upon nitrogen starvation or rapamycin treatment, Gln3p is dephosphorylated, enabling it to interact with importin-α, its nuclear import receptor. After nuclear import Gln3p, is responsible for nitrogen catabolite repression (Carvalho *et al.*, 2001).

In contrast, Swi6p, which is important for G1/S-specific gene expression, is able to enter the nucleus upon the phosphorylation of serine 160. Although highly phosphorylated in the cytoplasm, serine 160 is the exclusive residue directing Swi6p to the nucleus (Sidorova *et al.*, 1995). In general, phosphorylation, and not dephosphorylation, enhances or enables nuclear import in yeast. Other proteins accumulating in the nucleus in response to phosphorylation include Cdc48p (Madeo *et al.*, 1998), Hog1p (Warmka *et al.*, 2001), Mig1p (Smith et *al.*, 1999), Npl3p (Gilbert *et al.*, 2001), Pho4p (Kaffman *et al.*, 1998a,b), Snf1p (McCartney and Schmidt, 2001; Vincent *et al.*, 2001) and Swi5p (Moll *et al.*, 1991). The influence of phosphorylation-regulated nucleocytoplasmic transport on cellular processes will be discussed in a later section, using the phosphate nutrient sensing signalling pathway in yeast as an example.

2.5.1.3 NLS/NES masking and competition

Signal-bearing proteins might be prohibited from nuclear entry and/or exit by masking mechanisms that hide their NLS/NES motifs. A good example is the yeast transcription factor Yap1p, which is involved in the oxidative stress response. Under normal circumstances, Yap1p is exclusively cytoplasmic, due to rapid nuclear export mediated by Crm1p (Yan *et al.*, 1998). Intracellular hydrogen peroxide activates Yap1p by modifying its NES, masking it to remain nuclear. The mechnism involves two cysteine residues, which form a disulfide bond upon oxidation, causing conformational change that masks the NES from Crm1p recognition (Delaunay *et al.*, 2000).

Transcription factors may contain DNA-binding motifs inside or in close proximity of transport sequences. In a database search of yeast and higher eukaryotic proteins containing NLSs and DNA/RNA-binding domains in close proximity of each other, it was found that in >79% of cases, the domains overlapped (LaCasse and Lefebvre, 1995). This also is the case for the transcription factor Gal4p, which contains an NLS that overlaps with its DNA-binding domain. Nelson and Silver (1989) demonstrated that the first 29 amino acids of Gal4p fused to invertase were sufficient to direct the fusion protein to the nucleoplasm. *In vitro* evidence shows that Gal4p, when bound to its import mediator importin-β1, is not able to bind to DNA containing a Gal4p-binding

domain, or to perform the reverse process when bound to the Gal4p-binding DNA domain (Chan *et al.*, 1998). Similarly, DNA containing a TATA sequence (Pemberton *et al.*, 1999) stimulates the *in vitro* release of TATA-binding protein (TBP) from its import receptor Kap114p. Transcription factors may thus be released from their respective import receptors upon nuclear entry by means of DNA interaction, competing for a different binding site on the same locus.

2.5.2 REGULATION BY NUCLEAR SHUTTLING, AND NUCLEAR/CYTOPLASMIC SEQUESTRATION

Signalling events may affect various aspects of substrate nucleocytoplasmic transport or specific subcellular localisation. Many proteins have been shown to move continuously between the nucleoplasm and cytoplasm by means of rapid sequential nuclear import and export, referred to as nuclear shuttling. The mechanism of shuttling may be responsible for the relatively even distribution of factors between the nucleus and cytoplasm, or, by separate regulation of import and export dynamics, cause nuclear or cytoplasmic accumulation. Not involving shuttling, factors can be exclusively localised to the nucleus or cytoplasm by prevention of nuclear import and/or export. As previously discussed, this may occur at the level of transport recognition. In addition, factors are denied access to the NPC by means of physical anchoring in the nucleoplasm or cytoplasm, referred to as nuclear or cytoplasmic sequestration. These phenomena are discussed in the following section.

Shuttling factors continuously translocate in and out of the nucleus, either constitutively, or under specific conditions dependent on specific signalling. The already mentioned importin-α can be regarded as a shuttling factor, due to its continued cycling between the cytoplasm and the nucleus. Kap109p serves as the export receptor after importin-α has entered the nucleus via classical nuclear import (Hood and Silver, 1998; Kunzler and Hurt, 1998; Solsbacher *et al.*, 1998). Another previously mentioned component of the central transport apparatus, Yrb1p, has been shown to shuttle between the nucleus and the cytoplasm *in vivo* (Kunzler *et al.*, 2000), although the function of Yrb1p in the nucleus is not well understood.

Ace2p, a transcription factor first identified to activate *CUP1* in a copper dose-dependent fashion (Butler and Thiele, 1991; Thiele, 1988), shuttles constitutively between the nucleus and the cytoplasm due to an NLS and Crm1p-mediated export (Jensen *et al.*, 2000). Although it localises predominantly in the nucleus in a cell cycle-dependant manner during the G1 phase, similar to its cell cycle-regulated homologue Swi5p (Moll *et al.*, 1991), it is present in the nucleus at low levels during the rest of the cell cycle. The finding that Ace2p was required for basal *CUP1* transcription, which is not regulated in a cell cycle-dependent manner upon copper-mediated induction, provides evidence for this. Ace2p shuttling is thus not dependent on cell cycle-dependent regulation, but it influences the nuclear to cytoplasmic ratio of the transcription factor. Jensen *et al.* (2000) hypothesise that, during the G1

phase, Crm1p-mediated Ace2p export is inhibited, resulting in the nuclear accumulation of Ace2p.

Another shuttling protein, Npl3p, continuously binds mRNA in the nucleus and exports it to the cytoplasm (Flach *et al.*, 1994), after which it is imported back into the nucleus by the import receptor Kap111p (Senger *et al.*, 1998). Senger *et al.* (1998) showed that the nuclear release of Npl3p from Kap111p was dependent on the cooperative binding of Ran-GTP and newly synthesised mRNA.

Cdc24p, a guanine exchange factor for Cdc42p, is necessary for polarised growth (Nern and Arkowitz, 1998). Although it shuttles between the nucleus and cytoplasm, its specific functions depend on distinct mechanisms. Nuclear import of Cdc24p requires Far1p. In this case, Far1p anchors Cdc24p in the nucleus, preventing Cdc24p export (Nern and Arkowitz, 2000; Shimada *et al.*, 2000). Upon bud emergence, Far1p degradation is triggered in a cell cycle-dependent manner by Cdc28p, involving ubiquitination by Cdc4p (Blondel *et al.*, 2000). This enables Cdc24p to relocate to the cytoplasm. In contrast, Kap142p specifically exports the Cdc24p/Far1p complex upon treatment with mating pheromone. This mechanism was shown to be essential for the specific localisation of Cdc24p to the site of shmoo formation (Blondel *et al.*, 1999; Shimada *et al.*, 2000).

Apart from a compartmentalising function, the nuclear interior may perform roles in protein activation. Mahanty *et al.* (1999) showed that cytoplasmic Ste5p had to pass through the nucleus before being able to perform its cytoplasmic function. Ste5p functions as the scaffold protein for the mating pheromone receptor/signal transducer complex (Choi *et al.*, 1999; Whiteway *et al.*, 1995; for a review see Elion, 2001). Ste5p shuttles constitutively during vegetative growth, but translocates to the cytoplasm upon pheromone treatment. Remarkably, Ste5p that had not experienced nuclear exposure was unable to correctly locate to the cell periphery (Mahanty *et al.*, 1999). The activation of Ste5p in the nucleus is not yet understood, but the broad mechanism indicates another way of regulating the specific function of a protein.

2.5.3 SIGNALLING EVENTS

The subcellular localisation of some factors depends greatly on tightly regulated pathways affecting their accessibility to the nucleus and/or cytoplasm. Signalling pathways, for example, regulate the subcellular location of the two partially redundant transcription factors, Msn2p and Msn4p, which perform a central role in the general stress response. Stress conditions resulting from heat shock, osmotic shock and carbon-source starvation affect intracellular cAMP levels and PKA activity, causing the nuclear accumulation of these factors. Upon nuclear entry, they bind stress responsive elements (STRE), regulating the transcription of various genes involved in stress responses (Gorner *et al.*, 1998; Martinez-Pastor *et al.*, 1996). Interestingly, Stochaj *et al.* (2000) showed *in vivo* inhibition of the general classical import pathway under conditions of oxidative, starvation and heat shock stress.

2.6 NUCLEOCYTOPLASMIC TRANSPORT AND THE PHOSPHATE SYSTEM

Specific aspects of the process of nucleocytoplasmic communication were discussed in the previous section. With Pho4p as example, a more integrated view will be given of phosphate-regulated signalling controlling Pho4p intracellular location and function.

A network of receptors, phosphatases, signalling elements and transcription factors constitute the yeast phosphate regulon, which regulates intracellular inorganic phosphate (P_i) levels in response to extracellular P_i levels (reviewed by Oshima, 1997). The transcription factor Pho4p performs a central role in the regulation of this system (**Figure 2.8**).

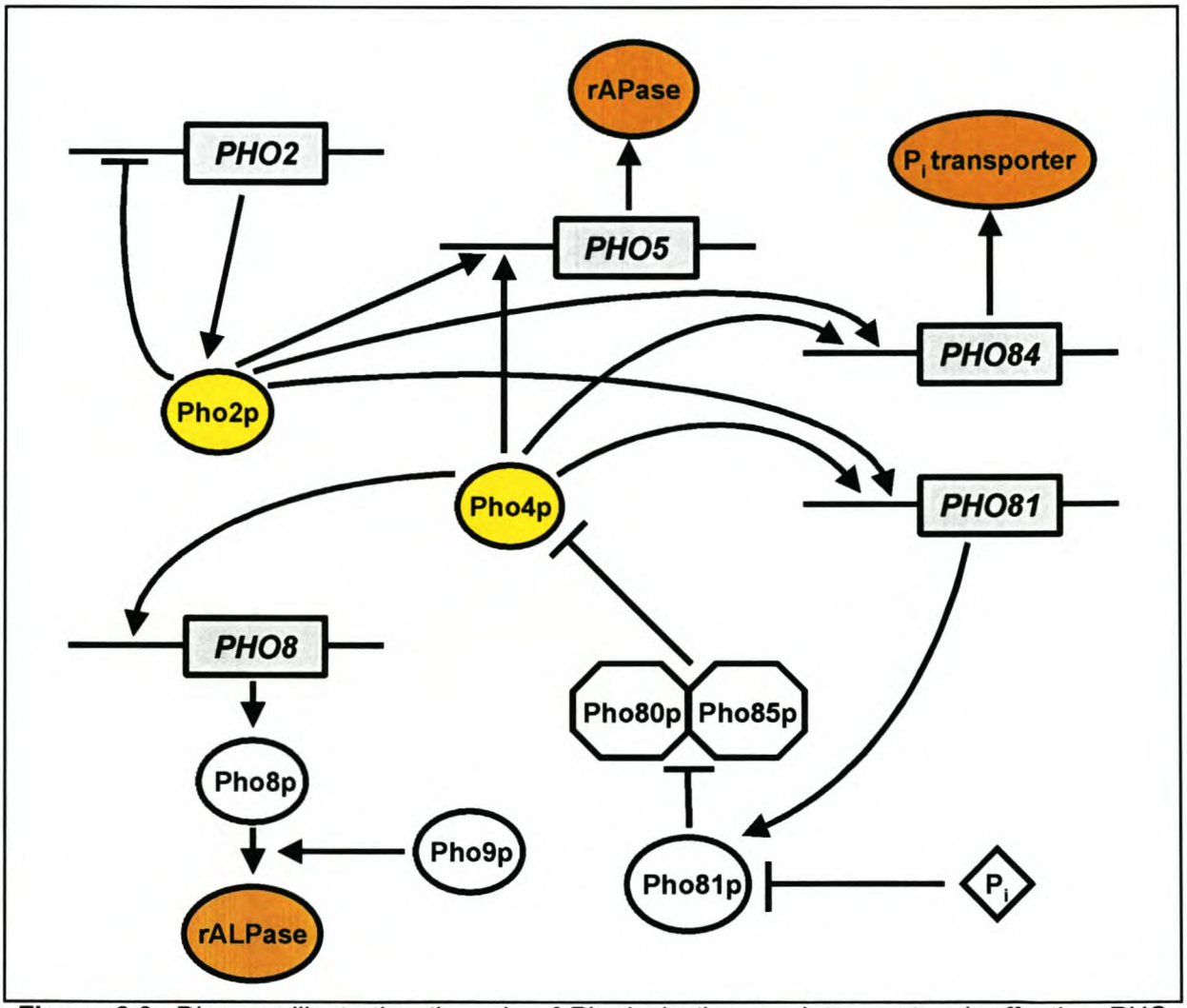

Figure 2.8 Diagram illustrating the role of Pho4p in the regulatory network affecting *PHO* genes. Abbreviations: rAPase (repressive acid phosphatase), rALPase (repressible alkaline phosphatase) (Oshima, 1997).

2.6.1 Pho4p STRUCTURE AND FUNCTION

Pho4p binds a consensus palindrome sequence (CACGTG), referred to as the E-box, which is present in certain promoters of genes involved in the phosphate

regulon. Crystallographic analysis shows that, upon homo-dimerisation of Pho4p, a C-terminal basic helix-turn-helix (bHLH) domain (Shimizu *et al.*, 1997) mediates DNA binding (**Figure 2.9**). An N-terminal acidic *trans*-activation domain of Pho4p was shown to be required for nucleosome remodelling, and subsequent transcriptional activation at the promoter of *PHO5,* which encodes an acid phosphatase (McAndrew *et al.*, 1998; Ogawa and Oshima, 1990; Svaren *et al.*, 1994). Pho4p also contains an NLS, a region essential for nuclear export (Kaffman *et al.*, 1998b), various serine-proline (SP) sites that are phosphorylated at the serine residue under low phosphate conditions, and a C-terminal DNA-binding domain (Komeili and O'Shea, 1999).

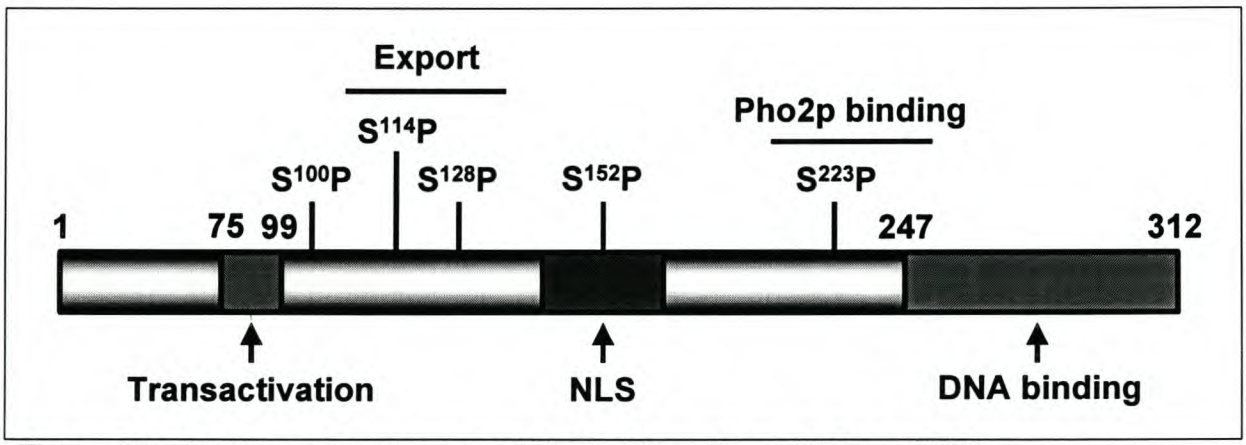

Figure 2.9 The secondary structure of Pho4p (Komeili and O'Shea, 1999)

2.6.2 Pho4p – Pho2p INTERACTION

Near the DNA-binding domain of Pho4p, a short 13 amino acid region was identified that is essential for Pho4p interaction with another transcription factor, Pho2p. This interaction was shown to be specifically required for *PHO5* transcription (Shao *et al.*, 1996). Protein-binding assays showed that Pho4p and Pho2p interact directly, and together are able to interact with certain general transcription factors (Magbanua *et al.*, 1997b). Pho4p and Pho2p bind co-operatively at two upstream activating sequence (UAS) sites of the *PHO5* promoter - Pho4p to the E-box sequence, and Pho2p to the AT-rich regions adjacent to the E-box (Barbaric *et al.*, 1996; Magbanua *et al.*, 1997a). This co-operative binding is essential for the efficient *trans*-activation of *PHO5* by Pho4p (Barbaric *et al.*, 1998). Other *PHO* genes are also activated by the Pho4p-Pho2p dimer, with the exception of *PHO8*. Only Pho4p was shown to be able to bind to the *PHO8* promoter, resulting in chromatin remodelling. This suggests that Pho2p may function as an important co-activator, considering that *PHO8* transcription levels are ten times lower than those of *PH05* (Munsterkotter *et al.*, 2000).

2.6.3 INORGANIC PHOSPHATE (P_i) REGULATION OF Pho4p

Yeast grown on a phosphate-rich medium displays a cytosolic location of Pho4p, but Pho4p concentrates in the nucleus when cells are starved of phosphate. This subcellular localisation was shown to be dependent on a heterodimeric complex consisting of the cyclin, Pho80p, and its associated cyclin-dependant kinase (CDK), Pho85p (O'Neill *et al.*, 1996). Biochemical evidence showed that the Pho80p-Pho85p complex phosphorylated Pho4p, correlating with the negative regulation of *PHO5* (Kaffman *et al.*, 1994). However, when yeast was grown on media depleted of P_i, Pho81p inhibited the phosphorylating activity of the Pho80p-Pho85p complex. Although Pho81p was found to be physically associated with Pho80p-Pho85p under low and high P_i conditions, it only exhibited inhibiting activity under low P_i conditions (Schneider *et al.*, 1994). Nuclear Pho80p-Pho85p, when not inhibited by Pho81p, phosphorylates nuclear Pho4p. Phosphorylated Pho4p is exported by the export receptor Kap142p, and is not able to enter the nucleus again. Nuclear export was shown to be dependent on the phosphorylation of Pho4p (Kaffman *et al.*, 1998a). The same group obtained *in vivo* evidence of Kap121p-mediated nuclear import of unphosphorylated Pho4p, which could not be exported again (Kaffman *et al.*, 1998b). For a schematic representation of the above-mentioned regulatory processes, see **Figure 2.10**.

Five serine-proline (SP) sites in Pho4p have been identified that are potentially phosphorylated at the serine residue. Mutating the serine residues at position 100, 114, 128, 152 and 223 to alanine, in various combinations, resulted in specific and separable behaviour of Pho4p (Komeili and O'Shea, 1999). Four phosphorylation sites are located in the area needed for nuclear export, the NLS, and the binding site for Pho2p (**Figure 2.9**). Phosphorylation of $S^{114}P$ and $S^{128}P$ was found to be essential for nuclear export, but phosphorylation of $S^{152}P$, located in the NLS, inhibited nuclear import. Phosphorylation of $S^{223}P$, located in the Pho2p binding site, also abolished Pho2p interaction (Komeili and O'Shea, 1999). Interestingly, the same group, by means of computer modelling of kinetic data, obtained evidence that Pho80p-Pha85p exhibits site preference when phosphorylating Pho4p. The highest preference was for $S^{223}P$, which abolished Pho4p transcriptional activity, whereas $S^{100}P$ was least likely to be phosphorylated (Jeffery *et al.*, 2001). The authors propose a model whereby rapid phosphorylation abolishes Pho4p function, whereas complete phosphorylation would result in Pho4p export.

2.7 CONCLUSION

Intracellular compartmentalisation clearly provides eukaryotes with an advantage when compared to prokaryotes. Intracellular compartmentalisation enables eukaryotes to exert more control over intracellular processes, allowing them to develop into more specialised multicellular organisms (Bird, 1995). In the nuclear compartment, processes like transcription and ribosome assembly are separated

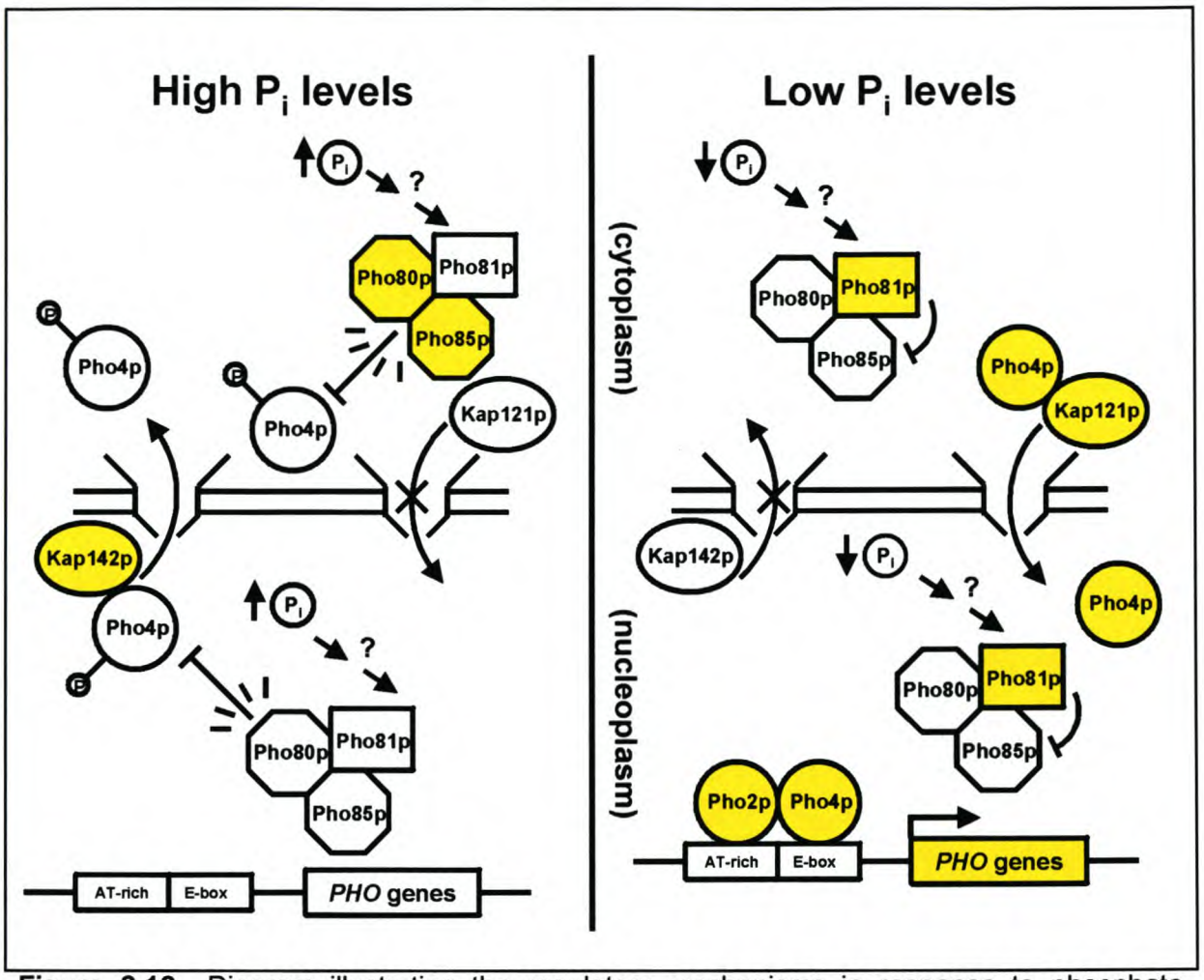

Figure 2.10 Diagram illustrating the regulatory mechanisms in response to phosphate availibilty.

from the cytoplasm. Nuclear processes, however, are coupled to processes in the cytoplasm through selective transport of components across the nuclear membrane barrier. Central to the regulation of nucleocytoplasmic communication between the cytoplasm and nucleoplasm are the NPC and its associated components, which directly affect the accessibility of soluble factors to the nuclear compartment. Signal transduction pathways control the subcellular location of proteins through processes involving covalent modification or the cytoplasmic/nucleoplasmic sequestration of these proteins. Covalent modifications include phosphorylation and oxidative events that directly influence the nuclear accessibility of the target proteins.

Yeast has proven to be a very useful model system for the dissection of the mechanisms affecting, and forming part of nucleocytoplasmic transport. From the level of signalling to the molecular mechanism of nuclear translocation, a considerable amount of knowledge is available. There are large differences, however, between yeast and higher eukaryotes regarding nuclear transport events. For example, the higher eukaryote NPC seems to contain an additional cytoplasmic and nucleocytoplasmic ring structure, and is estimated to be roughly twice the size of the yeast NPC (for reviews see Adam, 2001; Rout and Aitchison, 2001). Other differences include receptor and transport signal diversity. Being the best studied and

understood system so far, yeast can nevertheless still serve a useful purpose as a model system in attempts to understand higher eukaryote nucleocytoplasmic transport mechanisms.

2.9 LITERATURE CITED

Adam, S. A. (2001). The nuclear pore complex. *Genome Biology* **2,** 0007.1-.6.

Aitchison, J. D., Blobel, G. and Rout, M. P. (1996). Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science* **274,** 624-627.

Akey, C. W. and Goldfarb, D. S. (1989). Protein import through the nuclear pore complex is a multistep process. *The Journal of Cell Biology* **109,** 971-982.

Albertini, M., Pemberton, L. F., Rosenblum, J. S. and Blobel, G. (1998). A novel nuclear import pathway for the transcription factor TFIIS. *The Journal of Cell Biology* **143,** 1447-1455.

Alepuz, P. M., Matheos, D., Cunningham, K. W. and Estruch, F. (1999). The *Saccharomyces cerevisiae* RanGTP-binding protein msn5p is involved in different signal transduction pathways. *Genetics* **153,** 1219-1231.

Allen, T. D., Rutherford, S. A., Bennion, G. R., Wiese, C., Riepert, S., Kiseleva, E. and Goldberg, M. W. (1998). Three-dimensional surface structure analysis of the nucleus. *Methods in Cell Biology* **53,** 125-138.

Allen, N. P., Huang, L., Burlingame, A., and Rexach, M. (2001). Proteomic analysis of nucleoporin interacting proteins. *The Journal of Biological Chemistry* **276**, 29268-29274.

Amberg, D. C., Fleischmann, M., Stagljar, I., Cole, C. N. and Aebi, M. (1993). Nuclear PRP20 protein is required for mRNA export. *The EMBO journal* **12,** 233-241.

Andrade, M. A., Petosa, C., O'Donoghue, S. I., Muller, C. W. and Bork, P. (2001). Comparison of ARM and HEAT protein repeats. *Journal of Molecular Biology* **309**, 1-18.

Aris, J. P. and Blobel, G. (1989). Yeast nuclear envelope proteins cross-react with an antibody against mammalian pore complex proteins. *The Journal of Cell Biology* **108,** 2059-2067.

Barbaric, S., Munsterkotter, M., Goding, C. and Horz, W. (1998). Cooperative Pho2-Pho4 interactions at the PHO5 promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. *Molecular and Cellular Biology* **18,** 2629-2639.

Barbaric, S., Munsterkotter, M., Svaren, J. and Horz, W. (1996). The homeodomain protein Pho2 and the basic-helix-loop-helix protein Pho4 bind DNA cooperatively at the yeast PHO5 promoter. *Nucleic Acids Research* **24,** 4479-4486.

Bayliss, R., Kent, H. M., Corbett, A. H. and Stewart, M. (2000a). Crystallization and initial X-ray diffraction characterization of complexes of FxFG nucleoporin repeats with nuclear transport factors. *Journal of Structural Biology* **131,** 240-247.

Bayliss, R., Littlewood, T. and Stewart, M. (2000b). Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking. *Cell* **102,** 99-108.

Baxter, J., Merkenschlager, M. and Fisher, A. G. (2002). Nuclear organisation and gene expression. *Current Opinion In Cell Biology* **14,** 372-376.

Becker, J., Melchior, F., Gerke, V., Bischoff, F. R., Ponstingl, H. and Wittinghofer, A. (1995). RNA1 encodes a GTPase-activating protein specific for Gsp1p, the Ran/TC4 homologue of *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry* **270,** 11860-11865.

Belanger, K. D., Kenna, M. A., Wei, S. and Davis, L. I. (1994). Genetic and physical interactions between Srp1p and nuclear pore complex proteins Nup1p and Nup2p. *The Journal of Cell Biology* **126,** 619-630.

Belhumeur, P., Lee, A., Tam, R., DiPaolo, T., Fortin, N. and Clark, M. W. (1993). GSP1 and GSP2, genetic suppressors of the prp20-1 mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organization. *Molecular and Cellular Biology* **13,** 2152-2161.

Bird, A. P. (1995). Gene number, noise reduction and biological complexity. *Trends in Genetics* **11**, 94-100.

Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W. and Ponstingl, H. (1995). Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *The EMBO journal* **14,** 705-715.

Blondel, M., Alepuz, P. M., Huang, L. S., Shaham, S., Ammerer, G. and Peter, M. (1999). Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes and Development* **13,** 2284-2300.

Blondel, M., Galan, J. M., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies, R. J. and Peter, M. (2000). Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. *The EMBO journal* **19,** 6085-6097.

Buiatti, M. and Buiatti, M. (2001). The living state of matter. *Rivista di biologia* **94**, 59-82.

Butler, G. and Thiele, D. J. (1991). ACE2, an activator of yeast metallothionein expression which is homologous to SWI5. *Molecular and Cellular Biology* **11,** 476-485.

Carvalho, J., Bertram, P. G., Wente, S. R. and Zheng, X. F. (2001). Phosphorylation regulates the interaction between Gln3p and the nuclear import factor Srp1p. *The Journal of Biological Chemistry* **276,** 25359-25365.

Catimel, B., Teh, T., Fontes, M. R., Jennings, I. G., Jans, D. A., Howlett, G. J., Nice, E. C. and Kobe, B. (2001). Biophysical characterization of interactions involving importin-alpha during nuclear import. *The Journal of Biological Chemistry* **276,** 34189-34198.

Chan, C. K., Hubner, S., Hu, W. and Jans, D. A. (1998). Mutual exclusivity of DNA binding and nuclear localization signal recognition by the yeast transcription factor GAL4: implications for nonviral DNA delivery. *Gene Therapy* **5,** 1204-1212.

Chaves, S. R. and Blobel, G. (2001). Nuclear import of Spo12p, a protein essential for meiosis. *The Journal of Biological Chemistry* **276,** 17712-17717.

Chial, H. J., Rout, M. P., Giddings, T. H. and Winey, M. (1998). *Saccharomyces cerevisiae* Ndc1p is a shared component of nuclear pore complexes and spindle pole bodies. *The Journal of Cell Biology* **143,** 1789-1800.

Choi, K. Y., Kranz, J. E., Mahanty, S. K., Park, K. S. and Elion, E. A. (1999). Characterization of Fus3 localization: active Fus3 localizes in complexes of varying size and specific activity. *Molecular Biology of the Cell* **10,** 1553-1568.

