# Role of Rim101 in mitotic repression of the yeast sporulation-specific genes *DIT1* and *DIT2*

by

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## Abstract

The process of sporulation in cells of *Saccharomyces cerevisiae* depends on the sequential activation of temporally distinct subsets of genes. The adjacent and divergently transcribed *DIT1* and *DIT2* genes belong to the mid-late class of sporulation-specific genes. A *cis*-acting DNA element, $NRE^{DIT}$, prevents expression of these genes in mitotic cells. I confirmed that the downstream portion of $NRE^{DIT}$, termed NRE42, is an efficient operator that consists of two sub-sites. The two sites combine to give a much higher level of repression than that predicted by simple arithmetic summation of the operator function of each sub-site. Multimers of each element act co-operatively to give a high level of repression.

Repression mediated by the upstream and downstream portions of NRE42 is Rim101-independent and Rim101-dependent, respectively. My data support the model that a hypothetical protein and Rim101 bind to adjacent sites in NRE42 and recruit the Ssn6-Tup1 co-repressor complex.

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# Introduction

The focus of my thesis has been to further elucidate how a short negative regulatory element, NRE42, contributes to repression of two sporulation-specific genes, *DIT1* and *DIT2*, during mitotic growth in *Saccharomyces cerevisiae* cells. I begin the introduction with an overview of sporulation-specific events and relevant background on the regulation of expression of the mid-late genes, *DIT1* and *DIT2*. Previous experiments in our lab indicate that the Ssn6-Tup1 co-repressor complex contributes to the mitotic repression of *DIT1* and *DIT2*. Part of my introduction focuses on how the Ssn6-Tup1 complex is thought to mediate repression in yeast. Since Rim101 was identified in our lab as a protein required for full repression through $NRE^{DIT}$, I review what is known about Rim101 and compare Rim101 to its homologue, PacC, in *Aspergillus nidulans*. One of the aims of my thesis was to demonstrate that *in vivo* Rim101 binds to the NRE. In the last part of my introduction I outline why it is important to study protein-DNA interactions *in vivo*. Finally, I discuss one approach, *in vivo* cross-linking coupled with chromatin immunoprecipitation, which has been used to study transcriptional regulation under *in vivo* conditions.

## 1.A Sporulation: Landmark events

Initiation of sporulation in *S. cerevisiae* requires appropriate input into the cell-type and nutritional sensing pathways. In the presence of a non-fermentable carbon source and the absence of nitrogen, an **a**/α diploid cell will enter the sporulation program. Sporulation begins with one round of DNA replication followed by two consecutive meiotic divisions. The first or reductional meiotic division leads to the segregation of homologous chromosomes, whereas the second meiotic division leads to segregation of sister chromatids. The haploid meiotic products are encapsulated in a multi-layered structure, the spore wall. The resultant spores will germinate under favourable conditions.

**1.A.i Premeiotic DNA synthesis.** Premeiotic DNA synthesis, leading to a cell with a 4N DNA content, is the first landmark event of meiosis (reviewed in Kupiec *et al.*, 1997). At about the time that DNA synthesis begins, duplication of the spindle pole body (SPB) occurs. The SPB, which is embedded in the nuclear envelope with cytoplasmic (the outer plaque) and nucleoplasmic components, serves two major roles during sporulation. It is the nucleation site for the meiotic spindles and also plays a critical role in spore wall formation (see below). Thus, SPBs are instrumental in the segregation of both homologues and chromatids at meiosis I and II, respectively (reviewed in Kupiec *et al.*, 1997).

**1.A.ii Meiotic prophase.** Pairing of meiotic chromosomes during prophase is accompanied by the formation of the synaptonemal complex (SC). The SC is a proteinaceous structure consisting of two lateral elements (formerly the axial elements) and the region between homologues (reviewed in Roeder, 1995). Tentative pairing of homologous chromosomes occurs early during prophase, prior to SC formation. Recombination begins with DNA double-strand breaks (DSBs), occurring at about the time that axial elements (AEs) first appear. Unsynapsed AEs, running along the axes of sister chromatids begin to form periodic "association sites", where homologues are brought closer together (reviewed in Kleckner, 1996). These association sites may be where components of the central region of the SC begin to polymerize to generate fully synapsed homologues (reviewed in Roeder, 1995). At the end of prophase, resolution of DSBs to give either noncrossover or crossover products occurs and the synaptonemal complex breaks down. Chiasmata, the cytological manifestation of crossovers, are critical for proper disjunction of homologs; the tension exerted by the meiotic spindles is "counteracted" by the bonding of homologs at chiasmata, leading to the proper alignment of chromosomes between the two poles (reviewed in Kupiec *et al.*, 1997).

**1.A.iii Meiotic divisions.** At the end of pachytene, the SPBs begin to move to opposite poles to form the meiosis I spindle. Paired homologues, held together by chiasmata, align on the metaphase plate and move to opposite poles. At this point the SPBs duplicate and separate to form the MII spindles. Similar to mitotic division, in MII sister chromatids are pulled to opposite poles. At the end of the two meiotic divisions, yeast meiosis is still incomplete. The four haploid complements of chromosomes are still housed in the original nuclear membrane,

although the initial spherical nucleus has taken on a four-lobed structure (reviewed in Kupiec *et al.*, 1997).

**1.A.iv Spore wall formation.** At the end of MII, the cytoplasmic face of each outer plaque of the SPBs begins to thicken and widen. Cytoplasmic vesicles coalesce against the cytoplasmic surface of each outer plaque, forming a flattened sac that begins to encapsulate the haploid meiotic products. Eventually four nuclear compartments are pinched off, each with a double membrane that forms the prospore wall. Glucans and mannans are deposited within the lumen of the prospore membrane. This maturation process continues with the deposition of insoluble spore wall materials on the outer surface of the prospore membrane. Mature spore walls consist of four distinct layers with the innermost two layers consisting mainly of glucans and mannans, similar to the vegetative cell wall. The next layer is rich in chitosan and contains some chitin (reviewed in Briza *et al.*, 1990). The outermost layer of the spore wall, which consists of an insoluble macromolecule rich in cross-linked tyrosine of both the *LL* and *DL* stereoisomers, makes spores resistant to glusulase, ether and higher temperatures (Briza *et al.*, 1990; Briza *et al.*, 1994).

## 1.B Background on NRE$^{DIT}$

**1.B.i An overview of the regulation of gene expression during sporulation.** Underlying the genetic and morphological events that take a cell through sporulation is the sequential transcription of sporulation-specific genes. Based on their time of expression, sporulation specific genes can be grouped into at least four temporally distinct classes: early, middle, mid-late and late genes (reviewed in Mitchell, 1994). The time of expression of a sporulation gene coincides well with the process that it is required for. More recently, DNA microarray analysis suggests that the four temporal classes should be expanded into at least seven temporal classes (Chu *et al.*, 1998). For the purposes of brevity, only the broader, four temporal classes will be discussed. Briefly, early genes are needed for such processes as the recombination and segregation of homologues, whereas middle genes are required for both meiotic divisions and spore formation. The only well characterised mid-late genes, *DIT1* and *DIT2*, encode enzymes that catalyse the synthesis of the dityrosine precursor that is incorporated into the outer spore

wall (Briza *et al.*, 1990; Briza *et al.*, 1994). Products of the late genes are involved in proper spore wall maturation.

**1.B.ii Regulation of expression of the mid-late genes *DIT1* and *DIT2*.** The two mid-late genes, *DIT1* and *DIT2*, are adjacent and divergently transcribed. Their transcripts accumulate at the time of prospore enclosure. (Briza *et al.*, 1990; Friesen *et al.*, 1997). Friesen and colleagues (1997) analysed the 800 bp intergenic region between *DIT1* and *DIT2* and identified a 76 bp element responsible for keeping both genes off during vegetative growth. This element, named $NRE^{DIT}$, is located ~500 bp upstream of the *DIT1* transcription start site. $NRE^{DIT}$ is a potent operator, able to repress a gene driven by a heterologous UAS more than 500 fold in an orientation-independent manner. This repression is maintained during sporulation. Although $NRE^{DIT}$ does have a minimal UAS activity during sporulation, at least two other elements, both downstream of $NRE^{DIT}$ but before the *DIT1* transcription start site, are required for sporulation specific expression from the heterologous reporter. Friesen *et al.* (1997) demonstrated that repression of the reporter gene through $NRE^{DIT}$ is dependent on Ssn6 and Tup1. Similarly, repression of the chromosomal *DIT1* and *DIT2* genes is dependent on Ssn6 and Tup1. This confirms that repression by the Ssn6-Tup1 co-repressor complex is physiologically relevant. Although in the context of a heterologous reporter gene a sequence downstream of $NRE^{DIT}$ also shows Tup1-dependent repression, this repression is minor to that exhibited through $NRE^{DIT}$. Thus, in a chromosomal context most of the Ssn6-Tup1-dependent repression of *DIT1* and *DIT2* is mediated through $NRE^{DIT}$. In summary, this study established that repression of the *DIT1* and *DIT2* genes during vegetative growth is mediated through $NRE^{DIT}$ in an Ssn6-Tup1-dependent manner.

**1.B.iii Genetic screen to identify other proteins required for repression through $NRE^{DIT}$.** The co-repressor complex Ssn6-Tup1 mediates repression of a number of co-regulated genes. This complex does not bind DNA directly. Rather it is recruited by interactions with other promoter-specific DNA-binding proteins. Dr. Friesen carried out a genetic screen to identify other proteins that are required for repression through $NRE^{DIT}$, including a protein(s) that might directly bind to the operator. This screen took advantage of the ability of $NRE^{DIT}$ to prevent the expression of a $UAS^{CYC1}(NRE^{DIT})$-*lacZ* reporter gene. Following EMS mutagenesis, mutants

were identified that failed to repress the reporter gene. The repression abilities of these mutants, called *frds* to indicate that the wild type gene products *function in the repression of DIT* genes, were further analysed. In addition to their defect at repression through $NRE^{DIT}$, mutants were also classified according to their abilities to maintain two other kinds of repression: basal transcription and repression through the α2-Mcm1 operator, which is also Ssn6-Tup1 dependent. As expected, the genetic screen picked up mutations of general transcription factors, that led to defects in repression through all three types of promoters (Friesen *et al.*, 1998; Tanny, 1998b). The screen also identified mutations in *SSN6*, *TUP1*, as well as *SPE3*, which cause defects in repression through the α2-Mcm1 operator and through $NRE^{DIT}$ (Friesen *et al.*, 1998). The final class of mutants contained strains that are specifically defective in $NRE^{DIT}$-mediated repression. These mutants maintain repression of basal transcription and repression mediated through the α2-Mcm1 operator (Friesen *et al.*, 1998).

Jason Tanny, a former Master student, characterised *FRD5*, a gene that is specifically required for repression through $NRE^{DIT}$. The wild-type *FRD5* gene was isolated from a yeast DNA library by complementation of the *frd5* phenotype. Linkage analysis, or observing the segregation pattern of a marked *rim101* allele and *frd5*, confirmed that *frd5* is an allele of *RIM101*. Rim101 contains three N-terminal zinc fingers which are highly similar in sequence to the zinc fingers of PacC, a transcription factor form *Aspergillus nidulans*. Further analysis by Jason Tanny and myself suggests that Rim101 binds to $NRE^{DIT}$.

## 1.C Repression by the Ssn6-Tup1 co-repressor complex

**1.C.i Ssn6 and Tup1 as a co-repressor complex.** *SSN6* and *TUP1* were originally identified in distinct studies. The protein product of the *SNF1* gene is normally required for the derepression of many glucose repressible genes, including *SUC2*, which codes for invertase. *SSN6* was first identified in a screen designed to identify suppressors of *snf1* (*ssn*). Such suppressor mutations would allow *snf1* strains to ferment sucrose. However, Carlson and colleagues (1984) discovered that mutations in *SSN6* alone (in a *SNF1* background) cause constitutive expression of invertase irrespective of the presence or absence of glucose. These authors concluded that *SSN6* is not simply a suppressor of *snf1*, and that the *SSN6* product likely has a regulatory function (Carlson *et al.*, 1984).

Characterisation of a mutant that was unable to take up dTMP from the growth medium led to the identification of *TUP1* (thymidine uptake) (Wickner, 1974). As is the case for *ssn6* strains, the *SUC2* gene is expressed constitutively in *snf1Δtup1Δ* and *tup1Δ* strains with its expression being insensitive to glucose repression (Carlson *et al.*, 1984; reviewed in Williams and Trumbly, 1990). Mutations in either *SSN6* or *TUP1* lead to other common phenotypes, including slow growth, flocculation, decreased sporulation, and loss of mating in α strains. Many of these phenotypes result from derepression of certain classes of genes (reviewed in Smith and Johnson, 2000). Based on a DNA microarray analysis of gene expression profiles of wild type and *tup1Δ* cells, the Ssn6-Tup1 co-repressor complex appears to contribute to repression of >150 or about 3% of *Saccharomyces cerevisiae* genes (DeRisi *et al.*, 1997). Experimental evidence suggests that the Ssn6-Tup1 co-repressor complex mediates repression by interacting with specific DNA-binding proteins at different promoters (reviewed in Smith and Johnson, 2000, also see below). These DNA-binding proteins include α2, Mig1, Crt1, and Rox1, which are proteins that recognise promoters of cell-type specific, glucose repressible, DNA-damage-inducible, and hypoxia-induced genes, respectively (Tzamarias and Struhl, 1995; reviewed in Smith and Johnson, 2000). The Ssn6-Tup1 complex is recruited by proteins with different DNA-binding motifs that show no appreciable amino acid similarity to each other (reviewed in Smith and Johnson, 2000). Thus an obvious question is how is Ssn6-Tup1 able to specifically interact with these proteins. The characterisation of both Ssn6 and Tup1 will be discussed below, but first, I will outline a few key experiments that elucidated the structure of the Ssn6-Tup1 complex and I will briefly discuss how the complex is thought to function as a whole.

Ssn6 and Tup1 can be co-immunoprecipitated and have been shown to associate in a complex (Williams *et al.*, 1991). Complexes reconstituted from *in vitro* synthesized proteins and epitope-tagged complexes immunoprecipitated from cells have been analysed in a number of ways to determine their molecular weights. It appears that Tup1 and Ssn6 are present in a 4:1 ratio and that no other proteins are present in the complex (Smith and Johnson, 2000; reviewed in Varanasi *et al.*, 1996).

**1.C.ii An overview of repression by Ssn6 and Tup1.** In general, Tup1 is believed to provide the repression function, whereas Ssn6 allows the complex to interact with DNA-binding proteins. To investigate the repression abilities of both Ssn6 and Tup1, chimeric fusion proteins were

tested for their ability to repress heterologous reporter genes. A LexA-Ssn6 fusion protein was found to efficiently repress a heterologous reporter gene containing LexA binding sites. This repression was abrogated in *tup1Δ* cells (Keleher *et al.*, 1992). A LexA-Tup1 fusion protein also leads to significant repression of a reporter gene containing LexA binding sites, but this repression is maintained in an *ssn6Δ* strain (Tzamarias and Struhl, 1994). Through deletion analysis, and by making use of the LexA-Ssn6 and LexA-Tup1 chimeras, Tzamarias and Struhl (1994) identified the regions of both Ssn6 and Tup1 that are required for repression. Regions of Ssn6 and Tup1 that are required for complex formation were also identified by a yeast two-hybrid approach (Fields and Song, 1989). The region of Ssn6 that interacts with Tup1 is the same as that required for LexA-Ssn6-directed repression (see above). However, the Ssn6-interacting region of Tup1, the first 72 N-terminal amino acids, is separable from the two repression regions of Tup1 (Tzamarias and Struhl, 1994). Based on the above results, it was proposed that Ssn6 acts as an adaptor between the repressor complex and DNA-binding proteins and that Tup1 provides the repression function. The overall picture is likely a bit more complicated; for instance, an examination of Tup1 deletion derivatives (no LexA domain) at native promoters (including *SUC2*) revealed that the repression and the Ssn6 interaction domains are only partially sufficient for Tup1 function (see below). Other regions of Tup1 with unknown functions are also required for full repression (Tzamarias and Struhl, 1994).

**1.C.iii Analysis of Ssn6 and Tup1.** Characterisation of Ssn6 and Tup1 revealed motifs that are thought to be involved in protein-protein interactions. Ssn6 contains tetratricopeptide (TPR) motifs which make up its functional region. The WD repeats of Tup1, which fall outside the repression and Ssn6-interacting regions seems to allow Tup1 to interact with DNA-binding proteins.

The amino-terminal region of Ssn6, a 107 KDa protein, contains ten tandem repeats of the 34 amino-acid tetratricopeptide (TPR) motif (Sikorski *et al.*, 1990; reviewed in Schultz *et al.*, 1990). A C-terminal truncated Ssn6 that has only the TPR repeats retains wild-type function. Therefore, the TPRs make up the functional region of Ssn6 (Schultz *et al.*, 1990). The TPR motif, although degenerate, shows remarkable evolutionary conservation, being present in bacteria, fungi, insects, plants, and animals. It is present in a number of unrelated proteins that are involved in various processes including cell cycle control (part of the Anaphase Promoting

Complex, or APC), transcription (Ssn6-Tup1 complex), and protein folding (a number of co-chaperones contain TPR motifs) (Blatch and Lassle, 1999). The crystal structure of the three TPR repeats of protein phosphatase 5 (PP5) has been solved (Das *et al.*, 1998). Secondary structure predictions and the crystal structure of PP5 TPR repeats reveal that each TPR motif is composed of two antiparallel α-helical domains, A and B. It has been proposed that helices from tandem TPRs generate a right handed super-helical structure with an amphipathic channel that may accommodate target α-helices (Blatch and Lassle, 1999; reviewed in Das *et al.*, 1998). It is not yet clear how TPR motifs allow for protein-protein interactions, but presumably the tandem nature of the repeats and the degenerate nature of each repeat allow for multiple and diverse protein-protein interactions (reviewed in Blatch and Lassle, 1999).

For Ssn6, experimental evidence suggests that different TPRs (and to various extents) are involved in mediating different protein-protein interactions (Tzamarias and Struhl, 1995). For example, glucose repression by Ssn6 relies on TPR8, TPR9 and maybe TPR10, whereas repression of oxygen repressible genes by Ssn6 requires TPRs4-7 (Tzamarias and Struhl, 1995). Other experiments suggest that each of the TPR repeats of Ssn6 can weakly interact with target proteins, and enhanced specificity and affinity are achieved through multiple interactions between target proteins and different TPRs (Smith *et al.*, 1995). In theory, weak, multiple interactions would also allow for greater flexibility. Given the different contexts of promoters and 10 TPRs, Ssn6 can probably take on different orientations and still make the necessary contacts with DNA-binding proteins. Different promoter contexts could account for the preferential use of some TPRs by certain DNA-binding proteins, as described by Tzamarias and Struhl (1995).

Tup1, a 78 kDa protein, contains seven WD-40 repeats near its C-terminus (Komachi and Johnson, 1997). WD proteins, so named because they usually end in Trp-Asp (WD), appear to be confined to eukaryotes. WD repeats are also thought to mediate protein-protein interactions and have been found in proteins linked to diverse cellular functions. These functions include signal transduction (e.g. the Gβ subunit of heterotrimeric G proteins), RNA-processing, transcriptional regulation (e.g. TFIID subunit of RNAPII, Tup1), cytoskeletal assembly and others (Smith *et al.*, 1999). The crystal structure of Gβ, the best characterised of WD proteins, has been solved (Lambright *et al.*, 1996; Sondek *et al.*, 1996; Wall *et al.*, 1995). Each of its seven WD repeats corresponds to four β-strands, with the overall motif forming a seven-bladed

β-propeller fold and assuming a donut shape. The top, the bottom and the circumference of the donut are all potential interacting surfaces (references as above and as reviewed in Smith *et al.*, 1999).

Recruitment of the Ssn6-Tup1 co-repressor complex by DNA-binding proteins is thought to occur mainly through Ssn6 with Tup1 providing the repression domain. Both the repression and Ssn6-interacting regions of Tup1 are outside the WD repeats. However, the WD repeats of Tup1 can interact with α2 and these interactions are physiologically relevant (Komachi *et al.*, 1994). More detailed experiments revealed key residues of WD repeats of Tup1 that affect α2-mediated repression by interfering with a Tup1-α2 interaction (Komachi and Johnson, 1997). Point mutations in Tup1 that affect repression at the α2 operator also lead to decreased repression through other promoters; these include the promoters of *SUC2* (glucose-repressed), *ANB1* (hypoxia-induced), and *RNR2* (DNA damage-inducible) (Komachi and Johnson, 1997; Tzamarias and Struhl, 1994).

Given that each TPR and WD motif can individually interact with α2, one can extrapolate that both proteins allow the complex to interact with target DNA-binding proteins (Komachi *et al.*, 1994; also see above). Genetically some truncations and or mutations in either Ssn6 or Tup1 may not appear to significantly impair the complex's function. One reason may be the versatility and flexibility of this complex with both Ssn6 and Tup1 interacting with and being recruited by target proteins.

**1.C.iv Mechanisms of repression by the Ssn6-Tup1 complex.** Experimental evidence suggests that Ssn6-Tup1 achieves repression through two major mechanisms: 1) altering the local chromatin structure, and 2) directly interacting with components of the RNAPII transcription machinery (reviewed in Smith and Johnson, 2000). A third possible mechanism has been proposed for repression by Tup1; interference with activators (Gavin *et al.*, 2000; Huang *et al.*, 1997). Evidence for this mechanism is limited and will be discussed within the first two sections.

**1.C.iv.a Altering the local chromatin structure.** Early experiments demonstrated the presence of positioned nucleosomes at some Ssn6-Tup1 repressed genes. Subsequently the positioning of stable nucleosomes has been linked to repression by the Ssn6-Tup1 complex. Experiments

designed to elucidate how a-specific genes are repressed in α-cells gave the first insights into the mechanisms of Ssn6-Tup1 repression. In α cells, binding of the α2-Mcm1 complex at the α2 operator of the *STE6* and *BAR1* genes was found to correlate with the establishment of stable, precisely placed nucleosomes over essential promoter elements (Shimizu *et al.*, 1991). All endogenous α2 operators that have been analysed show this well organised nucleosomal array in α, but not a cells (Gavin *et al.*, 2000). This positioning effect has been noted on plasmid DNA and on reporter genes containing the α2 operator. Thus, nucleosomal positioning around the α2 operator appears to be context-independent, perhaps hinting that the positioning of nucleosomes may be important in repression (Morse *et al.*, 1992; Roth *et al.*, 1990; Simpson, 1990).

A link between repression by Ssn6-Tup1 and the positioning of a stable nucleosomal array was suggested by the observation that nucleosomal positioning around the *STE6* promoter region is disrupted in *ssn6* or *tup1* α cells (Cooper *et al.*, 1994). Stable, precise positioning of nucleosomes and full repression through the α2 operator also requires the N-terminal tail of histone H4 (Roth *et al.*, 1992). A direct interaction between Tup1 and intact amino termini of histones H3 and H4 has been demonstrated. Deletion of the N-termini of histones H3 or H4 or mutations of specific residues in the N-terminus of H4 lead to the partial derepression of a-cell specific and DNA damage-inducible genes. (Edmondson *et al.*, 1996). The repression domain of Tup1, which maps between amino acids 72 to 385, substantially overlaps with the H3 and H4 histone-binding-domain of Tup1 (Edmondson *et al.*, 1996; Tzamarias and Struhl, 1994). A repressive chromatin structure has also been implicated at other Ssn6-Tup1 repressed genes, such as the glucose repressible gene, *SUC2* (Matallana *et al.*, 1992). The above experiments suggest that Ssn6-Tup1 mediated repression involves the interaction of Tup1 with histones H3 and H4, and that this interaction contributes to the establishment of a repressive nucleosomal structure.

Is chromatin remodelling generally associated with Ssn6-Tup1-mediated repression? In contrast to the above, repression through the a1-α2 operator, which is also mediated through the Ssn6-Tup1 complex and keeps expression of haploid-specific genes off in diploid cells, does not appear to require precisely positioned nucleosomes. In the context of a series of related yeast plasmids, for which normal nucleosomal positioning has been determined, nucleosomal positioning around the α2, but not the a1-α2 operator can be readily demonstrated (Huang *et al.*, 1997; Roth *et al.*, 1990). However, the same mutations in histones H3 and H4 that lead to derepression of a-specific genes also lead to derepression of haploid-specific genes. In the

context of the *CYC1-lacZ* heterologous reporter, the presence of the a1-α2 operator leads to decreased activator binding to UAS sequences. A more general, repressive aspect of chromatin that may prevent activator binding might be employed for repression of haploid-specific genes. In this case the degree of chromatin organisation may not be so crucial. The positioning of nucleosomes at a-specific genes may depend on the extent of Ssn6-Tup1 binding and its architecture as dictated by the particular promoter. For example, the authors point out that two molecules of α2 associate at the α2 operator, whereas only one α2 protein binds at the a1-α2 operator (Huang *et al.*, 1997). More contact or varied sites for the Ssn6-Tup1 complex may facilitate the establishment of positioned nucleosomes around an operator.

*Linking the modification of histones to Tup1-mediated repression.* The acetylation state of histones may also be important for Ssn6-Tup1-mediated repression. The use of a chromatin immunoprecipitation (ChIP) approach with antibodies specific to certain acetylated isoforms of histones H3 and H4 showed that a strain that contains mutations in three histone deacetylases, Rpd3, Hos1, and Hos2, has increased acetylation of histones H3 and H4 at Tup1-repressed promoters. This strain is also defective in repression of the Tup1-repressed genes, *MFA2* and *SUC2*. A combination of mutations in other histone deacetylases led to different acetylation patterns and did not lead to derepression of Ssn6-Tup1 repressed genes (Watson *et al.*, 2000). Because Tup1 does not bind well *in vitro* to hyperacetylated histones H3 and H4, it has been proposed that Tup1 interacts with hypoacetylated histones H3 and H4 and then promotes nucleosomal positioning (Edmondson *et al.*, 1996). However, Watson and colleagues (2000) did not check for Tup1 occupancy of relevant promoters in the *rpd3hos1hos2* mutant background. Other experiments suggest that Tup1 is recruited to promoters and in turn recruits Hda1 to deacetylate histones. Deacetylation by Hda1 is important for repression at representative Tup1-regulated promoters. A direct *in vitro* interaction has also been demonstrated between Tup1 and Hda1 and Hda3, specifically between the N-terminal region of Tup1, which contains the repression domain, and Hda1 (Wu *et al.*, 2001). Considering that similar techniques were employed by the two research groups, it is difficult to reconcile the conflicting data as to which histone deacetylases are important.

*The importance of positioned nucleosomes and how they may affect transcription.* It is believed that stable, precisely placed nucleosomes (over regulatory regions) interfere with the abilities of activators and/or the general transcription machinery to contact promoter elements. A direct

examination of the binding of TBP (TATA binding protein) to promoter elements, by a Chromatin Immunoprecipitation (ChIP) approach, revealed a strong correlation between TBP occupancy at the promoter and the level of transcription of the respective gene. TBP occupancy at the Ssn6-Tup1 regulated promoters, *ANB1*, *SUC2*, and *MFA1*, is at background levels during repressive conditions. In a *tup1* strain, TBP occupancy increases (Kuras and Struhl, 1999). Thus, one way that Ssn6-Tup1 appears to exert repression is to deny TBP access to certain promoters, perhaps by helping to establish a repressive nucleosomal structure. There is also evidence to suggest that Ssn6-Tup1 acts by blocking the chromatin remodelling activity of activators (Gavin *et al.*, 2000). In contrast, it has been demonstrated that Ssn6-Tup1 cannot prevent the Gal4 activator from binding, yet robust repression is still achieved (Redd *et al.*, 1996; also see below).

Positioned nucleosomes, although present during repressive conditions, may not be as crucial for the repression of the hypoxic gene, *ANB1*. Nucleosomes, present over the TATA box in wild type cells, are absent in *rox1Δ* or *tup1Δ* cells and this does correlate with some degree of derepression. However, deletion of the N-terminal region of histone H4 does not lead to derepression even though nucleosomes are lost over the previously protected TATA box region (Deckert *et al.*, 1998; Kastaniotis *et al.*, 2000). Either repression at the *ANB1* promoter relies solely on another mode of repression, or chromatin remodelling plays a redundant role.

The importance of nucleosomes and an organised chromatin structure has been best demonstrated at the *STE6* promoter and coding region in α cells (see above). To determine more precisely the involvement of Tup1, the extent of Tup1 binding and the stoichiometry of Tup1 with assembled chromatin has been investigated (Ducker and Simpson, 2000). The authors demonstrated that Tup1 binding extends from the α2 operator to the 3' end of the *STE6* gene and that two molecules of Tup1 associate with each positioned nucleosome. Other investigators, however, find Tup1 limited to the promoter region, or detect Tup1 binding from the promoter region to the start of transcription of the *STE6* gene (Cassidy-Stone and Johnson, 2000; Wu *et al.*, 2001).

Even at promoters where the phasing of nucleosomes has been demonstrated, its correlation with repression is not absolute. As outlined above, at some promoters, disruption of nucleosomal phasing leads only to partial derepression, whereas at the *ANB1* promoter, it

appears dispensable. Clearly, the Ssn6-Tup1 co-repressor complex utilises other methods as well to achieve full repression.

**1.C.iv.b Interaction of the Ssn6-Tup1complex with the transcription machinery.** Experiments to determine if α2-repressed promoters are accessible to activators demonstrated that *in vivo*, robust repression is possible in the absence of a repressive nucleosomal structure. As outlined above, positioned nucleosomes may block TBP's access to the TATA box and thus prevent transcription. At the *CYC1* promoter, this correlation does not seem to hold; both DNase I footprinting and ChIP (chromatin immunoprecipitation) with HA-tagged TBP indicate the association of TBP with the *CYC1* TATA box at low gene transcription levels (Chen *et al.*, 1994; Kuras and Struhl, 1999). Previous experiments have indicated that nucleosomal positioning around the α2 operator is context-independent (see previous section). However, there is one exception. Positioning the α2 operator upstream of the *CYC1* minimal promoter (plasmid or chromosomal context) does not lead to the precise positioning of nucleosomes over the test promoter. A Gal4 binding site, positioned either upstream or downstream of the α2 operator, is also accessible during repressive conditions. It is possible that the persistent association of TBP with the TATA box blocks nucleosomal positioning. Despite the absence of positioned nucleosomes, repression through this reporter is robust, comparable to native, a-specific gene repression. Thus the α2 operator, which relies on the Ssn6-Tup1 complex for repression, must interfere with transcription after binding of the Gal4 activator and in the absence of positioned nucleosomes (Redd *et al.*, 1996).

Modest Ssn6-Tup1 mediated repression has been demonstrated *in vitro*, with requirements that mirror *in vivo* conditions. The test promoter employed in the *in vitro* assay contained two α2 operators upstream of a minimal *CYC1* promoter. Modest repression through this promoter was demonstrated when purified α2 protein was added to whole cell extracts from cells that had over-expressed Ssn6 and Tup1. The requirements of the *in vitro* repression system indicated that certain key *in vivo* conditions had been mimicked: the absolute requirement for α2 protein, and enrichment for Ssn6 and Tup1. The transcription system did not include a chromatin assembly step, indicating that repression can occur in the absence of chromatin organisation (Herschbach *et al.*, 1994). It appears that in the *in vitro* and *in vivo* experiments outlined above repression occurs through a direct interference with the general transcription

machinery. The Ssn6-Tup1 complex may interfere with the assembly of the machinery, or prevent initiation and/or elongation.

Genetic evidence supports the notion that Ssn6-Tup1 interferes with the RNAPII machinery. A genetic screen designed to uncover genes required for α2-mediated repression uncovered, as expected, alleles of *SSN6*, *TUP1*, and alleles of *SRB8*, *SRB10*, and *ROX3*, genes that encode for components of the RNA Polymerase II (RNAPII) holoenzyme (Carlson, 1997; reviewed in Wahi and Johnson, 1995). Sin4, another component of the RNAPII holoenzyme has also been previously linked to α2 mediated repression (Chen *et al.*, 1993). Suppressors of the *snf1* defect for *SUC2* derepression include mutations in *MIG1* (the DNA binding protein thought to recruit Ssn6-Tup1), *SSN6*, *SRB8-SRB11*, *SIN4*, and *ROX3* (Carlson *et al.*, 1984; Kuchin *et al.*, 1995; Song *et al.*, 1996). *SIN4*, *ROX3*, and *SRB8-11* encode genes that associate with the carboxy-terminal domain (CTD) of RNAPII, a portion of the holoenzyme that is required by a number of regulatory proteins (reviewed in Carlson, 1997). Srb10 and Srb11 function as a cyclin-dependent kinase (cdk)/cyclin pair, contributing to the phosphorylation of the CTD *in vitro* (Liao *et al.*, 1995). Phosphorylation of the CTD may prevent the transition from initiation to elongation (reviewed in Carlson, 1997). Repression by LexA-Tup1 or LexA-Ssn6 at a heterologous promoter is partially impaired in *srb10Δ*, *srb11Δ* and *ctk1Δ* strains (Kuchin and Carlson, 1998). Ctk1 is functionally related to Srb10 and with Ctk2, it also contributes to the phosphorylation of the CTD. The two cdk/cyclin pairs are not interchangeable, but rather have unique roles in repression and/or activation. The genetic interaction of LexA-Tup1 and LexA-Ssn6 with both cdk/cyclin pairs may reinforce the effect on the CTD or may allow Ssn6-Tup1 to differentially influence both modes of phosphorylation.

An interaction, by genetic and biochemical approaches, has been demonstrated between the Ssn6-Tup1 complex and Hrs1/Med3, a subunit of the Mediator that forms part of the RNAPII holoenzyme (Papamichos-Chronakis *et al.*, 2000). Various experiments suggest that the Ssn6-Tup1 complex may interact with Hrs1 and interfere with the ability of Hrs1 to interact with activators and recruit the RNAPII holoenzyme.

A more direct, physiologically relevant target of Tup1 appears to be Srb7, an essential holoenzyme component (Gromoller and Lehming, 2000). An *in vivo* interaction between Tup1 and Srb7 was demonstrated via the split-ubiquitin assay. To detect an interaction between Srb7 and Tup1 via this assay, Srb7 was fused to the C-terminal portion of ubiquitin, which in turn

contained a reporter gene, the green flourescent protein (GFP). Tup1 was fused to the N-terminal portion of ubiquitin. Expressing both proteins in the same cell led to the rapid degradation of the otherwise stable GFP reporter protein. 14 other holoenzyme components that were tested, including Srb10, showed no interaction with Tup1. Srb7 also interacts with Med6, another holoenzyme component. Tup1 and Med6 appear to compete for Srb7 since overexpressing Tup1 leads to a decreased Med6-Srb7 interaction. Thus, Tup1 binding to Srb7 may prevent a Med6-Srb7 interaction and interfere with transcription activation.

Recently, a role for the RNAPII holoenzyme, and in particular the Srb10/11kinase-cyclin pair in Tup1-mediated repression has been reaffirmed (Zaman *et al.*, 2001). Through the artificial recruitment of Tup1 and various activators, it has been demonstrated that classical activators (e.g. Gal4) are able to overcome Tup-1 mediated repression to a greater degree than nonclassical activators (e.g. Gal11)[1]. Telomeric repression, however, is equally effective against both types of activators. Furthermore, only Tup1-mediated and not telomeric repression shows Srb10/11 dependence in this context. The kinase activity is crucial, as a single mutation in the kinase domain abolishes Tup1-dependent repression. Based on genetic and biochemical evidence, the authors proposed that the interaction of Tup1 with Srb10 may destabilise the holoenzyme. Tup1-mediated repression may be less effective against classical activators for two major reasons: first, classical activators make a number of contacts with the transcriptional machinery and thus they may counteract the destabilising effect of Srb10's kinase action and allow transcription. Second, classical activators can also recruit histone acetyltransferase complexes and their actions may diminish Tup1's access to a promoter.

Finally, at different promoters the Ssn6-Tup1 co-repressor complex may interact differently with the basal transcription machinery. Srb10, implicated in repression through glucose-repressed genes (*SUC2*) and a-cell type specific genes (*MFA1*), does not play a major role in repression of *ANB1* (Kastaniotis *et al.*, 2000). In other independent experiments, Srb8-11 mutations (individually or combined) only affected *SUC2* expression and not *MFA1*, *MFA2* (a-cell specific genes), *ANB1* (oxygen pathway) or *RNR2* (DNA damage inducible gene) expression (Lee *et al.*, 2000).

[1] Classical activators, e.g. Gal4, Hap4, Gcn4, VP16, have acidic activating regions capable of contacting a number of targets in the transcriptional machinery. Furthermore, they are also believed to recruit histone acetyltransferase complexes to facilitate transcription. Nonclassical activators, e.g. Gal11, are components of the transcriptional machinery with DNA binding domains.

**1.C.iv.c Concluding remarks on Ssn6-Tup1 mediated repression.** How important are the two major forms of repression and how much redundancy exists between the two modes of repression? Do the present models account fully for repression by the Ssn6-Tup1 co-repressor complex? It is difficult to compare results from different experimental designs, especially with different promoter contexts, and arrive at a clear picture. Recently, the effects of combined mutations between certain Srbs (Srb8-11), Sin4 and deletions in histone tails H3 and H4 on natural promoters have been assessed (Lee *et al.*, 2000). Individually, mutations in the components of the RNAPII holoenzyme and deletions of the N-tails of histones H3 and H4 have only modest effects on repression by Ssn6-Tup1 (see above). If the uncovered components of Ssn6-Tup1-mediated repression represent redundant pathways, then the combination of different mutations should result in a synergistic loss of repression. Different combinations of mutations, however, did not reveal such a synergistic effect. It is possible that there is so much redundancy that a number of different pathways need to be disabled before full derepression occurs. However, the construction of strains containing multiple mutations is not feasible since certain combinations are severely debilitating to the cell. Another possible reason for the lack of synergy may be that chromatin remodelling is more important. However, removing all potential histone tail interacting sites might be lethal for the cell. Finally, another method of repression, not yet revealed by mutations, may also contribute to full repression. Given the versatility and robust nature of Ssn6-Tup1-mediated repression, it seems reasonable that redundancy and complexity would underlie the mechanism.

## 1.D Background on Rim101, a transcriptional regulator

As outlined above, repression through $NRE^{DIT}$, a DNA element required for repression of the *DIT1* and *DIT2* genes, is Ssn6-Tup1 dependent. A genetic screen, designed to identify mutants defective at repression through $NRE^{DIT}$ yielded, as expected, alleles of *SSN6* and *TUP1* as well as alleles of *ROX3* and *SIN4*[2]. Rox3 and Sin4 are general regulators of transcription that are thought to associate with the RNAPII holoenzyme (see above). Unexpectedly, *SPE3*, which

[2] *FRD10* is most likely an allele of *SIN4*, even though this has not been conclusively demonstrated through linkage analysis. *SIN4* is required for full repression through $NRE^{DIT}$ (Tanny, M.Sc. thesis, 1998b).

encodes spermidine synthase, was also identified. Spe3-dependent repression may reflect effects of spermidine on chromatin structure (Friesen *et al.*, 1998; Tanny, 1998b). *RIM101* was the only gene identified in this screen whose mutant allele led to $NRE^{DIT}$-specific loss of repression.

**1.D.i Characterisation of Rim101 and its involvement in meiosis.** Rim101, previously called Rim1, was initially identified in a screen for mutations that cause decreased expression of an *ime2-lacZ* fusion gene (hence Regulator of *IME2*). Subsequently, it was demonstrated that defects in *RIM101*, *RIM8*, *RIM9*, and *RIM13* diminish *IME1*expression. The *IME1* gene product, a key transcriptional activator required for entry into sporulation, contributes to the upregulation of a number of early meiotic genes, including *IME2*. Genetic analysis has revealed that besides the Rim pathway, three other pathways contribute to *IME1* expression (Li and Mitchell, 1997; Su and Mitchell, 1993a; Su and Mitchell, 1993b). The regulatory region of *IME1* is complex, encompassing at least 2 kb and responding to a number of signals: cell type, carbon source and nitrogen depletion (Sagee *et al.*, 1998). Although Rim101 is required for both basal and upregulated expression of *IME1*, it has not been demonstrated that it acts directly on the *IME1* regulatory region. Its effect on *IME1* expression may be indirect.

Attempts to show that Rim101 is an activator or a repressor of gene expression by the use of chimeric proteins have been unsuccessful. The ability of full-length Rim101 and portions of Rim101 fused to a heterologous DNA-binding domain to activate expression of a reporter gene *in vivo* has been assessed in the laboratory of Dr. A. Mitchell (Xu and Mitchell, personal communication). Jason Tanny tested the ability of a protein containing the LexA DNA-binding domain fused to Rim101 to prevent transcription *in vivo*. Only slight repression was observed. However, it should be noted that the fusion protein was not fully active (Tanny, M.Sc. thesis, 1998). Thus at this time there is no direct evidence that Rim101 can act on its own as an activator or repressor of gene expression.

Mutations in *RIM101*, as well as a null allele, lead to four major phenotypes: decreased sporulation efficiency, cold sensitivity, altered colony morphology, and defective haploid invasive growth (Li and Mitchell, 1997; Su and Mitchell, 1993b). The *RIM101* nucleotide sequence revealed that Rim101 contains three amino terminal zinc-finger motifs of the $Cys_2$-$His_2$ type and a carboxy-terminal acidic region (Su and Mitchell, 1993b). Mutations in the second cysteine residue of each zinc-finger abolish Rim101 activity whereas some C-terminally

truncated Rim101 proteins are still functional. Thus, the zinc-fingers, but not the acidic C-terminus, are essential for Rim101 function.

The $Cys_2$-$His_2$ type of zinc-finger motif is one of the most common eukaryotic DNA-binding motifs. The 31-amino acid consensus motif forms a globular structure, consisting of an anti-parallel β-sheet and an α-helix. Two cysteine residues in the β-sheet region and two histidines within the α-helical region co-ordinate the Zn ion, which is nestled in a hydrophobic core (Lee *et al.*, 1989; Parraga *et al.*, 1988). Although it is not possible to predict the target sequence site from the primary amino acid sequence of a Zn-finger, solved crystal structures of a few zinc-finger-DNA complexes give some guidelines. Generally, site-specific DNA-binding requires two or more zinc-fingers with each finger contacting three to five bases in the major groove. The amino acids shown to contact specific DNA-bases usually reside in the α-helical region. Finally, some zinc fingers do not contact DNA but rather stabilise the conformation of the DNA-binding protein (Berg and Shi, 1996; Fairall *et al.*, 1993; Pavletich and Pabo, 1991; reviewed in Pavletich and Pabo, 1993).