Cingolani, G., Petosa, C., Weis, K. and Muller, C. W. (1999). Structure of importin-beta bound to the IBB domain of importin-alpha. *Nature* **399,** 221-229.

Clarke, P. R. and Zhang, C. (2001). Ran GTPase: a master regulator of nuclear structure and function during the eukaryotic cell division cycle? *Trends in Cell Biology* **11,** 366-371.

Clarkson, W. D., Kent, H. M. and Stewart, M. (1996). Separate binding sites on nuclear transport factor 2 (NTF2) for GDP-Ran and the phenylalanine-rich repeat regions of nucleoporins p62 and Nsp1p. *Journal of Molecular Biology* **263,** 517-524.

Cokol, M., Nair, R. and Rost, B. (2000). Finding nuclear localization signals. *EMBO Reports* **1,** 411-415.

Conti, E. and Izaurralde, E. (2001). Nucleocytoplasmic transport enters the atomic age. *Current Opinion in Cell Biology* **13,** 310-319.

Conti, E. and Kuriyan, J. (2000). Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha. *Structure with Folding and Design* **8,** 329-338.

Conti, E., Uy, M., Leighton, L., Blobel, G. and Kuriyan, J. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. *Cell* **94,** 193-204.

Damelin, M., and Silver, P. A. (2000). Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex. *Molecular cell*, **5**, 133-140.

Danker, T. and Oberleithner, H. (2000). Nuclear pore function viewed with atomic force microscopy. *Pflugers Archiv European Journal of Physiology* **439,** 671-681.

De Bruyn-Kops, A. and Guthrie, C. (2001). An essential nuclear envelope integral membrane protein, Brr6p, required for nuclear transport. *The EMBO journal* **20,** 4183-4193.

Delahodde, A., Pandjaitan, R., Corral-Debrinski, M. and Jacq, C. (2001). Pse1/Kap121-dependent nuclear localization of the major yeast multidrug resistance (MDR) transcription factor Pdr1. *Molecular Microbiology* **39,** 304-312.

Delaunay, A., Isnard, A. D. and Toledano, M. B. (2000). H_2O_2 sensing through oxidation of the Yap1 transcription factor. *The EMBO journal* **19,** 5157-5166.

Dingwall, C. and Laskey, R. (1992). The nuclear membrane. *Science* **258,** 942-947.

El-Alami, M., Feller, A., Pierard, A. and Dubois, E. (2000). Characterisation of a tripartite nuclear localisation sequence in the regulatory protein Lys14 of *Saccharomyces cerevisiae*. *Current Genetics* **38,** 78-86.

Elion, E. A. (2001). The Ste5p scaffold. *Journal of Cell Science* **114,** 3967-3978.

Enenkel, C., Blobel, G. and Rexach, M. (1995). Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *The Journal of Biological Chemistry* **270,** 16499-16502.

Fahrenkrog, B., Hubner, W., Mandinova, A., Pante, N., Keller, W. and Aebi, U. (2000). The yeast nucleoporin Nup53p specifically interacts with Nic96p and is directly involved in nuclear protein import. *Molecular Biology of the Cell* **11,** 3885-3896.

Fahrenkrog, B., Hurt, E. C., Aebi, U. and Pante, N. (1998). Molecular architecture of the yeast nuclear pore complex: localization of Nsp1p subcomplexes. *The Journal of Cell Biology* **143,** 577-588.

Fahrenkrog, B., Stoffler, D. and Aebi, U. (2001). Nuclear pore complex architecture and functional dynamics. *Current Topics in Microbiology and Immunology* **259,** 95-117.

Feldherr, C., Akin, D., Littlewood, T. and Stewart, M. (2002). The molecular mechanism of translocation through the nuclear pore complex is highly conserved. *Journal of Cell Science* **115**, 2997-3005.

Ferrigno, P., Posas, F., Koepp, D., Saito, H. and Silver, P. A. (1998). Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *The EMBO journal* **17,** 5606-5614.

Flach, J., Bossie, M., Vogel, J., Corbett, A., Jinks, T., Willins, D. A. and Silver, P. A. (1994). A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. *Molecular and Cellular Biology* **14,** 8399-8407.

Fontoura, B. M., Dales, S., Blobel, G. and Zhong, H. (2001). The nucleoporin Nup98 associates with the intranuclear filamentous protein network of TPR. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 3208-3213.

Garcia, S. N. and Pillus, L. (1999). Net results of nucleolar dynamics. *Cell* **97,** 825-828.

Gilchrist, D., Mykytka, B. and Rexach, M. (2002). Accelerating the rate of disassembly of karyopherin-cargo complexes. *The Journal of Biological Chemistry* **277**, 18161-18172.

Gilbert, W., Siebel, C. W. and Guthrie, C. (2001). Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA dissociation. *RNA* **7,** 302-313.

Gorlich, D., Henklein, P., Laskey, R. A. and Hartmann, E. (1996). A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. *The EMBO journal* **15,** 1810-1817.

Gorlich, D., Prehn, S., Laskey, R. A. and Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* **79,** 767-778.

Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E. and Laskey, R. A. (1995). Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377,** 246-248.

Gorner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schuller, C. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes and Development* **12,** 586-597.

Grandi, P., Doye, V. and Hurt, E. C. (1993). Purification of NSP1 reveals complex formation with 'GLFG' nucleoporins and a novel nuclear pore protein NIC96. *The EMBO journal* **12,** 3061-3071.

Grandi, P., Emig, S., Weise, C., Hucho, F., Pohl, T. and Hurt, E. C. (1995a). A novel nuclear pore protein Nup82p which specifically binds to a fraction of Nsp1p. *Journal of cell biology, The* **130,** 1263-1273.

Grandi, P., Schlaich, N., Tekotte, H. and Hurt, E. C. (1995b). Functional interaction of Nic96p with a core nucleoporin complex consisting of Nsp1p, Nup49p and a novel protein Nup57p. *The EMBO journal* **14,** 76-87.

Grosshans, H., Deinert, K., Hurt, E. and Simos, G. (2001). Biogenesis of the signal recognition particle (SRP) involves import of SRP proteins into the nucleolus, assembly with the SRP-RNA, and Xpo1p-mediated export. *The Journal of Cell Biology* **153,** 745-762.

Groves, M. R., Hanlon, N., Turowski, P., Hemmings, B. A. and Barford, D. (1999). The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. *Cell* **96**, 99-110.

Hase, M. E., Kuznetsov, N. V. and Cordes, V. C. (2001). Amino acid substitutions of coiled-coil protein Tpr abrogate anchorage to the nuclear pore complex but not parallel, in-register homodimerization. *Molecular Biology of the Cell* **12,** 2433-2452.

Hellmuth, K., Lau, D. M., Bischoff, F. R., Kunzler, M., Hurt, E. and Simos, G. (1998). Yeast Los1p has properties of an exportin-like nucleocytoplasmic transport factor for tRNA. *Molecular and Cellular Biology* **18,** 6374-6386.

Herold, A., Truant, R., Wiegand, H. and Cullen, B. R. (1998). Determination of the functional domain organization of the importin alpha nuclear import factor. *The Journal of Cell Biology* **143,** 309-318.

Hood, J. K., Casolari, J. M. and Silver, P. A. (2000). Nup2p is located on the nuclear side of the nuclear pore complex and coordinates Srp1p/importin-alpha export. *Journal of Cell Science* **113,** 1471-1480.

Hood, J. K. and Silver, P. A. (1998). Cse1p is required for export of Srp1p/importin-alpha from the nucleus in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry* **273,** 35142-35146.

Hopper, A. K., Traglia, H. M. and Dunst, R. W. (1990). The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. *The Journal of Cell Biology* **111,** 309-321.

Hozak, P. (1996). The nucleoskeleton and attached activities. *Experimental Cell Research* **229,** 267-271.

Hubner, S., Xiao, C. Y. and Jans, D. A. (1997). The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *The Journal of Biological Chemistry* **272,** 17191-17195.

Huijbregts, R. P., Topalof, L. and Bankaitis, V. A. (2000). Lipid metabolism and regulation of membrane trafficking. *Traffic* **1**, 195-202.

Iovine, M. K., Watkins, J. L. and Wente, S. R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. *The Journal of Cell Biology* **131,** 1699-1713.

Izaurralde, E., Kutay, U., Von Kobbe, C., Mattaj, I. W. and Gorlich, D. (1997). The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *The EMBO journal* **16,** 6535-6547.

Jans, D. A., Ackermann, M. J., Bischoff, J. R., Beach, D. H. and Peters, R. (1991). p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins. *The Journal of Cell Biology* **115,** 1203-1212.

Jans, D. A. and Hubner, S. (1996). Regulation of protein transport to the nucleus: central role of phosphorylation. *Physiological Reviews* **76,** 651-685.

Jans, D. A. and Jans, P. (1994). Negative charge at the casein kinase II site flanking the nuclear localization signal of the SV40 large T-antigen is mechanistically important for enhanced nuclear import. *Oncogene* **9,** 2961-2968.

Jans, D. A., Xiao, C. Y. and Lam, M. H. (2000). Nuclear targeting signal recognition: a key control point in nuclear transport? *BioEssays* **22,** 532-544.

Jeffery, D. A., Springer, M., King, D. S. and O'Shea, E. K. (2001). Multi-site phosphorylation of Pho4 by the cyclin-CDK Pho80-Pho85 is semi-processive with site preference. *Journal of Molecular Biology* **306,** 997-1010.

Jensen, T. H., Neville, M., Rain, J. C., McCarthy, T., Legrain, P. and Rosbash, M. (2000). Identification of novel *Saccharomyces cerevisiae* proteins with nuclear export activity: cell cycle-regulated transcription factor ace2p shows cell cycle-independent nucleocytoplasmic shuttling. *Molecular and Cellular Biology* **20,** 8047-6058.

Kaffman, A., Herskowitz, I., Tjian, R. and O'Shea, E. K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. *Science* **263,** 1153-1156.

Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S. and O'Shea, E. K. (1998a). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature* **396,** 482-486.

Kaffman, A., Rank, N. M. and O'Shea, E. K. (1998b). Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. *Genes and Development* **12,** 2673-2683.

Kalderon, D., Richardson, W. D., Markham, A. F. and Smith, A. E. (1984a). Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311,** 33-38.

Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. (1984b). A short amino acid sequence able to specify nuclear location. *Cell* **39,** 499-509.

Kehlenbach, R. H. and Gerace, L. (2000). Phosphorylation of the nuclear transport machinery down-regulates nuclear protein import in vitro. *The Journal of Biological Chemistry* **275,** 17848-17856.

Kobe, B. (1999). Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha. *Nature Structural Biology* **6,** 388-397.

Komeili, A. and O'Shea, E. K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. *Science* **284,** 977-980.

Komeili, A., Wedaman, K. P., O'Shea, E. K. and Powers, T. (2000). Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. *The Journal of Cell Biology* **151,** 863-878.

Kosova, B., Pante, N., Rollenhagen, C., Podtelejnikov, A., Mann, M., Aebi, U. and Hurt, E. (2000). Mlp2p, a component of nuclear pore attached intranuclear filaments, associates with nic96p. *The Journal of Biological Chemistry* **275,** 343-350.

Kunzler, M., Gerstberger, T., Stutz, F., Bischoff, F. R. and Hurt, E. (2000). Yeast Ran-binding protein 1 (Yrb1) shuttles between the nucleus and cytoplasm and is exported from the nucleus via a CRM1 (XPO1)-dependent pathway. *Molecular and Cellular Biology* **20,** 4295-4308.

Kunzler, M. and Hurt, E. C. (1998). Cse1p functions as the nuclear export receptor for importin alpha in yeast. *FEBS Letters* **433,** 185-190.

Kunzler, M., Trueheart, J., Sette, C., Hurt, E. and Thorner, J. (2001). Mutations in the YRB1 gene encoding yeast ran-binding-protein-1 that impair nucleocytoplasmic transport and suppress yeast mating defects. *Genetics* **157,** 1089-1105.

Kutay, U., Izaurralde, E., Bischoff, F. R., Mattaj, I. W. and Gorlich, D. (1997). Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. *The EMBO journal* **16,** 1153-1163.

LaCasse, E. C. and Lefebvre, Y. A. (1995). Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. *Nucleic Acids Research* **23,** 1647-1656.

Lee, S. J., Imamoto, N., Sakai, H., Nakagawa, A., Kose, S., Koike, M., Yamamoto, M., Kumasaka, T., Yoneda, Y. and Tsukihara, T. (2000). The adoption of a twisted structure of importin-beta is essential for the protein-protein interaction required for nuclear transport. *Journal of Molecular Biology* **302,** 251-264.

Leger-Silvestre, I., Trumtel, S., Noaillac-Depeyre, J. and Gas, N. (1999). Functional compartmentalisation of the nucleus in the budding yeast *Saccharomyces cerevisiae*. *Chromosoma* **108,** 103-113.

Leslie, D. M., Grill, B., Rout, M. P., Wozniak, R. W. and Aitchison, J. D. (2002). Kap121p-mediated nuclear import is required for mating and cellular differentiation in yeast. *Molecular and Cellular Biology* **22,** 2544-2555.

Lorenz, M. C. and Heitman, J. (1998). Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. *Genetics* **150,** 1443-1457.

Madeo, F., Schlauer, J., Zischka, H., Mecke, D. and Frohlich, K. U. (1998). Tyrosine phosphorylation regulates cell cycle-dependent nuclear localization of Cdc48p. *Molecular Biology of the Cell* **9,** 131-141.

Magbanua, J. P., Fujisawa, K., Ogawa, N. and Oshima, Y. (1997a). The homeodomain protein Pho2p binds at an A/T-rich segment flanking the binding site of the basic-helix-loop-helix protein Pho4p in the yeast PHO promoters. *YeasT* **13,** 1299-1308.

Magbanua, J. P., Ogawa, N., Harashima, S. and Oshima, Y. (1997b). The transcriptional activators of the PHO regulon, Pho4p and Pho2p, interact directly with each other and with components of the basal transcription machinery in *Saccharomyces cerevisiae*. *Journal of Biochemistry* **121,** 1182-1189.

Mahanty, S. K., Wang, Y., Farley, F. W. and Elion, E. A. (1999). Nuclear shuttling of yeast scaffold Ste5 is required for its recruitment to the plasma membrane and activation of the mating MAPK cascade. *Cell* **98,** 501-512.

Malik, H. S., Eickbush, T. H. and Goldfarb, D. S. (1997). Evolutionary specialization of the nuclear targeting apparatus. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 13738-13742.

Marelli, M., Aitchison, J. D. and Wozniak, R. W. (1998). Specific binding of the karyopherin Kap121p to a subunit of the nuclear pore complex containing Nup53p, Nup59p, and Nup170p. *The Journal of Cell Biology* **143,** 1813-1830.

Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *The EMBO journal* **15,** 2227-2235.

Mattaj, I. W. and Englmeier, L. (1998). Nucleocytoplasmic transport: the soluble phase. *Annual Review of Biochemistry* **67,** 265-306.

Maurer, P., Redd, M., Solsbacher, J., Bischoff, F. R., Greiner, M., Podtelejnikov, A. V., Mann, M., Stade, K., Weis, K. and Schlenstedt, G. (2001). The nuclear export receptor Xpo1p forms distinct complexes with NES transport substrates and the yeast Ran binding protein 1 (Yrb1p). *Molecular Biology of the Cell* **12,** 539-549.

McAdams, H. H. and Arkin, A. (1999). It's a noisy business! Genetic regulation at the nanomolar scale. *Trends in Genetics* **15**, 65-69.

McAndrew, P. C., Svaren, J., Martin, S. R., Horz, W. and Goding, C. R. (1998). Requirements for chromatin modulation and transcription activation by the Pho4 acidic activation domain. *Molecular and Cellular Biology* **18,** 5818-5827.

McCartney, R. R. and Schmidt, M. C. (2001). Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *The Journal of Biological Chemistry* **276,** 36460-36466.

Michael, W. M., Choi, M. and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell* **83,** 415-422.

Moll, T., Tebb, G., Surana, U., Robitsch, H. and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. *Cell* **66,** 743-758.

Moore, J. D. (2001). The Ran-GTPase and cell-cycle control. *BioEssays* **23,** 77-85.

Morehouse, H., Buratowski, R. M., Silver, P. A. and Buratowski, S. (1999). The importin/karyopherin Kap114 mediates the nuclear import of TATA-binding protein. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 12542-12547.

Moy, T. I. and Silver, P. A. (1999). Nuclear export of the small ribosomal subunit requires the ran-GTPase cycle and certain nucleoporins. *Genes and Development* **13,** 2118-2133.

Munsterkotter, M., Barbaric, S. and Horz, W. (2000). Transcriptional regulation of the yeast PHO8 promoter in comparison to the coregulated PHO5 promoter. *The Journal of Biological Chemistry* **275,** 22678-22685.

Nachury, M. V. and Weis, K. (1999). The direction of transport through the nuclear pore can be inverted. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 9622-9627.

Nehrbass, U., Rout, M. P., Maguire, S., Blobel, G. and Wozniak, R. W. (1996). The yeast nucleoporin Nup188p interacts genetically and physically with the core structures of the nuclear pore complex. *The Journal of Cell Biology* **133,** 1153-1162.

Nelson, M. and Silver, P. (1989). Context affects nuclear protein localization in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **9,** 384-389.

Nern, A. and Arkowitz, R. A. (1998). A GTP-exchange factor required for cell orientation. *Nature* **391,** 195-198.

Nern, A. and Arkowitz, R. A. (2000). Nucleocytoplasmic shuttling of the Cdc42p exchange factor Cdc24p. *The Journal of Cell Biology* **148,** 1115-1122.

Newmeyer, D. D. and Forbes, D. J. (1988). Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell* **52,** 641-653.

Nigg, E. A. (1997). Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* **386,** 779-787.

Ogawa, N. and Oshima, Y. (1990). Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10,** 2224-2236.

Ohno, M., Fornerod, M. and Mattaj, I. W. (1998). Nucleocytoplasmic transport: the last 200 nanometers. *Cell* **92,** 327-336.

Olson, M. O., Dundr, M. and Szebeni, A. (2000). The nucleolus: an old factory with unexpected capabilities. *Trends in Cell Biology* **10,** 189-196.

O'Neill, E. M., Kaffman, A., Jolly, E. R. and O'Shea, E. K. (1996). Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. *Science* **271,** 209-212.

Oshima, Y. (1997). The phosphatase system in *Saccharomyces cerevisiae*. *Genes and Genetic Systems* **72,** 323-334.

Peifer, M., Berg, S. and Reynolds, A. B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* **76,** 789-791.

Pemberton, L. F., Rosenblum, J. S. and Blobel, G. (1999). Nuclear import of the TATA-binding protein: mediation by the karyopherin Kap114p and a possible mechanism for intranuclear targeting. *The Journal of Cell Biology* **145**, 1407-1417.

Pemberton, L. F., Rout, M. P. and Blobel, G. (1995). Disruption of the nucleoporin gene NUP133 results in clustering of nuclear pore complexes. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 1187-1191.

Percipalle, P., Butler, P. J., Finch, J. T., Jans, D. A. and Rhodes, D. (1999). Nuclear localization signal recognition causes release of importin-alpha from aggregates in the cytosol. *Journal of Molecular Biology* **292,** 263-273.

Philimonenko, V. V., Flechon, J. E. and Hozak, P. (2001). The nucleoskeleton: a permanent structure of cell nuclei regardless of their transcriptional activity. *Experimental Cell Research* **264,** 201-210.

Polizotto, R. S. and Cyert, M. S. (2001). Calcineurin-dependent nuclear import of the transcription factor Crz1p requires Nmd5p. *The Journal of Cell Biology* **154,** 951-960.

Quimby, B. B., Lamitina, T., L'Hernault, S. W. and Corbett, A. H. (2000). The mechanism of ran import into the nucleus by nuclear transport factor 2. *The Journal of Biological Chemistry* **275,** 28575-28582.

Radu, A., Blobel, G. and Wozniak, R. W. (1994). Nup107 is a novel nuclear pore complex protein that contains a leucine zipper. *The Journal of Biological Chemistry* **269,** 17600-17605.

Rexach, M. and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell* **83,** 683-692.

Richards, S.A., Carey, K. L. and Macara, I. G. (1997). Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. *Science* **276**, 1842-1844.

Ribbeck, K. and Gorlich, D. (2001). Kinetic analysis of translocation through nuclear pore complexes. *The EMBO journal* **20,** 1320-1330.

Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M. and Gorlich, D. (1998). NTF2 mediates nuclear import of Ran. *The EMBO journal* **17,** 6587-6598.

Richardson, W. D., Mills, A. D., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1988). Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell* **52,** 655-664.

Rihs, H. P., Jans, D. A., Fan, H. and Peters, R. (1991). The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. *The EMBO journal* **10,** 633-639.

Rihs, H. P. and Peters, R. (1989). Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the Simian virus 40 T-antigen. *The EMBO journal* **8,** 1479-1484.

Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64,** 615-623.

Rosenblum, J. S., Pemberton, L. F. and Blobel, G. (1997). A nuclear import pathway for a protein involved in tRNA maturation. *The Journal of Cell Biology* **139,** 1655-61.

Rout, M. P. and Aitchison, J. D. (2001). The nuclear pore complex as a transport machine. *The Journal of Biological Chemistry* **276,** 16593-16596.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y. and Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. *The Journal of Cell Biology* **148,** 635-651.

Rout, M. P., Blobel, G. and Aitchison, J. D. (1997). A distinct nuclear import pathway used by ribosomal proteins. *Cell* **89,** 715-725.

Schlaich, N. L., Haner, M., Lustig, A., Aebi, U. and Hurt, E. C. (1997). In vitro reconstitution of a heterotrimeric nucleoporin complex consisting of recombinant Nsp1p, Nup49p, and Nup57p. *Molecular Biology of the Cell* **8,** 33-46.

Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Gorlich, D., Ponstingl, H. and Bischoff, F. R. (1997). Yrb4p, a yeast ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. *The EMBO journal* **16,** 6237-6249.

Schneider, K. R., Smith, R. L. and O'Shea, E. K. (1994). Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. *Science* **266,** 122-126.

Seedorf, M., Damelin, M., Kahana, J., Taura, T. and Silver, P. A. (1999). Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase. *Molecular and Cellular Biology* **19,** 1547-1557.

Senger, B., Simos, G., Bischoff, F. R., Podtelejnikov, A., Mann, M. and Hurt, E. (1998). Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. *The EMBO journal* **17,** 2196-2207.

Shao, D., Creasy, C. L. and Bergman, L. W. (1996). Interaction of *Saccharomyces cerevisiae* Pho2 with Pho4 increases the accessibility of the activation domain of Pho4. *Molecular and General Genetics* **251,** 358-364.

Shimada, Y., Gulli, M. P. and Peter, M. (2000). Nuclear sequestration of the exchange factor Cdc24 by Far1 regulates cell polarity during yeast mating. *Nature Cell Biology* **2,** 117-124.

Shimizu, T., Toumoto, A., Ihara, K., Shimizu, M., Kyogoku, Y., Ogawa, N., Oshima, Y. and Hakoshima, T. (1997). Crystal structure of PHO4 bHLH domain-DNA complex: flanking base recognition. *The EMBO journal* **16,** 4689-4697.

Shulga, N., James, P., Craig, E. A. and Goldfarb, D. S. (1999). A nuclear export signal prevents *Saccharomyces cerevisiae* Hsp70 Ssb1p from stimulating nuclear localization signal-directed nuclear transport. *The Journal of Biological Chemistry* **274,** 16501-16507.

Shulga, N., Roberts, P., Gu, Z., Spitz, L., Tabb, M. M., Nomura, M. and Goldfarb, D. S. (1996). In vivo nuclear transport kinetics in *Saccharomyces cerevisiae*: a role for heat shock protein 70 during targeting and translocation. *The Journal of Cell Biology* **135,** 329-339.

Sidorova, J. M., Mikesell, G. E. and Breeden, L. L. (1995). Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. *Molecular Biology of the Cell* **6,** 1641-1658.

Siomi, H. and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. *The Journal of Cell Biology* **129,** 551-560.

Siomi, M. C., Fromont, M., Rain, J. C., Wan, L., Wang, F., Legrain, P. and Dreyfuss, G. (1998). Functional conservation of the transportin nuclear import pathway in divergent organisms. *Molecular and Cellular Biology* **18,** 4141-4148.

Smith, A., Brownawell, A. and Macara, I. G. (1998). Nuclear import of Ran is mediated by the transport factor NTF2. *Current Biology* **8,** 1403-1406.

Smith, F. C., Davies, S. P., Wilson, W. A., Carling, D. and Hardie, D. G. (1999). The SNF1 kinase complex from *Saccharomyces cerevisiae* phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1. *FEBS Letters* **453,** 219-223.

Smythe, C., Jenkins, H. E. and Hutchison, C. J. (2000). Incorporation of the nuclear pore basket protein nup153 into nuclear pore structures is dependent upon lamina assembly: evidence from cell-free extracts of Xenopus eggs. *The EMBO journal* **19,** 3918-3931.

Solsbacher, J., Maurer, P., Bischoff, F. R. and Schlenstedt, G. (1998). Cse1p is involved in export of yeast importin alpha from the nucleus. *Molecular and Cellular Biology* **18,** 6805-6815.

Stage-Zimmermann, T., Schmidt, U. and Silver, P. A. (2000). Factors affecting nuclear export of the 60S ribosomal subunit in vivo. *Molecular Biology of the Cell* **11,** 3777-3789.

Starr, C. M. and Hanover, J. A. (1991). A common structural motif in nuclear pore proteins (nucleoporins). *BioEssays* **13,** 145-146.

Stathopoulos-Gerontides, A., Guo, J. J. and Cyert, M. S. (1999). Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. *Genes and Development* **13,** 798-803.

Steggerda, S. M., Black, B. E. and Paschal, B. M. (2000). Monoclonal antibodies to NTF2 inhibit nuclear protein import by preventing nuclear translocation of the GTPase Ran. *Molecular Biology of the Cell* **11,** 703-719.

Stewart, M., Kent, H. M. and McCoy, A. J. (1998). Structural basis for molecular recognition between nuclear transport factor 2 (NTF2) and the GDP-bound form of the Ras-family GTPase Ran. *Journal of Molecular Biology* **277,** 635-646.

Stochaj, U., Rassadi, R. and Chiu, J. (2000). Stress-mediated inhibition of the classical nuclear protein import pathway and nuclear accumulation of the small GTPase Gsp1p. *FASEB journal, The official publication of the Federation of American Societies for Experimental Biology* **14,** 2130-2132.

Stoffler, D., Goldie, K. N., Feja, B. and Aebi, U. (1999a). Calcium-mediated structural changes of native nuclear pore complexes monitored by time-lapse atomic force microscopy. *Journal of Molecular Biology* **287,** 741-752.

Stoffler, D., Steinmetz, M. O. and Aebi, U. (1999b). Imaging biological matter across dimensions: from cells to molecules and atoms. *FASEB journal* **13 Suppl 2,** S195-200.

Strambio-de-Castillia, C., Blobel, G. and Rout, M. P. (1995). Isolation and characterization of nuclear envelopes from the yeast *Saccharomyces*. *The Journal of Cell Biology* **131,** 19-31.

Strambio-de-Castillia, C., Blobel, G. and Rout, M. P. (1999). Proteins connecting the nuclear pore complex with the nuclear interior. *The Journal of Cell Biology* **144,** 839-855.

Strawn, L. A., Shen, T. and Wente, S. R. (2001). The GLFG regions of Nup116p and Nup100p serve as binding sites for both Kap95p and Mex67p at the nuclear pore complex. *The Journal of Biological Chemistry* **276,** 6445-6452.

Strom, A. C. and Weis, K. (2001). Importin-beta-like nuclear transport receptors. *Genome Biology* **2,** 3008.

Strouboulis, J. and Wolffe, A. P. (1996). Functional compartmentalisation of the nucleus. *Journal of Cell Science* **109,** 1991-2000.

Sukegawa, J. and Blobel, G. (1993). A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell* **72,** 29-38.

Svaren, J., Schmitz, J. and Horz, W. (1994). The transactivation domain of Pho4 is required for nucleosome disruption at the PHO5 promoter. *The EMBO journal* **13,** 4856-4862.

Swain, P. S., Elowitz, M. B. and Siggia, E. D. (2002). Intrinsic and extrinsic contributions to stochasticity in gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12795-12800.

Thiele, D. J. (1988). ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. *Molecular and Cellular Biology* **8,** 2745-2752.

Titov, A. A. and Blobel, G. (1999). The karyopherin Kap122p/Pdr6p imports both subunits of the transcription factor IIA into the nucleus. *The Journal of Cell Biology* **147,** 235-246.

Truant, R., Fridell, R. A., Benson, R. E., Bogerd, H. and Cullen, B. R. (1998). Identification and functional characterization of a novel nuclear localization signal present in the yeast Nab2 poly(A)+ RNA binding protein. *Molecular and Cellular Biology* **18,** 1449-1458.

Trumtel, S., Leger-Silvestre, I., Gleizes, P. E., Teulieres, F. and Gas, N. (2000). Assembly and functional organization of the nucleolus: ultrastructural analysis of *Saccharomyces cerevisiae* mutants. *Molecular Biology of the Cell* **11,** 2175-2189.

Ullman, K. S., Powers, M. A. and Forbes, D. J. (1997). Nuclear export receptors: from importin to exportin. *Cell* **90,** 967-970.

Vetter, I. R., Arndt, A., Kutay, U., Gorlich, D. and Wittinghofer, A. (1999). Structural view of the Ran-Importin beta interaction at 2.3 A resolution. *Cell* **97,** 635-646.

Vincent, O., Townley, R., Kuchin, S. and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes and Development* **15,** 1104-1114.

Warmka, J., Hanneman, J., Lee, J., Amin, D. and Ota, I. (2001). Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. *Molecular and Cellular Biology* **21,** 51-60.

Weighardt, F., Biamonti, G. and Riva, S. (1995). Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. *Journal of Cell Science* **108,** 545-555.

Weis, K., Ryder, U. and Lamond, A. I. (1996). The conserved amino-terminal domain of hSRP1 alpha is essential for nuclear protein import. *The EMBO journal* **15,** 1818-1825.

Wen, W., Meinkoth, J. L., Tsien, R. Y. and Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82,** 463-473.

Whiteway, M. S., Wu, C., Leeuw, T., Clark, K., Fourest-Lieuvin, A., Thomas, D. Y. and Leberer, E. (1995). Association of the yeast pheromone response G protein beta gamma subunits with the MAP kinase scaffold Ste5p. *Science* **269,** 1572-1575.

Wozniak, R. W., Blobel, G. and Rout, M. P. (1994). POM152 is an integral protein of the pore membrane domain of the yeast nuclear envelope. *The Journal of Cell Biology* **125,** 31-42.

Wozniak, R. W., Rout, M. P. and Aitchison, J. D. (1998). Karyopherins and kissing cousins. *Trends in Cell Biology* **8,** 184-188.