Although Rim101 does not show extensive similarity to known proteins (Tanny, 1998b), the three zinc-fingers are highly similar to those found in a group of fungal proteins. These proteins are involved in regulation of genes in pH-response pathways (Denison, 2000; Lambert *et al.*, 1997; Tilburn *et al.*, 1995; reviewed in Wilson *et al.*, 1999). The best characterised of these proteins, PacC of *Aspergillus nidulans*, is 57% identical and 65% similar to Rim101 within the zinc-finger region (Tanny, 1998b; Tilburn *et al.*, 1995). PacC is a repressor and an activator of acid- and alkaline-expressed genes, respectively (Tilburn *et al.*, 1995). A model for how PacC contacts its cognate site has been proposed. This model is based on the crystal structures of solved zinc-finger protein-DNA complexes, especially the human oncoprotein GLI, as well as biochemical evidence from mutagenesis of selected residues in the PacC α helices (Espeso *et al.*, 1997; Tilburn *et al.*, 1995). Fingers two and three of PacC appear to be responsible for its DNA binding whereas finger one is most likely involved in intramolecular interactions to stabilise the protein. The most extensive identity between Rim101 and PacC is within the predicted α-helical regions of fingers two and three, the regions believed to be responsible for the specificity of DNA-binding. Except for one residue, the α-helical regions of the last two fingers are identical. One other peculiar feature of PacC that is also present in Rim101 is an extended linker region between fingers 1 and 2 that is almost identical between the two proteins. Given the extent of

identity between the zinc-fingers, it appeared plausible that Rim101 would recognise the same DNA site as PacC. Sequence inspection revealed that the high affinity binding site of PacC, 5'-TGCCAAGA-3', is present in the downstream portion of $NRE^{DIT}$ (Tanny, 1998b). In our lab, we refer to this as the $PacC^{DIT}$ site. There is much evidence to suggest that the regulation of the activity of PacC, Rim101 and other PacC homologues is similar, further strengthening our hypothesis that Rim101 is a transcriptional regulator acting through the $PacC^{DIT}$ site.

## 1.E Regulation of PacC, Rim101 and other homologues

**1.E.i The pH response pathway in *Aspergillus nidulans*.** The filamentous ascomycete, *Aspergillus nidulans* (*A. nidulans*) is able to adapt to a wide range of pH environments. By regulating the synthesis of permeases, secreted enzymes, and metabolites, such molecules are only synthesized if the ambient pH will allow them to function (reviewed in Denison, 2000). The pH response pathway, which was first characterised in *A. nidulans*, appears to be present in a wide range of fungi, including *S. cerevisiae*.

Genes encoding components of the pH response pathway in *A. nidulans* were first identified because their mutant alleles cause defects in the production of phosphatases (in phosphate minimal medium). Depending on their deficiencies, strains were classified as *pal*, alkaline phosphatase deficient, or *pac*, acid phosphatase deficient mutants (Dorn, 1965; reviewed in Denison, 2000). Later, it was demonstrated that some of these *pal* mutants share other pH dependent-phenotypes, underscoring the importance of pH regulation of a number of genes, not just those encoding phosphatases. Mutations in certain *pal* genes, such as *palA*, *B*, *C*, and *F*, mimic growth at acidic pH. In an alkaline environment these mutants behaved as if their ambient pH were acidic. Conversely, certain *pacC* mutations are alkaline mimicking. Because these mutations do not directly impact on the pH homeostatic mechanism, it was proposed that they regulate the expression of genes in response to pH. It was hypothesized that the products of the *palA*, *B*, *C*, and *F* genes are involved in a pathway that affects the synthesis or activity of a transcriptional regulator, the *pacC* product (Caddick *et al.*, 1986). Two other *pal* genes, *palH* and *palI* have since been identified as components of this pathway. Compared to wild-type, *palI* mutants grow more slowly at pH 8 whereas *palA*, *B*, *C*, *F* and *H* mutants do not grow at pH 8. Double *palI palA-H* mutants do grow slowly at pH 8. Genetic analyses suggest that mutations in

*palA, B, C, F* and *H* are epistatic to mutations in *palI* (Arst *et al.*, 1994). The order of action of *pal* gene products, however, has been difficult to determine with the exception that *pacC* appears to act furthest downstream in the pH pathway (Caddick *et al.*, 1986). This is consistent with the hypothesis that *pacC* encodes a transcriptional regulator, a hypothesis supported by a wealth of experiments. For example, it has been demonstrated that pH conditions and certain mutations have a direct impact on certain mRNA transcript levels (Espeso *et al.*, 1993; Tilburn *et al.*, 1995).

**1.E.ii Characterisation of PacC, a transcriptional regulator.** Cloning of the *pacC* gene indicated that the encoded 674 amino acid protein contains three putative N-terminal zinc fingers. Outside the zinc finger region, PacC does not show significant sequence similarity to other proteins (Tilburn *et al.*, 1995). Characterisation of PacC has helped explain why some mutations in *pacC* are acidity mimicking whereas other mutations are alkaline mimicking. The zinc fingers are essential for DNA binding and presumably for transcriptional regulation whereas the C-terminus is required for pH specific response (Orejas *et al.*, 1995 and references in this paragraph). A more detailed analysis of the promoter of the isopenicillin N synthase (*ipnA*) gene helped identify a PacC recognition sequence. A bacterially expressed GST-fusion polypeptide containing the PacC zinc fingers binds to the *ipnA* promoter region *in vitro*. Four different kinds of footprinting techniques and mutational analysis of the promoter region identified 5'-GCCARG-3' as a core consensus site that is protected by the PacC zinc fingers. The three PacC binding sites within the *ipnA* promoter were shown to be necessary and sufficient to confer pH-dependent expression *in vivo* (Espeso and Penalva, 1996; Tilburn *et al.*, 1995). A more detailed analysis revealed that bases flanking the core sequence, 5'-T**GCCAAGA**-3' (core sequence bolded), are required for high affinity binding (Espeso *et al.*, 1997).

In summary, the current hypothesis is that products of the *pal* genes (*palA, B, C, F, H,* and *I*) mediate the pH signal, affecting the activity of PacC, the transcriptional regulator. In response to alkaline ambient pH, PacC is activated; in its active form it represses acid-specific genes and activates the expression of alkaline-specific genes.

**1.E.iii Activation of PacC through carboxy-terminal proteolytic cleavage.** Under alkaline pH conditions, PacC is converted to a transcriptional regulator with the removal of about 400 C-

terminal amino acids. Electrophoretic mobility shift assays testing for binding of proteins in a crude extract of *A. nidulans* with the high affinity *ipnA2* PacC-binding site as probe revealed two complexes. By using anti-sera raised against the N-terminal and C-terminal portions of the PacC protein, it was shown that one of the protein-DNA complexes is formed with a PacC-derivative that lacks its C-terminus. Analysis of PacC present in crude extracts of cells grown at various pH conditions showed that a smaller version of the protein, ~27-29 kDa, predominates under alkaline growth conditions, whereas the full length protein, ~73 kDa, predominates under acidic growth conditions. Analysis of PacC present in various mutant strains indicated that the smaller, C-terminal truncated version of the protein predominates in alkaline-mimicking mutants, whereas proteolytic cleavage of PacC does not occur in acidity-mimicking *pal* mutants. Transient expression experiments have established that in the presence of an operational *pal* pathway full-length PacC is unstable, whereas the C-terminal truncated product is very stable (Mingot *et al.*, 1999). It is the truncated amino-terminal portion of the protein, the first ~248-250[3] amino acids, that is responsible for transcriptional regulation. The C-terminus of the protein is necessary for the proper pH-dependent activation of PacC (Mingot *et al.*, 1999; Orejas *et al.*, 1995; reviewed in Denison, 2000).

How is the proteolytic cleavage of PacC prevented under acidic growth conditions? How do the products of the *pal* genes contribute to PacC truncation and hence activation? The activity of the protease itself is not pH-dependent since gain-of-function mutations in PacC allow pH-independent processing (Denison *et al.*, 1995; Mingot *et al.*, 1999; Negrete-Urtasun *et al.*, 1997). A wealth of information from a plethora of *pacC* mutants has helped investigators not only to construct an overall view of the pH pathway but also to elucidate how PacC is activated. Mutations in *pacC* can be classified into three groups: alkalinity mimicking or gain-of-function mutations (*pacC*$^{c}$), acidity mimicking or loss of function mutations (*pacC*$^{+/-}$, or *pacC*$^{-}$ for a null allele), and neutrality-mimicking or constitutive-derepressed mutations (*pacC*$^{c/-}$) (Mingot *et al.*, 1999). Strains with neutrality-mimicking mutations in PacC do not respond to ambient pH conditions and exhibit both alkalinity- and acidity-mimicking phenotypes. Phenotypic and biochemical analyses of mutant strains and mutant proteins, respectively, have led to the following model: full-length PacC exists in two different types of conformations, "open" and

[3] Originially, *pacC* transcription was thought to start at Met1 to yield a 678 amino acid protein. However, as described in Mingot, *et al.*, 1999, the major translational product, which utilizes Met5, yields a 674 amino acid protein.

"closed". Under acidic pH conditions, intramolecular interactions between the N- and C-terminal moieties keep PacC in a "closed", or protease inaccessible conformation. In the presence of an operational *pal* pathway (e.g. wild-type cells at alkaline pH), PacC takes on an "open" conformation and proteolytic cleavage occurs (Espeso *et al.*, 2000; Mingot *et al.*, 1999). The gain-of-function phenotype caused by some C-terminal truncations in PacC is consistent with the notion that the C-terminus is critical for keeping full length PacC in a protease-inaccessible state in the absence of a pH signal. The protein appears to sense the pH and switch from a "closed" to an "open" conformation. The "open" vs. "closed" conformation model also predicts that certain point mutations or internal deletions might destabilize intramolecular interactions, leading to the constitutive processing of PacC. Other mutations might lock the protein into the "closed" conformation, making it inaccessible to the protease. Both types of mutations have indeed been identified. One-hybrid, two-hybrid and *in vitro* experiments show that there are three interacting regions: two regions downstream of the DNA-binding domain (zinc fingers), designated as A and B, that interact with region C near the C-terminus. Electrophoretic mobility supershift assays have confirmed interactions between wild type moieties; as expected from genetic analysis and depending on the type of mutation, critical mutations interfere with or enhance these interactions (Espeso *et al.*, 2000).

How does the protease recognise the proteolytic site? Cleavage normally occurs within the region from residues 231-260. Deletion of the normal proteolytic site, however, does not abolish cleavage but instead results in constitutive processing at a distinct site, giving a stable product indistinguishable in size from the processed wild-type PacC protein. This suggests that the sequence in the vicinity of the proteolytic site is not required to direct cleavage by the protease. The sequence at which cleavage normally occurs appears to protect against "unscheduled" proteolytic cleavage. Although the precise sequence has not been identified, analysis of deletion mutants suggests that the region upstream of amino acid 231 determines the specificity of the protease (Mingot *et al.*, 1999).

Analysis of mutant forms of PacC and GFP-tagged PacC moieties revealed that the pH signal also regulates the subcellular localization of PacC (Mingot *et al.*, 2001). Full-length PacC in the closed conformation is cytoplasmic, whereas the processed form is found in the nucleus. A small portion of the full-length form also has a nuclear localisation, but as revealed through mutational and biochemical analyses, the protein is in the open conformation. Proteolytic

processing is likely cytoplasmic, whereas nuclear localisation requires the protein to be in an open confirmation.

In conclusion, genetic and biochemical analyses have revealed that intramolecular interactions and a pH signal govern PacC's conformation and subcellular localisation. How pH and the *pal* gene products contribute to the conformational change, from "closed" to "open", is not yet understood.

**1.E.iv Regulation of Rim101 through C-terminal proteolytic cleavage.** In wild-type yeast cells, a C-terminally truncated form of Rim101 predominates. Mutations in *RIM8/9/13/20* or *RIM30* prevent proteolytic cleavage of Rim101. In such mutant strains, the overall levels of Rim101 are the same as in a wild-type strain but only the larger form of Rim101 accumulates. Analysis of HA-tagged, truncated forms of Rim101 revealed that the first ~530 amino acids (Rim101-HA2-531) of the 678 amino acid protein are sufficient for activity. The same truncation also leads to the suppression of mutations in *RIM 8, 9, 13, 20,* and *30.* This suggests that the products of the *RIM 8, 9, 13, 20,* and *30* genes are required for the proteolytic activation of Rim101. Consistent with this hypothesis, Rim101-HA2-531, does not undergo proteolytic cleavage in either wild-type or *rim* strains (Lamb *et al.*, 2001; Li and Mitchell, 1997; Xu and Mitchell, 2000).

In glucose grown cells, both the truncated and full-length forms of Rim101 are present, whereas in acetate grown cells only the truncated form of Rim101 is present. Increasing the glucose concentration of the medium, from 5% to 20%, leads to increased acidity of the medium as well as an increase in the full length form of Rim101. However, the amount of truncated Rim101 is not affected. Increased acetate concentrations maintain a neutral pH. Irrespective of the carbon source, acidic media promote the accumulation of both the full length and truncated versions of Rim101, whereas in more neutral pH media, only the truncated version of Rim101 accumulates. Thus, reminiscent of PacC regulation, external pH does have an effect on Rim101 regulation; however, this effect differs from and it is not as profound as for PacC. Whereas PacC cleavage is very much dependent on external alkaline pH, Rim101 is cleaved, regardless of ambient pH conditions (Li and Mitchell, 1997). Nonetheless, identity between *RIM101* and *pacC*, and between *RIM* and *pal* genes (see below) as well as the requirement for proteolytic cleavage of both Rim101 and PacC, suggests that a PacC-like pathway operates in *S. cerevisiae*.

However, this pathway may have evolved to undertake slightly different roles in the two organisms. In support of partial conservation, the processing protease does appear to be conserved between the two species. As outlined above, *pacC*$^{c}$ (gain-of-function mutations) strains bypass the need for the pH signal for processing of PacC. If the *A. nidulans pacC*$^{c}$ is expressed in *S. cerevisiae*, it is properly cleaved by an endogenous yeast protease, although some aberrant processing also occurs (Mingot *et al.*, 1999).

**1.E.v Proteins required for PacC and Rim101 processing in *A. nidulans* and *S. cerevisiae* are similar.** Analysis of one acidity-mimicking *pacC* mutant, unable to respond to a pH signal, suggests that amino acids 461-536 may be involved in pH signal transduction (Mingot *et al.*, 1999). It is not clear what kind of PacC modification occurs upon signalling through the *pal* genes. *A. nidulans* PalB appears to be a cysteine protease, similar to the catalytic subunits of calpains but lacks the calcium-binding domains. Although PalB is not responsible for the final proteolytic cleavage of PacC, it may lead to a more C-terminal proteolytic cleavage of PacC upon a pH signal. This may destabilise intramolecular interactions and facilitating full proteolytic cleavage of PacC (Denison *et al.*, 1995). Rim13 (also called Cpl1) is the only calpain-like gene in *S. cerevisiae*; it is 30% identical and 70% similar to the PalB cysteine-protease domain (Futai *et al.*, 1999). *In vitro* studies suggest that Rim13 does not act as a direct protease on Rim101 (Xu, 2000).

Two of the *pal* genes, *palI* and *palH*, code for putative membrane proteins, perhaps serving as sensors of ambient pH. PalI is predicted to have four transmembrane domains at its N-terminus, a region similar to the Rim9 protein in *S. cerevisiae* (Denison *et al.*, 1998; Li and Mitchell, 1997). The other predicted membrane protein, PalH, has seven putative N-terminal transmembrane domains and a large hydrophilic C-terminal tail. Its *S. cerevisiae* homologue, similar over transmembrane regions 2-6, is Rim30; no specific role has been assigned to Rim30 (Negrete-Urtasun *et al.*, 1999; Xu, 2000). Neither PalI nor PalH (or their homologues) have been located to the plasma membrane, so it is unknown, which (if either) may be involved in the primary signal reception.

An interaction, via the yeast-two-hybrid method, has been detected between Rim20 and the C-terminal region of Rim101 (Xu and Mitchell, 2000). The yeast protein most related to Rim20 is Bro1, a protein that contains an SH3 domain-binding motif. Mutations in *BRO1*

display similar phenotypes to perturbations in the protein kinase C (Pkc1)-MAP kinase pathway, a pathway required for maintenance of the cell wall. Bro1 may exert its effect by contacting SH3 domain containing proteins (e.g. Bem1) required for cell wall growth or maintenance (Nickas and Yaffe, 1996). PalA, is about 21% identical to Bro1, and also contains the SH3 domain-binding motifs, but again, a precise role for it has not been assigned (Negrete-Urtasun *et al.*, 1997).

The other two *pal* genes, *palF* and *palC*, have also been cloned, but their roles are even more elusive. The yeast homologue of PalF is Rim8, whereas PalC is the only pal protein with no putative *S. cerevisiae* homologue. Sequence inspection does not reveal their possible functions (Denison, 2000; Xu, 2000; Xu and Mitchell, 2000).

**1.E.vi Rim101 dependent pH-response pathways in *S. cerevisiae*.** An analysis of alkaline response genes in *S. cerevisiae* suggests a role for the Rim101 pathway in the regulation of some genes required or preferentially expressed at higher pH. With the use of a GeneFilter macroarray, gene expression at pH4 and pH8 was compared and results were confirmed through Northern blots and β-gal assays (*lacZ* fusion genes) (Lamb *et al.*, 2001). Expression of some of these alkaline response genes is partially or completely Rim101-dependent, whereas expression of others is Rim101-independent.

The Rim101-dependent[4] genes include *ARN4*, *YARO68W/YHR214W*, and *YOL154W*, whereas *ENA1* and *NRG2* show partial dependence on Rim101. Alkaline ambient pH disrupts the normal membrane proton gradient of a cell; usually, the proton gradient supplies the energy for translocation of other solutes (reviewed in van der Rest *et al.*, 1995). At higher pH, the cell relies on other ion pumps, including a plasma membrane $Na^+(Li^+)$-ATPase, encoded by *ENA1* (Garciadeblas *et al.*, 1993; reviewed in Mendoza *et al.*, 1994). Since *ENA1* expression shows partial Rim101-dependence, and *rim101* mutants are sensitive to elevated cation concentrations, it has been proposed that Rim101 is involved in general ion homeostasis (also see below).

Since yeast cells grow optimally under acidic conditions, it would make sense that some alkaline response genes would help cells cope with slower growth under higher pH conditions. The product of the *ARN4* gene may do that; it encodes a transporter that imports a bacterial

siderophore-iron complex, which may inhibit bacterial growth and help slower growing yeast compete at higher pH (Lamb *et al.*, 2001). This is reminiscent of the observation that expression of some genes required for penicillin synthesis in *A. nidulans* is alkaline pH-dependent (Espeso *et al.*, 1993; Then Bergh and Brakhage, 1998). Expression of *ANR4* requires Rim101, but this is not sufficient: the presence of a truncated version of Rim101 at acidic pH does not lead to the expression of *ANR4*. The Rim101 pathway is necessary and sufficient for *YOL154W* expression. *YOL154W* encodes for a zinc-metalloprotease-like protein, which is also expressed under zinc deficient conditions (Lyons *et al.*, 2000). This gene has homologs in *Candida albicans* and *Aspergillus nidulans* and expression of these homologues is pH-dependent (reviewed in Lamb *et al.*, 2001). In summary, Rim101 does appear to be involved in a pH response pathway in *S. cerevisiae*.

**1.E.vii Calcineurin-dependent regulation of *ENA1* expression.** The observation that expression of the ion pump gene, *ENA1*, is Rim101 dependent accounts, at least in part, for the sensitivity of *rim101* mutants to elevated cation concentrations (Futai *et al.*, 2000; Lamb *et al.*, 2001). Regulation of *ENA1* transcription is also dependent on calcineurin, a $Ca^{2+}$/calmodulin-dependent protein phosphatase. In *S. cerevisiae*, a number of parallel pathways are necessary for salt tolerance: calcineurin appears to regulate one of these pathways (reviewed in Rusnak and Mertz, 2000). Calcineurin, a heterodimeric protein, consists of a catalytic subunit, calcineurin A, and a regulatory subunit, calcineurin B. Calcineurin is not required for normal growth but contributes to tolerance of cells to high $Na^+/Li^+$, $Mn^{2+}$, and alkaline pH conditions, to recovery from pheromone-induced growth arrest, to calcium homeostasis, and to cell wall synthesis under specific circumstances (Matheos *et al.*, 1997; Stathopoulos-Gerontides *et al.*, 1999; reviewed in Rusnak and Mertz, 2000). Regulation by calcineurin is exerted at both transcriptional and post-translational levels. The four known genes whose expression is regulated by calcineurin are activated by the transcriptional regulator Crz1/Tcn1. *ENA1* is one of the genes that is activated by calcineurin-Crz1 (reviewed in Rusnak and Mertz, 2000). The predicted 678-amino acid Crz1 protein binds to its target site through three $C_2H_2$ zinc finger motifs at its C-terminus and activates transcription in a calcineurin-mediated manner through its amino-terminal region

[4] Rim101 dependence and independence was assessed by comparing expression of a gene in a WT vs. a *rim101Δ* strain at pH4 vs. pH8. Rim101 dependence was assumed if expression of the gene was not induced at pH8 in the *rim101Δ* strain.

(Matheos *et al.*, 1997). Calcineurin directly dephosphorylates Crz1 *in vitro* in a $Ca^{2+}$/calmodulin dependent manner and *in vivo* the $Ca^{2+}$ induced cytosol-to-nuclear localisation of Crz1 is calcineurin-dependent (Stathopoulos-Gerontides *et al.*, 1999). Although Crz1 mediated transcription is complex, depending on factors such as the strength of the $Ca^{2+}$ signal and the level of Crz1 protein, calcineurin also acts through other downstream targets (Matheos *et al.*, 1997). Rim101 appears to be another downstream component of the calcineurin-mediated salt stress response.

**1.E.viii Calcineurin mediated dephosphorylation of Rim101 is required for the salt stress response.** Two separate screens have identified *ENA1* as a transcriptional target of Rim101, strongly implicating Rim101's involvement in the salt stress response pathway (Futai *et al.*, 2000; Lamb *et al.*, 2001). Similar to calcineurin deficient mutants, *rim101* and *rim13* mutants are sensitive to high $Na^+$, $Li^+$, and pH conditions. Since mutations in *RIM13* prevent the proteolytic cleavage of Rim101, the truncated version of Rim101 appears to be required for salt stress and pH response. *In vitro* results demonstrate that calcineurin dephosphorylates Rim101 in a $Ca^+$-dependent manner. Under salt stress conditions *in vivo* Rim101 dephosphorylation exhibits calcineurin dependence. It is unclear how calcineurin exerts its effect since localisation studies with a GFP-Rim101 fusion protein reveal that Rim101 is a nuclear protein, even in the absence of calcineurin or salt stress signals. Since *rim13* mutants are sensitive to salt stress conditions and Rim101 processing occurs during high-$Na^+$ conditions, it appears that a dual activation system is acting on Rim101. It is not yet known if Rim101 phosphorylation or dephosphorylation plays a role in activating Rim101 in other pathways (Futai *et al.*, 2000).

## 1.F Studying protein-DNA interactions within a chromatin context

In the last portion of my thesis, I attempted to demonstrate the presence of Rim101 at the *DIT1* promoter *in vivo* by using the chromatin immunoprecipitation technique (ChIP). As background, I have included a brief overview of chromatin structure in eukaryotes and its relevance to trancriptional regulation. A discussion on ChIPing, including various applications of the technique, is also included.

**1.F.i An overview of chromatin structure in eukaryotes.** In eukaryotic organisms, DNA-templated processes, such as transcription, replication, recombination, and repair take place within the chromatin structure. At its least condensed level chromatin consists of nucleosomes arranged on DNA, resembling a "beads on a string"-like structure. The condensed metaphase chromosomes represent the highest level of compaction (reviewed in Kuo and Allis, 1999). In yeast, each nucleosome encompasses 147 basepairs of DNA wrapped 1 3/4 turns around a histone core. Two molecules each of histones H2A, H2B, H3 and H4 constitute the cylindrical core. In higher eukaryotes, linker histones H1 and H5, which contact DNA entering and exiting the nucleosome, as well as non-histone chromatin-associated proteins, such as the HMG proteins, have a role in the further compaction of chromatin. In yeast, the organisation of more condensed chromatin appears to differ. The homologue of linker histone H1 does not appear to have such a prominent role in chromatin structure and function, and HMG proteins are less abundant in yeast than in higher eukaryotes (reviewed in Churchill *et al.*, 1999; Ryan *et al.*, 1999).

Nuclease digestion experiments indicate that regularly positioned nucleosomes within promoter regions correlate with repressed gene activity, whereas hypersensitivity to nucleases signals increased gene activation (reviewed in Gregory and Horz, 1999; *for e.g.* Matallana *et al.*, 1992). It is thought that one way that nucleosomes exert their repressive function is by occluding certain DNA sequences and thus keeping transcription factors and/or activators away from their DNA-binding regions. Chromatin, however, also appears to enhance transcription; in some cases activation *in vivo* may be a thousand-fold, but only five- to ten-fold with naked, *in vitro* DNA templates (reviewed in Kornberg and Lorch, 1995).

**1.F.ii Modifying chromatin structure.** Nucleosomal arrays are highly dynamic structures. Histones can undergo a number of post-translational modifications, including ubiquitination, methylation, phosphorylation, ADP-ribosylation, and acetylation (reviewed in Grunstein, 1997; Vettese-Dadey *et al.*, 1996). Transcriptional control that is mediated by regulation of nucleosomal structure results from modifications of the N-terminal tails of histones, which protrude from the nucleosome surface. Acetylation and deacetylation of the ε-amino group of lysine residues within these tails have been most strongly linked to transcriptional regulation. It is believed that acetylation, which neutralises positively charged lysine residues, decreases the

interaction between N-terminal histone tails and DNA, making the DNA more accessible to activators or transcription factors (reviewed in Grunstein, 1997).

Numerous histone modifying complexes that are involved in regulation of transcription have been identified in yeast and other eukaryotes (reviewed in Struhl, 1998). The yeast protein Gcn5, which was genetically identified as a transcriptional co-activator, was later demonstrated to possess histone acetylase (HAT) activity. In yeast cells Gcn5 is found in at least two multi-protein complexes, Ada and SAGA. Although recombinant, purified Gcn5 can acetylate free H3 and H4 histones, it cannot do so in a nucleosomal context, implying that proteins in the complexes potentiate the activity of Gcn5 *in vivo* (reviewed in Struhl, 1998; Wu, 1997). Homologues of Gcn5 and a number of other HATs are present in multicellular organisms: these include the nuclear receptor co-activators ACTR and SRC-1 (they associate with nuclear receptors in a hormone-dependent manner), and p300/CBP, which associates tightly with RNAPII. Another HAT that is found in all eukaryotes, TAF130/250, is a subunit of TFIID and thus a component of the RNAPII transcription machinery (reviewed in Struhl, 1998). TATA elements in yeast are often found in or near nucleosomes; one mode of activation of HATs may be the disruption of nucleosomes around the TATA box to allow TBP access to the promoter (reviewed in Grunstein, 1997). Activators also appear to interact more easily with more acetylated nucleosomes. By using isolated hypo-and hyperacetylated nucleosomes, it has been demonstrated *in vitro* that Gal4 and USF1 (two activators) bind more strongly to acetylated than hypoacetylated nucleosomes (Vettese-Dadey *et al.*, 1996). Histone deacetylases (HDACs) have been typically associated with repression of genes. There are five known histone deacetylases in yeast, including Rpd3. Rpd3 can interact with Ume6, a DNA-binding protein, and in a complex with Sin3 represses transcription of certain meiosis specific genes, such as *IME2* (reviewed in Grunstein, 1997; Rundlett *et al.*, 1998).

Chromatin remodelling complexes have also been characterised in eukaryotes. In yeast, some SWI/SNF products form complexes that disrupt chromatin structure in an ATP-dependent manner. The genetic and biochemical evidence is extensive. Originally, five genes (*SWI1*, *SWI2*, *SWI3*, *SNF5*, and *SNF6*) were identified as important for transcriptional activation of certain genes (for example, *SWI* genes for expression of *HO* and *SNF* genes for expression of *SUC2*). Some mutations that suppress *swi* and *snf* mutations are in genes encoding for histones H3 and H4, at residues believed to be important for the stability of the core octamer. Decreased

amounts of histones H2A and H2B also alleviate the need for certain *SWI/SNF* gene products. An analysis of the nucleosomal structure of the *SUC2* promoter in *snf5* and *swi2* mutants indicates decreased micrococcal nuclease sensitivity. The purified SWI/SNF complex is able to disrupt nucleosomes in an ATP-dependent manner (reviewed in Kingston *et al.*, 1996; Kornberg and Lorch, 1995). All of this evidence indicates that the SWI/SNF complex contributes to activation by disrupting chromatin structure.

**1.F.iii Studying transcriptional regulation under *in vivo* conditions.** Given the role of the chromosomal structure in various cellular processes, a concern is how to study transcriptional regulation in more *in vivo*-like conditions. To more closely resemble an *in vivo* background, some *in vitro* transcription assays have been performed on reconstituted nucleosomal templates. For example, nucleosomes were reconstituted on the *Drosophila melanogaster* heat shock promoter, *hsp70* using an *in vitro* nucleosomal assembly-system. This allowed investigators to study ATP-dependent nucleosomal remodelling at this promoter (Tsukiyama *et al.*, 1994). Extracts from *Xenopus* eggs have been extensively used to study the mechanisms of eukaryotic chromosomal replication. For example, coupled with chromatin fractionation and immunoblotting, it has been possible to study the association of certain proteins, e.g. Xcdc6, with chromatin during the replication cycle (Coleman *et al.*, 1996).

With the use of special antibodies, it has been possible to specifically immunoprecipitate acetylated histones. Because core histone-DNA and histone-histone interactions are stable, unfixed chromatin can be immunoprecipitated. Isolated nuclei are first digested with MNase, oligonucleosomes are fractionated and the resulting chromatin fragments are subjected to immunoprecipitation with antibodies that recognise acetylated histones. Then, the DNA content of the immunofractionated chromatin is determined (reviewed in Chang *et al.*, 1999; Crane-Robinson *et al.*, 1997). Hebbes and colleagues employed this technique to demonstrated that active or poised genes from chicken embryo erythrocytes ($\beta^A$ and $\beta^p$ from the chicken β-globin locus) are enriched in the acetylated histone fraction (Hebbes *et al.*, 1994; Hebbes *et al.*, 1992).

**1.F.iv Immunoprecipitation of cross-linked chromatin.** When examining precise histone-DNA or protein-DNA interactions, cross-linking reagents have been employed to preserve the *in vivo* architecture of DNA-protein contacts. Nonhistone proteins often interact transiently with

DNA and linker proteins and HMG proteins can shift during chromatin fractionation (reviewed in Crane-Robinson *et al.*, 1997; Kuo and Allis, 1999). Historically, UV and formaldehyde cross-linking have been extensively used. Cross-linking with formaldehyde has many advantages. Formaldehyde is water soluble, penetrating cells easily, and it is active over a wide range of buffer and temperature conditions. Because it is a small molecule that reacts with amino and imino groups found in proteins (especially with lysines, arginines, and histidines) and DNA (mainly with adenines and cytosines), tight (2Å) DNA-protein and protein-protein crosslinks are formed. In this manner a protein does not have to be in direct contact with DNA to be detected through the chromatin immunoprecipitation (ChIP) technique. Furthermore, formaldehyde cross-links are fully and selectively reversible: DNA-protein links can be broken under milder conditions than protein-protein links. This allows both the DNA and protein content of immunoprecipitates to be analysed (reviewed in Hecht and Grunstein, 1999; Kuo and Allis, 1999; Orlando, 2000).

One of the initial uses of formaldehyde was to cross-link isolated chromatin or nuclei and study the distribution of newly synthesised histones on replicated DNA (reviewed in Kuo and Allis, 1999). In the early 1980's, Jackson and Chalkley treated whole cells with formaldehyde prior to the isolation of replicated chromatin (Jackson and Chalkley, 1981). One of the major reasons of using a cross-linker is to prevent protein-DNA rearrangements. Previously, it had been suggested that the formaldehyde treatment itself may introduce rearrangements. To address this concern, Jackson and Chalkey demonstrated *in vitro* the rearrangement of histones from labelled chromatin onto free DNA in the absence but not in the presence of formaldehyde cross-linking (Jackson and Chalkley, 1981). Formaldehyde cross-linking and immunoprecipitation of chromatin (X-ChIP) was further simplified in experiments carried out with the single cell eukaryote *Tetrahymena thermophila*. Short cross-linking time (minutes vs. hours) was demonstrated to be sufficient and chromatin was simply sheared and then immunoprecipitated (Dedon *et al.*, 1991). This same basic method has been adapted in yeast. In *Drosophila*, due to its larger genome size, cross-linked and non-cross-linked chromatin is separated through CsCl density centrifugation and only the cross-linked chromatin is analysed (reviewed in Orlando *et al.*, 1997).

In yeast, *Drosophila*, and mammalian cells, X-ChIP has been used to investigate a number of issues, including the acetylation state of histones, the association of large complexes

with DNA and binding of transcription factors *in vivo* (reviewed in Kuo and Allis, 1999). To demonstrate the usefulness of this technique, I will outline a few key experiments.

In yeast, transcriptional silencing occurs at the silent mating type loci (*HML* and *HMR*) and at telomeres. At these DNA sites, X-ChIP links transcriptional silencing with reduced nucleosomal acetylation at histone H4. Mutating key genes, *SIR2* and *SIR3*, disrupts silencing and leads to an increase in the co-immunoprecipitation of acetylated histone H4 with silent loci-specific DNA (Braunstein *et al.*, 1993). These results were refined with the use of more specific anti-acetylated histone antibodies. It has been demonstrated that histones H3 and H4 show the same acetylation pattern in chromatin spanning the silent mating-type cassettes in yeast as in heterochromatin in *Drosophila melanogaster* (Braunstein *et al.*, 1996). Silencing requires a number of Sir proteins (Sir2, Sir3 and Sir4) which bind to nucleosomes or interact with Rap1, a DNA-binding protein. With the use of appropriate antibodies and mutant strains, X-ChIP is contributing to the understanding of how the repressive chromatin structure is established. For example, expressing different amounts of Sir3 leads to the spreading of Sir3 occupancy around silent chromosomal regions and telomeres. It has been postulated that Rap1 and histone H4 may be necessary for the binding of Sir3, and that Sir3's function may be to spread silencing beyond the focal point (Hecht *et al.*, 1996; Strahl-Bolsinger *et al.*, 1997).

*In vivo* cross-linking has been used to understand the structure of the budding yeast centromere. Gene products that are genetically or biochemically linked to the centromere, such as Ndc10 and Cbf1, have been shown to localise to centromeric DNA (Meluh and Koshland, 1997). DNA replication complexes have also been investigated through the X-ChIP technique. The ORC complex is believed to associate with origins throughout the cell cycle, but the association of Mcm proteins, Cdc6, and Cdc45 is cell-cycle dependent (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). The association of certain proteins, within the context of large complexes, with chromatin has included the study of mismatch repair proteins, such as Msh2, at recombination intermediates, and the association of Ndd1 at G2/M-specific promoters (Evans *et al.*, 2000; Koranda *et al.*, 2000).

Although there are numerous other examples of the application of the chromatin immunoprecipitation technique, one final example demonstrates another useful aspect of this approach and also the need to combine it with other techniques. Sometimes, transcription factors that belong to the same family can recognise the same DNA sequence *in vitro*. In some instances

kinetic studies may suggest which ones act at certain promoters *in vivo*. Hypothetically, the lack of binding specificity *in vitro* may indicate redundancy, or more complex control *in vivo*. Members of the basic helix-loop-helix leucine zipper family (bHLHzip), including c-Myc and USF1, can bind the E box motif found in some promoters, such as the cad promoter, in mammalian cells. By using the X-ChIP technique, continuous binding of USF1 at the cad promoter has been demonstrated. Myc associates with the promoter after serum stimulation of quiescent cells. However, expression of a cad-reporter construct revealed that there is very little activation upon USF1 binding and that activation by Myc is only effective when the E box is located near the cad core promoter (Boyd *et al.*, 1998). Thus, in this case occupancy alone does not determine which protein is responsible for activation.

The above examples point out the versatility of chromatin immunoprecipitation with the use of formaldehyde. The last example also indicates that data from X-ChIP experiments alone may not be sufficient, and other complementary experiments should also be undertaken.

As a final note, recently the effects of histone H4 depletion and hence nucleosomal disruption have been assessed on global gene expression in *S. cerevisiae*. Silencing at telomeres is substantially compromised (in agreement with previous genetic and biochemical analyses), and 15% of genes are more strongly expressed, whereas 10% of genes show reduced expression compared to wild-type. Expression of the majority of yeast genes (75%) appears normal, suggesting that nucleosomes may not be the major regulators of gene expression. At certain genes, activators and repressors, much like in prokaryotic genomes, may be dominant to the effects of chromatin (Wyrick *et al.*, 1999).

## 1.G Thesis Rationale

As outlined in this introduction, we believe that Rim101 contributes to repression of the mid-late sporulation specific genes, *DIT1* and *DIT2*. A 76-bp DNA element, $NRE^{DIT}$, located in the *DIT1/DIT2* intergenic region, contributes to the transcriptional repression of *DIT1* and *DIT2* in vegetatively growing cells (Friesen *et al.*, 1997). By inserting $NRE^{DIT}$ into a reporter gene, Helena Friesen carried out a genetic screen to identify mutants that are defective at repression through this element. Jason Tanny analysed some of these mutants, and showed that a mutant allele of *RIM101* reduces $NRE^{DIT}$-mediate repression (Tanny, 1998b). His analyses also

demonstrated that the downstream portion of NRE76, NRE44, retained significant repression. Whereas repression mediated by NRE44 was somewhat Rim101 dependent, repression mediated by NRE30, was very much Rim101-dependent. Jason's data seemed to indicate that a minimal NRE could be defined, one that mediated both Rim101-dependent and –independent repression. An unidentified protein, Protein X, was also thought to contribute to repression. Repression was always Tup1-dependent. Figure 1 represents an abbreviated version of Jason Tanny's model for NRE-mediated repression.

By using a heterologous reporter gene containing various portions of $NRE^{DIT}$, I have confirmed that the minimal NRE is 42 bp and that it contains two sub-elements. Repression by each element depends on its multimerisation. Both elements require Ssn6-Tup1 to mediate repression. Only one element, which contains a Rim101-binding site, requires Rim101 to mediate repression. Since Rim101's activity is controlled through C-terminal proteolytic cleavage, I have determined whether cleavage is required for Rim101-mediated repression through $NRE^{DIT}$.

Our hypothesis is that Rim101 mediates repression by binding to $NRE^{DIT}$ and recruiting the Ssn6-Tup1 co-repressor complex. Previous work by Jason Tanny and Cosimo Commisso has shown that Rim101 binds to $NRE^{DIT}$ *in vitro*. Cosimo Commisso has also demonstrated that a protein containing the zinc-fingers of Rim101 binds to a plasmid-borne sub-region of $NRE^{DIT}$. By using the chromatin immunoprecipitation technique, I have attempted to demonstrate the direct binding of Rim101 at the genomic $NRE^{DIT}$ site.

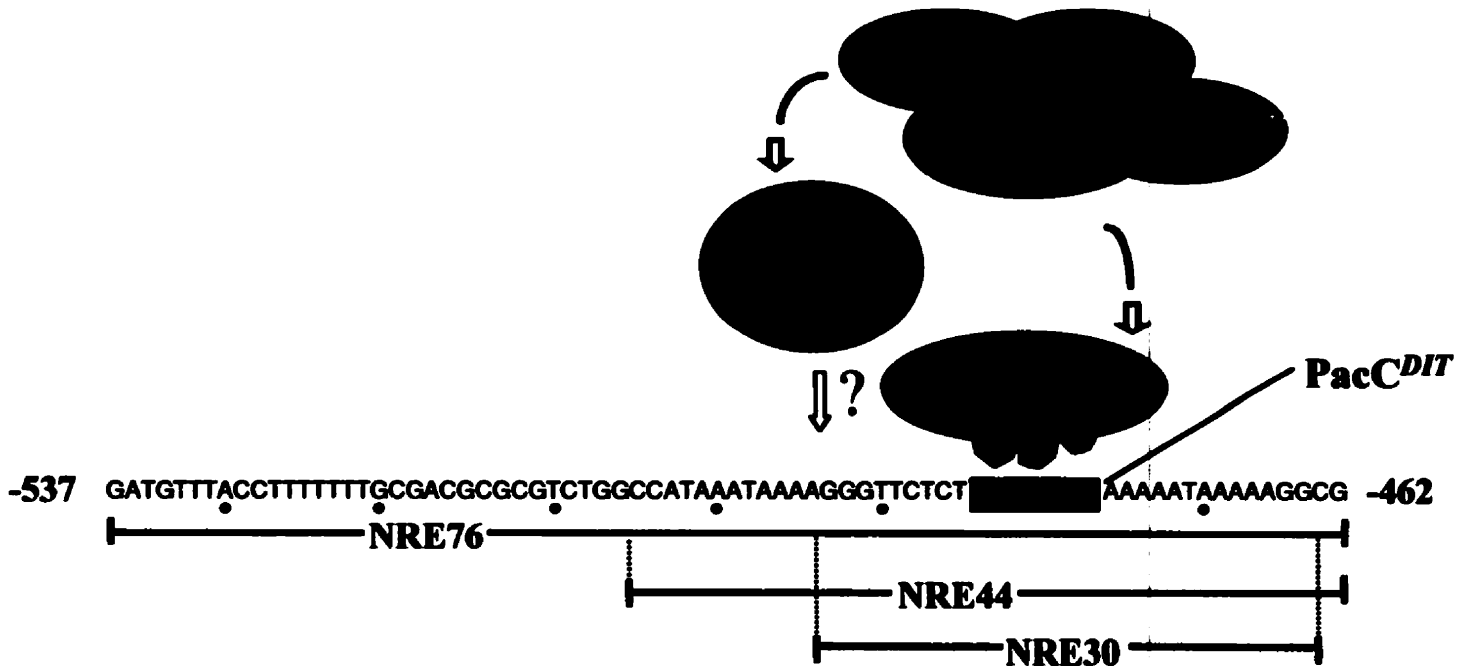



**Figure 1. Initial model for NRE-mediated repression.**

Jason Tanny's experimental results supported a model where Rim101contributes to NRE$^{DIT}$-mediated repression by binding to the downstream portion of NRE76. NRE44 retained significant repression, indicating that perhaps a minimal NRE can be defined. Repression through both NRE76 and NRE44 was partially Rim101-dependent. Mutational analysis of the PacC$^{DIT}$ site within the context of NRE30 and NRE76 also supported the idea that in the absence of Rim101, another protein, Protein X could mediate repression in an Ssn6-Tup1-dependent manner.