Xiao, C. Y., Hubner, S., Elliot, R. M., Caon, A. and Jans, D. A. (1996). A consensus cAMP-dependent protein kinase (PK-A) site in place of the CcN motif casein kinase II site simian virus 40 large T-antigen confers PK-A-mediated regulation of nuclear import. *The Journal of Biological Chemistry* **271,** 6451-6457.

Xiao, C. Y., Hubner, S. and Jans, D. A. (1997). SV40 large tumor antigen nuclear import is regulated by the double-stranded DNA-dependent protein kinase site (serine 120) flanking the nuclear localization sequence. *The Journal of Biological Chemistry* **272,** 22191-22198.

Xiao, C. Y. and Jans, D. A. (1998). An engineered site for protein kinase C flanking the SV40 large T-antigen NLS confers phorbol ester-inducible nuclear import. *FEBS Letters* **436,** 313-317.

Xiao, C. Y., Jans, P. and Jans, D. A. (1998). Negative charge at the protein kinase CK2 site enhances recognition of the SV40 large T-antigen NLS by importin: effect of conformation. *FEBS Letters* **440,** 297-301.

Yan, C., Lee, L. H. and Davis, L. I. (1998). Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. *The EMBO journal* **17,** 7416-7429.

Yano, R., Oakes, M., Yamaghishi, M., Dodd, J. A. and Nomura, M. (1992). Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations, in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **12,** 5640-5651.

Yoshida, K. and Blobel, G. (2001). The karyopherin Kap142p/Msn5p mediates nuclear import and nuclear export of different cargo proteins. *The Journal of cell biology* 152, 729-740.

Zaccolo, M., Magalhães, P. and Pozzan, T. (2002). Compartmentalisation of cAMP and Ca^{2+} signals. Current Opinion in Cell Biology 14, 160-166.

CHAPTER 3

RESEARCH RESULTS

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FUNCTIONAL CHARACTERISATION OF Mss11p, A *Saccharomyces cerevisiae* TRANSCRIPTIONAL REGULATOR

3.1 SUMMARY

The yeast *Saccharomyces cerevisiae* alters its growth behaviour in response to nutritional signals. In the case of nutrient limitation, this typically involves cell elongation, the formation of pseudohyphae and the invasion of agar-containing medium. *FLO11*, encoding a cell surface protein, is critical for these processes and displays partial homology to *STA2*, encoding a glucoamylase that enables yeast to utilise starch as sole carbon source. Extensive promoter homology exists between the promoters of *FLO11* and *STA2*, up to ~3 kb upstream of the transcriptional START site. Despite this homology, the transcriptional regulation of *FLO11* and *STA2* differs significantly. Previously, it was shown that the transcriptional regulators Flo8p, Msn1p and Mss11p co-regulate the transcription of *FLO11* and *STA2*. Results indicate that Mss11p function is central to other regulatory proteins. Here we further characterise Mss11p function. We assess the ability of Mss11p to induce invasive growth and flocculation, as well as its effect on cell shape. Furthermore, we identify additional phenotypes associated with different Mss11p expression levels. In particular, we show that differential expression of *MSS11* affects growth in media containing fermentable or non-fermentable carbon sources. We also show that a deletion in *MSS11* leads to the suppression of a severe growth defect on non-fermentable carbon sources of yeast from the ∑1278b genetic background carrying the hyperactive $RAS2^{val19}$ allele. Our biochemical data indicate that Mss11p is a 97 kDa nuclear protein maintained in the cell at low levels.

3.2 INTRODUCTION

The yeast *Saccharomyces cerevisiae* undergoes developmental differentiation in response to nutrient limitation or starvation. The limited availability of carbon and/or nitrogen stimulates the morphological change from an ovoid-shaped "yeast" form to an elongated cell. The normal bipolar budding pattern of diploid strains is replaced by unipolar budding and the cells tend to remain attached to each other after completion of the budding process to form pseudohyphae (Gimeno *et al.*, 1992; Kron *et al.*, 1994). Under the same conditions, cells are able to grow invasively into the agar medium. Although invasive growth has been associated with haploid genotypes, and pseudohyphal growth with diploid genotypes, both processes are essentially the same and most likely perform the same function. The current hypothesis is that this morphological transition enables yeast to grow towards regions with a higher nutrient content. The intracellular signalling mechanisms or proteins involved in the regulation of this change in morphology have been found to be required, in part, for other cellular processes, such as mating, flocculation and biofilm formation. To date, two clear pathways have been identified. One involves a MAP kinase cascade that is at least partially shared with the pheromone response pathway, and the other involves the cAMP-PKA pathway (reviewed in Bauer and Pretorius, 2001; Gancedo, 2001;

Palecek *et al.*, 2002; Pan *et al.*, 2000). Proteins performing regulatory roles in the above mentioned processes that do not fit into clear signalling pathways include Ash1p (Chandarlapaty and Errede, 1998), Elm1p (Edgington *et al.*, 1999), Msn1p (Gagiano *et al.*, 1999a,b; Lambrechts *et al.*, 1996a; Lorenz and Heitman, 1998), Mss11p (Gagiano *et al.*, 1999a,b; 2002; Lorenz and Heitman, 1998), Phd1p (Gimeno and Fink, 1994; Lorenz and Heitman, 1998), Sok2p (Pan and Heitman, 2000; Ward *et al.*, 1995) and Spt3p (Laprade *et al.*, 2002).

Flo8p functions down stream of the cAMP-PKA pathway (Rupp *et al.*, 1999), and, together with Msn1p and Mss11p, has been shown to also regulate the ability of yeast that possesses any of the glucoamylase-encoding *STA1-3* genes (reviewed in Pretorius *et al.*, 1991) to utilise starch as a sole carbon source (Lambrechts *et al.*, 1996a,b; Gagiano *et al.*, 1999a,b; Webber *et al.*, 1997). Gagiano *et al.* (1999a) specifically showed that these factors all act on the promoters of *STA2* and *FLO11*, which encodes a cell surface protein involved in flocculation and which is required for pseudohyphal and invasive growth (Lambrechts *et al.*, 1996a; Lo and Dranginis, 1996; Lo and Dranginis, 1998; Guo *et al.*, 2000). The promoters of *STA2* and *FLO11* are highly homologous, with a similarity stretching over ~3kb. Flo8p, Msn1p and Mss11p were shown to act specifically through an upstream activating sequence (UAS1) of *STA2* and *FLO11*. Although the UAS1 sequence of both genes is virtually identical, the small sequence differences between them account for very significant differences in transcriptional activation, as shown by means of reporter gene studies (Gagiano *et al.*, 1999a).

Flo8p, Msn1p and Mss11p were originally identified as regulators of a diverse selection of processes. Flo8p was identified as a dominant activator of flocculation (Kobayashi *et al.*, 1996; Yamashita and Fukui, 1983), a process in which cells clump together and thus sediment more readily from liquid media (Stratford, 1993). *FLO8* mutation (Liu *et al.*, 1996) and disruption (Kobayashi *et al.*, 1996) reduce the ability of yeast to flocculate. Flo8p acts as a transcription factor downstream of the cAMP signalling pathway (Pan and Heitman, 1999; Rupp *et al.*, 1999), and also positively regulates the transcription of *FLO1* (Kobayashi *et al.*, 1996; Kobayashi *et al.*, 1999) and *FLO11* (Gagiano *et al.*, 1999a; Kobayashi *et al.*, 1999; Pan and Heitman, 1999; Pan and Heitman, 2002; Rupp *et al.*, 1999), which all belong to the family of dominant flocculation genes (Teunissen and Steunsma, 1995). Msn1p, on the other hand, has previously been implicated in the ability to overcome temperature-sensitive *snf1* (Estruch and Carlson, 1990) and *swi6* mutants (Sidorova and Breeden, 1999). Furthermore, it is able to enhance yeast growth on iron-limited media (Eide and Guarente, 1992) and is required for proper osmotic stress-induced transcription (Rep *et al.*, 1999). Multiple copies of *MSN1* were found to be able to suppress the effect of *FLO8* deletion on *STA2* (Lambrechts *et al.*, 1996b) and *FLO11* transcription (Lambrechts *et al.*, 1996a). Finally, Mss11p was first identified as a regulator of starch metabolism (Webber *et al.*, 1997) and, more recently, has been implicated in cell cycle control (Stevenson *et al.*, 2001). Evidence suggests that

Mss11p performs a central role in the transcriptional regulation of these genes, since no other factor was able to completely suppress a mutation in *MSS11* (Gagiano *et al.*, 1999a,b). Recently, it was shown that specific domains of Mss11p, fused to the DNA-binding domain of the Gal4p transcription factor, are able to activate transcription of a reporter gene *in vivo* (Gagiano *et al.*, 2002).

Previous work showed that multiple copies of *MSS11* could suppress mutations in components of both the cAMP-PKA and MAPK pathways to induce pseudohyphal development, invasive growth and the utilisation of starch (Gagiano *et al.*, 1999a,b). In this study, we further investigate the central role of Mss11p in the regulation of these processes. By overexpressing Mss11p from transcriptionally strong promoters, we identify Mss11p as a strong activator of cell elongation and invasive growth. Employing *MSS11* overexpression and deletion, we also implicate *MSS11* in previously unknown physiological processes. Mss11p strongly induces flocculation and regulates vegetative growth on fermentable and non-fermentable carbon sources in a dosage-dependent mechanism. *MSS11* deletion leads to improved biomass formation and suppresses the growth defect on non-fermentable carbon sources of ∑1278b carrying the hyperactive $RAS2^{val19}$ allele. Antibodies raised against Mss11p detected Mss11p protein levels in direct relation to *MSS11* expression levels. Furthermore, GFP-tagged Mss11p localised to the interior of the nucleus in the form of distinct, concentrated "spots".

3.3 MATERIALS AND METHODS

3.3.1 YEAST STRAINS AND CULTURE CONDITIONS

The yeast strains used in this study and descriptions of their relevant genotypes are listed in **Table 3.1**. Standard molecular genetic and yeast techniques were used in generating and handling yeast strains (Ausubel *et al.*, 1994, Sherman *et al.*, 1991). Plasmids were transformed into yeast using the lithium acetate method (Ausubel *et al.*, 1994), and transformants were propagated on selective synthetic media for plasmid maintenance. All selective synthetic media contained 0.67% yeast nitrogen base (NH_4SO_4 supplemented) as nitrogen source, with the required amino acids added, depending on the auxotrophic markers present in each strain. Carbon sources were added as follow: 2% glucose for SCD, 3% ethanol and 3% glycerol for SCGE, and 2% galactose for SCD_{GAL}. Solid media contained 2% agar.

3.3.2 PLASMID CONSTRUCTION

Standard procedures for the isolation and manipulation of DNA were used throughout this study (Ausubel *et al.*, 1994, Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity polymerase used in the enzymatic manipulation of DNA were obtained from Roche Diagnostics (Randburg, South Africa) and used according to the instructions of the supplier. All DNA fragments obtained by the

Table 3.1 Yeast strains used in this study.

Strain	Genotype	Source or reference
BY4742	*MATα his3 leu2 lys2 ura3*	Euroscarf deletion library
Y13694	*MATα his3 leu2 lys2 ura3 Δydr335w::KanMX4*	Euroscarf deletion library
Y14231	*MATα his3 leu2 lys2 ura3 Δydr395w::KanMX4*	Euroscarf deletion library
Y16108	*MATα his3 leu2 lys2 ura3 Δyer110c::KanMX4*	Euroscarf deletion library
Y14384	*MATα his3 leu2 lys2 ura3 Δygl016w::KanMX4*	Euroscarf deletion library
Y14608	*MATα his3 leu2 lys2 ura3 Δygl241w::KanMX4*	Euroscarf deletion library
Y15055	*MATα his3 leu2 lys2 ura3 Δykl205w::KanMX4*	Euroscarf deletion library
Y10734	*MATα his3 leu2 lys2 ura3 Δymr153w::KanMX4*	Euroscarf deletion library
Y12127	*MATα his3 leu2 lys2 ura3 Δypl125w::KanMX4*	Euroscarf deletion library
Y16896	*MATα his3 leu2 lys2 ura3 Δyjr074w::KanMX4*	Euroscarf deletion library
FY23	*MATa leu2 trp1 ura3*	Winston *et al.*, 1995
FY23Δmss11	*MATa trp1 ura3 Δmss11::LEU2*	Gagiano *et al.*, 1999b
ISP15	*MATa his3 leu2 thr1 trp1 ura3*	This laboratory
ISP15Δmss11	*MATa his3 thr1 trp1 ura3 Δmss11::LEU2*	Webber *et al.*, 1997
ISP20	*MATa leu2 thr1 trp1 ura3 Δmss11::LEU2*	This laboratory
ISP20Δmss11	*MATa thr1 trp1 ura3 Δmss11::LEU2*	Gagiano *et al.*, 1999b
Σ272 (Σ1278b)	*MATa his3 leu2 trp1 ura3*	H.U. Mosch
Σ272Δmss11	*MATa his3 trp1 ura3 Δmss11::LEU2*	D. van Dyk

polymerase chain reaction (PCR) were directly cloned into plasmids pGEM-T or pGEM-T-Easy (pGEM-T cloning kit, Promega), rendering them more manageable for subcloning purposes. All plasmids and constructs were verified by restriction enzyme analysis. *E. coli* DH5α (Gibco BRL/Life Technologies) was used as host for the construction and propagation of plasmids and was grown in Luria-Bertani (LB) broth at 37°C. Primers used to generate DNA fragments by PCR are listed in **Table 3.2**. Likewise, all plasmids used in this study are listed in **Table 3.3**. Diagrammatic illustrations of the cloning strategies described in the following section are included in the Appendix, where indicated.

The open reading frame (ORF) of a mutated form of green fluorescent protein (GFP), *GFPmut2*, with a *Bam*HI and *Bgl*II restriction site introduced at the 5' and 3' ends respectively, was generated by PCR using primers GFP2-Bam-F and GFP2-Bgl-R, and plasmid pKEN (Cormack *et al.*, 1996) as template. A 3439 bp fragment containing the ORF and native promoter of *FLO8* was generated by PCR, using genomic DNA from strain ISP15 as template and primers FLO8-F and FLO8-Bam-R, introducing a *Bam*HI restriction site in front of the STOP codon. Following insertion of the phosphoglycerate kinase (*PGK*) terminator (PGK_T), isolated from pHVX2 (Volschenk *et al.*, 1997) as a 227 bp *Xho*I - *Hin*dIII fragment, into the

Table 3.2 Primers used in this study. Nucleotides homologous to the template are shown in capital letters and those introducing restriction sites are shown in small underlined capital letters.

Primer name	Sequence
Flo8-ORF	5'-ggcggatccATGAGTTATAAAGTGAATAGTTCGTATCC-3'
FLO8-Bam-R	5'-cacgtgggatccGCCTTCCCAATTAATAAAATTGAA-3'
FLO8-BamRS	5'-agtggatccTTAGCCTTCCCAATTAATAAAATTGAA-3'
FLO8-F	5'-CTTTCCCACCCAATCTTAGGCACCT-3'
GEX2T-mutF	5'-gatccgaattcaagctttctagagtcgactgactgactga-3'
GEX2T-mutR	5'-aatttcagtcagtcagtcgactctagaaagcttgaattcg-3'
GFP2-Bam-F	5'-aagataggatccATGAGTAAAGGAGAAGAACTTTTC-3'
GFP2-Bgl-R	5'-aagtctagatctTTATTTGTATAGTTCATCCATGCC-3'
GFP2-Xba-R	5'-aagtcttctagaTTATTTGTATAGTTCATCCATGCC-3'
Msn1-F	5'-AGAATGTCGATCAGGCACGT-3'
Msn1-BamR	5'-taggatccCTTCAAAGTCTCTGGAATATGAGA-3'
Msn1-BamF	5'-attaggatccATGGCAAGTAACCAGCACATA-3'
Msn1-EcoR	5'-tgaattcCCTTCAAAGTCTCTGGAATATGAGA-3'
MSS11-EXPF	5'-tggatccATGGATAACACGACCAATATTAATA-3'
MSS11-Bam-R	5'-taatagggatccGCTATCCATTAGATCAGGAGAAAA-3'
Mss11-EcoR	5'-tgaattcCGCTATCCATTAGATCAGGAGAAA-3
MSS11ex-F	5'-ccggaattcATGGATAACACGACCAATATTAATACA-3'
MSS11ex-R	5'-atgcatgtcgacTTAGCTATCCATTAGATCAGGAGAAAAGTCACCAAT-3'
MSS11-F	5'-GATGCCATAACCGACTAGAC-3'

2μm shuttle vector YEpLac112 (Gietz and Sugino, 1988) digested with *Sal*I and *Hin*dIII, a 714 bp *Bam*HI – *Bgl*II-digested fragment of *GFPmut2* was inserted into the unique *Bam*HI site of the same plasmid. The *FLO8* ORF and native promoter were subsequently inserted into the above-mentioned plasmid digested with *Nae*I and *Bam*HI, as two fragments, *Nae*I – *Spe*I and *Spe*I – *Bam*HI, thus generating an in-frame fusion of *GFPmut2* to the 3' end of *FLO8* under transcriptional control of $FLO8_P$. $FLO8_P$-*FLO8-GFPmut2-*PGK_T was further subcloned into the unique *Sac*I and *Xba*I sites of the centromeric plasmids YCpLac22, YCpLac33 and YCpLac111 (Gietz and Sugino, 1988), and the episomal plasmids YEpLac181 and YEpLac195 (Gietz and Sugino, 1988) (see **Figure A** for cloning strategy). A 1468 bp fragment containing the ORF and native promoter of *MSN1* with a *Bam*HI restriction site introduced in front of the ORF STOP codon was PCR generated using primers Msn1-F and Msn1-Bam-R, and plasmid YEpLac112-MSN1 as template. In a strategy similar to generating the *FLO8* fusion, *Bam*HI – *Pvu*II-digested $MSN1_P$-*MSN1* was inserted into *Bam*HI – *Sma*I-digested YEpLac112, which already contained

Table 3.3 Plasmids used in this study

Plasmid name	Relevant genotype	Source/reference
B2255	*CEN4 URA3 RAS2*^{val19}	M. Vanoni
pGEM-T		Promega
pGEM-T-Easy		Promega
pGEM-T-H1F-H1R	*MSS11*$_{35\rightarrow112}$	Gagiano *et al.*, 2002
pGEM-T-H1F-H2R	*MSS11*$_{35\rightarrow168}$	Gagiano *et al.*, 2002
pGEM-T-H2F-H2R	*MSS11*$_{146\rightarrow168}$	Gagiano *et al.*, 2002
pGEM-T-QF-ID1R	*MSS11*$_{330\rightarrow420}$	Gagiano *et al.*, 2002
pGEM-T-QF-ID2R	*MSS11*$_{330\rightarrow511}$	Gagiano *et al.*, 2002
pGEM-T-QF-NR	*MSS11*$_{330\rightarrow604}$	Gagiano *et al.*, 2002
pGEM-T-ID3F-NR	*MSS11*$_{504\rightarrow604}$	Gagiano *et al.*, 2002
pGEX-2T		Pharmacia Biotech
pGEX-2T-FLO8	*FLO8*$_{1\rightarrow758}$	This study
pGEX-2T-MSN1	*MSN1*$_{1\rightarrow758}$	This study
pGEX-2T-MSS11	*MSS11*$_{1\rightarrow758}$	This study
pGEX-2T+		This study
pGEX-2T+-H1	*MSS11*$_{35\rightarrow112}$	This study
pGEX-2T+-H1-2	*MSS11*$_{35\rightarrow168}$	This study
pGEX-2T+-H2	*MSS11*$_{146\rightarrow168}$	This study
pGEX-2T+-ID1	*MSS11*$_{330\rightarrow420}$	This study
pGEX-2T+-ID1-2	*MSS11*$_{330\rightarrow511}$	This study
pGEX-2T+-ID1-3	*MSS11*$_{330\rightarrow604}$	This study
pGEX-2T+-ID3	*MSS11*$_{504\rightarrow604}$	This study
pHVX2	2μm *LEU2 PGK*$_P$ *PGK*$_T$	Volschenk *et al.*, 1997
pJC1	2μm *URA3 PGK*$_P$ *PGK*$_T$	Crous *et al.*, 1995
pKEN-GFP2	*GFPmut2*	Cormack *et al.*, 1996
pYES2	2μm *URA3 GAL1*$_P$ *CYC1*$_T$	Invitrogen
pYES2-MSS11	2μm *URA3 GAL1*$_P$ *MSS11 CYC1*$_T$	T. Crane
pYES2(CEN)	*CEN4 URA3 GAL1*$_P$ *CYC1*$_T$	This study
pYES2(CEN)-MSS11	*CEN4 URA3 GAL1*$_P$ *MSS11 CYC1*$_T$	This study
pYES-mtBFP	2μm *URA3 GAL1*$_P$ *BFP*	Westermann and Neupert, 2000
YCpLac22	*CEN4 TRP1*	Gietz and Sugino, 1988
YCpLac22-FLO8-GFP	*CEN4 TRP1 FLO8*$_P$ *FLO8 GFPmut2 PGK*$_T$	This study

Table 3.3 Plasmids used in this study (continued)

Plasmid name	Relevant genotype	Source/Reference
YCpLac22-MSN1-GFP	*CEN4 TRP1 MSN1*$_P$ *MSN1 GFPmut2 PGK*$_T$	This study
YCpLac22-MSS11-GFP	*CEN4 TRP1 MSS11*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study
YCpLac33	*CEN4 URA3*	Gietz and Sugino, 1988
YCpLac33-FLO8-GFP	*CEN4 URA3 FLO8*$_P$ *FLO8 GFPmut2 PGK*$_T$	This study
YCpLac33-MSN1-GFP	*CEN4 URA3 MSN1*$_P$ *MSN1 GFPmut2 PGK*$_T$	This study
YCpLac33-MSS11-GFP	*CEN4 URA3 MSS11*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study
YCpLac111	*CEN4 LEU2*	Gietz and Sugino, 1988
YCpLac111-FLO8-GFP	*CEN4 LEU2 FLO8*$_P$ *FLO8 GFPmut2 PGK*$_T$	This study
YCpLac111-MSN1-GFP	*CEN4 LEU2 MSN1*$_P$ *MSN1 GFPmut2 PGK*$_T$	This study
YCpLac111-MSS11-GFP	*CEN4 LEU2 MSS11*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study
YEpLac112	2µm *TRP1*	Gietz and Sugino, 1988
YEpLac112-3-12	2µm *TRP1*	M. Gagiano
YEpLac112-3-13	2µm *TRP1*	M. Gagiano
YEpLac112-14-10	2µm *TRP1 STA2/MUC1*$_{P\text{-}1199\rightarrow1074}$	This study
YEpLac112-15-10	2µm *TRP1 STA2/MUC1*$_{P\text{-}1160\rightarrow1074}$	This study
YEpLac112-M3-10	2µm *TRP1 MUC1*$_{P\text{-}1479\rightarrow1136}$	This study
YEpLac112-S3-10	2µm *TRP1 STA2*$_{P\text{-}1390\rightarrow1074}$	This study
YEpLac112-FLO8-GFP	2µm *TRP1 FLO8*$_P$ *FLO8 GFPmut2 PGK*$_T$	This study
YEpLac112-MSN1-GFP	2µm *TRP1 MSN1*$_P$ *MSN1 GFPmut2 PGK*$_T$	This study
YEpLac112-MSS11-GFP	2µm *TRP1 MSS11*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study
YEpLac112-MSN1	2µm *TRP1 MSN1*$_P$ *MSN1 MSN1*$_T$	Gagiano *et al.*, 1999b
YEpLac112-MSS11	2µm *TRP1 MSS11*$_P$ *MSS11 MSS11*$_T$	Gagiano *et al.*, 1999b
YEpLac112-PGK$_{PT}$	2µm *TRP1 PGK*$_P$ *PGK*$_T$	This study
YEpLac112-PGK$_{PT}$-GFP	2µm *TRP1 PGK*$_P$ *GFPmut2 PGK*$_T$	This study
YEpLac112-PGK$_{PT}$-MSS11	2µm *TRP1 PGK*$_P$ *MSS11 PGK*$_T$	This study
YEpLac112-PGK$_{PT}$-MSS11-GFP	2µm *TRP1 PGK*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study
YEpLac181	2µm *LEU2*	Gietz and Sugino, 1988
YEpLac181-FLO8-BFP	2µm *LEU2 FLO8*$_P$ *FLO8 BFP PGK*$_T$	This study
YEpLac181-FLO8-GFP	2µm *LEU2 FLO8*$_P$ *FLO8 GFPmut2 PGK*$_T$	This study
YEpLac181-MSN1-GFP	2µm *LEU2 MSN1*$_P$ *MSN1 GFPmut2 PGK*$_T$	This study
YEpLac181-MSS11-BFP	2µm *LEU2 MSS11*$_P$ *MSS11 BFP PGK*$_T$	This study
YEpLac181-MSS11-GFP	2µm *LEU2 MSS11*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study

Table 3.3 Plasmids used in this study (continued)

Plasmid name	Relevant genotype	Source/reference
YEpLac195-FLO8-BFP	2μm *URA3 FLO8*$_P$ *FLO8 BFP PGK*$_T$	This study
YEpLac195-FLO8-GFP	2μm *URA3 FLO8*$_P$ *FLO8 GFPmut2 PGK*$_T$	This study
YEpLac195-MSN1-GFP	2μm *URA3 MSN1*$_P$ *MSN1 GFPmut2 PGK*$_T$	This study
YEpLac195-MSS11-GFP	2μm *URA3 MSS11*$_P$ *MSS11 GFPmut2 PGK*$_T$	This study

GFPmut2- PGK$_T$, thus generating an in-frame fusion of *GFPmut2* to the 3' end of *MSN1* under transcriptional control of *MSN1*$_P$. *MSN1*$_P$-*MSN-GFPmut2-PGK*$_T$ was further subcloned into the unique *Eco*RI and *Hin*dIII restriction sites of YCpLac22, YCpLac33, YCpLac111, YEpLac181 and YEpLac195 (see **Figure B** for cloning strategy). A 2870 bp fragment containing the ORF and native promoter of *MSS11,* with a *Bam*HI restriction site introduced in front of the STOP codon, was PCR generated using primers MSS11-F and MSS11-Bam-R and genomic DNA from strain ISP15 as template. The fragment was digested with *Eco*RI and *Bam*HI, and ligated to *Eco*RI – *Bam*HI-digested YEpLac112 already containing *PGK*$_T$. Finally, *Bam*HI – *Bgl*II-digested *GFPmut2* was inserted into the unique *Bam*HI restriction site to generate an in-frame fusion of *GFPmut2* to the 3' end of *MSS11* under the transcriptional control of *MSS11*$_P$. *MSS11*$_P$-*MSS11-GFPmut2-PGK*$_T$ was further subcloned into the unique *Eco*RI – *Hin*dIII restriction sites of YCpLac22, YCpLac33, YCpLac111, YEpLac181 and YEpLac195 (see **Figure C** for cloning strategy).

The ORF of another mutant form of GFP, referred to here as blue fluorescent protein (BFP), was PCR amplified with pYES-mtBFP (Westermann and Neupert, 2000) as template. Two different primer sets, GFP2-Bam-F & GFP2-Bgl-R and GFP2-Bam-F & GFP2-Xba-R, were used, yielding fragments that were both 714 bp in size, with a *Bam*HI site introduced at the 5' end and a *Bgl*II or *Xba*I site at the 3' end respectively. The *BFP* ORF encodes a 238 amino acid protein with two amino acid changes, Y66→H and Y145→F, resulting in an emission wavelength shift to 445 nm or blue light with 49% of the quantum yield of wt GFP (Heim and Tsien, 1996). BFP is ~200 times less luminescent than GFPmut2. The *GFPmut2* ORF in YEpLac181-FLO8-GFP and YEpLac195-FLO8-GFP was removed by *Bam*HI – *Xba*I digestion and replaced by the *BFP* ORF using the same restriction sites, yielding YEpLac181-FLO8-BFP and YEplac195-FLO8-BFP (see **Figure D** for cloning strategy). To create YEpLac181-MSS11-BFP, an *Eco*RI – *Bam*HI-digested fragment containing the MSS11 ORF and native promoter was inserted into the unique *Eco*RI and *Bam*HI restriction sites of YEpLac181 that already contained *PGK*$_T$, as mentioned above for YEpLac112. The *BFP* ORF was finally inserted into the unique *Bam*HI site as a *Bam*HI –*Bgl*II fragment (see **Figure E** for cloning strategy).

A 1711 bp fragment containing a multiple cloning site flanked by *PGK*$_P$, a strong constitutive promoter, and *PGK*$_T$ was isolated from YEp352-PGK$_{PT}$ (Crous *et al.*, 1995) and inserted into the *Bam*HI and *Nar*I sites of YEpLac112 to give

YEpLac112-PGK$_{PT}$. The *MSS11* ORF, with an *Eco*RI site introduced before the ATG codon and an *Sal*I site introduced after the STOP codon by means of PCR using YEpLac112-MSS11 as template and primers MSS11ex-F and MSS11ex-R respectively, was ligated into the unique *Eco*RI and *Xho*I sites in the multiple cloning site of the *PGK* expression cassette to obtain YEpLac112-PGK$_{PT}$-MSS11. The *GFPmut2* ORF was cloned as a *Bam*HI-*Bgl*II fragment into the *Bgl*II site of YEpLac112-PGK$_{PT}$ to give YEpLac112-PGK$_{PT}$-GFP (see **Figure F** for cloning strategy). Using PCR and primers MSS11ex-F and GFP2-Bgl-R, an in-frame fusion between *MSS11* ORF and *GFPmut2* ORF was obtained using YEpLac112-MSS11-GFP as template, with an *Eco*RI restriction site introduced before the ATG codon of *MSS11* and a *Bgl*II site introduced after the STOP codon of *GFPmut2,* and subsequently cloned into the *Eco*RI and *Bgl*II sites of YEpLac112-PGK$_{PT}$ to obtain YEpLac112-PGK$_{PT}$-MSS11-GFP (see **Figure G** for cloning strategy).

In order to convert the 2μm-based plasmid pYES2 (Invitrogen) to a centromeric plasmid, the 2μm part was excised with *Nhe*I and *Sna*BI and replaced by a fragment containing the *CEN4* and *ARS1* sequences isolated from YCpLac33. This was done by digesting it with *Nsi*I, followed by blunt-end generation by Klenow enzyme and, finally, digestion with *Spe*I, generating pYES2 (CEN). The *MSS11* ORF with ATG was inserted as an *Eco*RI-*Sal*I fragment into pYES2 (CEN) digested with *Eco*RI and *Xho*I, generating pYES2(CEN)-MSS11 (see **Figure H** for cloning strategy). The promoter fragments present in pHP41 (Gagiano *et al.*, 1999a) were subcloned into the unique *Sal*I site of YEpLac112 as *Sal*I-*Xho*I-digested fragments, generating YEpLac112-14-10 and YEpLac112-15-10, and as *Bam*HI-digested fragments into the unique *Bam*HI site of YEpLac112, generating YEpLac112-M3-10 and YEpLac112-S3-10.