# Materials and Methods

**2A. Media, culture conditions, and general methods.** Yeast rich medium (YPD) contained 1% yeast extract, 2% bacto-peptone, and 2% glucose. Yeast semi-minimal medium (SK) contained 0.7% yeast nitrogen base without amino acids, 2% glucose, 16 μg of uracil per ml, 20 μg each of tryptophan, histidine, and methionine per ml, 30 μg each of tyrosine and lysine per ml, 40 μg each of adenine, and arginine per ml, 50 μg of phenylalanine per ml, 60 μg of leucine per ml, and 200 μg of threonine per ml. Dropout medium was SK with selected amino acid(s) omitted. All solid yeast media contained 2% agar. Yeast strains were grown at 30°C and transformations were carried out via the lithium-acetate method (Gietz *et al.*, 1992). Standard methods were employed for mating, sporulation, and tetrad analysis (Sherman, 1991; Sherman and Hicks, 1991). Bacterial LB medium contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar for solid medium. Ampicillin was added as required to a concentration of 0.1 mg/ml. I purified plasmid DNA from bacteria by the alkaline lysis method (Sambrook *et al.*, 1989). Synthetic oligonucleotides were purchased from ATGC and GIBCO/BRL Life Technologies. DNA sequencing was carried out with the dideoxy chain termination method (Sanger *et al.*, 1977) with reagents from a kit (Amersham). Most enzymes were from New England Biolabs.

**2B. Strains.** Yeast strains used in this study are described in Table 1. The parental haploid strains W3031A and W3031B were constructed by R. Rothstein. LP112 is the a/α diploid strain obtained by mating W3031A and W3031B. The *rim101Δ* strain (Y104), the *tup1Δ* strain (Y169), and strains Y195 and Y197, which were derived from haploid progeny of the diploid strain obtained by mating Y104 with W3031B, were constructed by Jason Tanny (Tanny, 1998b).

I used a PCR-mediated gene disruption technique (Longtine *et al.*, 1998) to introduce a *rim9Δ::kan*$^r$ allele into W3031A and a *tup1Δ::kan*$^r$ allele into strain Y104. pFA6a-kanMX6 served as the template for amplification of the *kan*$^r$ gene with the Expand™ Long Template PCR System (Boehringer Mannheim). For construction of the of *rim9Δ::kan*$^r$ allele (or

**Table 1.** ***Saccharomyces cerevisiae*** **strains**

| Strain | Genotype |
|---|---|
| **W303-1A** | ***MATa ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100*** |
| **W303-1B** | ***MATα ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100*** |
| **LP112** | ***MATa ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100***<br>***MATα ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100*** |
| **Y104** | ***MATa ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 rim101Δ::ura3-jt*** |
| **Y197** | ***MATα; ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 rim101Δ::ura3-jt*** |
| **Y195** | ***MATa ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 rim101Δ::ura3-jt***<br>***MATα ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 rim101Δ::ura3-jt*** |
| **Y169** | ***MATα ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 tup1Δ::TRP1*** |
| **W303-1A *rim9Δ*** | ***MATa ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 rim9Δ::kan$^r$*** |
| **W303-1A *rim101Δtup1Δ*** | ***MATa ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 can1-100 rim101Δ::ura3-jt tup1Δ::kan$^r$*** |

*tup1Δ::kan*$^r$ allele), one primer had a 5' extension that contained sequence from upstream of the *RIM9* (or *TUP1*) ORF and the other primer had a 5' extension that contained sequence from downstream of the *RIM9* (or *TUP1*) ORF. In this way the amplified *kan*$^r$ cassette could be used to replace the *RIM9* (or *TUP1*) locus by homologous recombination. The Rim9FD and Rim9RD primers were 5'-TAC GGT TGA GAC AGA TTC ATT GAG GAA AAG AGG AGA **ATG** GCG GAT CCC CGG GTT AAT TAA-3' and 5'-AAG CCG ATT GGC AAA CCG ATG TAA CGT GCA AAA TGA CAC AGA ATT CGA GCT CGT TTA AAC-3', respectively, with the start codon of *RIM9* in bold and the 20 bases complementary to the ends of the *kan*$^r$ cassette being underlined. The Tup1FD and Tup1RD primers were 5'-TAA GCA GGG GAA GAA AGA AAT CAG CTT TCC ATC CAA ACC ACG CAT CCC CGG GTT AAT TAA-3' and 5'-GTA AAG TGT TCC TTT TGT GTT CTG TTC TTA ATT TGG CGC TGA ATT CGA GCT CGT TTA AAC-3', respectively, with the 20 bases complementary to the ends of the *kan*$^r$ cassette being underlined. The ~1.6 kb PCR products were gel-purified and the *rim9Δ::kan*$^r$-containing fragment was used to transform W3031A cells and the *tup1Δ::kan*$^r$-containing fragment was used to transform Y104 cells. Transformants were replica-plated from YPD medium to YPD medium containing 40 μg G418/ml. Surviving colonies were again replica-plated to G418-containing medium to select for stable kanamycin-resistant strains. The replacement of the *RIM9* and *TUP1* genes with *kan*$^r$ was then confirmed by PCR analysis of genomic yeast DNA. The *RIM9*-specific primers were Rim9FCH (5'-AGT GAT GAG CGT TGA GAA CC) and Rim9RCH (5'- CCG ATT GGC AAA CCG ATG TA-3'), which begin 94 bases upstream of the *RIM9* start codon and 46 bases downstream of the *RIM9* stop codon, respectively. The *TUP1*-specific primers were Tup1FCH (5'-GAA CAA CTG GCT GAA CAC GT-3') and Tup1RCH (5'-GCG TAC CTG GAT CAT AAC ATA A-3') which start 166 bases upstream of the *TUP1* start codon and 120 bases downstream of the *TUP1* stop codon, respectively. The *kan*$^r$-specific primers were kan+375T (5'CTG CGC CGG TTG CAT TCG-3') and kan+392B (5'-CGA ATG CAA CCG GCG CAG-3') as forward and reverse primers, respectively. The expected lengths of the PCR products for the *tup1Δ::kan*$^r$ allele were 920 bp with Tup1FCH and kan+392B as primers, 890 bp with Tup1RCh and kan+375T as primers, and 1693 bp with Tup1FCH and Tup1RCH, as primers. The expected lengths of PCR products for *rim9Δ::kan*$^r$ allele were 851 bp with Rim9FCH and kan+392B as primers, 794 bp with Rim9RCH and kan+375T as primers, and 1528 bp with Rim9FD and Rim9RD as primers.

**2.C Plasmids.** The high-copy plasmid pLG312Bgl, which contains a *CYC1-lacZ* reporter gene, was constructed by A. Mitchell and is described in Hepworth *et al.* (1995). In pLG312MCS#3, which was derived from pLG312(Bgl), the *Xho*I-*Sal*I-*Bgl*II-*Sal*I-*Xho*I sequence that is present between the *CYC1* UASs and TATA box of *CYC1-lacZ* reporter gene in pLG312(Bgl) has been replaced with an *Xho*I-*Bgl*II-*Kpn*I-*Sal*I polylinker as follows. The synthetic oligonucleotides, MCS TOP (5'-**TCG** AGA GAT CTG GTA CCG TCG AC-3') and MCS BOT (5'-**TCG** AGT CGA CGG TAC CAG ATC TC-3') containing *Xho*I overhangs (bold) were phosphorylated at their 5'-ends and annealed. The double-strand oligonucleotide was then ligated into the *Xho*I site of pLG312(Bgl). Sequencing the insert revealed that a cloning artefact led to mutation of two bases within the downstream overhang. Thus the insert is 5'-TCG AGA GAT CTG GTA CCG TCG ACC CCA-3' with the last four bases being CCCA rather than TCGA). Because these two changes did not affect the unique restriction sites, pLG312MCS#3 was retained as a cloning vector. This new multiple cloning site allowed pLG312MCS#3(+insert) constructs to be easily converted to UASless (or pLGΔSS) constructs. It should be noted that the region encompassing the *CYC1* UAS1 and UAS2 in pLG312MCS#3 can be readily deleted without removal of any inserts cloned into the *Bgl*II site. The nomenclature that I have used to describe this plasmid and others in this series is as follows: pLGnX, with pLGn referring to pLG312MCS#3 and X referring to the fragment(s) inserted into the polylinker. Some previously cloned NRE constructs are in pLG312Bgl; these are indicated as pLGX, with pLG referring to pLG312Bgl and X referring to the fragment(s) inserted in the polylinker.

The construction of pLGNRE76, pLGNRE76m, pLGNRE53, pLGNRE30, and pLG3xNRE30 has been described (Friesen *et al.*, 1997; Tanny, 1998b). The parent vector for these constructs was pLG312Bgl. I used two additional constructs, pLGNRE29 and pLG3xNRE29, both cloned by Helena Friesen (see Table 2). Other NRE-containing fragments were cloned into the *Bgl*II site of pLG312MCS#3 as listed in Table 2. Plasmids were constructed as follows. Synthetic oligonucleotides were purified by sequential precipitations with LiCl and an ethanol-acetone mixture. The resuspended oligonucleotides were treated with T4 polynucleotide kinase to phoshporylate their 5' ends, annealed, and the duplexes were ligated into pLG312Bgl or pLG312MCS#3. These vectors had been digested with *Bgl*II, gel-purified,

**Table 2.** Plasmids

| Name | Description of plasmids and synthetic oligonucleotides used (if applicable) | Source |
|---|---|---|
| pLG312Bgl | UAS$^{CYC1}$(MCS)TATA$^{CYC1}$-*lacZ* reporter construct | Guarente and Mason (1983) |
| pLG312MCS#3 | Same construct as above but original MCS was replaced with a shorter version (look under Plasmids for details) | Friesen, H.; described in this work |
| pLGNRE76 | pLG312Bgl with nt –537 to –462 of *DIT1* inserted into *Bgl*II site of MCS. | Friesen, *et al.*, 1997 |
| pLGNRE53 | pLG312Bgl with nt –537 to –485 of *DIT1* inserted into *Bgl*II site of MCS. | Friesen, *et al.*, 1997 |
| pLGnNRE42 | pLG312MCS#2 with nt –505 to –464 of *DIT1* inserted into *Bgl*II site of MCS. The following synthetic oligonucleotides were used: NRE42T, 5' to 3', **GAT** CCC ATA AAT AAA AGG GTT CTC TTG CCA AGA AAA AAT AAA AAG G, and NRE42B, 5' to 3', **GAT** CCC TTT TTA TTT TTT CTT GGC AAG AGA ACC CTT TTA TTT ATG G (overhangs compatible with *Bgl*II site are in bold). | This work |
| pLGNRE30 | pLG312Bgl with nt –493 to –464 of *DIT1* inserted into *Bgl*II site of MCS. | Jason Tanny, M.Sc., 1998 |
| pLG3xNRE30 | pLG312Bgl with three copies of nt –464 to –493 of *DIT1* inserted into *Bgl*II site of MCS. Please note that this is in the opposite orientation to the insert in pLGNRE30 (*DIT2* orientation). | Jason Tanny, M.Sc., 1998 |
| pLGnNRE30 | pLG312MCS#3 with nt –493 to –464 of *DIT1* inserted into *Bgl*II site of MCS. The synthetic oligonucleotides used to create this plasmid have been described (Jason Tanny, M.Sc., 1998). | This work |
| pLGn3xNRE30 | pLG312MCS#3 with one copy of nt –493 to –464 (*DIT1* orientation) and two copies of nt –464 to -493 of *DIT1* (*DIT2* orientation) inserted into *Bgl*II site of MCS. | This work |
| pLGNRE29 | pLG312Bgl with nt –505 to –477 of *DIT1* inserted into *Bgl*II site of MCS. The following synthetic oligonucleotides were used: NRE477, 5' to 3', **GAT** CTT GGC AAG AGA ACC CTT TTA TTT ATG, and NRE479, 5' to 3', **GAT** CCA TAA ATA AAA GGG TTC TCT TGC CAA. | Helena Friesen |

**Table 2.** Plasmids (continued)

| | | |
|---|---|---|
| pLG3xNRE29 | pLG312Bgl with three copies of nt –505 to –477 of *DIT1* inserted into *Bgl*II site of MCS. | Helena Friesen |
| pLGn2xNRE23 | pLG312MCS#3 with two copies of nt –477 to –499 (*DIT2* orientation) of *DIT1* inserted into *Bgl*II site of MCS. The following synthetic oligonucleotides were used: NRE23-T, 5' to 3', GAT CAT AAA AGG GTT CTC TTG CCA AGA G and NRE23-B, 5' to 3', GAT CCT CTT GGC AAG AGA ACC CTT TTA T. | This work |
| pLGn2xNRE22U | pLG312MCS#3 with one copy of nt –484 to –505 (*DIT2* orientation) and one copy of nt –505 to –484 of *DIT1* inserted into *Bgl*II site of MCS. The following synthetic oligonucleotides were used: NRE22U-T, 5' to 3', GAT CCA TAA ATA AAA GGG TTC TCT T and NRE22U-B, 5' to 3', GAT CAA GAG AAC CCT TTT ATT TAT G. | This work |
| pLGnNRE25 | pLG312MCS#3 with one copy of nt –505 to –481 of *DIT1* inserted into *Bgl*II site of MCS. The following synthetic oligonucleotides were used: NRE25-T, 5' to 3', GAT CCA TAA ATA AAA GGG TTC TCT TGC C, and NRE25-B, 5' to 3', GAT CGG CAA GAG AAC CCT TTT ATT TAT G. | This work |
| pLGn3xNRE25 | pLG312MCS#3 with two copies of nt –481 to –505 (*DIT2* orientation) and one copy of nt –505 to –481 of *DIT1* inserted into *Bgl*II site of MCS. | This work |
| pLGnNRE22D | pLG312MCS#3 with one copy of nt –486 to –464 of *DIT1* inserted into *Bgl*II site of MCS. The following synthetic oligonucleotides were used: NRE22D-T, 5' to 3', GAT CCT TGC CAA GAA AAA ATA AAA AG, and NRE22D-B, 5' to 3', GAT CCT TTT TAT TTT TTC TTG GCA AG. | This work |
| pLGn3xNRE22D | pLG312MCS#3 with three copies of nt –505 to –481 of *DIT1* inserted into *Bgl*II site of MCS. | This work |
| pCEN*RIM1* | pRS314 (*CEN-ARS, TRP1* single-copy plasmid) carrying the full *RIM101* ORF and ~1 kb of upstream sequence. | Su and Mitchell, 1993b |
| pAS1-CYH2 (or pAS2) | Multi-copy (2μ plasmid) *TRP1* plasmid that carries a *GAL4(BD)-HA* fusion gene under the *ADH* promoter. This was used in the Chromatin immunoprecipitation experiments as a control. | The parent plasmid, pAS1 is described in Durfee, *et al.*, 1993; pAS2 is described in the Clonetech Catalog. |

and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) prior to purification by extraction with phenol/chloroform and precipitation with ethanol. Insertions containing multiple copies of a fragment were obtained by varying the ratio of insert DNA:vector in the ligation reactions. Table 2 lists the plasmids used in this study and, as appropriate, the synthetic oligonucleotide pairs used to create them. Because I found that the *CYC1-lacZ* reporter gene was expressed at a slightly higher level in pLG312MCS#3 than in pLG312Bgl, the oligonucleotides PAC-T and PAC-B that were previously use to construct pLG312NRE30 (Tanny, 1998b) were also used in this study to construct pLGnNRE30 and pLGn3xNRE30.

Four other plasmids used in this study have been described elsewhere. pCEN*RIM101* contains the *RIM101* gene including 1 Kb of upstream sequence on a CEN-ARS low copy plasmid (Su and Mitchell, 1993a). pASII (Clonetech) carries a *GAL4(BD)-HA* fusion gene on a multicopy, 2 μ plasmid. pWL40 and pWL41 are multi-copy plasmids that carry HA-tagged *RIM101* alleles (Li and Mitchell, 1997). In pWL40 three HA-cassettes, each with three HA-epitopes, are inserted after codon 312; in pWL41 one HA-cassette is inserted after codon 473.

**2.D β-galactosidase assays.** Liquid β-galactosidase assays were carried out essentially as described (Hepworth *et al.*, 1995). Cells were grown in 10 ml of YPD or SK dropout medium for 3 generations, harvested, washed with water and frozen in two aliquots. Cells from flocculent strains such as Y169 (*tup1Δ*) were washed in 20 mM Tris-HCl (pH 7.5)-10 mM EDTA to disrupt clumps prior to freezing. The frozen cell pellet was resuspended in Z-buffer and the cells were broken by vortexing on an Eppendorf shaker (Model 5432) in the presence of glass beads for 15 minutes at 4°C. This shaking procedure was just as effective as manual vortexing in breaking cells, as judged by tandem assays carried out on duplicate samples. After the addition of additional Z-buffer, the samples were adjusted to 0.005% SDS and 0.02% $CHCl_3$ and spun for 5 minutes at 10 000 g at 4°C. β-galactosidase activity was measured in aliquots from the supernatant. I found that assays performed with clarified lysates gave more producible results than those performed with the original turbid cell lysates. The activities reported are averages from assays performed on three independent transformants analysed at the same time. I repeated each experiment from two to five times and found that the relative levels of β-galactosidase activities were similar from one experiment to the next. β-galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) cleaved per minute per

milligram of protein at 28°C. I used a BioRad Protein assay, based on the Bradford protein assay, to determine the concentration of protein in the samples prior to freezing. In this case, lysates were kept on ice to avoid precipitation of protein and assayed as soon as possible. The data are presented as a ratio (fold repression) of β-galactosidase activity measured in cells containing the plasmid-borne *CYC1-lacZ* reporter gene with no insert to the activity measured in cells from the same strain containing a plasmid-borne *CYC1*-NRE-*lacZ* reporter gene.

I performed qualitative β-galactosidase assays on colonies growing on agar plates by overlaying the colonies on each plate with ten ml of a solution containing 0.5 M potassium phosphate buffer (pH 7.0), 0.1% SDS, 6 % dimethyl formamide, 0.5% agar and from 3 to 5 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). After the agar had solidified, the plates were incubated at 30°C for 4-5 hours at which time colour development was generally complete. However, if there was no significant colour change at this time, I left the plates at 30°C overnight. I found that this extended incubation did not lead to any further colour development. Within each experiment, I found that duplicate or triplicate colonies derived from different transformants gave comparable results. I repeated each experiment at least twice and obtained similar results.

**2.E Chromatin immunoprecipitation (ChIP).** For my ChIP experiments, I followed the protocol described by Hecht and Grunstein (1999) with some minor modifications. I used 75 ml, rather than 50 ml, cultures of cells grown to an $O.D._{600}$ of 0.75-0.8 and scaled up subsequent steps accordingly. If cells were treated with formaldehyde, the cross-linking reagent was added to a concentration of 1% and the reactions were quenched by the addition of glycine to 125 mM. I then washed the cells twice with ice-cold PBS and resuspended the cell pellet in 600 µl of lysis buffer to which protease inhibitors had been added (Hecht and Grunstein, 1999). I transferred the solution of cells to chilled eppendorf tubes containing ~400 µl of glass beads. I then lysed the cells by vortexing them on an Eppendorf shaker (Model 5432) at 4°C for 45 minutes. I monitored the extent of lysis microscopically and found that this shaking procedure was as effective as vortexing for nine two-minute intervals with samples being left on ice between the periods of vortexing. I added an additional 600 µl of lysis buffer containing protease inhibitors to the lysates. The samples were then sonicated for five 10-second pulses, interrupted by two-minute cooling intervals on ice. This yielded chromatin fragments from 500 bp to 1 kb in length.

Throughout my series of experiments, I checked that sonication was reproducibly generating fragments of this size. At this point, I removed a 50 µl aliquot of the sheared lysate, also referred to as the whole cell extract (WCE), for subsequent PCR analysis of the DNA and stored 50 µl of the lysate in SDS-PAGE sample buffer at –20°C for subsequent Western blot analysis.

I found that 4 µg of anti-HA antibody (Clone 16B12, BabCO) per ml of WCE allowed for optimal immunoprecipitation of Rim101-HA as assessed by Western blot analysis. I incubated the antibody with the WCE for 3 hours and then added 45 µl of a 50% suspension of protein A-Sepharose beads (Sigma) per ml of lysis buffer. Immune complexes were recovered by low speed centrifugation (3 000 xg for about 30 seconds) and washed twice with one ml of lysis buffer and twice with one ml of PBS. Protein and any cross-linked DNA was recovered from the immunoprecipitate by addition of 100 µl of TE buffer containing 1% SDS and heating at 65°C for 10 minutes. The beads were spun at 4 000 xg, for two minutes and washed with 150 µl of TE buffer containing 0.67% SDS and again spun at 4 000 xg for 2 minutes. The pooled supernatants, as well as an aliquot of the WCE, were kept at 65°C for 10 minutes to reverse protein-DNA cross-links and the DNA was purified as described (Hecht and Grunstein, 1999). DNA recovered from the WCEs and from the immunoprecipitates was resuspended in 50 µl and 20 µl of TE buffer, respectively, for analysis by PCR (see below). For recovery of proteins for subsequent analysis, the washed immunoprecipitates were treated with 50 µl of TE buffer containing 1% SDS. The eluted protein was stored in SDS-PAGE sample buffer at -20°C.

For more stringent washing of protein A-bound immune complexes, I used the following protocol (Kuo and Allis, 1999). The immunoprecipitates were washed twice with 1 ml of lysis buffer for 7 minutes each time, twice with 1 ml of lysis buffer containing 500 mM NaCl and 0.1% SDS for 7 minutes each time, once for ten minutes with buffer containing 10 mM Tris-HCl, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, and 1 mM EDTA, and once with 1 ml of TE buffer (pH 8.0) for 10 minutes.

**2.F PCR analysis of Immunoprecipitated DNA.** The primer pairs that I used are listed in Table 3. To obtain similar amounts of amplified DNA with DNA from the WCE as template I used 60 pmoles of *GAL1* and *ACT1* primer pairs and 40 pmoles of *DIT1* and *IME1* primer pairs in the same reactions. Recommended amounts of $MgCl_2$, dNTPs, and Taq DNA polymerase (Amersham) were added to PCR reactions (Hecht and Grunstein, 1999). Twenty-five cycles of

**Table 3.** Primers for ChIP experiments

| Primer pairs | Primer description | Expected PCR product |
|---|---|---|
| *ACT1*-F1 and *ACT1*-R2 | Designed to amplify *ACT1* sequence (upstream of ORF), serving as a negative control in IP's (ChIP). Forward primer 5' to 3' GCT GCC ACA GCA ATT AAT GC and reverse primer 5' to 3' CGA GTT TGG TTT CAA AAC GGT T. | 253 nt |
| *GAL1*-F1 and *GAL1*-R2 | Designed to amplify *GAL1* sequence (upstream of ORF), serving as a negative and positive control in IP's for ChIP experiments (See Results). Forward primer 5' to 3' CGT TCC TGA AAC GCA GAT GT and reverse primer 5' to 3' CTT GAC GTT AAA GTA TAG AGG T. | 362 nt |
| *DIT1*-F2 and *DIT1*-R2 | DNA amplified by these primers encompassed the $PacC^{DIT}$ site. These primers were used to test for enrichment of *DIT1* DNA in IP's. Forward primer 5' to 3' AGG ATG ACC AGT CAT TCC TC and reverse primer 5' to 3' GCT CTT CAT TGA GGT TGC ATA. | 324 nt |
| *IME1*-F1 and *IME2*-R1 | Although this served as a negative control, it was originally designed to test for the presence of Rim101 within the *IME1* regulatory sequence (See Results). Forward primer 5' to 3' CAT CCG CTA TTA CCT CTC CT and reverse primer 5' to 3' ACC ATG ACG CTT CCT TGA TG. | 295 nt |

amplification were performed, following the PCR parameters described by Hecht and Grunstein (1999), to obtain semi-quantitative results reflecting relative amounts of DNA template present.

**2.G Western blots.** Western blotting was carried out following standard techniques. Anti-HA monoclonal antibody (BAbCO, Clone 16B12) was used as the primary antibody at a 1:5000 dilution and goat anti-mouse antibody conjugated to horse radish peroxidase (Bio-Rad) was used as the secondary antibody at a 1:2500 dilution. Alternatively, an anti-HA polyclonal antibody (Y-11, *sc-805*; Santa Cruz Biotechnology) was used as the primary antibody at a 1:100 dilution and goat anti-rabbit antibody conjugated to horse radish peroxidase (Bio-Rad) was used as the secondary antibody at a 1:2500 dilution.

# Results

## 3.A Defining the minimal NRE$^{DIT}$ and analysing its repression abilities

**3.A.i. Confirmation that NRE42 is a potent operator.** A 76-bp fragment, referred to as NRE76, is an Ssn6-Tup1-dependent negative regulatory sequence that serves to prevent expression of the sporulation-specific genes *DIT1* and *DIT2* in mitotic cells (see Introduction). This sequence is present between nt -537 and -462 of the *DIT1* gene, with +1 being the start site of transcription. The initial characterisation of NRE76 as an operator sequence that can down-regulate expression promoted by a heterologous UAS suggested that the entire region was required for maximal repression (Friesen *et al.*, 1997). On re-examination of this element, Jason Tanny found, however, that the downstream portion of NRE$^{DIT}$, which was previously observed to have only modest repression activity, was as efficient an operator as NRE76 (Tanny, 1998b). J. Tanny also demonstrated that *RIM101* is required to achieve full repression through this element (Tanny, 1998b).

As the first step in my thesis research, I constructed a new version of the *CYC1(UAS)-lacZ* reporter gene containing the downstream portion of NRE76 to reassess the ability of this sequence to act as an efficient operator. For this and subsequent studies, I used a modified version of the vector pLG312Bgl, referred to as pLG312MCS#3, which contains a modified polylinker between the UAS and the TATA box of the *CYC1(UAS)-lacZ* reporter gene. This new polylinker facilitated the manipulation of constructs (see Fig. 2B and Materials and Methods). I then cloned NRE42, containing the sequence from nt -505 to nt -464, into the polylinker of pLG312MCS#3 to obtain pLGnNRE42. I first used a qualitative overlay assay to assess the abilities of NRE42 to prevent expression of the *CYC1-lacZ* reporter gene in cells of both a wild-type strain (W3031A) and an otherwise isogenic *rim101Δ* strain (Y104). In this visual assay for expression of β-galactosidase, colonies that have grown up on solid medium are overlaid with a solution containing melted agar and the chromogenic substrate X-Gal. After the plates have been incubated for an additional interval of time, the relative extent of blueness of the colonies is visually monitored (see Materials and Methods). Colonies of wild-type cells containing pLG312MCS#3 became dark blue shortly after being overlaid with X-Gal-containing

agar whereas colonies of wild-type cells containing pLG312Bgl with a single copy of NRE76 inserted between the UAS and TATA box of the *CYC1-lacZ* reporter gene remained white in this assay (Figure 2A, line 1). In agreement with the results reported by J. Tanny, I found that NRE42 had repression activity similar to NRE76 and that this repression was partially *RIM101*-dependent (Fig. 2A, lines 1 and 2). It remains unclear why a fragment spanning this region did not provide efficient repression in the earlier study of Friesen *et al.* (1997).

**3.A.ii. Demonstration that NRE42 is bipartite.** J. Tanny's studies had also suggested that NRE42 consisted of two distinct sub-elements. In particular, J. Tanny showed that a single copy of a 30-bp fragment, referred to as NRE30, that spans nt -493 to -464, supports a modest level of Rim101- and Tup1-dependent repression (Tanny, 1998b). Consistent with the notion that the operator function of NRE30 depends on Rim101 binding to the PacC site present in this fragment (see Introduction), J. Tanny showed that a single point mutation within this element abolishes its ability to reduce expression of the reporter gene (Tanny, 1998b). Based on these observations, I set out to explore further the notion that NRE42 contains two potentially distinct operator elements with at least one element requiring the putative DNA-binding protein Rim101 and the Ssn6-Tup1 repression complex. With the use of the overlay assay, I qualitatively assessed the ability of two or three tandem copies of various oligonucleotides spanning different portions of NRE42 to repress expression of the *CYC1*(UAS)*-lacZ* reporter gene in wild-type and *rim101Δ* cells. As expected, colonies of wild-type cells containing pLGn3xNRE30, a pLG312MCS#3 derivative with three copies of NRE30 inserted between the UAS and TATA box of the *CYC1-lacZ* reporter gene, were light blue in this assay (moderate repression) (Fig. 2, line 4). In contrast, the presence of a single copy of NRE30 had minimal effect on expression of the *CYC1-lacZ* reporter gene as assessed in this assay (Fig. 2A, line 3). Colonies of *rim101Δ* cells containing pLGNRE76, pLGnNRE42, or pLGn3XNRE30 that were overlaid with X-gal containing agar turned blue, consistent with the Rim101-dependence of repression.

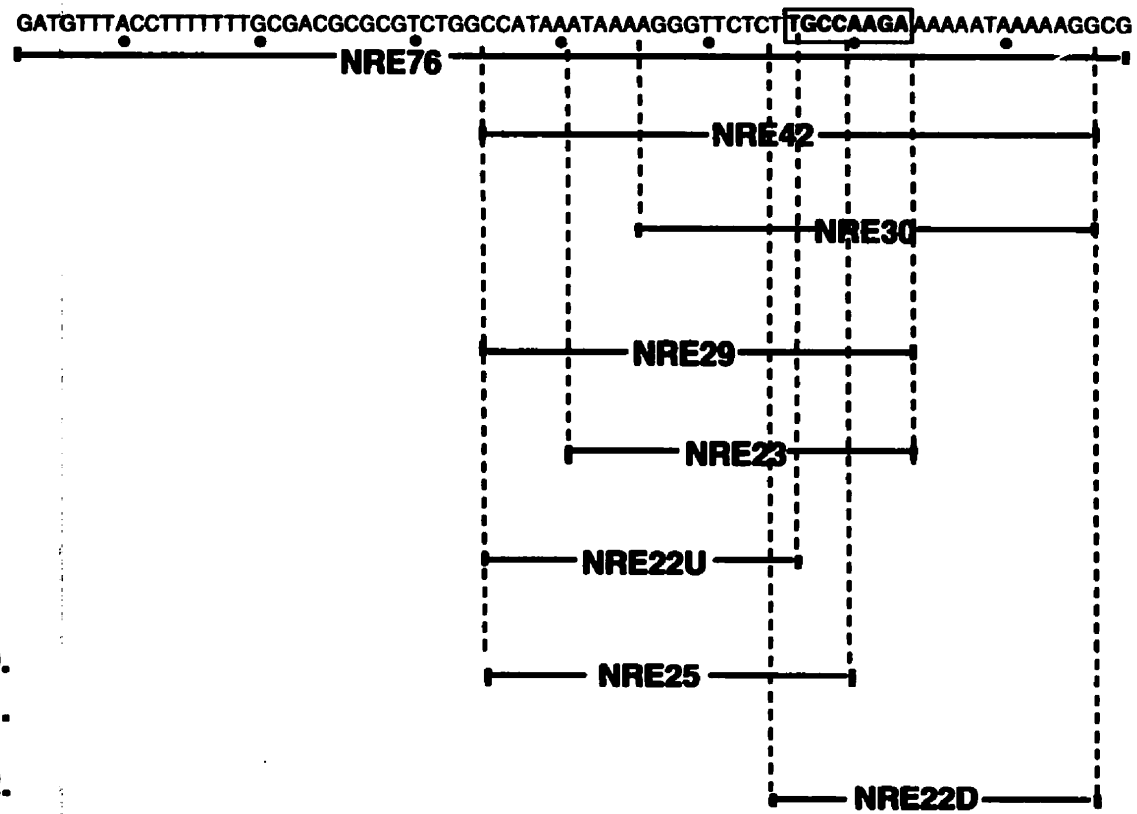
**Figure 2. Repression abilities and Rim101-dependence of NRE fragments.**

(A) Summary of the ability of various fragments to act as operators. Various oligonucleotides were inserted in one, two, or three copies into the BglII site of pLG312MCS#3 and were tested for their ability to repress expression of the *CYC1*(UAS)-*lacZ* reporter gene in wild-type and *rim101Δ* cells. Expression of β-galactosidase was monitored by a qualitative overlay assay of colonies (see Materials and Methods). The results are summarised as either strong repression, indicating that the colonies remained white after being overlaid with X-Gal-containing medium; moderate repression, indicating that the colonies turned light blue, and weak repression, indicating that the colonies turned blue. A lower level of repression of the reporter gene in *rim101Δ* background than in wild-type cells was taken to denote Rim101-dependence of repression.

(B) Schematic representation of the *CYC1-lacZ* reporter gene present in pLG312MCS#3. From left to right the reporter construct contains the *CYC1* upstream activation sequences (UAS1 and UAS2), a multiple cloning site (MCS) into which the NRE fragments were inserted, and a minimal *CYC1* TATA-box to drive the expression of the reporter gene, *lacZ*. The reporter plasmid, which carries the *URA3* selectable marker, was maintained by selection on medium lacking uracil.

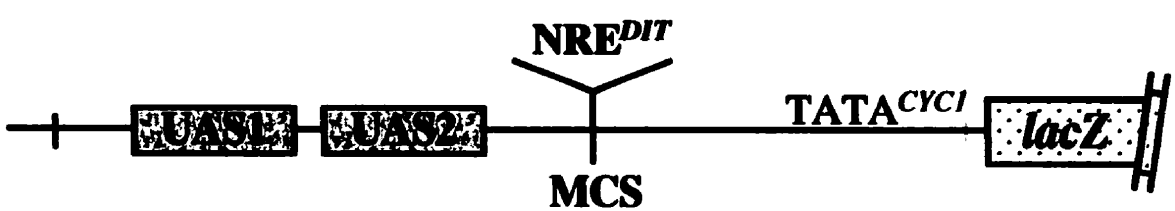
50

A




| | No. of copies | Repression | Rim101 dependence |
|---|---|---|---|
| 1. | 1X | Strong | Yes |
| 2. | 1X | Strong | Yes |
| 3. | 1X | Weak | Yes |
| 4. | 3X | Moderate | Yes |
| 5. | 1X | Weak | No |
| 6. | 3X | Moderate | No |
| 7. | 2X | Weak | No |
| 8. | 1X | Weak | No |
| 9. | 2X | Moderate | No |
| 10. | 1X | Weak | No |
| 11. | 3X | Strong | No |
| 12. | 1X | Weak | Yes |
| 13. | 3X | Strong | Yes |

B



I next tested a fragment, NRE29, that encompasses the region from nt -505 to –477 for operator function. This fragment contains the upstream portion of NRE42 and includes the PacC site (nt -484 to -477) but does not contain any sequence downstream of this site. The presence of one copy of NRE29 in the polylinker of pLG312Bgl did not affect expression of the reporter gene whereas the presence of three copies of this fragment led to moderately reduced expression of the *CYC1-lacZ* reporter gene (Fig. 2A, lines 5 and 6). Although the extent of repression mediated by 3xNRE29 was similar to that mediated by 3xNRE30, only repression by the former fragment was Rim101-dependent. I concluded that neither of the overlapping sequences, NRE29 or NRE30, which together spanned NRE42, could provide the same level of repression as NRE42 but that multiple copies of either element afforded significant repression. I also concluded that NRE42-mediated repression was achieved by both a Rim101-dependent and a Rim101-independent mechanism and that the minimal PacC site was not sufficient to mediate Rim101-dependent repression. My data suggest that at least some nucleotides immediately downstream of the PacC site contribute to its function (Fig. 2A, *cf.* lines 4 and 6).

To investigate further the notion that NRE42 contains two distinct operator sites that combine to give a much higher level of repression than expected from a simple additive effect, I tested additional fragments for operator function. The overlapping fragments NRE23 and NRE22U, which together span the sequence present in NRE29, act as modest operators when present in duplicate copies (Fig. 2A, lines 7 and 9). The overlapping fragments NRE25 and NRE22D, which together span the region represented by NRE42, each afforded strong repression when present in multiple copies but not when present in a single copy (Fig. 2A, lines 10 to 13). Repression by NRE22D, which contains the PacC site and downstream sequence, was Rim101-dependent, whereas repression by NRE25, which contains only a portion of the PacC site was Rim101-independent. From this qualitative study, I concluded that the NRE42 operator was bipartite in nature with both sub-sites combining to give a much higher level of repression than that predicted by simple arithmetic summation of the operator function of each sub-site. I also found that multimers of each element could act co-operatively to give a high level of repression. This type of synergistic effect afforded by a combination of promoter elements or by tandem repeats of the same promoter element has been well documented in yeast (*e.g.* Giniger and Ptashne, 1988).

**3.A.iii. Quantitative characterisation of the operator function of NRE25 and NRE22D.** To assess in greater detail the operator activities of NRE25 and NRE22D I carried out quantitative, liquid β-galactosidase (β-gal) assays with extracts of cells containing various reporter genes. First I compared repression provided by a single copy of NRE76, NRE42, NRE25 and NRE22D inserted between the UAS and TATA box of the *CYC1-lacZ* reporter gene of pLG312MCS#3[5]. The data are given as fold-repression relative to expression of β-galactosidase from pLG312MCS#3. As was apparent from the qualitative assays, NRE42, which provided 500-fold repression in this experiment, was almost as efficient an operator as NRE76, which provided 645-fold repression in this experiment (Fig. 3A, grey bars). These quantitative assays also demonstrated that NRE76 and NRE42 maintained a low level of repression in the absence of Rim101, 38- and 32-fold, respectively (Fig 3A, grey bars). I also tested the activity of these operators in diploid cells. Repression by NRE76 and NRE42, although approximately two-fold less than in haploid cells, remained very efficient (~300-fold) (Fig. 3A, black bars). In contrast, a single insert of NRE22D and NRE25 gave only 8- and 10-fold repression, respectively, in haploid cells (Fig. 3A). I next confirmed that multimerization of NRE22 or NRE25 leads to more efficient repression. In this particular experiment (Fig. 3B) the presence of NRE76 reduced expression of the reporter gene ~1200-fold. Increasing the number of copies of NRE25 from two to three, increased the efficiency of repression by this element from 70-fold to 165-fold (Fig. 3B). The effect of increasing the number of copies of NRE22D from two to three was quite dramatic. Repression increased from ~200-fold to ~1600-fold (Fig. 3B).

In a distinct experiment, I next compared repression by NRE76, NRE42, three copies of NRE25 and three copies of NRE22D in wild type (W3031A), *rim101Δ*, and *tup1Δ* backgrounds (Fig. 3C). It should be noted that for each experiment the data are an average of β-galactosidase activities obtained from two to three different transformants (see Material and Methods) with standard errors being less than 10-15%. Although the absolute extent of repression mediated by NRE76, NRE42, 3xNRE25, and 3xNRE22D in wild-type cells varied two- to three-fold between experiments (*cf.* panels A to C of Fig. 3), nonetheless, the conclusions made by comparison of the relative levels of repression were similar between experiments.

The most dramatic observation from the quantitative results presented in Fig. 3C was that whereas repression by NRE76 and NRE42 was greatly reduced in a *rim101Δ* strain and

[5] Note, as described earlier, I have used pLGNRE76, which had been cloned earlier.

**Figure 3. Rim101-dependent and independent repression through NRE25 and NRE22D.**

(A, B, C) Results of quantitative, liquid β-galactosidase assays of cells of the indicated strains containing pLG312Bgl or pLG312MCS#3 with the indicated fragments inserted into the *BglII* site of the *CYC1-lacZ* reporter gene (see Materials and Methods). The data are summarised as fold repression, which is the ratio of the β-galactosidase units measured in cells containing pLG312MCS#3 to the β-galactosidase units measured in cells containing pLG312MCS#3 with the indicated NRE fragments inserted into the reporter gene.