MSS11, amplified with Mss11-BamF and Mss11-EcoR to introduce a 5' *Bam*HI and 3' *Eco*RI restriction site, was inserted into the unique *Eco*RI and *Bam*HI sites of the bacterial expression vector pGEX-2T (Pharmacia Biotech) to create pGEX-2T-MSS11. By inserting fragments into the multiple cloning site of pGEX-2T, in-frame fusions were made to the DNA encoding for glutathione-S-transferase (GST). *MSN1*, amplified with Msn1-BamF and Msn1-EcoR, was inserted into pGEX-2T in the same way to generate pGEX-2T-MSN1. *FLO8*, amplified by FLO8-ORF and FLO8-BamRS to introduce *Bam*HI sites at both ends of the fragment, was inserted into the *Bam*HI site of pGEX-2T to give pGEX-2T-FLO8. Primers GEX2T-mutF and GEX2T-mutR were annealed to each other to form a linker with sticky ends compatible with *Bam*HI and *Sal*I sticky ends, and was thus inserted into pGEX-2T digested with *Bam*HI and *Sal*I to generate pGEX-2T+. The insertion of the linker conserves the original *Bam*HI restriction site, destroys the original *Eco*RI site, and adds *Eco*RI, *Hin*dIII, *Xba*I and *Sal*I restriction sites in the same frame as the *Bam*HI site, as well as three STOP codons in all three frames 3' of the *Sal*I site. Fragments encoding for Mss11p domains H1, H2, H1-2, ID1, ID1-2, ID1-3 and ID3

were removed from the respective pGEM-T plasmids by *Eco*RI and *Sal*I digestion and ligated to pGEX-2T+ digested with *Eco*RI and *Sal*I, to create the respective plasmids listed in **Table 3.3** (see **Figure I** for cloning strategy and *MSS11* domain architecture).

3.3.3 INVASIVE GROWTH PLATE ASSAY

Three independent transformed colonies were inoculated into 5 ml of SCD media and grown to an OD_{600} of approximately 1 unit. 10 µl were then spotted on agar plates. For the assessment of invasive growth, cells were washed off the agar surface with a gloved finger and running water. Thus, only cells that had grown invasively into the agar medium remained on the plate.

3.3.4 FLOCCULATION ASSAY

To determine the extent of Ca^{2+}-dependent flocculation, an aqueous solution of EDTA (pH 8.0) was added to a 5 ml liquid yeast culture to a final concentration of 30 mM, followed by vigorous agitation (vortex at maximum speed setting) until the clumps of cells were in homogeneous suspension, determined by visual inspection. The optical density (OD_{600}) was immediately determined by removing 100 µl of the liquid culture and adding it to 900 µl of 20 mM EDTA solution (pH 8.0), followed by spectrophotometric measurement (measurement "A") at a wavelength of 600 nm. Flocculation of a Ca^{2+}-dependent nature was then induced by transferring 1 ml of liquid culture to a microcentrifuge tube, separating the cells from the growth medium by quick centrifugation, removing the supernatant and adding 1 ml of an aqueous solution of 5 mM $CaCl_2$. Microcentrifuge tubes were vigorously agitated (vortex at maximum speed setting) for 10 seconds and left undisturbed for 60 seconds. A second spectrophotometric measurement (measurement "B") was performed on a 100 µl sample taken from just below the meniscus in the microcentrifuge tube, as described above. The extent of Ca^{2+}-dependent flocculation was then calculated by the following formula:

$$\%\text{Flocculation} = \left(\frac{A-B}{A}\right) \times 100$$

Images of flocculating liquid cultures were taken after vigorously agitating the culture tubes (vortex at top speed for ~10 seconds.), followed by leaving the cultures undisturbed for 1 minute.

3.3.5 GROWTH CURVE ANALYSIS

Population growth was monitored by determining the optical density, at 600 nm, of samples taken periodically from 25 ml cultures in 250 ml flasks shaking at 160 rpm on a rotary shaker at 30°C. Single colonies of transformants were inoculated in 5 ml

of SCD and grown for two days to serve as starter cultures. Cultures were inoculated to an initial OD_{600} of 0.1 units for SCD and 0.2 units for SCGE. For each different transformation, three individual transformed colonies were taken and further processed as described above. The growth rate constant (***k***) was calculated assuming that the optical density of a culture directly correlates to cell numbers, using the following equation:

$$k = \frac{n}{t} = \frac{\log N_1 - \log N_0}{\log 2t}$$

where

t = time of measured interval in hours
N_0 = initial population number = initial OD_{600} reading
N_1 = population number at time ***t*** = OD_{600} reading at time ***t***
n = number of generations in time ***t***
k = mean growth rate constant in number of generations (***n***) per hour

Source data used for the determination of ***k*** were only taken from data points corresponding to populations in true exponential growth. This was achieved by selecting for data that arrange in a linear fashion when plotted on a semi-logarithmic (logarithmic-linear) scale. The mean generation time (***g***) in hours per generation corresponds to k^{-1}.

3.3.6 RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

Recombinant expression of GST fusion proteins from the strong inducible *TAC* promoter was performed using the pGEX bacterial expression system (Pharmacia) (**Figure 3.1A**). *E. coli* BL21-DE3 (pLysS) (Novagen) was used as the host for protein production. Transformed colonies inoculated in 5 ml of "terrific broth" (TB), supplemented with 0.1% glucose as well as ampicillin and chloramphenicol (100 μg and 34 μg end concentration respectively), were grown overnight at 37°C and served as starter cultures. 5 ml of TB (0.1% glucose) with antibiotics was inoculated to an OD_{600} not greater than 0.1 units, and grown at 37°C to an OD_{600} of not greater than 1.0 units. 200 ml of TB (0.1% glucose) with antibiotics in a 1 L erlenmeyer flask was subsequently inoculated by adding the total secondary starter culture, and was grown at 37°C and 300 rpm on a rotary shaker to an OD_{600} of 0.6-0.8 units, at which point the expression system was induced by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 0.4 mM. Following induction, the cultures were grown further for three hours. Cultures were quick-cooled in an ice slurry, the cells were harvested at 5000 rpm and 4°C for 5 minutes, followed by resuspension of the cell pellet in ice cold 20 mM Tris-Cl (pH 7.5). After the addition of lysozyme to a final concentration of 100 μg/ml, the resulting cell suspension was incubated at 30°C for 15 min., with periodic mild agitation. Cells were sonicated until the completion of full lysis, with cooling on ice between sonication bursts. Soluble and insoluble fractions

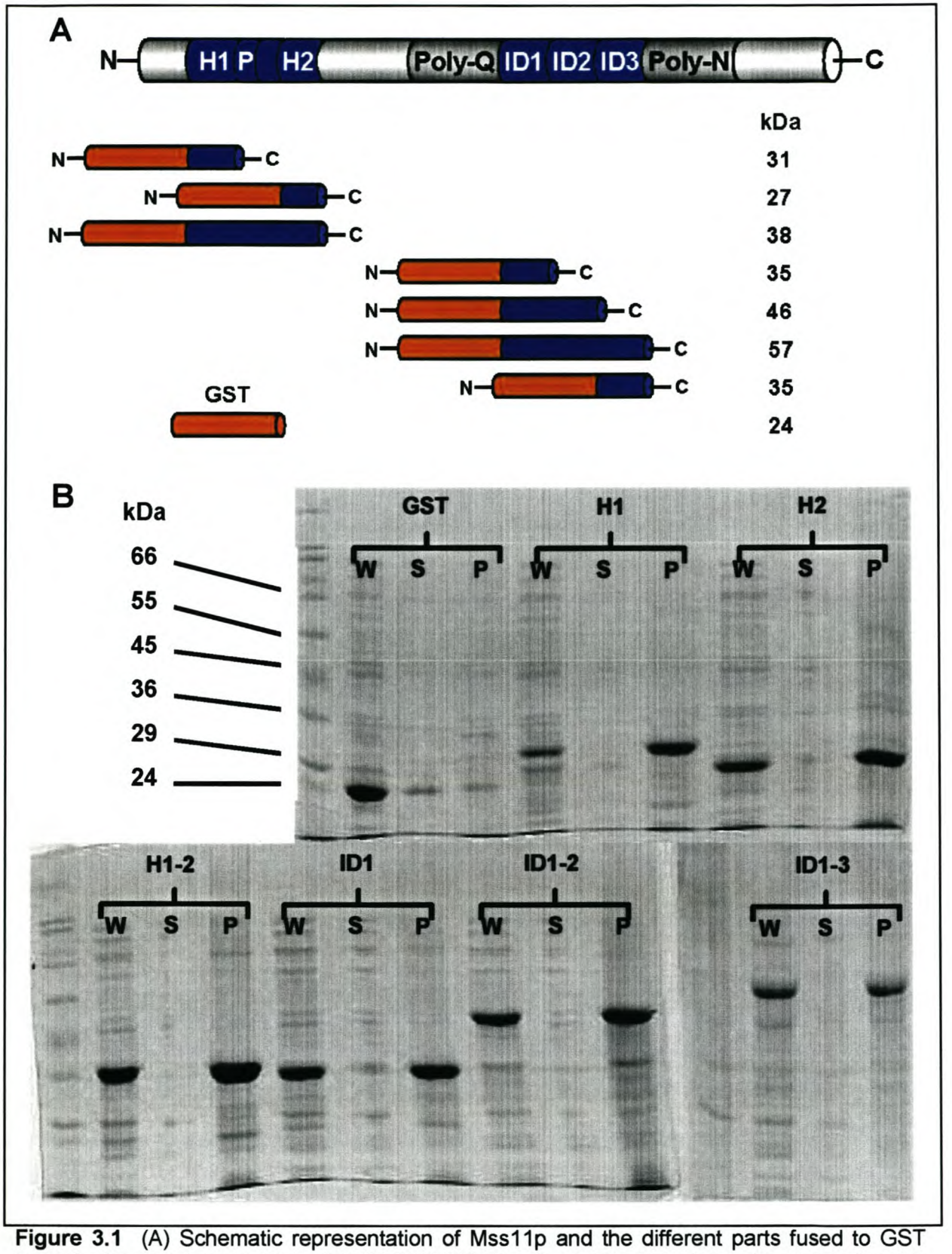

Figure 3.1 (A) Schematic representation of Mss11p and the different parts fused to GST used for bacterial expression. (B) SDS-PAGE of cell extracts from bacteria expressing the different fusion proteins. GST refers to the tag expressed alone, and the rest to the relevant Mss11p domain fused to GST (W – whole cell protein extracts, S – soluble protein extract after sonication and P – insoluble protein extract after sonication).

were separated by centrifugation at 12000 rpm and 4°C for 30 minutes. Soluble fractions were frozen for further analysis and the insoluble pellet was dissolved in

sample treatment buffer (0.062 mM Tris-Cl, 5% β-mercaptoethanol, 10% glycerol, pH 6.8), with sodium dodecyl sulfate (SDS) added to a final solution of 2% at 100°C with periodic mild agitation until the pellet was completely dissolved as determined by visual inspection (10-30 min.). All expressed proteins were found to be in the insoluble fraction, except GST alone (**Figure 3.1B**). Dissolved insoluble fractions were resolved on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Sambrook *et al.*, 1989) and visualised by staining in an aqueous solution of Coomassie Brilliant Blue R250 (5 mg/ml), followed by quick de-staining in water. The desired over expressed fusion protein-containing bands were excised, followed by electro-elution and concentration of the fusion protein in Biotrap BT1000 elution chambers (Schleicher and Schuel) in pre-chilled 1x Tris-glycine buffer (Sambrook *et al.*, 1989) at 200 V and 8°C. Eluted samples were further concentrated using Centricon YM-3 filters (Millipore) at 7500 rpm and 22°C for 2 hours. 2 ml of 1x phosphate buffered saline (PBS) was added to the sample and it was concentrated as described above. This process was repeated an additional six times to remove salts and detergent from the samples. Samples were analysed by SDS-PAGE and the protein concentration was determined using BIO-RAD protein assay reagent (**Figure 3.2A**).

3.3.7 IMMUNISATION OF RABBIT FOR ANTIBODY PRODUCTION

Immunisation of rabbit was carried out using naked bacteria as immune carriers, as described by Bellstedt *et al.* (1988), following a schedule of three sets of three injections each over the indicated time period (**Figure 3.2C**). For each set, differently sized antigens (**Figure 3.1A**) (purified fusion protein) were used to minimise the generation of antibodies against bacterial protein contaminants. All antigens used could be detected by Western blot analysis using primary polyclonal antibodies raised against Mss11p (**Figure 3.2B**).

3.3.8 WESTERN BLOT ANALYSIS

Proteins were separated by SDS-PAGE and transferred to Hybond-C nitro-cellulose membranes (Amersham Pharmacia Biotech), using a vertical wet electro-blot apparatus (BIO-RAD) following standard procedures (Sambrook *et al.*, 1989). Membranes were blocked for 1 hour in 1x PBS containing 5% bovine serum albumin (BSA) and 0.1% Tween20. For detection purposes, the ECL-system (Amersham) was used in accordance with the specifications provided by the supplier. All solutions for washing and antibody incubation contained 1x PBS and 0.1 % Tween20. The final dilutions were 1:50 000 for the primary antibodies and 1:30 000 for the secondary antibodies. Antibody incubations were ~ 1 hour in duration, and all steps for detection were performed at room temperature.

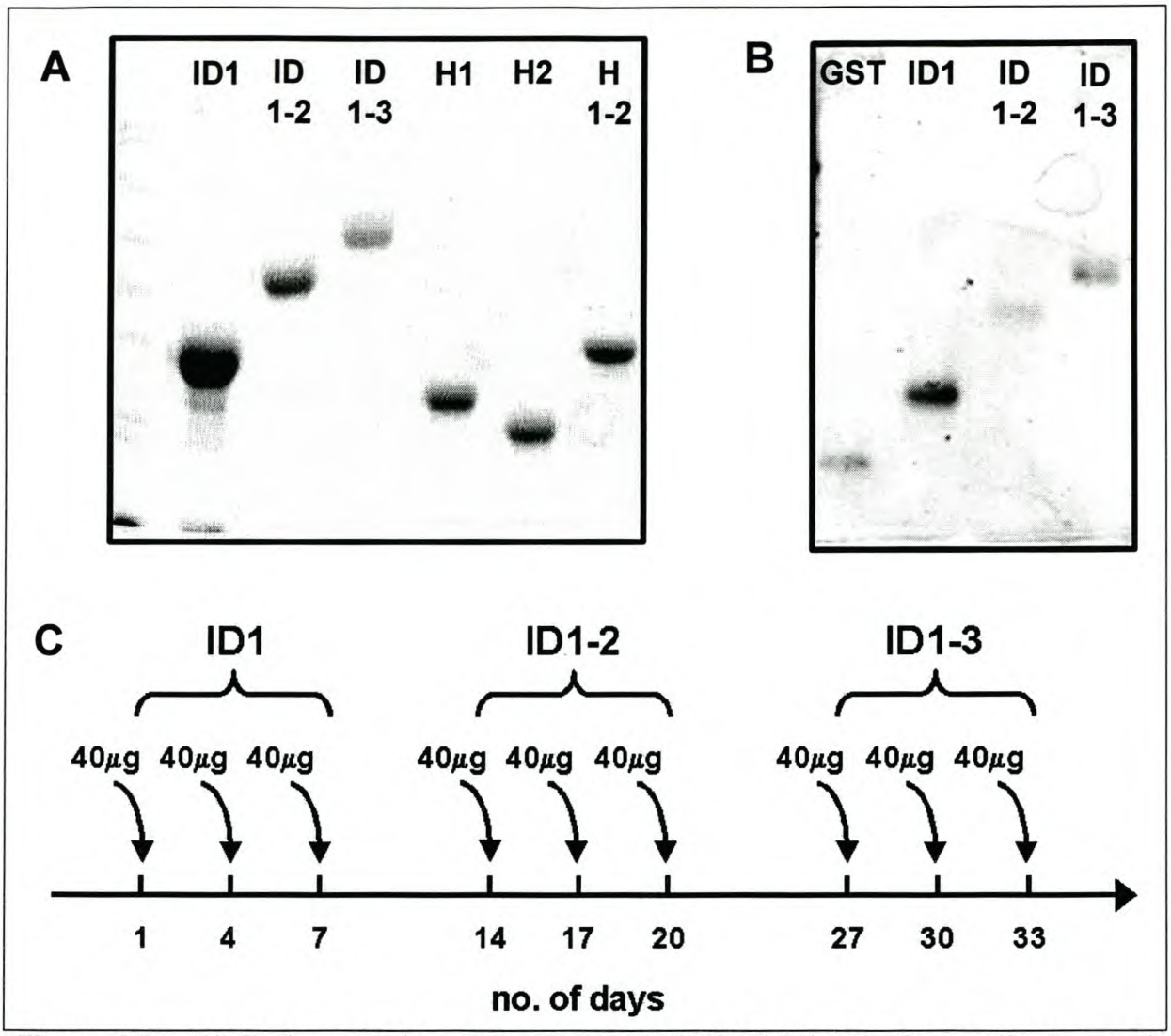

Figure 3.2 (A) Fusion proteins analysed on an SDS-PAGE gel after purification and concentration. (B) Western blot analysis of fusion proteins used for antigen and the GST tag alone after separation on SDS-PAGE, using final serum from rabbit after immunisation. (C) The immunisation schedule using different antigens for each set of injections.

3.3.9 YEAST PROTEIN PREPARATION

Yeast whole cell protein was prepared by quick centrifugation of a 1 ml yeast culture in a 1.5 ml eppendorf tube. After supernatant removal, 600 µl breaking buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA (pH 8.0)) and 300 µl glass beads were added to the cell pellet and agitated vigorously by vortexing at 8°C for 10 min. SDS was then added to a final concentration of 1.5%, after which the suspension was incubated at 100°C for 5 min. Following a 1 min. centrifugation at 12 000 rpm, the supernatant was used for further analysis.

3.3.10 FLUORESCENT MICROSCOPY

For direct fluorescent visualisation of yeast nuclei and mitochondria (**Figure 3.3A**), intracellular DNA was stained with 4',6'-diamidino-2-phenylindole dihydrochloride

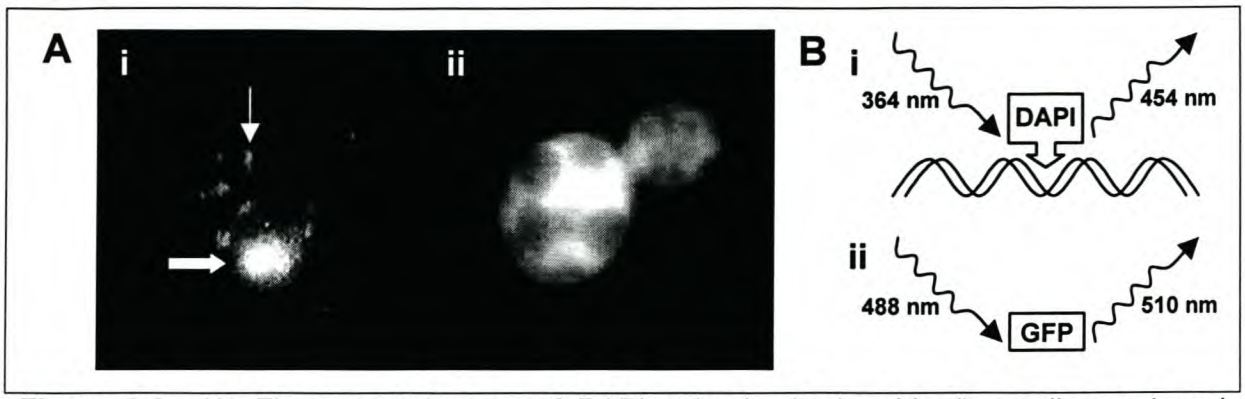

Figure 3.3 (A) Fluorescent images of DAPI-stained mitochondria (i, small arrow) and nucleus (i, big arrow), and GFP expressed from PGK_P (ii). (B) Diagram showing the excitation and emission wavelengths of DNA-bound DAPI (i) and GFP (ii).

hydrate (DAPI) (aqueous solution of 0.5 μg/ml). Yeast cells were dehydrated in an aqueous solution of 70% ethanol at room temperature for 10 min., followed by rehydration in the DAPI solution. DNA bound DAPI emits light (emission) of 454 nm when excited with 364 nm light (excitation), while GFPmut2p excitation and emission wavelengths are 488 nm and 510 nm respectively (**Figure 3.3B**). A Nikon E400 microscope with UV source and appropriate filter sets was used to visualise the fluorescence. Images were taken with either a Nikon COOLPIX 990 digital camera or a COHU CCD video camera. Scion Image for Windows was used to capture video images, and Microsoft Photo Editor ver. 3.0 was used to edit the images. The bright field images in **Figure 3.5 D** were taken using a Nikon Optiphot microscope fitted with a Nikon FDX-35 camera.

3.3.11 SEQUENCE RETRIEVAL AND ANALYSIS

Sequences from various *Saccharomyces* strains encoding for homologs/orthologs of *S. cerevisiae* genes were retrieved from a database of fully or partially sequenced genomes provided by the Saccharomyces genome database (SGD). Alignments of sequences were performed using the ClustalX program (ver. 1.81) (Thompson *et al.*, 1997). The degree of homology between sequences was scored using the following nomenclature:

"*" indicates positions that have a single, fully conserved residue
":" indicates that one of the following groups of residues is fully conserved:
STA; NEQK; NHQK; NDEQ; QHRK; MILV; MILF; HY; FYW
"." indicates that one of the following groups of residues is fully conserved:
CSA; ATV; SAG; STNK; STPA; SGND; SNDEQK; NDEQHK;
NEQHRK; FVLIM; HFY

3.4 RESULTS

3.4.1 Mss11p AFFECTS HAPLOID INVASIVE GROWTH

In order to determine the extent of haploid invasive growth in different genetic backgrounds, ISP15, S288C and ∑1278b, wild type or Δ*mss11*, were grown on solid rich media. After only four days, both ∑1278b strains showed signs of agar invasion, with clear discrepancies in the extent of agar invasion between ∑1278b wt and ∑1278bΔ*mss11* (**Figure 3.4**). This is in accordance with the previous identification of ∑1278b as the laboratory strain that readily invades agar-containing rich media (Stanhill *et al.*, 1999). When working with the ISP15 background, media containing limited amounts of nitrogen have to be used to detect discrepancies in invasive growth between strains (data not shown). For this reason, ∑1278b was chosen as genetic background to study the effect of high levels of Mss11p on invasive growth. 2μ-*MSS11* under transcriptional control of the *GAL1* promoter was transformed into ∑1278b wild type and ∑1278bΔ*mss11*. *GAL1*$_P$ is repressed when glucose is present, and is induced when galactose is provided as the sole carbon source. Transformants were spotted on SDC and SCD$_{GAL}$ plates to compare non-induced and induced conditions respectively. Induced conditions clearly result in agar invasion in both wild type and Δ*mss11* (**Figure 3.5A**), compared to the absence of agar invasion of the same transformants under non-induced conditions (**Figure 3.5B**). Not evident from the plate photos is the slight difference in agar invasion between wild type and Δ*mss11* transformed with *GAL1*$_P$-*MSS11* under induced conditions. The extent of agar invasion was found to be marginally more in the wild type than in Δ*mss11*. Although the importance of a single copy of *MSS11* for invasive growth in haploid genomes has already been shown (Gagiano *et al.*, 1999a,b and Figure 3.4), it is surprising that the influence of *MSS1* deletion is so apparent under conditions of Mss11p overproduction.

3.4.2 Mss11p INDUCES Ca^{2+}-DEPENDENT FLOCCULATION IN THE FLOCCULATION DEFICIENT S288C GENETIC BACKGROUND

The increased expression of Mss11p in the ISP15, S288C and ∑1278b backgrounds led to flocculation of the cells in liquid media, the extent of which was highly variable between strains and dependent on the availability of Ca^{2+} ions in the media (data not shown). The highest percentage of flocculation was measured in the S288C background, which is known to be deficient in its ability to flocculate, due to a point mutation creating a premature STOP codon in *FLO8* (Liu *et al.*, 1996) (**Figure 3.6 A**). The same mutation renders yeast that contains one or more of the *STA* genes unable to utilise starch as sole carbon source (Gagiano *et al.*, 1999a). In order to quantify Ca^{2+}-dependent flocculation, an assay was used that employs controlled Ca^{2+}-dependent flocculation induction after binding of all the bivalent ions by EDTA in the growth medium. Expressing *MSS11* from *PGK*$_P$ and *MSS11*$_P$ on multicopy

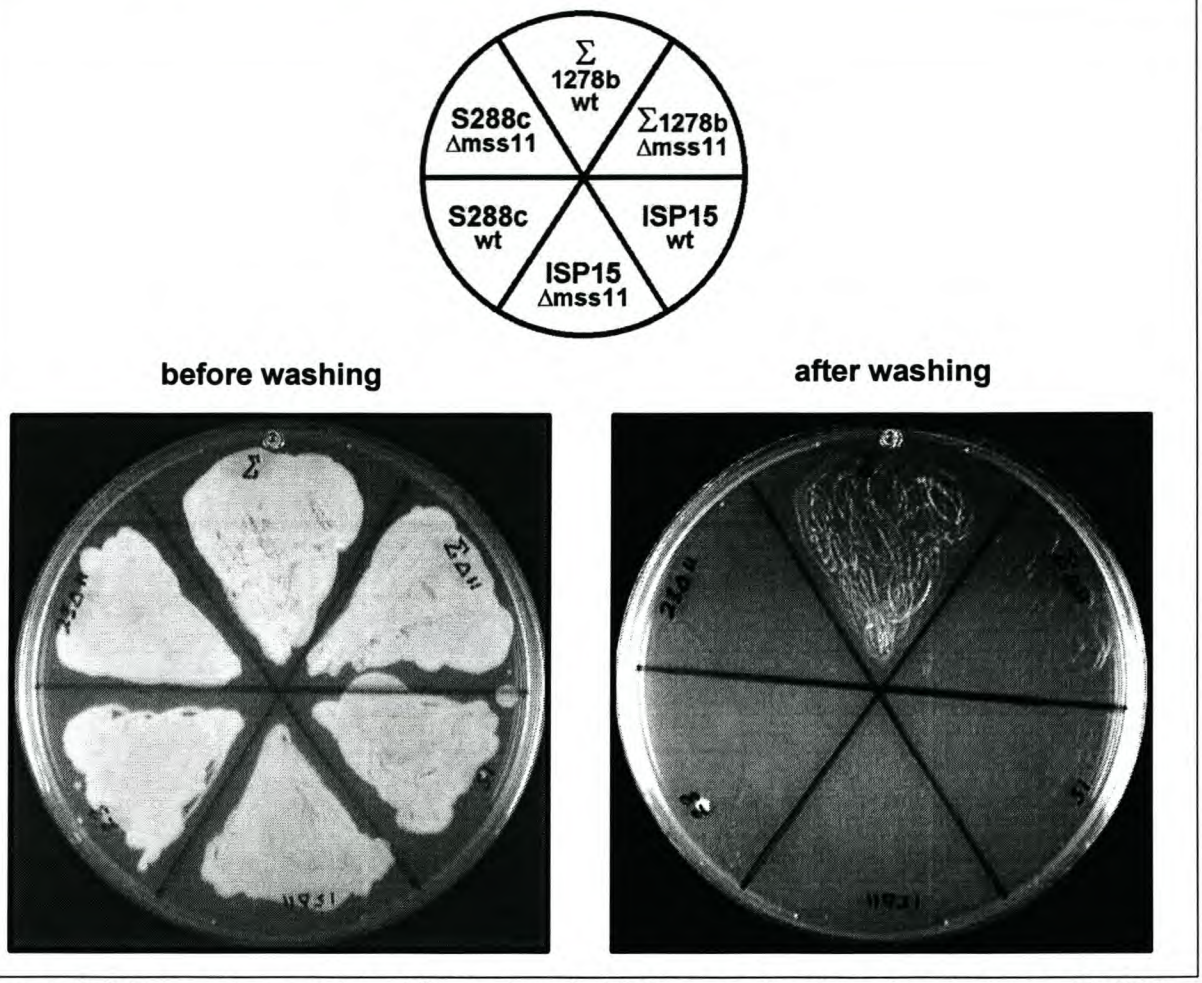

Figure 3.4 Comparison of the ability to invade YPD agar after four days between ∑1278b, S288c and ISP15 backgrounds, and the effect of deleting *MSS11* in these backgrounds. Cells were washed off after four days.

plasmids resulted in ~100% and ~60% flocculation respectively. Cells, both wild type or Δ*mss11*, transformed with vector alone did not exceed 10% flocculation on average (**Figure 3.6B**). The possibility that the detection system was saturated when measuring the percentage of flocculation in yeast constitutively overexpressing Mss11p should be noted, considering that some values are very close to 100%. Yeast cultures used for the flocculation assays were all propagated in liquid SCD medium.