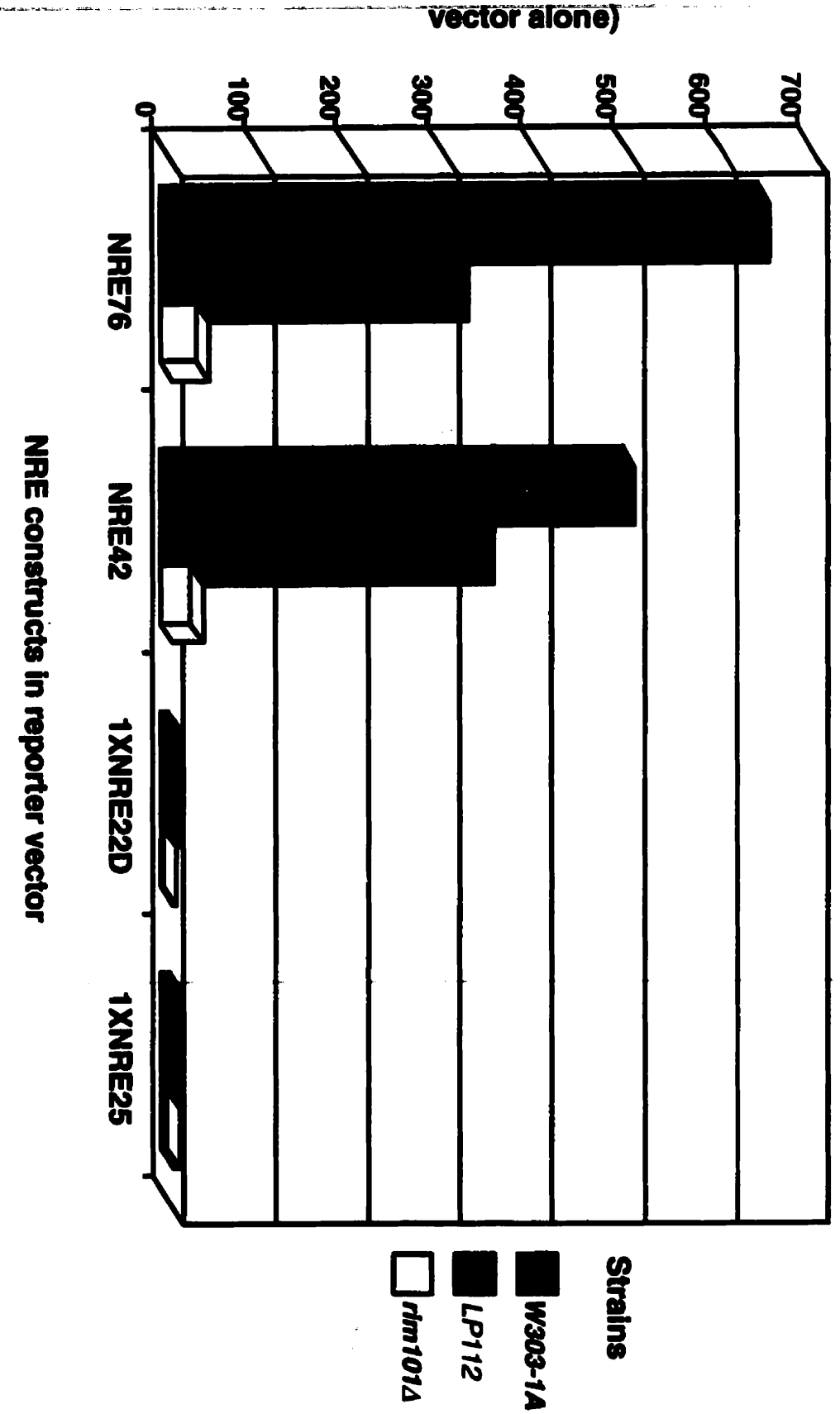
(A) Comparison of the extent of repression of reporter gene expression mediated by NRE76, NRE42, 1xNRE22D and 1xNRE25 in wild-type haploid cells (W3031A; light grey bars), wild-type diploid cells (LP112; dark grey bars), and haploid *rim101Δ* cells (open bars).

(B) Comparison of the extent of repression reporter gene expression mediated by NRE76, NRE42, 2xNRE25, 3xNRE25, 2xNRE22D, and 3xNRE22D in wild-type cells.

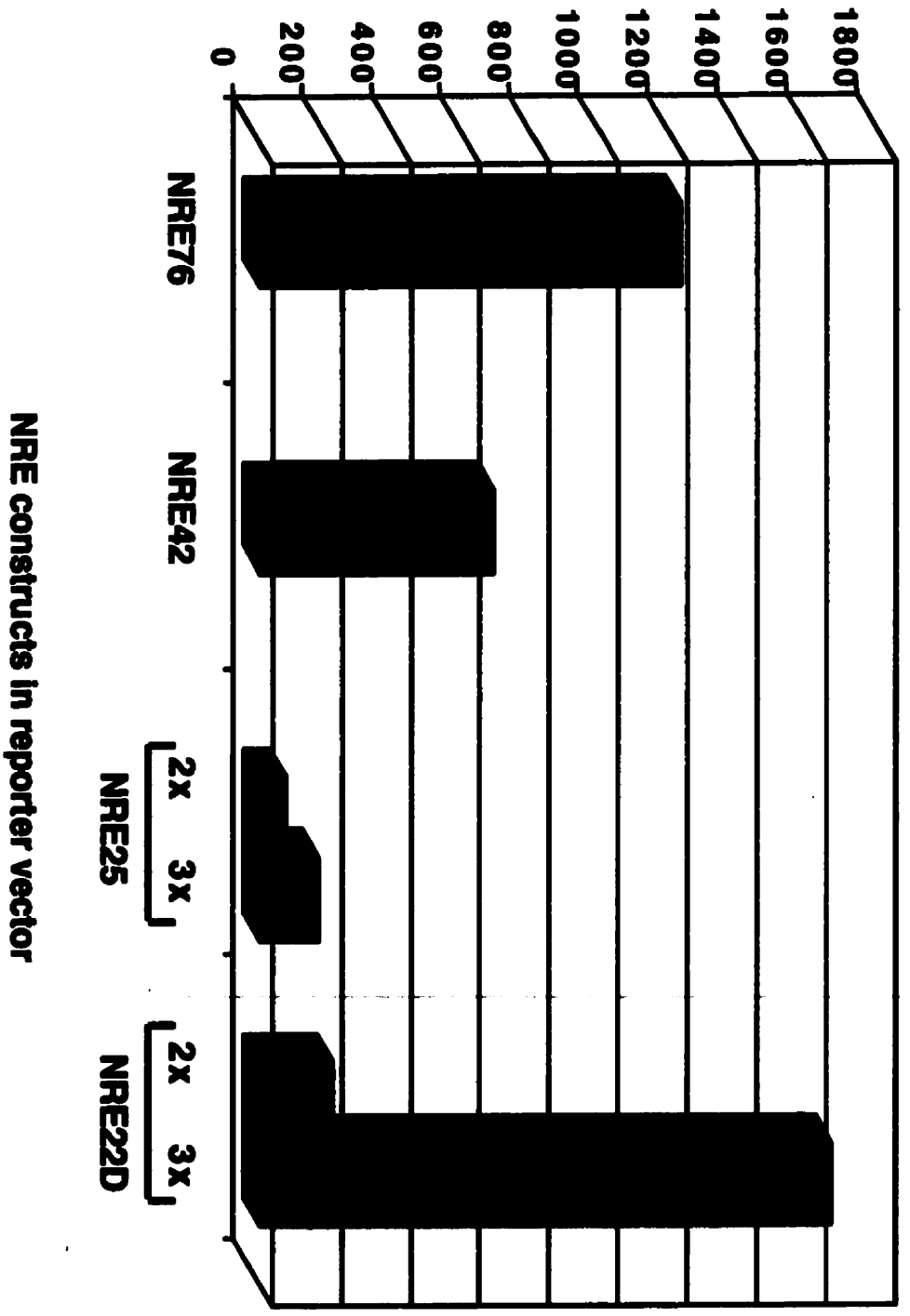
(C) Comparison of the extent of repression reporter gene expression mediated by NRE76, NRE42, 3xNRE25, and 3xNRE22D in wild-type cells (W3031A; light grey bars), *rim101Δ* cells (dark grey bars) and *tup1Δ* cells (white bars).

(D) Portion of the *DIT1/DIT2* intergenic region spanning NRE76. The regions encompassed by NRE42, NRE25 and NRE22D are indicated. The $PacC^{DIT}$ site is boxed.

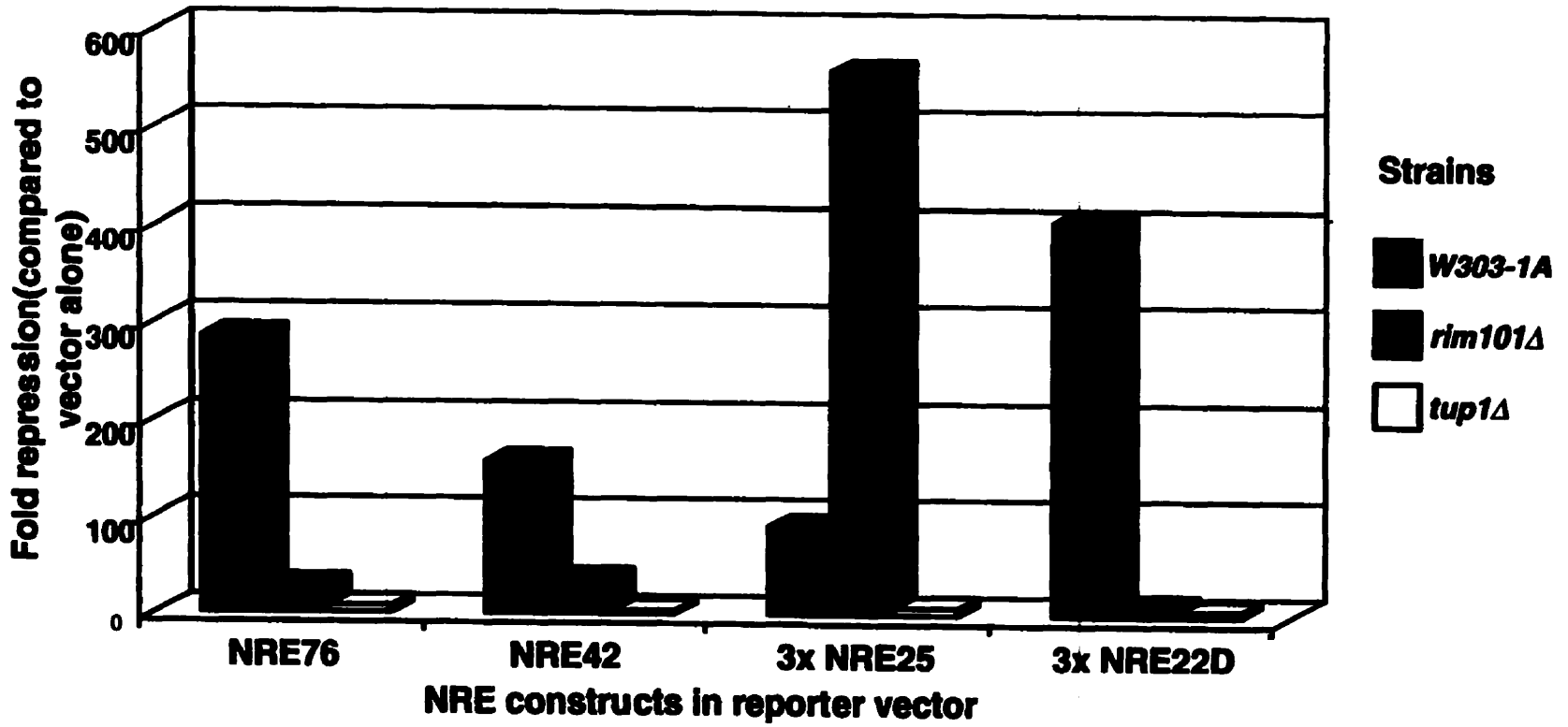
A


Fold repression(compared to vector alone)
0
100
200
300
400
500
600
700
NRE76
NRE42
1XNRE22D
1XNRE25
NRE constructs in reporter vector
Strains
W303-1A
LP112
rim101Δ

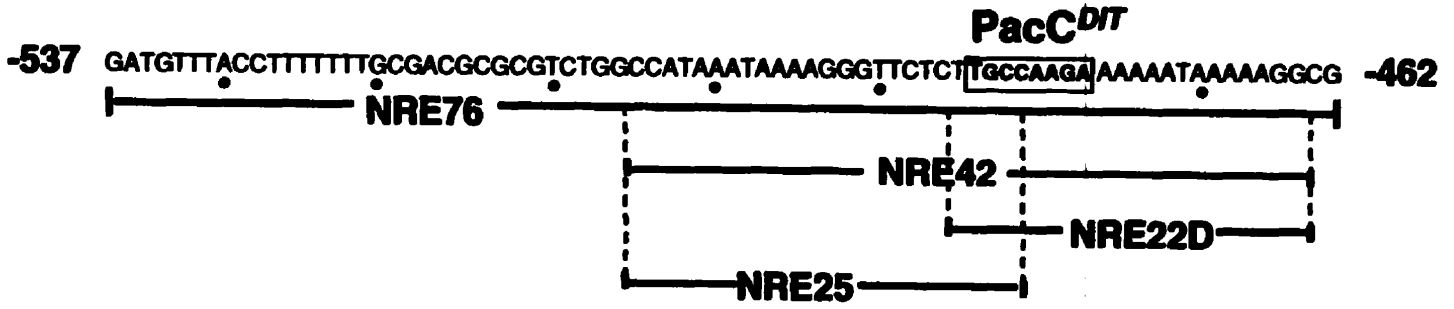
B


Fold repression(compared to vector alone)
0
200
400
600
800
1000
1200
1400
1600
1800
NRE76
NRE42
2x
3x
NRE25
2x
3x
NRE22D
NRE constructs in reporter vector

C


Fold repression(compared to vector alone)
600
500
400
300
200
100
0
NRE76
NRE42
3x NRE25
3x NRE22D
NRE constructs in reporter vector
Strains
W303-1A
rim101Δ
tup1Δ

D


PacC^DIT
-537
GATGTTTACCTTTTTTTGCGACGCGCGTCTGGCCATAAATAAAAGGGTTCTCTTGCCAAGAAAAATAAAAAGGCG
-462
NRE76
NRE42
NRE22D
NRE25

repression by 3xNRE22D was almost abolished in this strain, repression by 3xNRE25 was enhanced on mutation of *RIM101*. Once again, although the extent of enhancement varied from experiment to experiment, repression mediated by 3xNRE25 was always consistently higher in a *rim101Δ* strain than in a *RIM101* strain. This effect is discussed further below. Finally repression by 3xNRE25 and 3xNRE22D was Tup1-dependent (Fig. 3C), as is the case for NRE76 and NRE42 (Tanny, 1998b) (and Fig. 3C).

In summary this analysis has shown that the repression activity of NRE42 is similar to that of NRE76 and that NRE42 represents the minimal element that will mediate full repression. My data indicate that there are two sites within NRE42 that contribute to repression. The region represented by NRE22D contributes to Rim101-dependent repression. The region represented by NRE25 contributes to Rim101-independent repression. Repression by both these sub-sites requires Tup1. These data support the model (Tanny, 1998b) that a yet-to-be identified factor, referred to as protein X, binds to an element within NRE25 and acts with Rim101 bound to the sequence encompassing the PacC site in NRE22D to recruit the Ssn6-Tup1 repression complex. The simultaneous presence of both protein X and Rim101 leads to a synergistic effect on repression. Surprisingly NRE25 is more efficient in repression in the absence of Rim101.

## 3.B The short form of Rim101 mediates repression through NRE$^{DIT}$

The products of the *RIM8*, *RIM9*, *RIM13*, *RIM20* and *RIM30* genes contribute to the proteolytic cleavage of Rim101, removing approximately 70 residues from the carboxy-terminus (see Introduction). The majority of Rim101 present in wild-type cells is in the truncated or short form. However, some unprocessed Rim101 is also present (Li and Mitchell, 1997). Mutation of either *RIM8*, *RIM9*, *RIM13*, *RIM20* or *RIM30* blocks proteolytic cleavage of Rim101 and generates a mutant phenotype that is similar to that of a *rim101* strain. This suggests that it is the short form of Rim101 that is functional. To determine whether cleavage of Rim101 is required for repression through NRE$^{DIT}$, I tested the activity of various NREs in a *rim9Δ* strain in which processing is blocked (Li and Mitchell, 1997). I used the qualitative overlay assay to compare the extent of NRE76- , NRE42-, 3xNRE22D-, and 3xNRE25-mediated repression in wild-type (W3031A) cells and in otherwise isogenic *rim101Δ*, *rim9Δ*, and *tup1Δ* strains. Colonies of each

of these strains harbouring pLG312MCS#3, which contains the parental *CYC1-lacZ* reporter gene, became dark blue shortly after being overlaid with X-gal-containing agar (Fig. 4, column 1). As expected from my previous results (Fig. 2 and Fig. 3C), colonies of wild-type cells containing pLGNRE76, pLGnNRE42, pLGn3xNRE22D, or pLGn3xNRE25 remained white (Fig. 4, top row) and colonies of *tup1Δ* cells containing these plasmids became dark blue (Fig. 4, bottom row). Colonies of *rim101Δ*cells containing pLGn3xNRE22D became dark blue, colonies of *rim101Δ*cells containing pLGn3xNRE25 remained white, and colonies of *rim101Δ* cells containing pLGNRE76 or pLGnNRE42 became light blue (Fig. 4, row 2). This is consistent with the notion that NRE22D-mediated repression is Rim101- and Tup1-dependent and that NRE25-mediated repression is Rim101-independent and Tup1-dependent. The combination of these sites in NRE76 and NRE42 leads to efficient repression that is mediated by both Rim101-dependent and Rim101-independent mechanisms, both of which depend on Tup1.

In the *rim9Δ* strain, 3xNRE25 maintained full repression whereas 3xNRE22D-mediated repression was reduced. This is consistent with the short form of Rim101 being responsible for repression mediated by NRE22D. It should be noted however that repression by NRE76, NRE42, and 3xNRE22D appeared marginally more efficient in the *rim9Δ* strain than in the *rim101Δ* strain. The Rim9 protein is predicted to have four transmembrane domains; thus, it is possible that it may be a pH/signal sensor protein. Some Rim9-independent signalling may also occur and a small portion of Rim101 (not detectable through a Western) may be cleaved. It is also possible that full-length Rim101 has a low level of repressor activity that becomes fully functional on cleavage of the protein.

I also tested the efficiency of NRE-mediated repression in a strain that expresses only a truncated version of Rim101 (see Materials and Methods). A qualitative comparison showed that the extent of repression mediated by NRE76, NRE42, 3xNRE22D, and 3xNRE25 in cells expressing only a truncated version of Rim101 was similar to the extent of repression mediated by these fragments in wild-type cells (data not shown). These data indicated, as was suggested by the experiments carried out in the *rim9Δ* strain, that the short form of Rim101 is functional in mediating NRE-dependent repression.

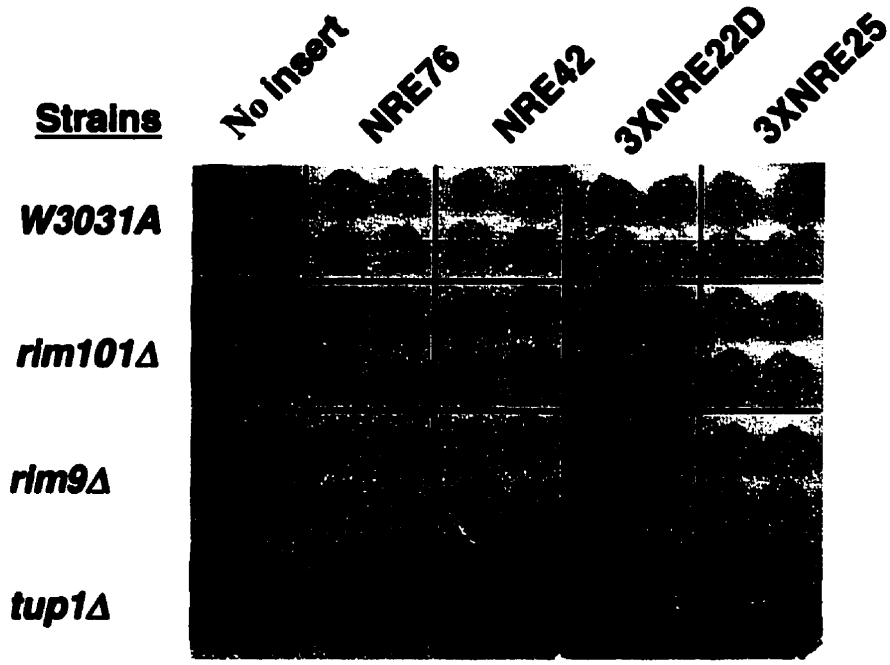



**Figure 4. The short form of Rim101 mediates repression through NRE$^{DIT}$.**

In the context of the *CYC1-lacZ* reporter, NRE76, NRE42, 3xNRE22D, and 3xNRE25 were tested for their abilities to repress in *W3031A*, *rim101Δ*, *rim9Δ*, and *tup1Δ* backgrounds. The results of an X-gal overlay experiment are presented above (See Materials and Methods and Figures 2B, and 3D for more details on contructs).

## 3.C Testing for *in vivo* binding of Rim101-HA to $NRE^{DIT}$ by a chromatin coimmunoprecipitation approach.

**3.C.i. *DIT1* DNA only co-immunoprecipitates with Rim101-HA in extracts of cells that have not been treated with formaldehyde.** One of the major goals of this project was to demonstrate that Rim101 mediates repression by acting directly through $NRE^{DIT}$. Jason Tanny demonstrated that a bacterially expressed GST-tagged polypeptide that contains the three zinc fingers of Rim101 binds *in vitro* to the NRE30-containing oligonucleotide as assessed by an electrophoretic mobility shift assay (EMSA) (Tanny, 1998a). Cosimo Commisso, who was an undergraduate project student in our lab, continued this series of experiments. He showed that an *in vitro*-synthesised version of Rim101 containing the amino-terminal 289 residues of Rim101, which includes the three zinc fingers, binds specifically to the NRE22D-containing oligonucleotide as assessed by EMSA. The wild-type oligonucleotide but not a version of the fragment containing an A to T mutation within the $PacC^{DIT}$ site could compete for Rim101 binding (Commisso, 2000). Furthermore, Cosimo Commisso demonstrated that the zinc finger region of Rim101 binds to a plasmid-borne NRE22D site *in vivo*. He found that expression in yeast cells of a $Rim101^{1\text{-}289}$-$Gal4^{AD}$ fusion protein, but not a $Rim101^{1\text{-}289}$ polypeptide, leads to activation of an NRE22D-*lacZ* reporter gene as assessed by a colony overlay assay (Commisso, 2000). The ability of the Gal4 portion of the fusion protein, which contains an activation domain, to activate the reporter gene is presumed to reflect the binding of the Rim101 portion of the fusion protein to the NRE22D site in the promoter region of the reporter gene.