3.4.3 DIFFERENTIAL EXPRESSION OF MSS11p AFFECTS YEAST GROWTH AND VIABILITY

When attempting to overproduce Mss11p from PGK_P in the ∑1278b background, it was found that wild type 2μm-PGK_P-*MSS11* transformants could never be obtained (data not shown). Transforming ∑1278bΔ*mss11* with the same construct, however, did yield transformants, as did transformation with 2μm-based $MSS11_P$-*MSS11* on both ∑1278b or ∑1278bΔ*mss11*. This suggests that strong expression of *MSS11* may be lethal in the ∑1278b background. This effect could not be detected in any other genetic background, although wild type cells of the ISP15 and S288C

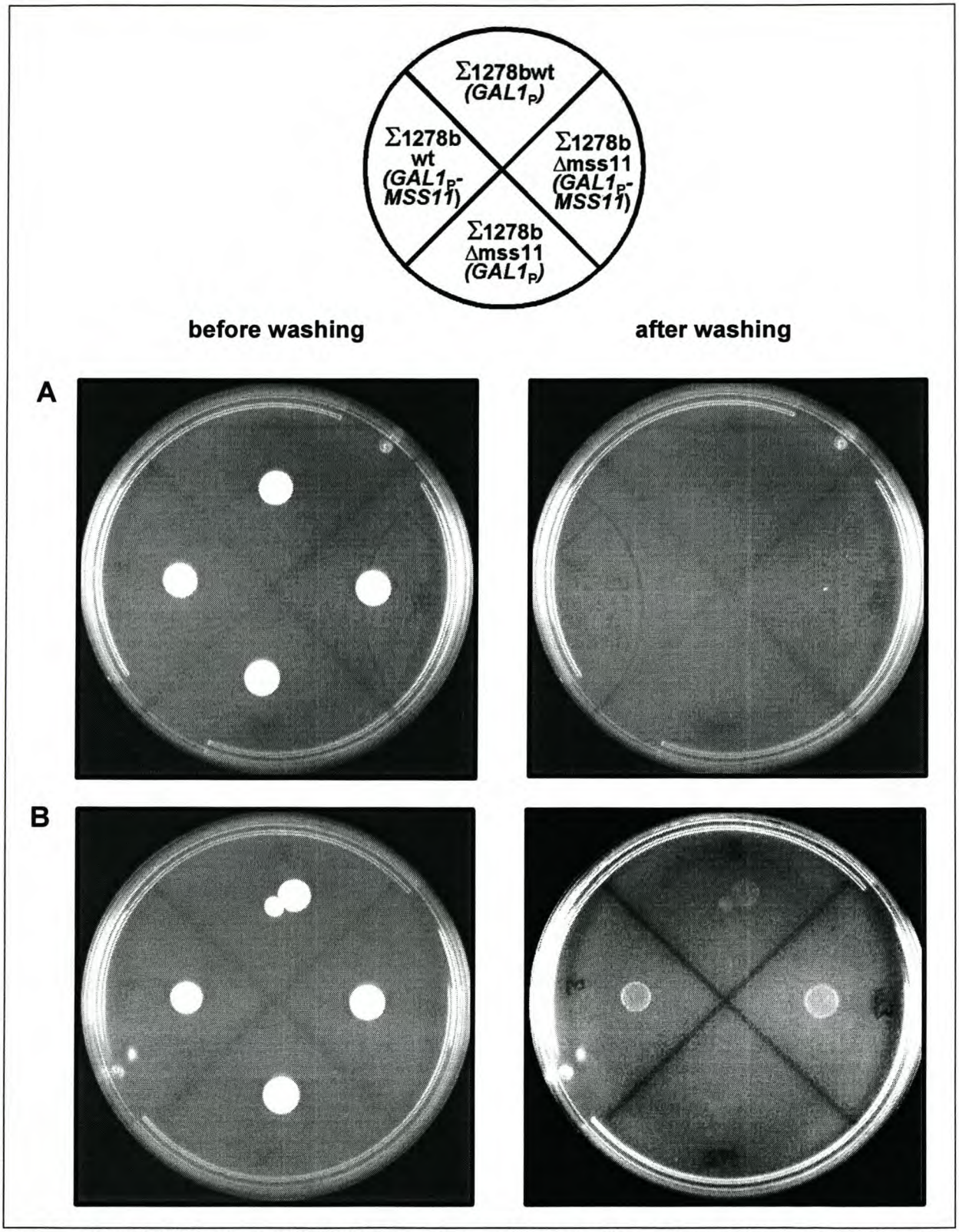

Figure 3.5 ∑1278bwt and ∑1278bΔmss11 transformed with multicopy plasmids containing *MSS11* under transcriptional control of *GAL1*$_P$, or just vector with *GAL1*$_P$. Cultures were spotted on SCD (A) or SCD$_{GAL}$ (B) agar, and washed off after four days of growth.

backgrounds carrying 2μm-*PGK*$_P$-*MSS11* displayed slower growth on plates following transformation when compared to other transformants of the same genotype (data not shown).

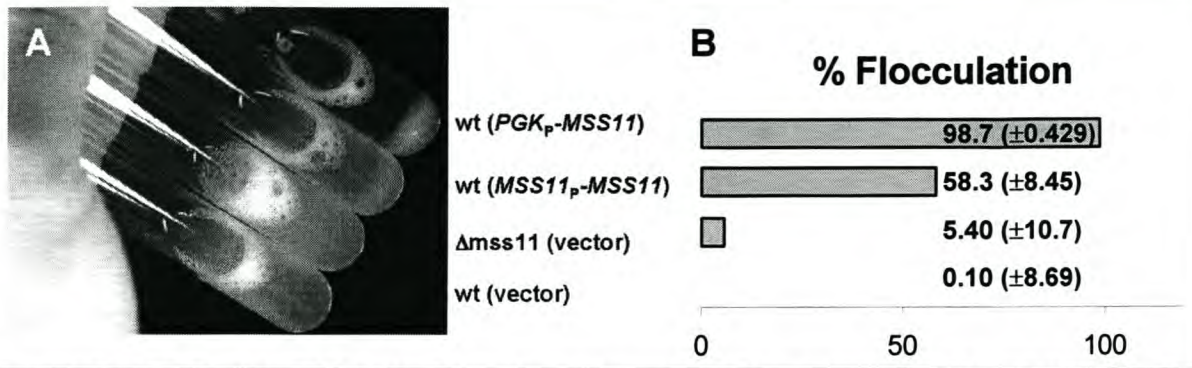

Figure 3.6 Multicopy *MSS11* under native and constitutive transcriptional control induces flocculation in the flocculation-deficient S288C genetic background when grown in SCD to stationary phase. (A) Photo taken vertically of liquid cultures at an angle of ~45°. (B) Mean Ca^{2+}-dependant % flocculation values of corresponding S288C transformants, with the amount values for average variation in brackets.

To monitor the effect of *MSS11* deletion and differential expression on population growth in liquid culture, ISP15Δ*mss11* transformed with vector, and ISP15 wild type transformed with vector, *MSS11*$_P$-*MSS11* and *PGK*$_P$-*MSS11* were inoculated in SCD (fermentable carbon source) and SCGE (non-fermentable carbon source). The mean generation time in the exponential phase for all the transformants was essentially the same, with only minor differences (**Figure 3.7C, D** and **G**). Transformants expressing Mss11p from its native promoter growing in SCD and those with the vector deleted in *MSS11* growing in SCGE displayed prolonged lag phases compared to the ISP15 wild type. The most striking effect was that of *MSS11* deletion on yeast culture density in the stationary phase (**Figure 3.7A, B** and **F**). On both fermentable and non-fermentable carbon sources, *MSS11* led to an increase in cell density. This effect was confirmed in the S288C and ∑1278b backgrounds on non-fermentable carbon sources (**Figure 3.8A, B** and **C**). The manner in which *MSS11* affects yeast growth is still unclear, but an increase in optical density of a liquid culture can almost certainly be associated with an increase in culture biomass. Thus, a deletion in *MSS11* appears to improve biomass production of yeast growing on fermentable or non-fermentable carbon sources in all the genetic backgrounds tested.

3.4.4 *MSS11* DELETION SUPPRESSES THE GROWTH DEFECT OF ∑1278b TRANSFORMED WITH THE HYPERACTIVE *RAS2*^{val19} ALLELE ON NON-FERMENTABLE CARBON SOURCES

Introduction of the hyperactive allele of *RAS2*, *RAS2*^{val19}, into yeast from different genetic backgrounds results in diminished growth, a phenotype that is more prominent in the ∑1278b background (data not shown). This might be due to the elevated intracellular cAMP levels found in the ∑1278b background (Stanhill *et al.*, 1999). Considering the effect of *MSS11* deletion on growth, and its inability to transform ∑1278b with *PGK*$_P$-*MSS11*, we set out to test whether *MSS11* function is in any way related to the growth defect introduced by RAS2^{val19}. ISP15, S288C and ∑1278b wild type and Δ*mss11* backgrounds were transformed with the hyperactive

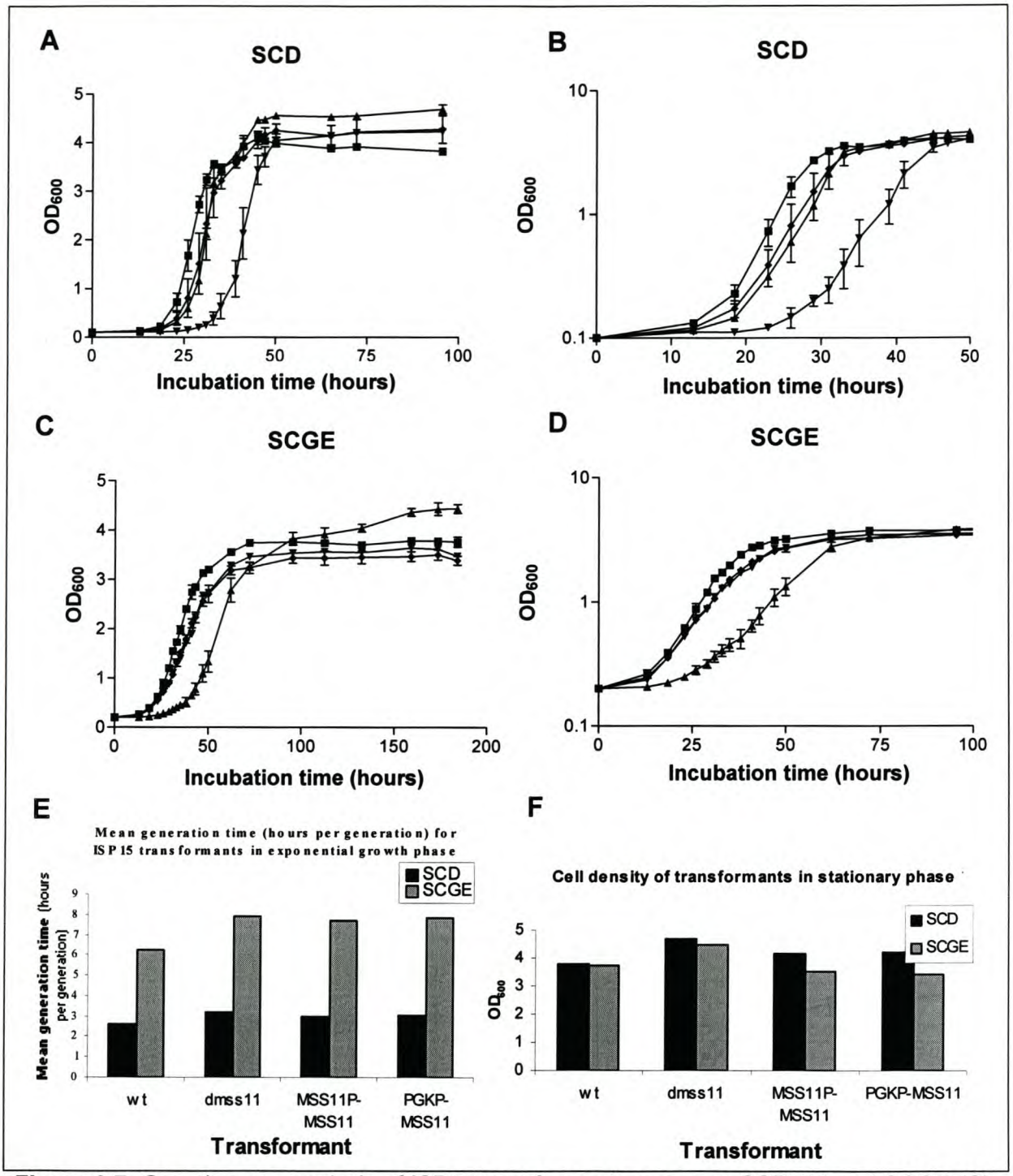

Figure 3.7 Growth curve analysis of ISP15 transformants grown on SCD (A) and SCGE (C), and the corresponding logarithmic-linear plots of these graphs (B and D) (■ - wt transformed with vector, ▲ - Δmss11 transformed with vector, ▼ - wt transformed with *MSS11*$_P$-*MSS11*, ♦ - wt transformed with *PGK*$_P$-*MSS11*). (E) Cell density of transformants after reaching the stationary phase. (F) Mean generation time of transformants in true exponential phase.

RAS2 allele or vector alone. In the ISP15 and S288C backgrounds, when comparing the difference between wild type and Δ*mss11* with the difference between wild type *RAS2*^{val19} and Δ*mss11 RAS2*^{val19}, the ratio of the difference in growth remains roughly the same, indicating that the effects of *RAS2*^{val19} on growth is not coupled to *MSS11* in these backgrounds (**Figure 3.8A** and **B**). When performing the same experiment in the ∑1278b background, Δ*mss11 RAS2*^{val19} nearly reached the optical

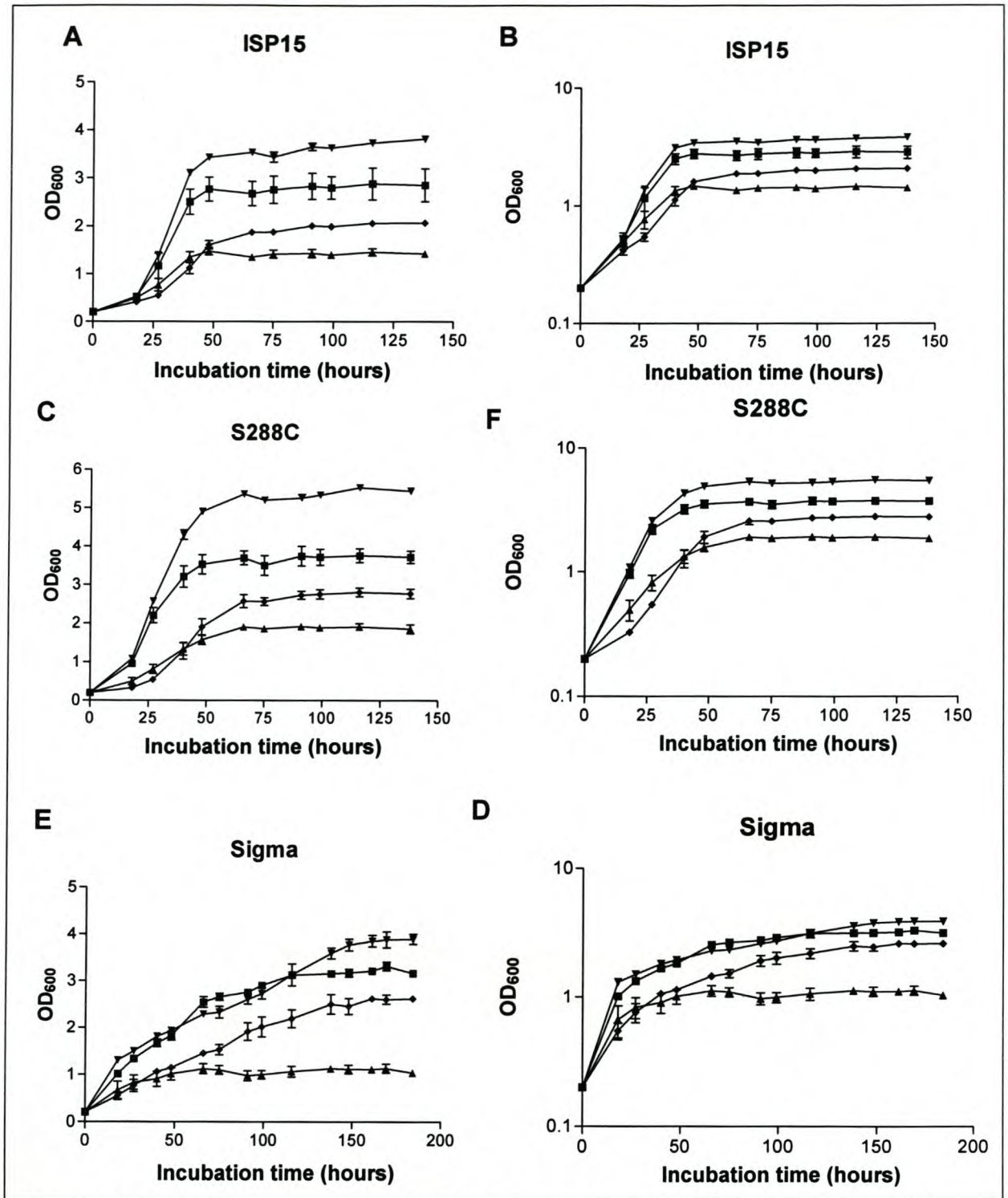

Figure 3.8 Growth curve analysis of *MSS11* deletion, and the effect of the *RAS2*^{val19} allele on ISP15, S288C and ∑1278b grown on SCGE (A, C and E), with the corresponding logarithmic-linear plots (B, D and F) (■- wt transformed with vector, ▲- wt transformed with *RAS2*^{val19}, ▼- Δ*mss11* transformed with vector, ♦- Δ*mss11* transformed with *RAS2*^{val19}).

density of the wild type strain transformed with vector alone. Wild type transformed with *RAS2*^{val19} showed a severe growth defect, remaining below an optical density of 1.0 (**Figure 3.8C**). This shows that a deletion in *MSS11* in the ∑1278b genetic background suppresses the growth defects of a hyperactive *RAS2* mutant.

3.4.5 MULTIPLE COPIES OF *UAS1*$_{STA2/MUC1}$, AND FRAGMENTS THEREOF, DO NOT AFFECT Mss11p-DEPENDENT REGULATION OF HAPLOID INVASIVE GROWTH

It has previously been shown by using a reporter system that Mss11p acts on a specific region, referred to as the UAS1 region, of the highly similar *FLO11* and *STA2* promoters (Gagiano *et al.*, 1999a). Suspecting that this might occur by direct or indirect binding, we argued that the presence of multiple copies of promoter fragments in the nucleus could block phenotypes created by Mss11p overexpression, by physically titrating out Mss11p levels. To assess this hypothesis, *GAL1*$_P$-*MSS11* present on a centromeric plasmid was co-transformed with 2 μm-based promoter fragments of complete UAS1$_{FLO11}$ or UAS1$_{STA2}$, or parts thereof. Using the shift from glucose to galactose as the induction system for Mss11p production, a clear difference in agar invasion was observed on galactose plates with *GAL1*$_P$-*MSS11* transformants when comparing them to normal vector transformants (data not shown). However, there was no observable difference in agar invasion between *GAL*$_P$-*MSS11* co-transformed with vector, and *GAL*$_P$-*MSS11* co-transformed with 2μm promoter fragments (data not shown). This experiment was repeated several times in the haploid backgrounds FY23, FY23Δ*mss11*, ISP20 and ISP20Δ*mss11*, with similar results.

3.4.6 IMMUNOLOGIAL ANALYSIS OF Mss11p

Whole cell protein extracts were prepared from yeast transformed with YEpLac112-PGK$_{PT}$-MSS11, thus overexpressing Mss1p, resolved on SDS-PAGE and transferred to a membrane. Mss11p was detected by incubating the membrane with polyclonal antibodies raised against the region corresponding to ID1, ID2 and ID3 (**Figure 3.1A**). Mss11p was found to migrate with proteins roughly 97 kDa in size (**Figure 3.9A**). This is roughly 15 kDa larger than the predicted size of 85.5 kDa. This difference could be due to Mss11p post-translational modification, or because the poly-asparagine and poly-glutamine stretches affect protein migration through SDS-PAGE.

To determine Mss11p levels in the ISP15 background, whole cell protein extracts from cells expressing *MSS11* were analysed by means of Western blot (**Figure 3.9A**). No Mss11p could be detected in the wild type, Δ*mss11*, or the cells carrying multiple copies of *MSS11*$_P$-*MSS11*. However, the antibodies detected a protein band in extracts from cells expressing *MSS11* from the *PGK* promoter, as has already been mentioned. Interestingly, extracts from Σ1278b cells expressing 2μ-*MSS11* from its native promoter contained detectable levels of Mss11p (**Figure 3.9B**).

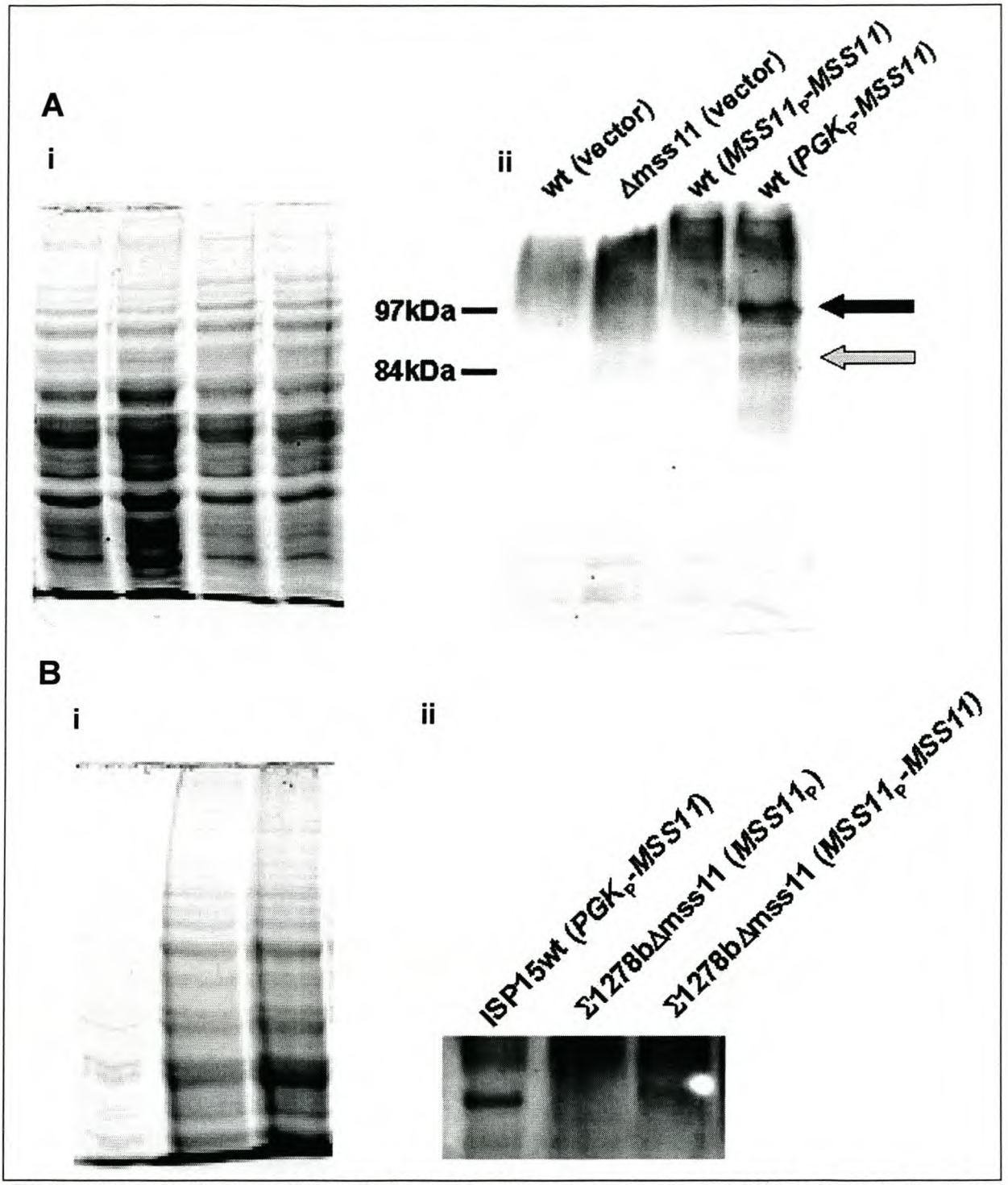

Figure 3.9 (A) Western blot to indicate Mss11p expression and peptide size (black arrow indicates detected size, and grey arrow indicates expected size) in ISP15 background (ii), with corresponding Coomassie stained SDS-PAGE gel to indicate protein quantities transferred to the membrane (i). (B) Western blot (ii) with corresponding resolved stained gel (i) to indicate slight detection of Mss11p in ∑1278b *MSS11*$_P$-*MSS11* transformants.

3.4.7 *IN VIVO* INTRACELLULAR LOCATION FOR GFP-TAGGED Flo8p, Msn1p AND Mss11p, AND CORRELATION WITH CELL PHENOTYPES

In order to determine the intracellular location of the three proteins Flo8p, Msn1p and Mss11p *in vivo*, each was C-terminally tagged with GFP. Multicopy shuttle vectors

carrying in-frame fusions of *GFP* to the 3' end of *FLO8*, *MSN1* and *MSS11,* under the transcriptional control of their respective native promoters, were introduced into haploid yeast (ISP15, FY23 (S288C) and BY4742 (S288C), carrying genomic deletions of the respective open reading frames. In order to determine if the fusion proteins were functional and behaved similarly to the *wt* protein counterparts, transformants carrying 2μm vectors with *wt* or fusion genes under native transcriptional control were compared in respect of previously defined phenotypes. Flo8p, Msn1p and Mss11p were previously found to positively regulate agar invasion and starch degradation (Gagiano *et al.*, 1999a,b, 2002; Lambrechts *et al.*, 1996a,b). Yeast expressing fusion proteins behaved similarly to cells expressing *wt* proteins as regards agar invasion, starch degradation and flocculation (data not show). As shown in **Figure 3.10A**, Flo8p-GFP, Msn1p-GFP and Mss11p-GFP localise to the nucleus, as indicated by DAPI co-staining. Under no circumstances could fluorescence be detected in other subcellular compartments other than the nucleus when expressing the fusion proteins from their native promoters. Except for Mss11p, for which the subcellular location was unknown, these results are in accordance with previously published findings. Triple myc, or GFP-tagged Flo8p under control of its native promoter and present on a multicopy vector, was shown to be nuclear (Liu *et al.*, 1996 and Pan and Heitman, 2002 respectively). Likewise, Msn1p fused to β-galactosidase under control of its native promoter on a multicopy plasmid (Estruch and Carlson, 1990), or tagged at the C-terminal by the V5 epitope (see http://medapps00.med.yale.edu/YGAC_Cellimages/pYES2/OEF24H9.jpg; Kumar *et al.*, 2002), was also previously localised to the nuclear interior. Thus, localising Flo8p and Msn1p to the nucleus serves as an internal control for the method employed to localise proteins, and localising tagged proteins expressed from their native promoter on multicopy plasmid seems to be a true reflection of their respective intracellular location. It is thus proposed that the Mss11p protein is imported into the nucleus to perform its function.

Figure 3.10B shows populations of the same transformants used in **Figure 3.10A** to indicate the number of cells in a given population of yeast cells that exhibits nuclear fluorescence. All transformants expressing Msn1p-GFP were found to display nuclear fluorescence, indicating that the fusion protein was present in the nucleus in all the cells at a relatively high concentration. Nuclear fluorescence was observed throughout exponential growth to early stationary phase (data not shown). In contrast, very few cells expressing Flo8p-GFP or Mss11p-GFP displayed nuclear fluorescence, with *FLO8-GFP* transformants having a lower fluorescence frequency than *MSS11-GFP* transformants. The less frequent fluorescent cells were mostly associated with cells that flocculate or that have undergone morphological change. Flo8p-GFP and Mss11p-GFP fluorescence were absent in stationary phase cells, but peaked in frequency in cells that had reached late logarithmic growth phase (**Figure 3.11A**). Whether *FLO8-GFP* induction can be correlated with the induction

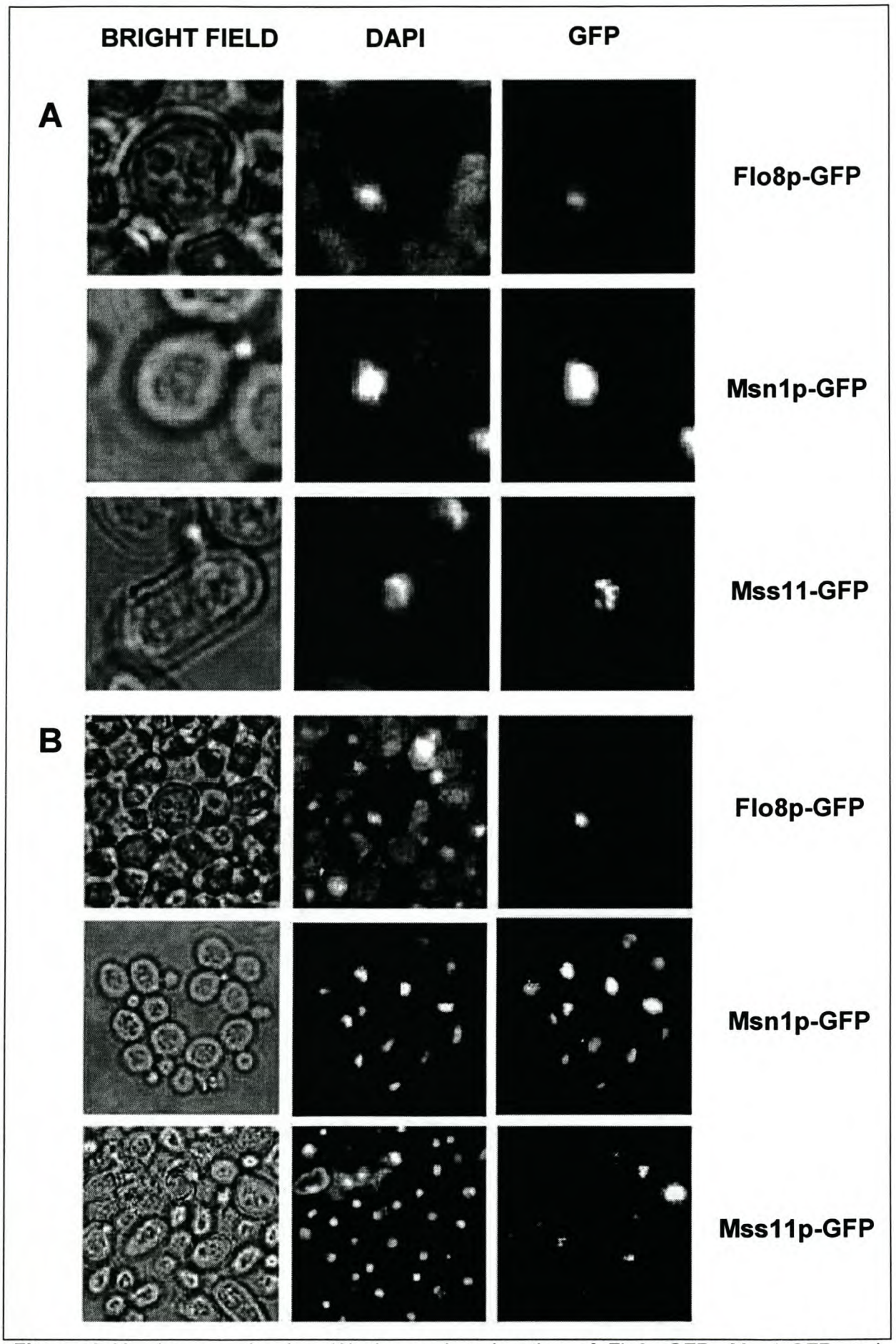

Figure 3.10 Images showing (A) the nuclear location of Flo8p-GFP, Msn1-GFP and Mss11p-GFP, and (B) the proportion of cells displaying fluorescence.

and maintenance of flocculation, or possibly with a cellular response to some form of nutrient deprivation inside flocculation clumps, is not clear.

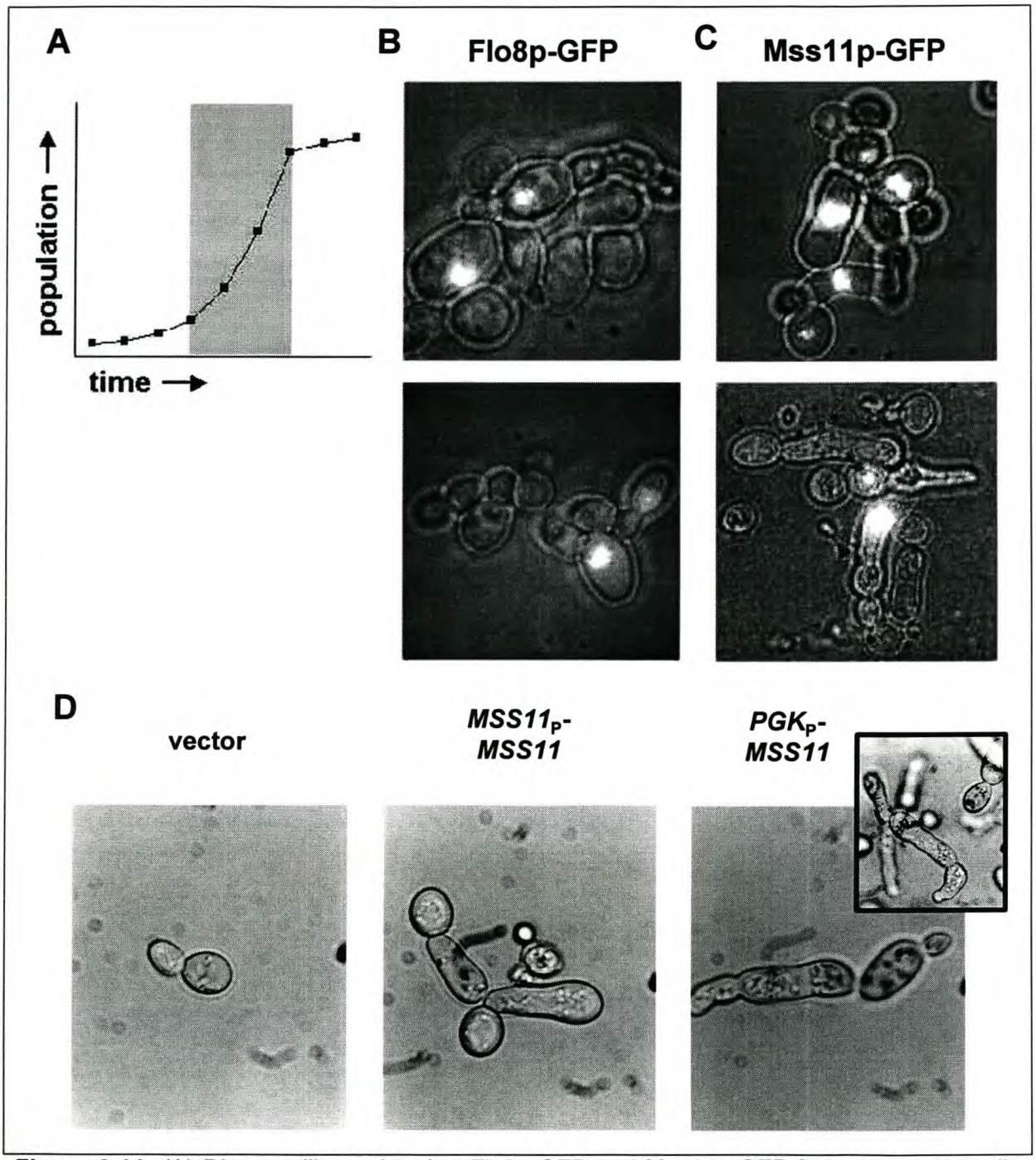

Figure 3.11 (A) Diagram illustrating that Flo8p-GFP and Mss11p-GFP fluorescence in cells in liquid culture was observed in late exponential phase only. Cells photographed with GFP filter sets and moderate normal light illumination show that cells displaying fluorescence expressing *FLO8-GFP* (B) and *MSS11-GFP* (C) are associated with enlarged or elongated cells. (D) Investigation of the effect of differential *MSS11* expression on cell morphology in liquid culture. Insert shows cells that have physically grown into the solid agar media.

MSN1-GFP transformants resemble *wt* cells (also referred to as the "yeast form") and display no abnormal growth characteristics when grown in liquid or on solid media. Certain cells expressing Flo8p-GFP and Mss11p-GFP, however, displayed

changed cell morphology of an enlarged and/or elongated form. In transformed populations expressing Mss11p-GFP, most of the fluorescent cells could be associated with the non-"yeast form" morphology (**Figure 3.11C**). This, however, was not the case for transformed populations expressing Flo8p-GFP, in which case only certain fluorescent cells displayed an increase in cell size (**Figure 3.11B**).

Cell elongation due to higher expression of *MSS11* was further investigated by putting *MSS11* under the transcriptional control of the strong constitutive *PGK* promoter. Expression from both the native and *PGK* promoters resulted in marked cell elongation of haploid yeast when compared to wild type (**Figure 3.11D**), with clear induction of agar invasion in the case of yeast transformed with PGK_P-*MSS11* (**Figure 3.11D** insert). It is important to note the unipolar budding pattern, which is typical of cells that undergo "hyphal" growth, of the cells expressing *MSS11* from $MSS11_P$ and PGK_P. Clearly, the invading cells depicted in **Figure 3.11D** resemble cells in the transition to pseudohyphal development.

Expressed from their native promoters, GFP-tagged Flo8p and Msn1p localised to the entire interior of the nucleus (**Figure 3.10A**) in a seemingly homogeneous, dispersed fashion. Mss11p-GFP, however, localised in distinct condensed subnuclear areas, varying in number between two to twelve per nucleus (**Figure 3.10A** and **Figure 3.12B**). In all cells in which fluorescence could be detected, this localisation pattern was observed when Mss11p-GFP was expressed from its native promoter.

Contrary to the fluorescent data obtained with Mss11p-GFP expressed from $MSS11_P$, the same fusion protein overexpressed from the strong constitutive *PGK* promoter did not result in the same subnuclear fluorescent "spots" observed previously. GFP fluorescence was detected in most of the cells, except the cells in stationary phase. Fluorescence could be detected throughout the whole cell (**Figure 3.12A**), much like GFP expressed on its own from PGK_P (**Figure 3.3A**).

To screen for possible nuclear import and/or export receptors of Flo8p, Msn1p and Mss11p, haploid yeast deleted in transporters, or genes involved in transport, were screened for the mis-localisation of GFP-tagged proteins under native transcriptional control. The transporters screened included *KAP114, KAP120, KAP122, KAP123, LOS1, MOG1, MSN5*, *NUP53* and *SXM1.* Yeast deleted in *MOG1* generally displayed less nuclear fluorescence of GFP-tagged Msn1p, with a few cells displaying abnormally high nuclear fluorescence (data not shown). Mog1p does not belong to the import receptor family (Strom and Weis, 2001), but is required for efficient nuclear import and interacts with Ran (Baker *et al.*, 2001; Oki and Nishimoto, 1998; see Chapter 2). Cells deleted in *KAP123* displayed very low occurrence of nuclear GFP-tagged Mss11p fluorescence. Previously, *KAP123* was shown to function in the nuclear import of histones H3 and H4 (Mosammaparast *et al.*, 2002), as well as in the import of certain ribosomal proteins prior to ribosomal assembly in the nucleus (Rout *et al.*, 1997). It appears that Mss11p, in the absence of Kap123p, is not able to efficiently enter the nucleus (data not shown). All mis-localisations were

compared to the localisation pattern in *wt*.

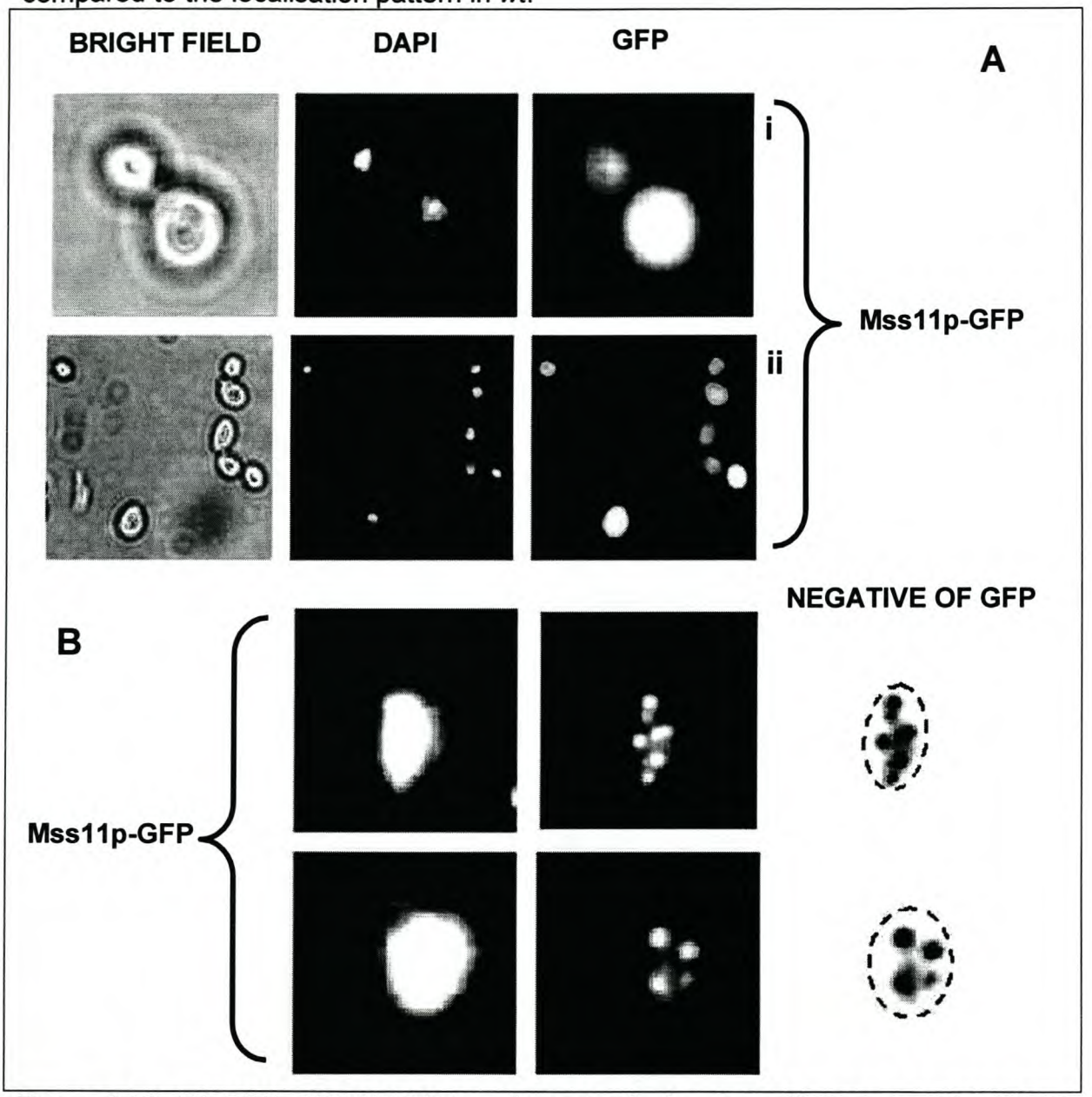

Figure 3.12 (A) *PGK*$_P$*-MSS11-GFP* transformants display strong fluorescence throughout the cell (i) in al the cells of an active growing population (ii). (B) Distinct intranuclear fluorescence in the form of two to twelve "spots" in *MSS11*$_P$*-MSS11-GFP* transformants. For better visualisation the negative image of the subnuclear fluorescence is shown in context of the approximate location of the nuclear envelope.

3.4.8 PROTEIN SEQUENCE COMPARISON BETWEEN Mss11p ORTHOLOGS FROM FOUR *SACCHAROMYCES* STRAINS OF THE *SENSU STRICTO* GROUP

Recently, genome sequence information on *Saccharomyces* species belonging to the *sensu stricto* group (**Figure 3.13**) was made available through the Saccharomyces Genome Database (SGD). Although this information was generated by the "shotgun" technique, and thus is liable to contain many errors and missing data (Palmer and McCombie, 2002), it still proved useful to determine the extent of homology of the predicted Mss11p between these species. Alignment of the

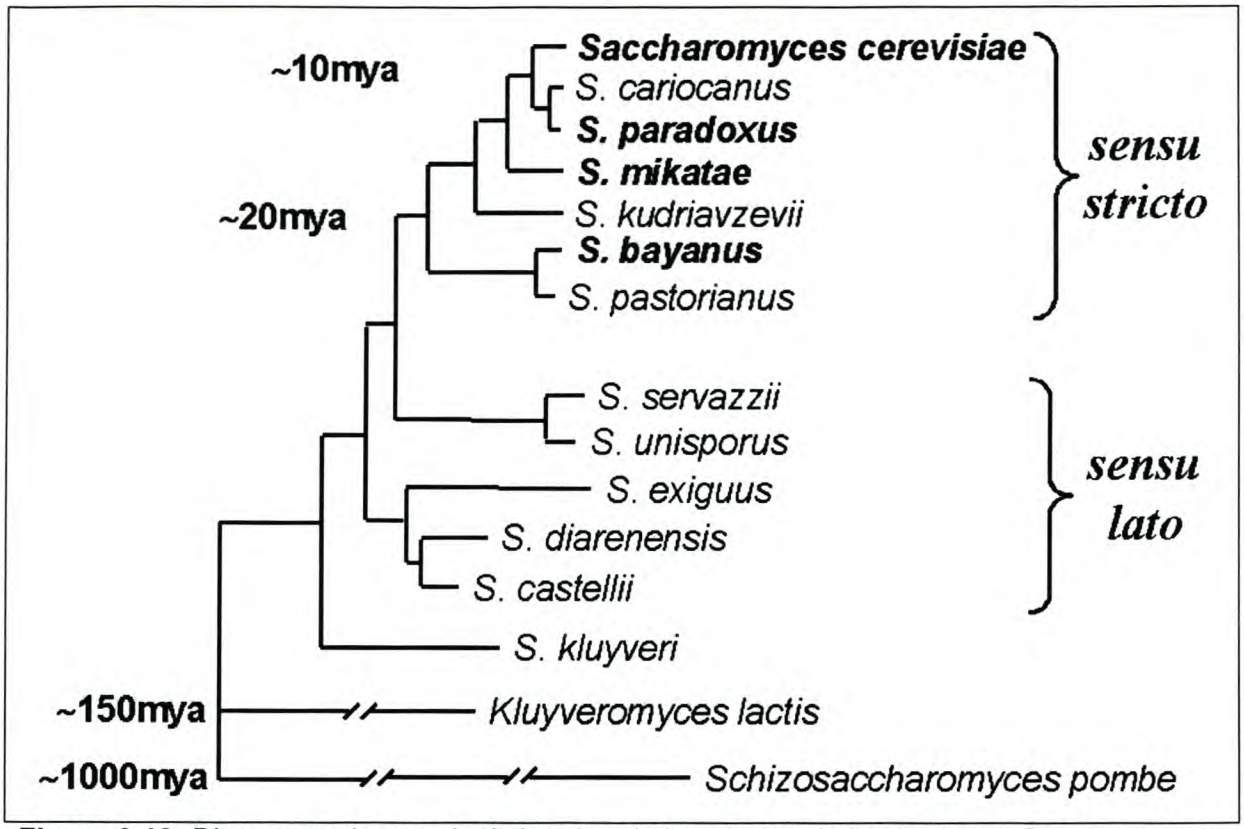

Figure 3.13 Diagrammatic tree depicting the phylogenetic relation between *Saccharomyces* yeast species. Species from which sequence information was used for Mss11p alignment analysis is shown in bold.

translation of *MSS11* orthologs found in *Saccharomyces bayanus*, *S. mikatae* and *S. paradoxus* shows that, apart from occasional amino acid substitutions and the poly-asparagine and poly-glutamine domains, great homology exists between these sequences (**Figure 3.14**). Although much sequence information is still lacking, it does seem clear that the poly-asparagine and poly-glutamine domains are the most variable parts. *S. cerevisiae* Mss11p is the only sequence with an extended poly-asparagine domain, containing 33 mostly uninterrupted asparagine residues, compared to the three to four residues in other sequences. Although the sequence for the poly-glutamine domain is missing, or does not exist, in the *S. mikatae* and *S. bayanus* sequences, it is very surprising to find that the already exceptionally long stretch of 47 glutamine residues in *S. cerevisiae* is extended to 127 glutamine residues in *S. paradoxus*. When comparing the Mss11p sequence from different *S. cerevisiae* laboratory strains, it was found that the poly-asparagine and poly-glutamine domains were of the same size than those derived from the first sequenced S288C strain (Gagiano *et al.*, 2002). The function of long stretches of these amino acids is still unclear, since they appear to perform no specific role in transcriptional activation/regulation in *S. cerevisiae* (Gagiano *et al.*, 2002). It is likely that these amino acid stretches function in protein-protein interaction, although the extent of variability between *sensu stricto* strains regarding these stretches makes this possibility less likely. Clearly, the poly-asparagine and poly-glutamine domains are more susceptible to sequence variability than "normal" heterogeneous and less

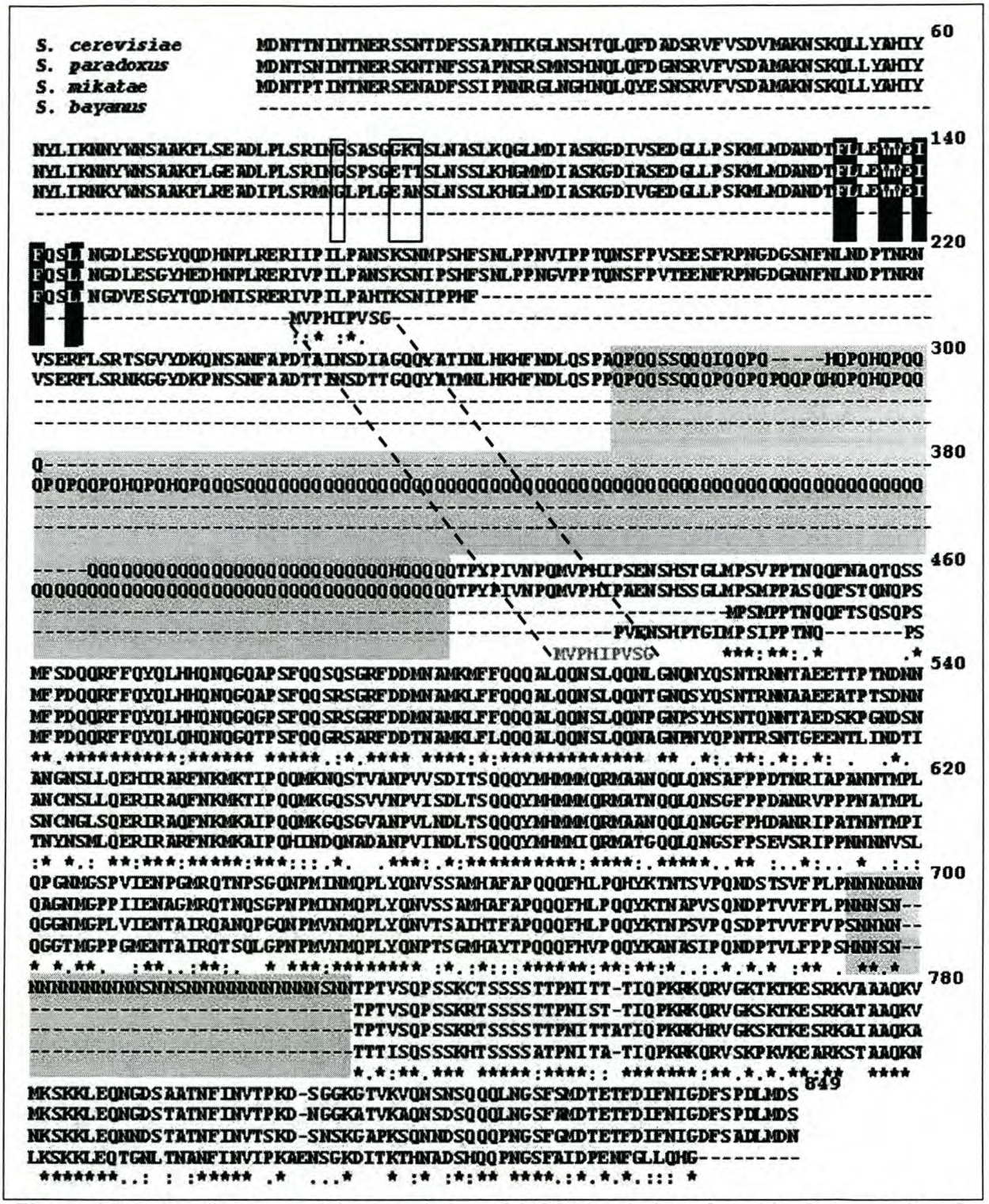

Figure 3.14 An alignment of the translation of *MSS11* orthologs found in *S. cerevisiae*, *S. bayanus*, *S. mikatae* and *S. paradoxus*. Homology is indicated as described in Materials and Methods. Shaded areas indicate the poly-asparagine and poly-glutamine domains, the putative P-loop region is outlined and the conserved amino acid residues in H2 in white with black background. A part of the *S. bayanus* sequence believed to align better behind the poly-glutamine domain is indicated in grey with dashed lines.

repetitive sequences when comparing orthologs of closely related species. This can also be observed when aligning *FLO8* and *MSN1* orthologs: relatively few amino acid changes are observed, except for the variability in length of the poly-glutamine domain found near the N-terminus of Flo8p (**Figure J,K**). The residues identified to

be similar between Flo8p and Mss11p, which had been shown to be of importance for Mss11p activation function (Gagiano *et al.*, 2002), as well as the surrounding amino acids, are highly conserved between *sensu stricto* strains. The same, however, cannot be said for the previously identified putative P-loop (Gagiano *et al.*, 2002), consisting of Gly98-...Gly103-Lys104. Gly98 is conserved, but not Gly103 and Lys104. In Mss1p, it was shown that the putative P-loop performs no function under the conditions tested (Gagiano *et al.*, 2002). Thus, the extent of homology between the evolutionarily close *sensu stricto* strains is a possible tool for identifying important residues/motifs.

3.5 DISCUSSION

3.5.1 *MSS11* EFFECTS ON YEAST PHYSIOLOGY

3.5.1.1 The effect of Mss11p is concentration dependent

In this study, we identified Mss11p as an important regulator of invasive growth, pseudohyphal development, cell morphology and flocculation. Previous work relied on *MSS11* under the transcriptional regulation of its native promoter and being present in multiple copies in the cell to study the effect of "high" levels of Mss11p (Gagiano *et al.*, 1999a,b; Webber *et al.*, 1997). By overexpressing *MSS11* from the strong *GAL1* and *PGK* promoters, we observed greater influences on yeast physiology than in the above-mentioned native expression system. From a comparison of the physiological data from yeast expressing *MSS11* from strong or native promoters with yeast deleted in *MSS11,* it is evident that the magnitude of the physiological effect is directly related to Mss11p levels. We thus conclude that Mss11p regulates processes in a concentration-dependent manner.

3.5.1.2 *MSS11* affects flocculation

Previously, it was reported that a mutation or deletion of *FLO8* abolished any flocculent behaviour (Liu *et al.*, 1996). We provide data suggesting that Mss11p can suppress the inability of yeast from the S288C background, containing a non-functional copy of *FLO8*, to flocculate. Whether the role of *MSS11* in the regulation of flocculation is central in epistatic relation to other factors, as is the case with the regulation of invasive growth and starch degradation (Gagiano *et al.*, 1999a,b), must still be investigated.

3.5.1.3 Deletion of *MSS11* leads to improved biomass formation

In all genetic backgrounds, we observed an increase in the ability of yeast deleted for *MSS11* to form biomass on both fermentable and non-fermentable carbon sources when compared to the wild type. Higher biomass at the end of the logarithmic growth phase was not the result of an increased rate of exponential growth, but rather of an

extension of growth before entering the stationary phase. Culture growth was measured by means of optical density. Additional experiments thus will focus on the relationship between an increase in biomass and possibilities such as higher cell count or altered cell morphology. Previously, *MSS11* was identified in an overexpression screen to regulate the cell cycle (Stevenson *et al.*, 2001). *MSS11* therefore could influence biomass formation by regulating the rate at which new cells are formed.

3.5.1.4 Deletion of *MSS11* suppresses the growth defect of *ras2*^{VAL19}

Yeast carrying the hyperactive allele of *RAS2*, *RAS2*^{val19}, is unable to grow efficiently on non-fermentable carbon sources. In the ISP15 and S288C backgrounds, a deletion in *MSS11* could not suppress this growth defect, at least not to a greater effect than had been observed in relation to improved biomass formation following *MSS11* deletion. In the ∑1278b background, however, this growth defect was clearly suppressed by a deletion in *MSS11*. ∑1278b is reported to have an overactive Ras2p-sigalling pathway, thus possibly resulting in a hyperactive cAMP-PKA signalling pathway (Stanhill *et al.*, 1999). With even more Ras2p activity provided by the *RAS2*^{val19} allele, yeast therefore is not able to grow efficiently on non-fermentable carbon sources. It is most likely that this hyperactive nature of the *RAS2* signalling pathway enabled us to observe the phenotypical suppression of this growth defect, providing evidence for a direct genetic link between *MSS11* and cAMP signalling. In further support of this relationship is the inability to obtain ∑1278b transformants containing 2μ-*MSS11* under transcriptional control of the *PGK* promoter. It appears that a high dosage of Mss11p, in combination with high cAMP levels, renders yeast unable to grow, although *GAL1*$_P$-*MSS11* transformants were able to grow under induced conditions. Promoter differences, or the stress experienced during yeast transformation, could explain this difference in viability.

3.5.2 MSS11p SIZE, INTRACELLULAR LOCATION, ABUNDANCE AND PROMOTER BINDING

We identified Mss11p to be of an apparent size of 97 kDa when separated on an SDS-PAGE gel. With the antibody used, Mss11p levels are generally below detection levels, except when overproduced. Thus Mss11p appears to be maintained in low levels in yeast. We further identified the *in vivo* location of Mss11p to be nuclear, but experienced the same detection problems when trying to detect GFP-tagged Mss11p expressed from single copy plasmids, thus confirming low expression levels of *MSS11*. Previous attempts in our laboratory to detect *MSS11* mRNA levels were unsuccessful.

Unlike nuclear proteins in general, which are found throughout the nuclear interior, GFP-tagged Mss11p was found to localise to distinct spots inside the nucleus. Whether this is due to intranuclear aggregation or specific location is unclear. It is unlikely that this specific localisation pattern is due to GFP, because the

expression of GFP-tagged Mss11p in yeast had the same physiological effects on invasive growth, flocculation and starch degradation when compared to the expression of un-tagged Mss11p. When GFP-tagged Mss11p was overproduced, the fusion protein was detected throughout the cell. This difference in fluorescent patterns could be due to the following: (1) Under native control *MSS11-GFP* may be strongly induced under specific conditions, or, cytoplasmically accumulated Mss11p-GFP, which, at low concentration, might be undetectable by the methods used, could be imported and maintained in the nucleus. Under constitutive control, high levels of Mss11p-GFP are clearly detectable, although not specifically in the nucleus. In this case it is likely that the nuclear transport machinery could be "overpowered" by such a high amount of protein, giving the false impression that Mss11p-GFP is maintained in the cytoplasm. (2) Mss11p-GFP nuclear import could be generally prohibited, so that nuclear accumulation is induced only in certain cells under certain conditions. (3) High levels of Mss11p-GFP could continuously "shuttle" in and out of the nucleus at a steady-state level, so as to create the impression that nuclear accumulation does not occur.

Expressing *MSS11* in the presence of promoter fragments present in multiple copies did not diminish the effect of *MSS11* expression on flocculation and invasive growth. This included using small promoter fragments that had been shown to be essential for Mss11p transcriptional regulation. Thus, the possibility exists that Mss11p does not confer transcriptional regulation through direct promoter binding. Previous attempts using whole cell soluble protein extracts from yeast could not detect any promoter binding by Mss11p using gel retardation assays. Considering the amount of physiological processes that *MSS11* has been implicated in (invasive growth, pseudohyphal development, flocculation, starch degradation, cell cycle control, biomass formation and suppression of the $RAS2^{val19}$ growth defect), Mss11p possibly could act as a mediator in the transcriptional control of various genes.

3.5.3 Mss11p ORTHOLOG VARIABILITY BETWEEN *sensu stricto* STRAINS AND THE IMPORTANCE OF POLY-GLUTAMINE TRACTS

Mss11p displayed variability in the poly-asparagine and poly-glutamine domains between orthologs from *Saccharomyces sensu stricto* strains. The same can be seen in a short poly-glutamine stretch present in *FLO8*. Furthermore, the amino acid sequences in Mss11p are of exceptional length when compared to similar repeats in other eukaryotes (Karlin *et al.*, 2002). The expansion of glutamine repeats is commonly found in diseases such as Huntington's disease (Chen *et al.*, 2002; for a review see Zogbi and Orr, 1999), and often causes aggregation of proteins in the nucleus. This might explain the subnuclear location of fluorescently-tagged Mss11p. Poly-glutamine aggregation is dependent on the length of poly-glutamine, its composition and the presence of chaperone proteins (Krobitsch and Lindquist, 2000). Poly-glutamine expansion may occur at the transcriptional level. Using yeast as an expression system, it was shown that the transcription of CAG repeat-containing

DNA resulted in mRNA that was several kilobases longer than expected (Fabre *et al.*, 2002). The authors propose that exceptionally long mRNAs are formed by transcriptional slippage of the transcription machinery. Furthermore, yeast expressing poly-glutamine was shown to have the same transcriptional repression profiles as yeast carrying deletions in components of the histone acetylase complex, Spt/Ada/Gcn5 acetyltransferase (SAGA). Poly-glutamine peptides had to be targeted to the nucleus to display this effect and proved to be toxic to cells deleted in *SPT3*. Single deletions in this SAGA component were found to be otherwise viable (Hughes *et al.*, 2001). The toxicity of Mss11p overproduction might thus be due to the high amount of poly-glutamine present in the nucleus.

Interestingly, all attempts failed to heterologously express full-length Mss11p in *E. coli*, using various bacterial expression systems. However, when *GST*-fused fragments of *MSS11* was put into the expression system, high amounts of heterologous protein were obtained. We thus suspect that the expression of full length Mss11p might be toxic to bacterial cells. This is supported by the observation that the expression of poly-glutamine-containing proteins is toxic to *E. coli* cells (Onodera *et al.*, 1996).

The poly-asparagine and poly-glutamine domains present in Mss11p are not able to induce transcription of a reporter gene when fused to the Gal4p DNA-binding domain (Gagiano *et al.*, 2002). It therefore would make sense that these domains are involved in protein-protein interaction. Indeed, it has been shown that poly-glutamine binds sequences that are rich in polar amino acid residues (Imafuku *et al.*, 1998). Overexpression of poly-glutamine-containing proteins thus could titrate out factors in the nucleus by physical binding, thereby affecting multiple processes and signalling pathways.

3.6 LITERATURE CITED

Ausubel, F. M., Brent, R. Kingston, R. E., Moore, D. D., Seidman, J. A. and Struhl, K. (1994). Current Protocols in Molecular Biology, John Wiley & Sons, New York, N. Y.

Baker, R. P., Harreman, M. T., Eccleston, J. F., Corbett, A. H. and Stewart, M. (2001). Interaction between Ran and Mog1 is required for efficient nuclear protein import. *Journal of Biological Chemistry* **276**, 41255-41262.

Bauer, F. F. and Pretorius, I. S. (2001). Pseudohyphal and invasive growth in *Saccharomyces cerevisiae*. *Focus on Biotechnology – Applied Microbiology* **2**, 109-133.

Bellstedt, D. U., Van der Merwe, K. J. and Galanos, C. (1988). Immune carrier properties of acid-treated *Salmonella minnesota* R595 bacteria. The immune response to TNP-bacterial conjugates in rabbits and mice. *Journal of Immunological Methods* **108,** 245-254.

Chandarlapaty, S. and Errede, B. (1998). Ash1, a daughter cell-specific protein, is required for pseudohyphal growth of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **18,** 2884-2891.

Chen, S., Ferrone, F. A. and Wetzel, R. (2002). Huntington's disease age-of-onset linked to poly-glutamine aggregation nucleation. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 11884-11889.

Cormack, B. P., Valdivia, R. H. and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33-38.

Crous, J. M., Pretorius, I. S. and Van Zyl, W. H. (1995). Cloning and expression of an *Aspergillus kawachii* endo-1,4-beta-xylanase gene in *Saccharomyces cerevisiae*. *Current Genetics* **28,** 467-473.

Edgington, N. P., Blacketer, M. J., Bierwagen, T. A. and Myers, A. M. (1999). Control of *Saccharomyces cerevisiae* filamentous growth by cyclin-dependent kinase Cdc28. *Molecular and Cellular Biology* **19,** 1369-1380.

Eide, D. and Guarente, L. (1992). Increased dosage of a transcriptional activator gene enhances iron-limited growth of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **138,** 347-354.

Estruch, F. and Carlson, M. (1990). Increased dosage of the MSN1 gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase. *Nucleic Acids Research* **18,** 6959-6964.

Fabre, E., Dujon, B. and Richard, G.-F. (2002). Transcription and nuclear transport of CAG/CTG trinucleotide repeats in yeast. *Nucleic Acids Research* **30**, 3540-3547.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999a). Divergent regulation of the evolutionarily closely related promoters of the *Saccharomyces cerevisiae* STA2 and MUC1 genes. *Journal of Bacteriology* **181,** 6497-6508.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999b). Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Molecular Microbiology* **31,** 103-116.

Gagiano, M., Bester, M. C., Van Dyk, D., Franken, C. J., Bauer, F. F. and Pretorius, I. S. (2002). Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability. *Molecular Microbiology* **47**, 119-134.

Gancedo, J. M. (2001). Control of pseudohyphae formation in Saccharomyces cerevisiae. *FEMS Microbiology Reviews* **25,** 107-123.

Gietz, R. D. and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenised yeast genes lacking six-base pair restriction sites. *Gene* **74,** 527-534.

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. and Fink, G. R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and *RAS*. *Cell* **68**, 1077-1090.

Gimeno, C. J. and Fink, G. R. (1994). Induction of pseudohyphal growth by overexpression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Molecular and Cellular Biology* **14,** 2100-2112.

Guo, B., Styles, C. A., Feng, Q. and Fink, G. R. (2000). A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 12158-163.

Heim, R. and Tsien, R. Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Current Biology* **6,** 178-182.

Hughes, R. E., Lo, R. S., Davis, C., Strand, A. D., Neal, C. L., Olson, J. M. and Fields, S. (2001). Altered transcription in yeast expressing expanded polyglutamine. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 13201-13206.

Imafuku, I., Waragai, M., Takeuchi, S., Kanazawa, I., Kawabata, M., Mouradian, M. M. and Okazawa, H. (1998). Polar amino acid-rich sequences bind to poly-glutamine tracts. *Biochemical and Biophysical Research Communications* **253**, 16-20.

Karlin, S., Brocchieri, L., Bergmen, A., Mrázek, J. and Gentles, A. J. (2002). Amino acid runs in eukaryotic proteomes and disease associations. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 333-338.

Kobayashi, O., Suda, H., Ohtani, T. and Sone, H. (1996). Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Molecular and General Genetics* **251**, 707-715.

Kobayashi, O., Yishimoto, H. and Sone, H. (1999). Analysis of the genes activated by the *FLO8* gene in *Saccharomyces cerevisiae*. *Current Genetics* **36**, 256-261.

Krobitsch, S. and Lindquist, S. (2000). Aggregation of huntington in yeast varies with the length of the poly-glutamine expansion and the expression of chaperone proteins. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 1589-1594.

Kron, S. J., Styles, C. A. and Fink, G. R. (1994). Symmetric cell division in pseudohyphae of the yeast *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **5**, 1003-1022.

Kumar, A., Agarwal, S., Heyman, J. A., Matson, S., Heidtman, M., Piccirillo, S., Umansky, L., Drawid, A., Jansen, R., Liu, Y., Cheung, K. H., Miller, P., Gerstein, M., Roeder, G. S. and Snyder, M. (2002). Subcellular localization of the yeast proteome. *Genes and development* **16,** 707-719.

Laprade, L., Boyartchuk, V. L., Dietrich, W. F. and Winston, F. (2002). Spt3 plays opposite roles in filamentous growth in *Saccharomyces cerevisiae* and *Candida albicans* and is required for *C. albicans* virulence. *Genetics* **161**, 509-519.

Lambrechts, M. G., Bauer, F. F., Marmur, J. and Pretorius, I. S. (1996a). Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 8419-8424.

Lambrechts, M. G., Sollitti, P., Marmur, J. and Pretorius, I. S. (1996b). A multicopy suppressor gene, MSS10, restores STA2 expression in *Saccharomyces cerevisiae* strains containing the STA10 repressor gene. *Current Genetics* **29,** 523-529.

Liu, H., Styles, C. A. and Fink, G. R. (1996). *Saccharomyces cerevisiae* S288C has a mutation in FLO8, a gene required for filamentous growth. *Genetics* **144,** 967-978.

Lo, W. S. and Dranginis, A. M. (1996). FLO11, a yeast gene related to the STA genes, encodes a novel cell surface flocculin. *Journal of Bacteriology* **178,** 7144-7151.

Lo, W. S. and Dranginis, A. M. (1998). The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **9,** 161-171.

Lorenz, M. C. and Heitman, J. (1998). Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. *Genetics* **150,** 1443-1457.

Mosammaparast, N., Guo, Y., Shabanowitz, J., Hunt, D. F. and Pemberton, L. F. (2002). Pathways mediating the nuclear import of histones H3 and H4 in yeast. *Journal of Biological Chemistry* **277**, 862-886.

Oki, M and Nishimoto, T. (1998). A protein required for nuclear-protein import, Mog1p, directly interacts with GTP-Gsp1p, the *Saccharomyces cerevisiae* ran homologue. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 15388-15393.

Onodera, O., Roses, A. D., Tsuji, S., Vance, J. M., Strittmatter, W. J. and Burke, J. R. (1996). Toxicity of expanded poly-glutamine-domain proteins in *Escherichia coli*. *FEBS Letters* **399**, 135-139.

Palecek, S. P., Parikh, A. S. and Kron, S. J. (2002). Sensing, signalling and integrating physical processes during *Saccharomyces cerevisiae* invasive and filamentous growth. *Microbiology* **148,** 893-907.

Palmer, L. E. and McCombie, W. R. (2002). On the importance of being finished. *Genome Biology* **3**, 2010.1-.4.

Pan, X and Heitman, J. (1999). Cyclic AMP-dependant protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, **19**, 4874-4887.

Pan, X., Harashima, T. and Heitman, J. (2000). Signal transduction cascades regulating pseudohyphal differentiation of *Saccharomyces cerevisiae*. *Current Opinion in Microbiology* **3,** 567-572.

Pan, X. and Heitman, J. (2000). Sok2 regulates yeast pseudohyphal differentiation via a transcription factor cascade that regulates cell-cell adhesion. *Molecular and Cellular Biology* **20,** 8364-8372.

Pan, X. and Heitman, J. (2002). Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation. *Molecular and Cellular Biology* **22,** 3981-3993.

Pretorius, I. S., Lambrechts, M. G. and Marmur, J. (1991). The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus*: an overview. *Critical Reviews in Biochemistry and Molecular Biology* **26,** 53-76.

Rep, M., Reiser, V., Gartner, U., Thevelein, J. M., Hohmann, S., Ammerer, G. and Ruis, H. (1999). Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. *Molecular and Cellular Biology* **19,** 5474-5485.

Rout, M. P., Blobel, G. and Aitchison, J. D. (1997). A distinct nuclear import pathway used by ribosomal proteins. *Cell* **89,** 715-725.

Rupp, S., Summers, E., Lo, H. J., Madhani, H. and Fink, G. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. *The EMBO journal* **18,** 1257-1269.

Sambrook, J., Fritch, E. F. and Maniates, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Sherman, F., Fink, G. R. and Hicks, J. (1991). Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Sidorova, J. and Breeden, L. (1999). The MSN1 and NHP6A genes suppress SWI6 defects in *Saccharomyces cerevisiae*. *Genetics* **151,** 45-55.

Stanhill, A., Schick, N. and Engelberg, D. (1999). The yeast ras/cyclic AMP pathway induces invasive growth by suppressing the cellular stress response. *Molecular and Cellular Biology* **19,** 7529-7538.

Stevenson, L. F., Kennedy, B. K. and Harlow, Ed. (2001). A large-scale overexpression screen in *Saccharomyces cerevisiae* identifies previously uncharacterized cell cycle genes. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3946-3951.

Stratford, M. (1993). Yeast flocculation: flocculation onset and receptor availability. *Yeast* **9**, 85-94.

Strom, A. C. and Weis, K. (2001). Importin-beta-like nuclear transport receptors. *Genome biology* **2,** 3008.

Teunissen, A. W. and Steensma, H. Y. (1995). Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new sub-telomeric gene family. *Yeast* **11,** 1001-1013.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25,** 4876-4882.

Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R. E., Young, R. A., Lonvaud, A., Denayrolles, M. and Van Vuuren, H. J. (1997). Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. *Nature Biotechnology* **15,** 253-257.

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

Webber, A. L., Lambrechts, M. G. and Pretorius, I. S. (1997). MSS11, a novel yeast gene involved in the regulation of starch metabolism. *Current Genetics* **32,** 260-266.

Westermann, B. and Neupert, W. (2000). Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. *Yeast* **16,** 1421-1427.

Winston, F., Dollard, C. and Ricupero-Hovasse, S. L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11,** 53-55.

Yamashita, I. and Fukui, S. (1983). Mating signals control expression of both starch fermentation genes and a novel flocculation gene FL08 in the yeast *Saccharomyces*. *Agric Biol Chem* **47,** 2889-2896.

Zogbi, H. Y. and Orr, H. T. (1999). Poly-glutamine diseases: protein cleavage and aggregation *Current Opinion in Neurobiology* **9**, 566-570.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

CHAPTER 4

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4.1 CONCLUDING REMARKS

Limiting processes that regulate genome organisation and transcription to the nuclear compartment have provided eukaryotes with an evolutionarily selective advantage over organisms lacking such an organelle. The transport mechanisms that regulate the accessibility of factors to the nuclear interior and/or exterior are tightly regulated and occur at the site of the NPC, which is located in the nuclear membrane. Various translocation pathways through the NPC exist and these are functionally connected to multiple intracellular signal transduction pathways. Transcription factors or regulators, activated by the appropriate signalling pathways, are transported into the nucleus in order to function in the transcriptional regulation of specific genes.

In this study, we locate three proteins, Flo8p, Msn1p and Mss11p, to the nucleus. These factors have previously been shown to regulate the promoters of *MUC1* and *STA2* (Gagiano *et al.,* 1999a,b). The results clearly show that the *in vivo* nuclear location of GFP-tagged Flo8p and Mss11p can be directly correlated with agar invasion, cell elongation and the formation of flocculation cell aggregates. Furthermore, fluorescently-tagged Mss11p localises to distinct intranuclear spots when expressed from its native promoter on a multicopy plasmid.

Mss11p appears to play a central role in regulating the ability of yeast to invade agar and utilise starch (Gagiano *et al.*, 1999a,b, 2002; Webber *et al.,* 1997). For this reason, Mss11p function was further characterised in this study. We identified Mss11p as a strong activator of invasive growth and flocculation when expressed at high levels from the strong PGK_P and $GAL1_P$ promoters. Furthermore, we show that Mss11p greatly influences the extent of yeast growth on fermentable and non-fermentable carbon sources. A deletion in *MSS11* enabled yeast to reach a higher cell density under fermentative and non-fermentative conditions. The same deletion also partially overcame the severe growth defect under non-fermentative conditions of a ∑1278b strain that has been transformed with the hyperactive $RAS2^{val19}$ allele.

The diversity of cellular events in which Mss11p can be implicated suggests that Mss11p does not necessarily function in a single signalling pathway. In this study, we identified Mss11p as a major role player in the regulation of agar invasion, starch degradation, flocculation and population growth. It is plausible that Mss11p functions downstream of the MAPK, cAMP-PKA and other less well defined pathways. Furthermore, although Mss11p truncations fused to the Gal4p DNA-binding domain are able to recruit the general transcriptional machinery (Gagiano *et al.*, 2002), no evidence for specific DNA binding of Mss11p has been obtained thus far by our research group (data not shown). We thus hypothesise that Mss11p performs a regulatory role, downstream of several signalling pathways, but upstream of various transcription factors. This could include a mediating role for Mss11p between transcription factors, transcription machinery and chromatin remodelling and histone

modifying complexes. Considering this hypothesis, the distinct intra-nuclear pattern of GFP-tagged Mss11p fluorescence could be explained as Mss11p-GFP localising to various areas on the genome to affect the transcription of multiple genes.

Further investigation of Mss11p function would require more detailed physical analysis, as the amount of phenotypical data available for *MSS11* does not make it possible to specify Mss11p molecular function. Two-hybrid analysis and co-immunoprecipitation experiments could be used to identify proteins interacting with Mss11p. To further investigate targets of Mss11p regulation, microarray analyses of yeast deleted in *MSS11* or overproducing Mss11p will have to be compared.

4.2 LITERATURE CITED

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999a). Divergent regulation of the evolutionarily closely related promoters of the Saccharomyces cerevisiae STA2 and MUC1 genes. *Journal of Bacteriology* **181,** 6497-6508.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999b). Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in Saccharomyces cerevisiae. *Molecular Microbiology* **31,** 103-116.

Gagiano, M., Bester, M. C., Van Dyk, D., Franken, C. J., Bauer, F. F. and Pretorius, I. S. (2002). Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability. *Molecular Microbiology* **47**, 119-134.

Webber, A. L., Lambrechts, M. G. and Pretorius, I. S. (1997). MSS11, a novel yeast gene involved in the regulation of starch metabolism. *Current Genetics* **32,** 260-266.

APPENDIX

SUPPLEMENTAL INFORMATION

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I DIAGRAMMATIC REPRESENTATIONS OF CLONING STRATEGIES

Diagrammatic representations are provided to aid in the understanding of the cloning strategies employed in plasmid construction. The symbol "✂" indicates the use of restriction enzyme(s), and "()" refers to the use of the polymerase chain reaction (PCR).

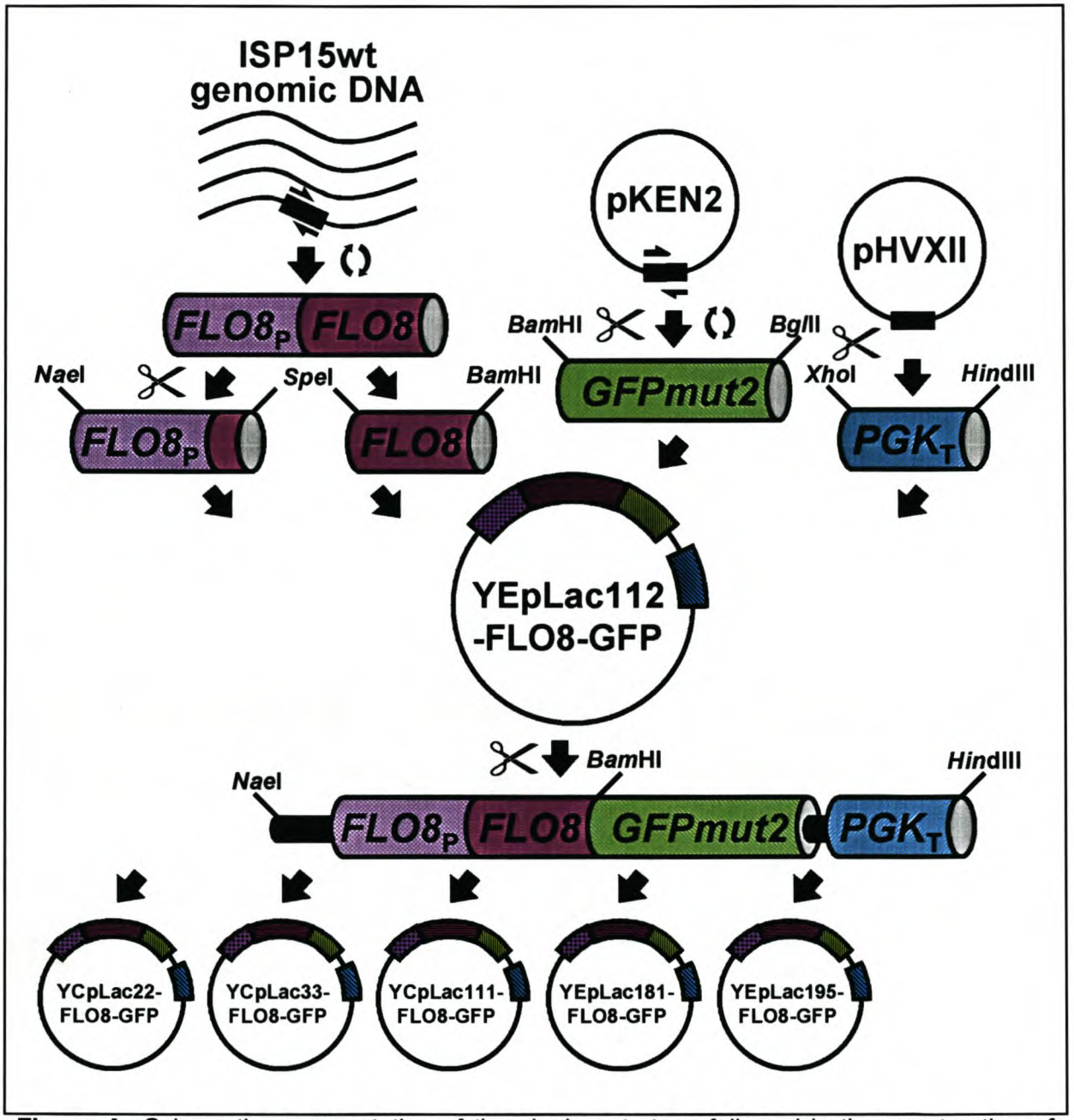

Figure A Schematic representation of the cloning strategy followed in the construction of YCpLac22-, YCpLac33-, YCpLac111-, YEpLac112-, YEpLac181- and YEpLac195-FLO8-GFP.

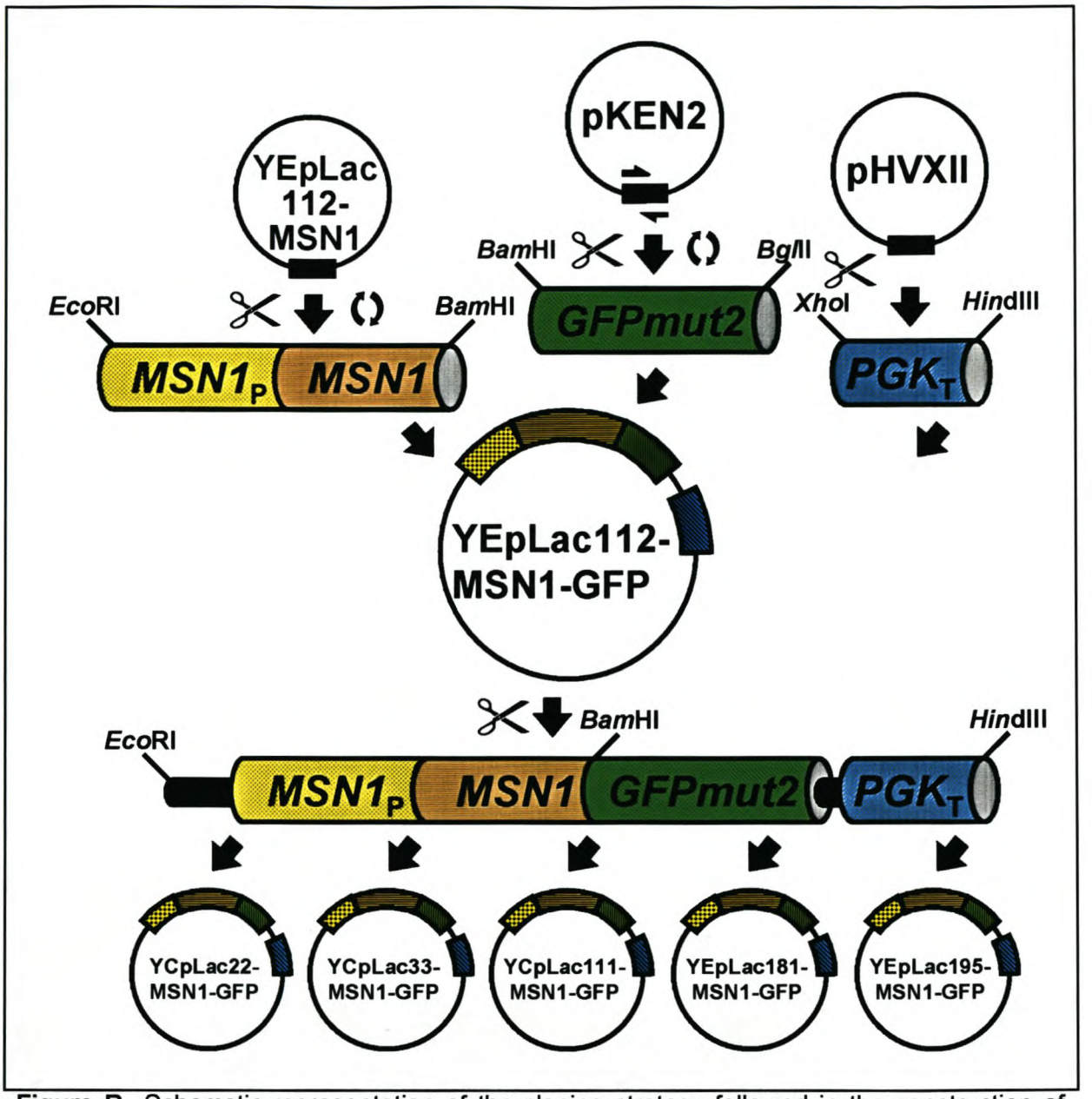

Figure B Schematic representation of the cloning strategy followed in the construction of YCpLac22-, YCpLac33-, YCpLac111-, YEpLac112-, YEpLac181- and YEpLac195-MSN1-GFP.

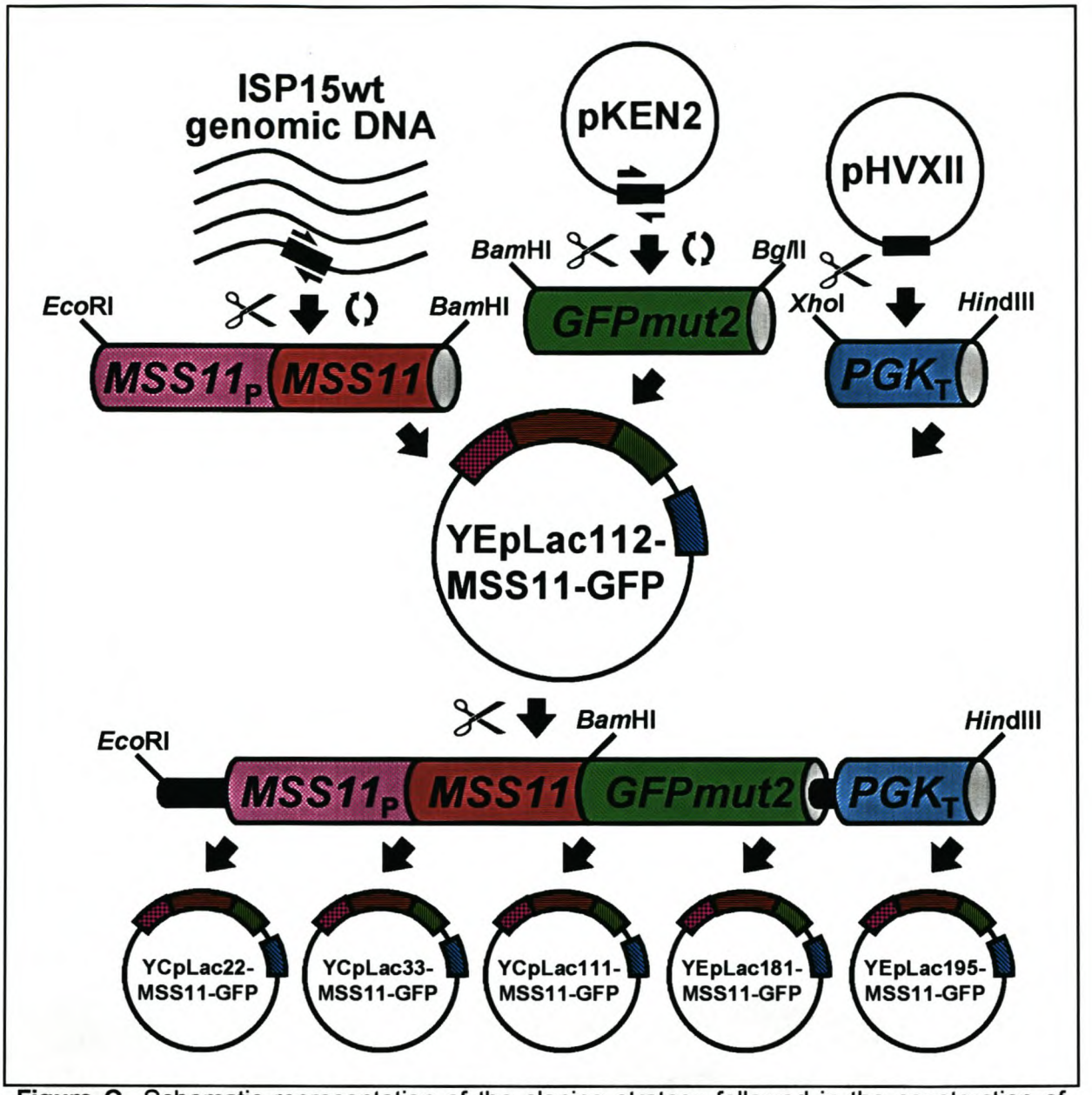

Figure C Schematic representation of the cloning strategy followed in the construction of YCpLac22-, YCpLac33-, YCpLac111-, YEpLac112-, YEpLac181- and YEpLac195-MSS11-GFP.

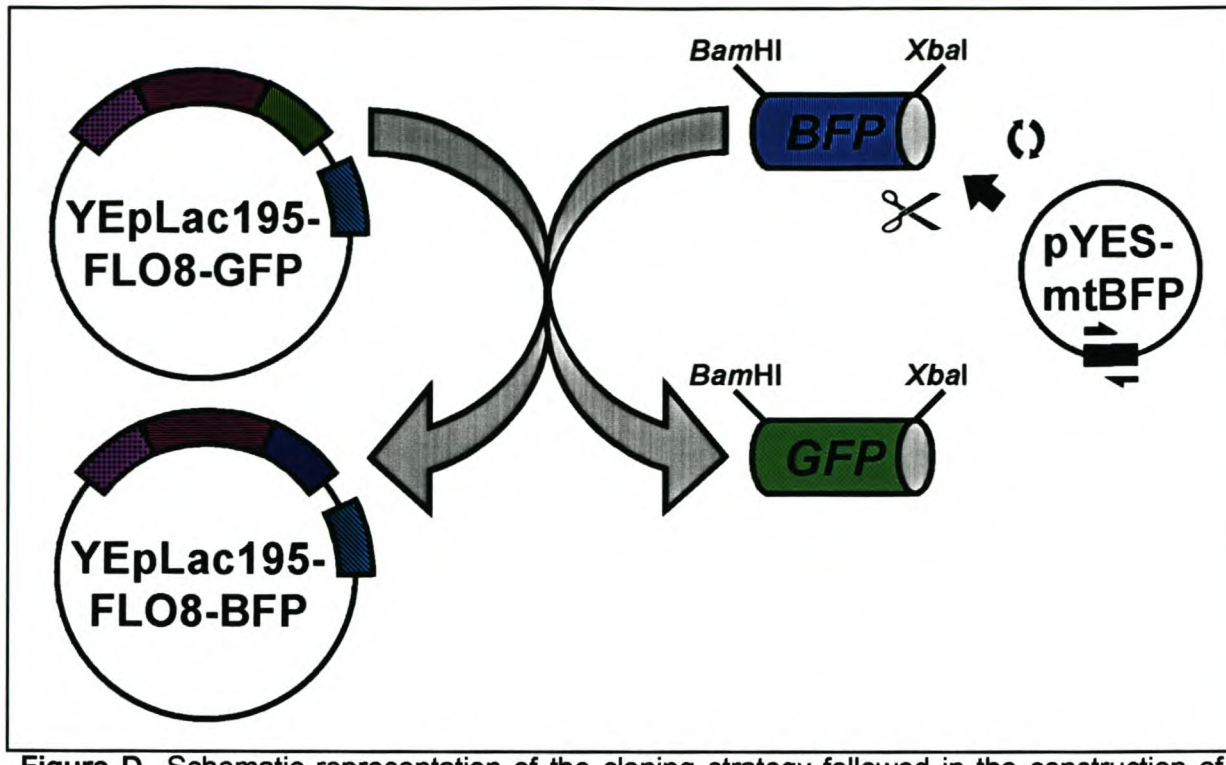

Figure D Schematic representation of the cloning strategy followed in the construction of YEpLac195-FLO8-BFP.

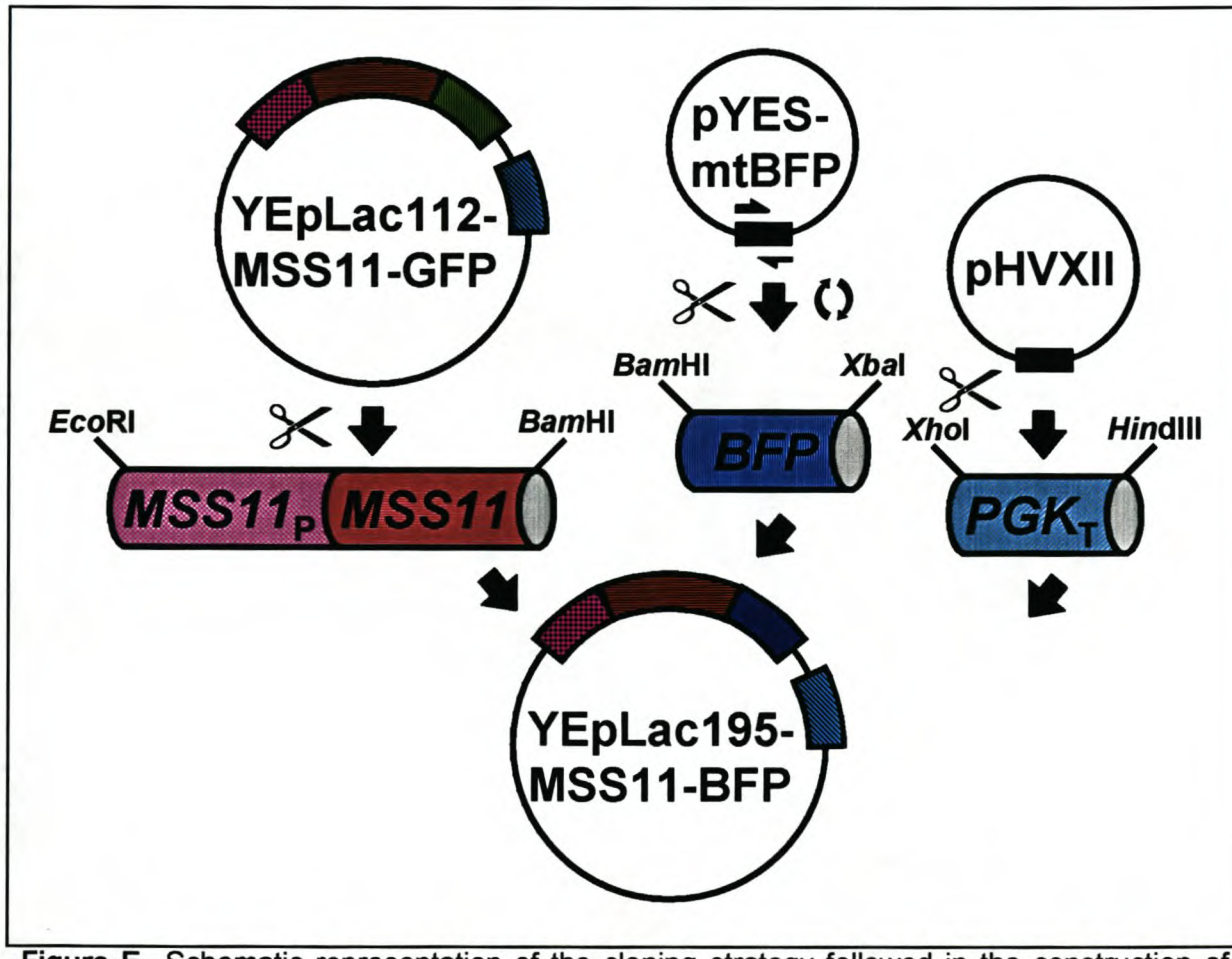

Figure E Schematic representation of the cloning strategy followed in the construction of YEpLac195-MSS11-BFP.

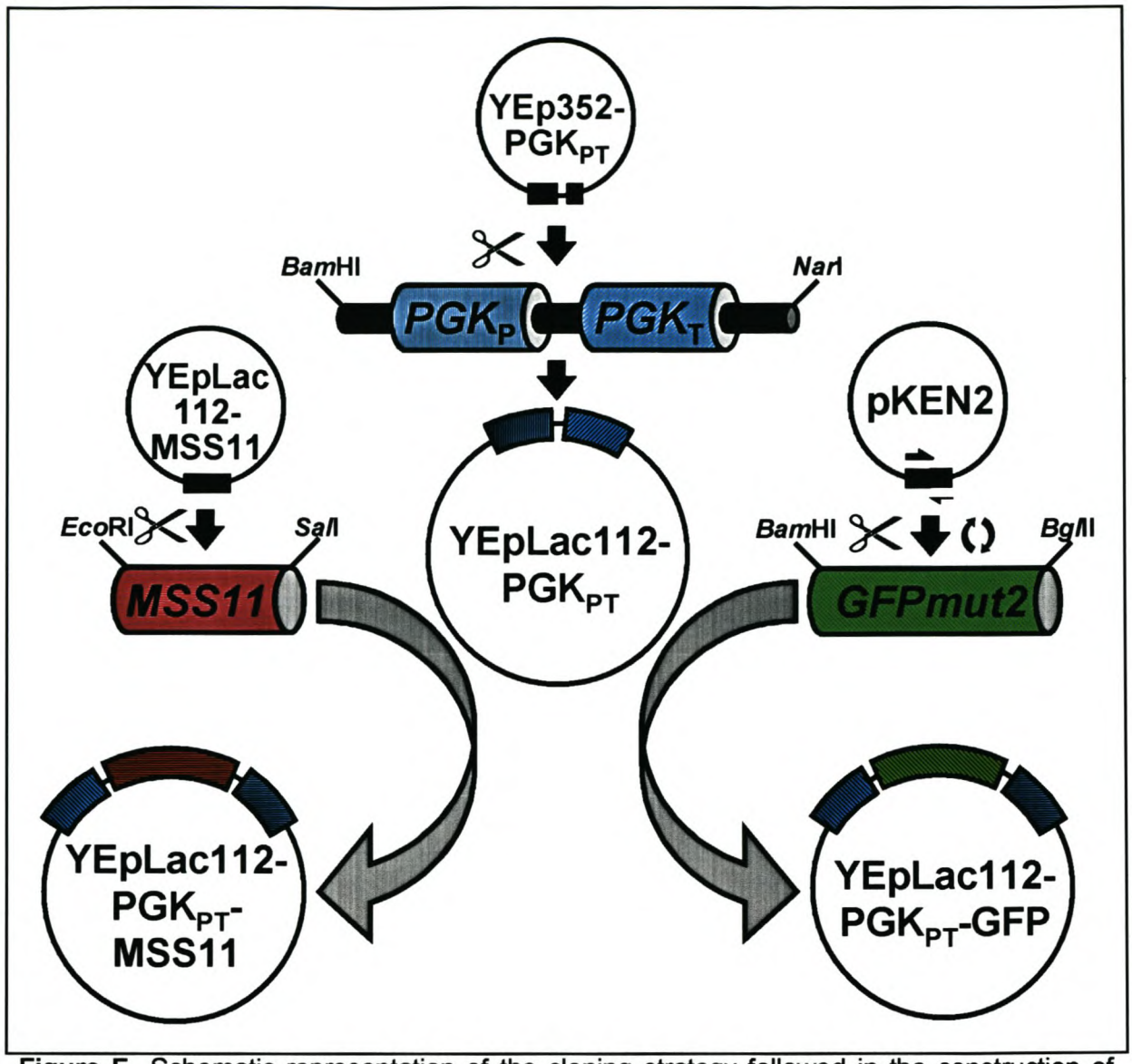

Figure F Schematic representation of the cloning strategy followed in the construction of YEpLac112-PGK$_{PT}$-MSS11 and YEpLac112-PGK$_{PT}$-GFP.

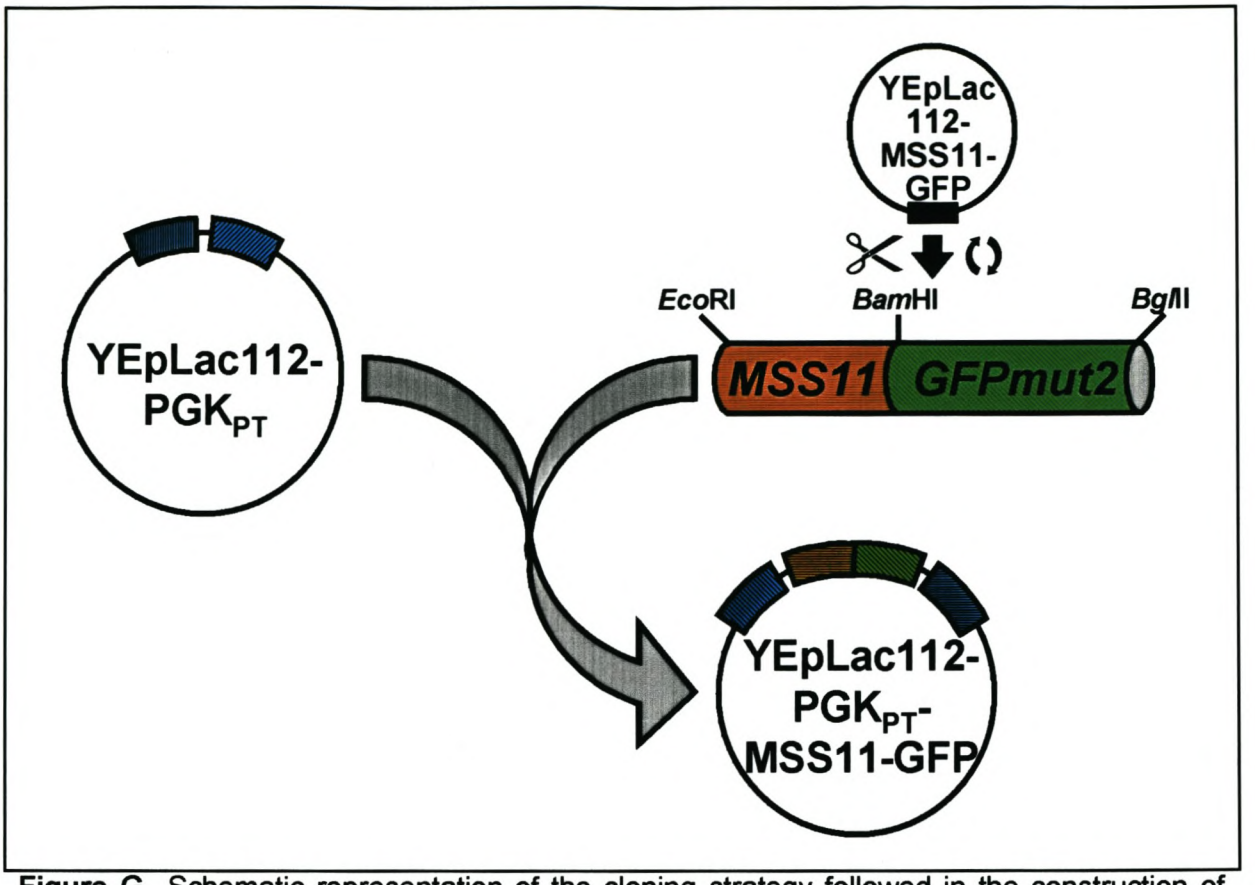

Figure G Schematic representation of the cloning strategy followed in the construction of YEpLac112-PGK$_{PT}$-MSS11-GFP.

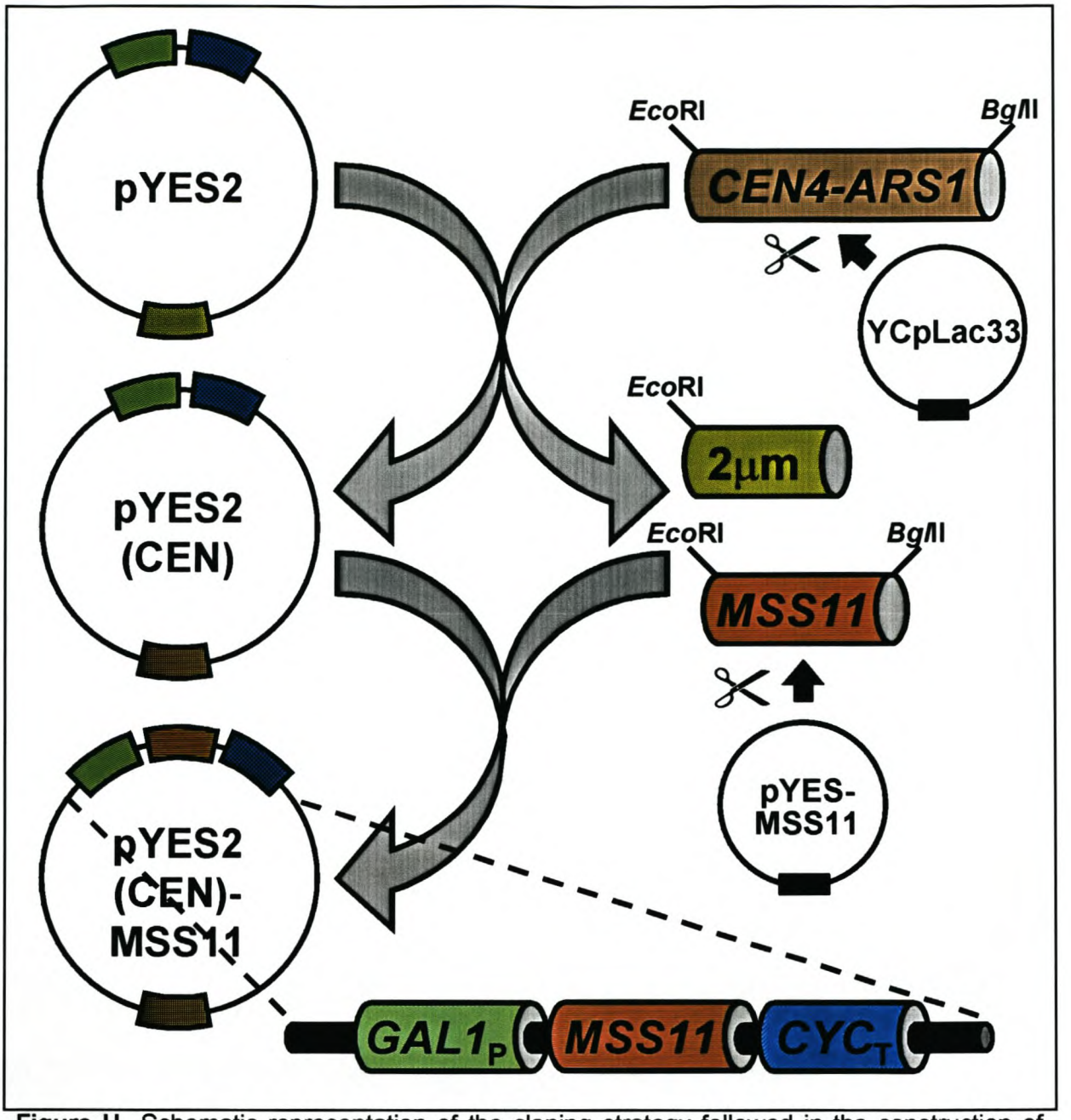

Figure H Schematic representation of the cloning strategy followed in the construction of pYES2(CEN) and pYES2(CEN)-MSS11.

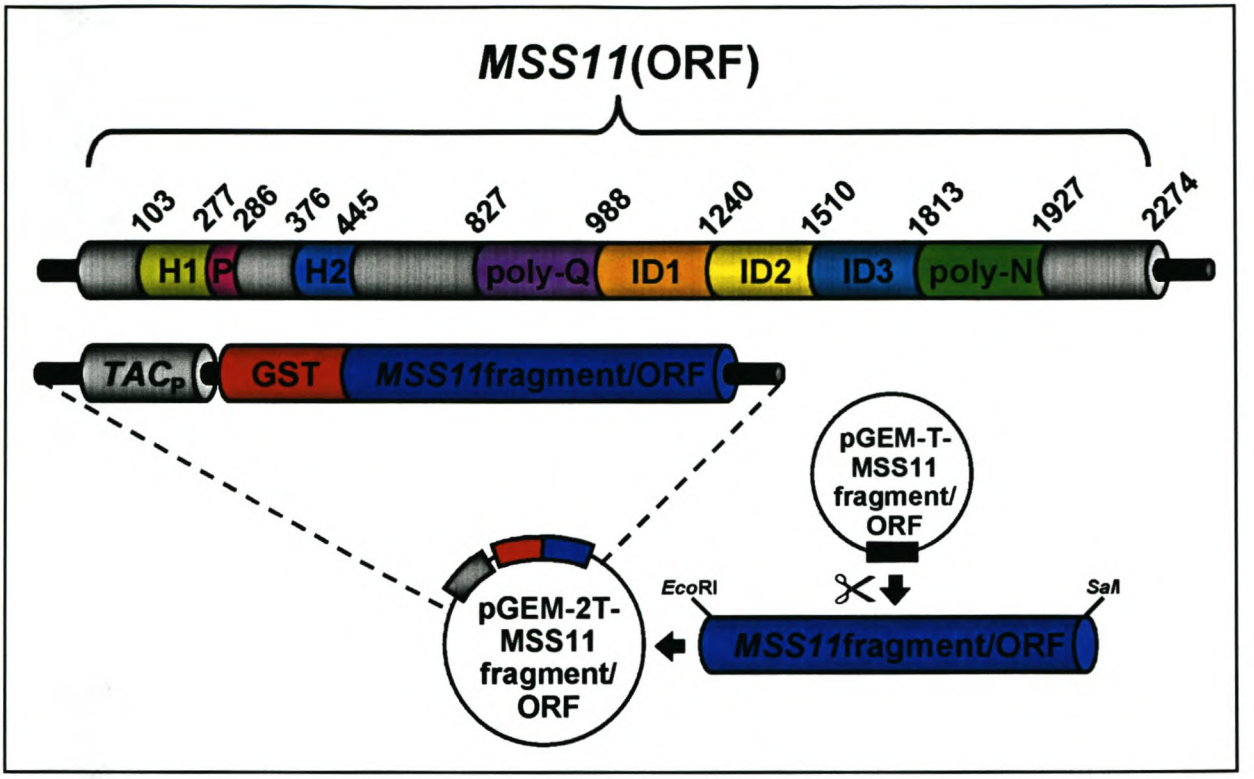

Figure I Schematic representation of the domain structure of *MSS11* as defined in Gagiano *et al.* (2002; see Chapter 3 reference list), and the cloning strategy followed in the construction of *GST*-fused *MSS11* fragments.

II ADDITIONAL ALLIGNMENTS

Alignments of *FLO8* and *MSS11* orthologs from *Saccharomyces sensu stricto* yeasts are provided. For an explanation of the symbols used, see *Chapter 3: Materials and Methods*.

```
S. cerevisiae       MSYKVNSSYPDSIPPTEQPYMASQYKQDLQSNIAMATNSEQQRQQQQQQQQQQQQWINQP  60
S. paradoxus        MSYKVSNSYPDSIPPADQPYIASQYKQDLQSNIAMATNNEQQRQQQPQQQQQ---WINHP
S. mikatae          MNYKVSSSYPDSIPSAEQPYVASQYKQDLQSNISMATNNAQQRQQQ---QQQ---WIKQP
S. bayanus          MGYKANSSFPDSIPPADTPYSSNDYRHGLQNNIPMSANSEQQHQQH---QQQ---WKSEA
                    *.**...*:*****.:: ** :.:*::.**.**.*::*. **:**:   ***    * ...
TAENSDLKEKMNCKNTLNEYIFDFLTKSSLKNTAAAFAQDAHLDRDKGQNPVDGPKSKENNGNQNTFSKVVDTPQGFLYE 140
TVENSDLKEKMNCKNTLNEYIFDFLTKSSLKNTAAAFAQDAQLDRDIGHEPVDGPNPKENNGKQSTLSKVVDTPQGFLYE
AVENPDMKEKINCKKALNEYILDFLTKSSLKSTAAAFAQDAQLDRDIDHEPEDEPTSKENTGSQSPLTKVVDTPQGFLYE
VLESPDLKERTNCKKALNQYIFDFLSKSSLKNTAAAFAQDAQLNRDTNDKQLGGNNYKEKTPGQSPMLEVVDAPQGFLFE
. *..*:**: ***::**:**:***:*****.*********:*:** ..:  .  . **:.  *..: :***:*****:*
W-QIFWDIFNTSSSRGGSEFAQQYYQLVLQEQRQEQIYRSLAVHAARLQHDAERRGEYSNEDIDPMHLAAMMLGNPMAPA 220
WWQIFWDIFNTSSSRGGSEFAQQYYQLVLQEQRQEQIYRSLAVHAARLQHDAERRGEYINEDIDPMHLAAMMLGNPMPPA
WWQIFWDIFNTSSSRGGSEFAQQYYQLVLQEQRQEQIYRSLAVHAARLQHDAERRGEYTNEDIDPMHLAAMMLGNPMPSP
WWQIFWDIFNTSSSRGGSESAQQYYQLVLQEQRQEQIYRSLAVHAARLQHDAERRGEFVNEEVDPMHLAAMMLGNSMPPA
* ********************************************************: **::************.*... 300
VQMRNVNMNPIPIPMVGNPIVNNFSIPPYNNANPTTGATAVAPTAPPSGDFTNVGPTQNRSQNVTGWPVYNYPMQPTTEN
VQMRNVNMNAIPIPIVGNPIVNNFSIPPYNNANPTAGATTVAATAPPSGDFTNVAPTQNRSQNVTGWPVYNYPMQPTTEN
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ASMQNVNVNPMPIPIVSHPIVNNFSVPPYSNTNPTTGATTVTAAAPPSGDFSNAAPIQNRNQNVTGWPVYNYPMQPTTEN
. *:***:*.:** :*.:*:*****:***.*:***:**::*..:*******:*..* ***.*****************:** 380
PVGNPCNNNTTNNTTNNKSPVNQPKSLKTMHST--DKPNNVPTSKSTRSRSATSKAKGKVKAGLVAKRRRKNNTATVSAG
SMVNPCNNNTTNNIANNKSPVNQPKSLKTMHST--DRPNNVPTSKSTRSRSATSKAKSKAKAGPVAKRRRKNNTATVSAG
LMGNPCNSNTTNNTINNKSPVNQPKNLKTMHST--DKPNNVVTSKSIRSRSATSKAKGKATIGPASKKRKKNNTATTSAG
PVGIPCNNNTT---TNNKSPVNPSKNLKNIHSSSIDKSSNLVMPKSTRSRSATSKSKGKTTNGPGTRKRRKITATAVSAG
 :  ***.***    ******* .*.**.:**:  *:..*:  .** ********:*.*.. *  :::*:* .::::.***
STNACSPNITTPGSTTSEPAMVGSRVNKTPRSDIATNFRNQAIIFGEEDIYSNSKSSPSLDGASPSALASKQPTKVRKNT 460
STNAGSPNITTPGSTTSEPAMVGSRVNKTPRSDIATNFRNQAIIFGEEDIYSNSKSSPSLDAASPSISVTKQPTKVRKNI
STNAGSPNITTPGSTTSEPAMVNSRLNKTPRCDIATNYRNQAIIYGEEDIYSNVKSSPSLGAASPSTAITKQPTKIRKNT
STNAGSPNITTPGSTTSEPAMVNSRLNKTPKSDMTAPHRSQAIVFGDEDAYSNKKASPLISAASPSTIVAKQPVKARKNT
**** ****************.**:****:.*::: .*.***::*:** *** *:** :..****   :***.* ***
KKASTSAFPVES---TNKLGGNSVVTGKKRSPPNTRVSRRKSTPSVILNADATKDENNMLRTFSNTIAPNIHSAPPTKTA 540
KKASTSAFPVES---SNKLNSNSGVTGRKRSPPNTRVSRRKSTPSVILTADATKDENDMLRTFSNTTTPIVHSAPPTKTT
KKALTSAFPVES---ANKSSSGSGVVGKKRGSLNTRVSRRKSTPSVILNVDSTKDENNMLRTLSNTTTPNMHSAPPPKTI
KKASTSAFSVESTQPTNKGSSNNGISGKRRSPPSSRVSRRKSTPSVILNDNTAEDANDMLRTHSNNTTPNIHSAPPSRST
*** ****.***   :** .... : *::*.. .:************. ::::* *:**** **. :* :*****.::
NSLPFPGINLGSFNKPAVSSPLSSVTESCFDPESGKIAGKNGPKRAVNSKVSASSPLSIATPRSGDAQKQRSSKVPGNVV 620
NPLPFSGINLGSFNKPAVSSPLSSVTESCFDPESAKVATKSGPKRAVNSKVSASSPLSIATPPSGDAQKQRSSKAAGNVV
NPLPFSAINLGSFNKPAVSSPLSSVTESCFDPESAKVVTKGGPKRAVNSKVTASSPLNIATPPSGDTQKQKNPKIAGNVI
NPLPFSGVNLGGFNKPAVSSPLSSVTETCFDPDSGKLVTKGGPKRTVNSKVSASSPLNISTPPSGDAQKQRNSKPTGSAV
*.***..:***.***************:****:*.*:. *.****:*****:*****.*:** ***:***:..* .*..:
IKPPHGFSTTNLNITLKNSKIITSQNNTVSQELPNGGNILEAQVGNDSRSSKGNRNTLSTPEEKKP-SSNNQGYDFDALK 700
IKPPHGFSTTNLNITLKSSKIIPSQNTTVPQELPTGGNPLEAQVGNDSRNNKGNRDALSTPEEKKL-NNTNQAYDFEALK
IKPPHGFSTTNLNITLKSSKIITSQNNTVSQELPPGGHPPEVHAGNDLRSNKGIRNALSTPEETKPPSSTNQTYDFEVSK
IKPPQGFSTTNLNITLKSSKIIPSKNNIVPQELPTT-KPLEAQMAKDLRSNKGNRNAF-TPDGTKPPNNTNQVYEFENVK
****:************.****.*:*. *.****    : *.: .:* *..** *::: **: .*  ...** *:*:  *
NSSSLLFPNQAYASNNRTPNENSNVADETSASTNSGDNDNTLIQPSSNVGTTLGPQQTSTNENQNVHSQNLKFGNIGMVE 780
NSSSLLFPNQSCVPTNRTPNGNSNVANETYASTNNGDNQKASVQPSSTVGTTLGPQQTGTNGHQNQQSQNMKFGNIGMVE
NSNSLPFPNQAYPSNNRTPNENLNVDDENSASINNGDGDSALVQPVSNVETALDPQRTSTGEHQNPQSQSMKFGNIGVIE
NPNSLMLPNQIFTSNNGTPNENSNPGN--TTSSNKGNSDSASVQPASNIGTTLGPHQASTTEQQNSQPQEMKLDNVGIVE
*..** :***   ..* *** * *  :   :* *.*:.:.: :** *.: *:*.*::*.*  :** :.*.:*:.*:*:*::*
DQGPDYDLNLLDTNENDFNFINWEG 805
DQGPDYDLNLLDTNENDFNFINWEG
DQGPDYDLNLLDTNENDFNFINWEG
GQGTDFGLNLLDTNENDFNFINWEG
.**.*:.******************
```

Figure J Alignment of the translation of *FLO8* orthologs found in *S. cerevisiae, S. bayanus*, *S. mikatae* and *S. paradoxus*. Shaded area indicates the poly-glutamine domain.

```
S. cerevisiae       MASNQHIGASNLNENEAILTNRVAELERRMSMFEGIFHALSNRLDLHFKKYDVVVNSQQQ  60
S. paradoxus        MANNQHMGASNLNENEAMLTNRVAELERRMSMFEGIFHALSNRLDLHFKKYDVVVNSQQQ
S. mikatae          MANNQHMGTSNLNENEAMLTNRVAELERRMSMFEGIFHALSNRLDLHFKKYDVVVNSQQQ
S. bayanus          MANNQHMGTSNLNENEAILTNRVAELERRMSMFEGIFHALSSRLDLHFKKYDVVVNSQQQ
                    **.***:*:********:**********************.******************
QINELTAFLSTLLNDQQRHAEILSEKLSGTLHGVSATSISLSQTLDPQGFTDGTTAPGAPRNYTSVPMNNDQTAHPQNEG 140
QINELTAFLSTLLNDQQRHAEILSEKLSGTLHGVSATSISLSQTLDPQGFTDGATAPGAQRNYSSVSMNNDETAHPQNEG
QINELTAFLSTLLNDQQRHAEILSERLAGTLHGVSATSISLSQTLEPQGFTDGPTAANTQRNYSSVSINNNETAHSQNGA
QINELTAFLSTLLNDQQRHAEILSEKLSGTLHGVSATSISLSQTLDPQGFGDGPSAPNTQRNYGPVSLNSSEAANTQNEG
**************************:*:*****************:**** **.:*..: *** .*.:*..::*:.***. 220
AVSNETLFEDILNGNSQENDKSQQQTNSSNSISQENNSTNPSVDTRFNKPQNYNSNLVPSLEEYSANPPNNDGGQSQGLY
AVSNETLFEDILNGNSQENDKGQQQANSSNPITQEN-STNPSVDTRFNKPQNYNPNLVPSLEEYSANPPNNDGDQSQGLY
AVSNETLFEDILNGNSQENDKSQQQPNSSNQITQEN-NNNPSVNTRFSKPQNYNPSLVTSLEDYSTNPPNNDNGQSQGLY
TVSNETLFEDILNGNSQENDKNQQHPNGPNPINQEN-NPNPSVDGRFNKPQNYNSNLVTTLEDYTTNPPNNDGGQSQGLY
:*******************.**:.*..* *.*** . ****: **.******..**.:**:*::******..****** 300
ISSNSSQSRQSPNLQKVSPNHENAVESNAQESVPTFEEEQYETKTGLKRKRIVCTRPFEFIKSPHSVMEVWKEYTEGVNG
ISSTSSQSRQSPNLQKISPNRENAVEPNVQDSVPTFEEEQYETKTGLKRKRIVCTRPFEFIKSPHSVMEVWKEYTEGVNG
MSSTSSQSQQSPNLQKISPNRENAAESNVQESVPTFEEEQYETKTGLKRKRIVCTRPFEFIKSPHSVMEVWKEYTEGVNG
ISSTSSQSQQSPNLQKISPEHESAVEPNAQEGVPTFEEEQYETKTGLKRKRIVCTRPFEFIKSPHSVMEVWKEYTEGVNG
:**.****:*******:**::*.*.*.*.*:.************************************************ 380
QPSIRKMEALYQTAWRRDPAVNKRYSRRKVLWKAIQTGLNRGYSLNYVVEILENSRYVNDKQKVKQPIGWLCHSSHIPET
QPSIRKMEALYQTAWRRDPAVNKRYSRRKVLWKAIQTGLNRGYSLNYVVEILENSRYVNDKQKVKQPIGWLCHSSHIPET
QPSIRKMEALYQTAWRRDPAVNKRYSRRKVLWKAIQTGLNRGYSLNYVVEILENSRYVNDKQKVKQPIGWLCHSSHIPET
QPSIRKMEALYQTAWRRDPAVNKRYSRRKVLWKAIQTGLNRGYSLNYVIEILENSRYVNDKQKVKQPIGWLCHSSHIPEI
**************************************************:******************************
LK 382
LK
LK
LK
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Figure J Alignment of the translation of *MSN1* orthologs found in *S. cerevisiae, S. bayanus*, *S. mikatae* and *S. paradoxus.*