I have used a chromatin immunoprecipitation (ChIP) approach (reviewed in the Introduction) to test for *in vivo* binding of Rim101 to the *DIT1-DIT2* intergenic region. In this approach, cells are treated with formaldehyde to cross-link DNA-bound proteins to their target sites *in vivo*. This cross-linking prevents dissociation of protein-DNA complexes on preparation of chromatin extracts and subsequent shearing of the chromatin. Specific protein-DNA complexes are then immunoprecipitated and DNA in the pellet is identified by PCR amplification.

As the first step for my ChIP experiments, I transformed *rim101Δ* cells with pWL41, a 2μ plasmid that encodes Rim101-HA (see Material and Methods). This HA-tagged Rim101 has been shown to restore expression of an *ime2-lacZ* reporter gene and to increase the efficiency of

spore formation in a *rim101Δ* strain (Li and Mitchell, 1997). I confirmed that this HA-tagged Rim101 also complemented the defect in NRE-mediated repression that is observed in *rim101Δ* cells. Indeed, I found that the presence of pWL41 in *rim101Δ* cells restored NRE76- and NRE22D-mediated repression to the same extent as did the presence of a low-copy plasmid expressing untagged Rim101 (data not shown). Similarly, as expected from my previous observations described above, expression of Rim101-HA in *rim101Δ* cells reduced NRE25-mediated repression.

I next showed that Rim101-HA could be immunoprecipitated with a monoclonal anti-HA antibody from extracts prepared from formaldehyde-treated cells. After determining the optimal dilution of antibody for maximal recovery of Rim101-HA in the immunoprecipitate, I made a qualitative estimate based on inspection of Western blots that approximately 1/100 of the Rim101-HA that was present in cells could be immunoprecipitated (data not shown).

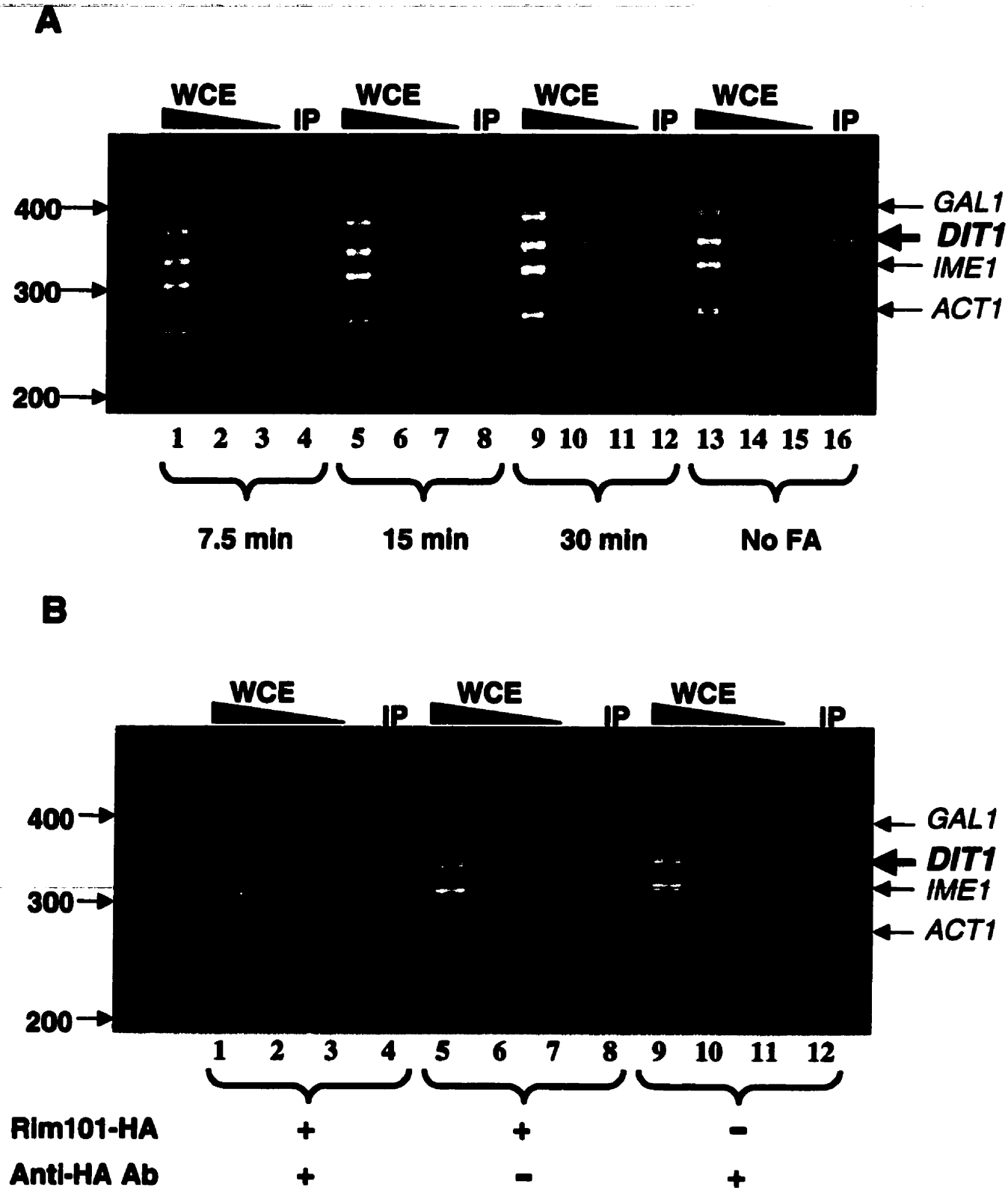
To assess binding of Rim101-HA to the genomic *DIT1-DIT2* intergenic region in mitotic cells, I harvested cells from log-phase cultures that had been treated with formaldehyde and prepared cell lysates (see Materials and Methods). After the chromatin in the cell lysate had been sheared to give 0.5-1 kb fragments (as determined in a pilot experiment; data not shown), monoclonal anti-HA antibody was added to a portion of the sheared lysate (which is also referred to as the whole cell extract, WCE), to allow recovery of Rim101-HA-DNA complexes. An aliquot of the WCE and the immunoprecipitate (IP) was then treated to break the formaldehyde-induced protein-DNA cross-links (see Materials and Methods). I then used semi-quantitative PCR to determine the relative amounts of various DNAs in the immunoprecipitates. In addition to a set of primers for amplification of DNA spanning $NRE^{DIT}$, I included three other sets for primers for amplification of DNA in the promoter regions of the *IME1*, *ACT1*, and *GAL1* genes. All primer pairs had very similar melting temperatures and led to the amplification of DNA fragments of similar size (see Table 3). I adjusted primer concentrations (ranging from 40-60 pmoles/reaction) such that all four primer pairs yielded similar amounts of product when DNA from a whole cell extract was used as template.

I included the *IME1* primer pair in the ChIP experiments because *RIM101* was originally identified as a positive regulator of *IME1* expression (Su and Mitchell, 1993b) and a low affinity PacC site is located 1100 bases upstream of the *IME1* transcription start site (Friesen, 1998). An Upstream Control Region (UCS2) that contains both repressive and activating elements has been

**Figure 5. Use of a chromatin immunoprecipitation approach to test for *in vivo* binding of Rim101 to the genomic NRE$^{DIT}$ site.**

(A) PCR analysis of two-fold serially diluted samples of DNA from whole cell extracts (lanes 1 to 3, 5 to 7, 9 to 11, and 13 to 15) and of an aliquot of DNA recovered from the anti-HA immunoprecipitates (lanes 4, 8, 12, and 16) of chromatin from *rim101Δ*cells that had been treated with formaldehyde for the indicated times (lanes 1 to 12) or from cells that had not been treated with formaldehyde (lanes 13-16). All cells contained pWL41, which encodes Rim101-HA. See the Materials and Methods for details of the chromatin immunoprecipitation procedure and for a description of the PRC amplification. Four primer pairs were used in the same reaction: primer pairs for amplification of regions in the promoters the of the *GAL1*, *IME1*, and *ACT1* genes and a primer pair for amplification of the NRE$^{DIT}$-containing region (see Table 3). The PCR products were analysed by electrophoresis in agarose gels. The positions of the 400, 300, and 200 bp molecular markers are indicated at the left of the gel. WCE, whole cell extract. IP, immunprecipitate.

(B) PCR analysis of two-fold serially diluted samples of DNA from whole cell extracts (lanes 1 to 3, 5 to 7, and 9 to 11) and of an aliquot of DNA recovered from the anti-HA immunoprecipitate (lanes 4, 8, and 12) of chromatin from *rim101Δ* cells that had not been treated with formaldehyde. Cells for the experiments of lanes 1 to 8 contained pWL41, which encodes Rim101-HA. The anti-HA antibody was omitted from the immunoprecipitation carried out with the sample of lane 8. The PCR products were analysed by electrophoresis in agarose gels. The positions of the 400, 300, and 200 bp molecular markers are indicated at the left edge of the gel.

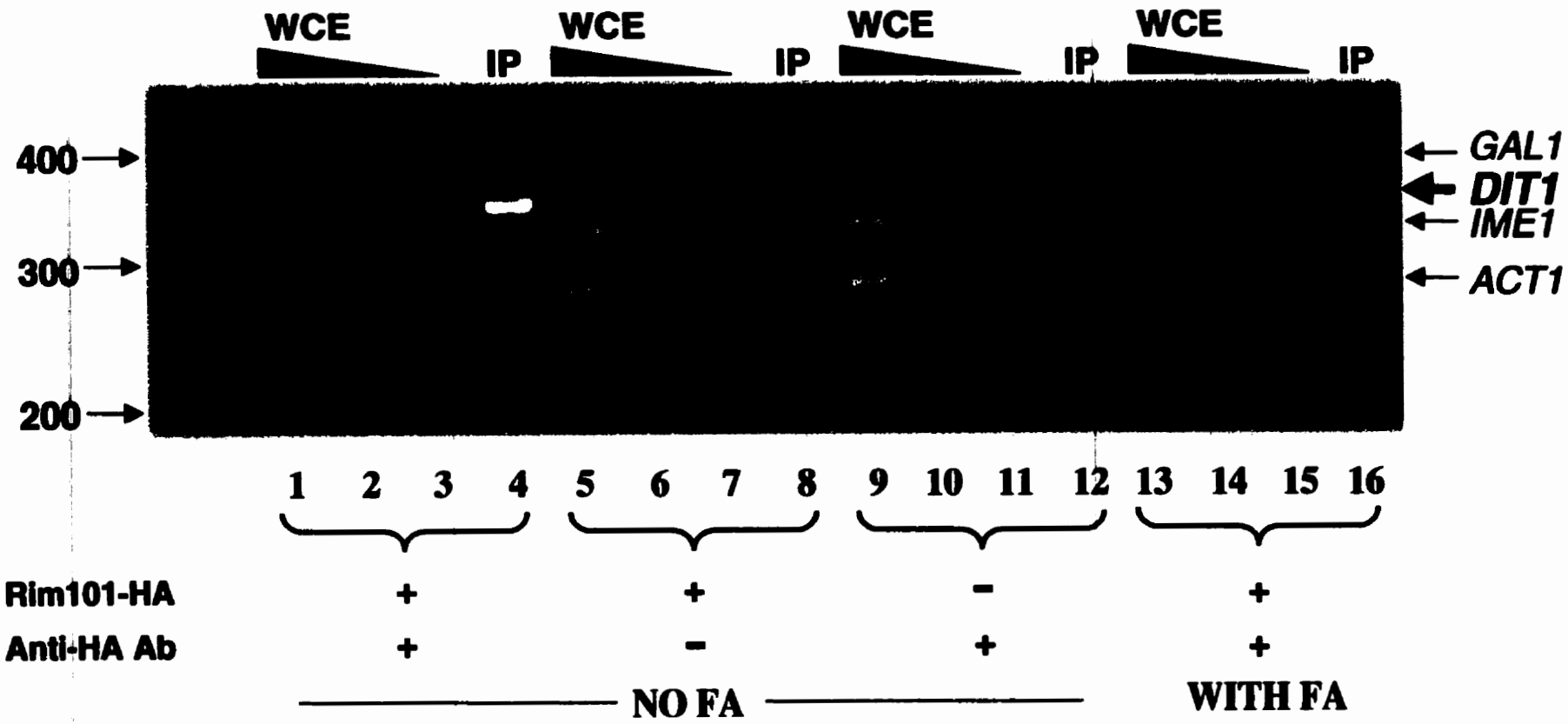

A
WCE
IP
WCE
IP
WCE
IP
WCE
IP
400
300
200
GAL1
DIT1
IME1
ACT1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
7.5 min
15 min
30 min
No FA
B
WCE
IP
WCE
IP
WCE
IP
400
300
200
GAL1
DIT1
IME1
ACT1
1 2 3 4 5 6 7 8 9 10 11 12
Rim101-HA + + -
Anti-HA Ab + - +

mapped between nucleotides –1369 to –621 of the *IME1* promoter region. Further dissection of this region revealed the presence of a repressive element around –1100 (Sagee *et al.*, 1998). The region from –1215 to –915 contains a Tup1-dependent operator site as well as a UAS that is only active in *tup1* cells (Mizuno *et al.*, 1998). I did not detect any enrichment for *DIT1* DNA, nor *IME1* DNA, in the immunoprecipitates of lysates prepared from cells that had been treated with formaldehyde for various periods of time. Representative results are shown in Fig. 5A for cells that had been exposed to 1% formaldehyde for 7.5, 15, and 30 minutes (lanes 1-12). I discovered, however, that *DIT1* DNA was selectively recovered in the anti-HA immunoprecipitate of cells that had not been treated with the cross-linker prior to harvesting (Fig. 5A, lanes 13-16). This enrichment of *DIT1* DNA in the immunoprecipitate depended on the expression of Rim101-HA in the cells (Fig. 5B, lanes 9-12) and the use of the anti-HA antibody (Fig. 5B, lanes 5-8). This observation that selective recovery of *DIT1* DNA in immunoprecipitates of chromatin required that the cells not be exposed to cross-linker was unexpected.

**3.C.ii. *DIT1* DNA is not recovered in immunoprecipitates of extracts of formaldehyde-treated *tup1Δ* cells that express Rim101-HA.** In our model for the mechanism of $NRE^{DIT}$-mediated repression, we speculate that DNA-bound Rim101 recruits the Ssn6-Tup1 repression complex to the *DIT1* promoter. It was conceivable that recruitment of the 440 kDa co-repressor complex (Varanasi *et al.*, 1996), which contains one molecule of Ssn6 and four molecules of Tup1, might occlude the HA epitope of Rim101-HA. In this case, formaldehyde-induced, *in vivo* cross-linking of the proteins in the complex might prevent recognition of the HA epitope by the anti-HA antibody *in vitro*. As outlined above, I had determined that about 1/100 of the total Rim101-HA can be immunoprecipitated from formaldehyde-treated cells. Since short formaldehyde treatments (minutes vs. hours) leave some proteins unlinked, it is possible that even in formaldehyde-treated, *TUP1* cells, only the non-cross-linked Rim101-HA can be detected through Western blotting. To test the possibility that the Tup1 complex could occlude the HA-epitope, I repeated the ChIP experiments in *tup1Δ* cells that expressed Rim101-HA. I found that the absence of Tup1 did not lead to enrichment for *DIT1* DNA in the immunoprecipitates of extracts of cells that had been treated with formaldehyde (Fig. 6, lanes 13-

**Figure 6. *DIT1* DNA is not recovered in chromatin immunoprecipitates from extracts of formaldehyde-treated *tup1Δ* cells that express Rim101-HA.**

To determine if Rim101-HA could be detected at the NRE in the absence of Tup1, lysates were prepared from *rim101Δ tup1Δ* cells that had not been treated with formaldehyde (lanes 1 to 12) or that had been treated with formaldehyde (lanes 13 to 16). Cells analysed in lanes 1 to 8 and 13 to 16 contained pWL41, which encodes Rim101-HA. PCR was performed with two-fold serially diluted samples of DNA from whole cell extracts (lanes 1 to 3, 5 to 7, 9 to 11 and 13 to 15) and of an aliquot of DNA recovered from the anti-HA chromatin immunoprecipitates (lanes 4, 8, 12 and 16). PCR primer pairs were used for the amplification of regions in the promoters of the *GAL1*, *DIT1*, *IME1*, and *ACT1* genes. The anti-HA antibody was omitted from the immunoprecipitation carried out with the sample of lane 8. The positions of the 400, 300, and 200 bp molecular size markers are indicated at the left edge of the gel.


WCE
IP
WCE
IP
WCE
IP
WCE
IP
400
300
200
GAL1
DIT1
IME1
ACT1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Rim101-HA + + - +
Anti-HA Ab + - + +
NO FA
WITH FA

16). However, as was observed for *TUP1* cells, *DIT1* DNA was clearly enriched in the immunoprecipitates of extracts of *tup1Δ* cells that had not been treated with formaldehyde (Fig. 6, lanes 1-4). The recovery of *DIT1* DNA from these cells required the expression of Rim101-HA (Fig. 5, lanes 9-12) and presence of the anti-HA antibody (Fig. 6, lanes 5-8). Thus, it is not the presence of Tup1 in genomic Rim101-HA-NRE$^{DIT}$ complexes that prevents recovery of *DIT1* DNA in anti-HA immunoprecipitates obtained from extracts of formaldehyde-treated *TUP1* cells. This experiment does, however, argue against the possibility that Tup1 acts indirectly to control the expression of Rim101 as Rim101 is still present in *tup1Δ* cells.

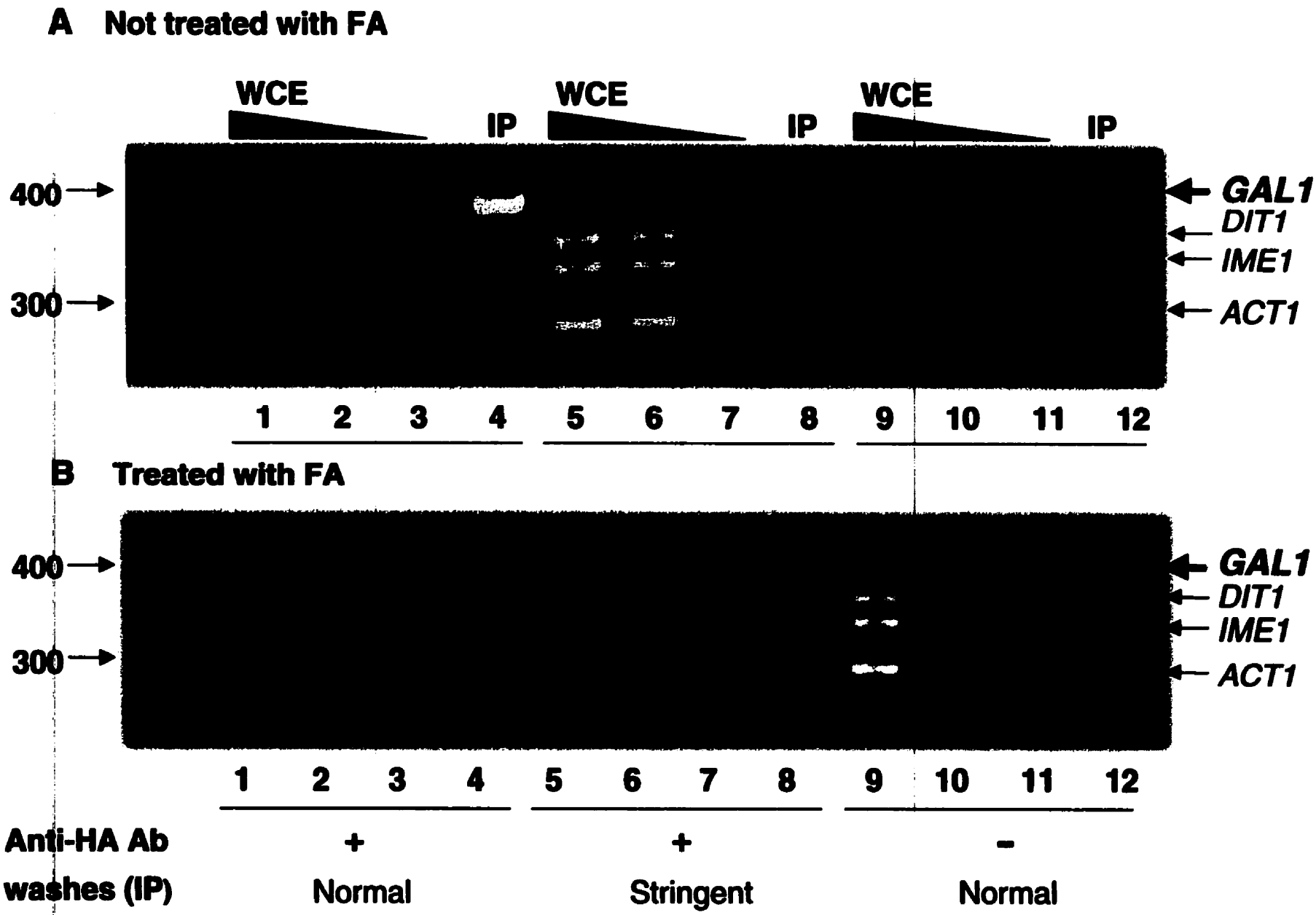
## 3.D *GAL1* DNA co-immunoprecipitates with GAL4(BD)-HA in the absence and presence of formaldehyde linking

Treatment of cells with formaldehyde followed by chromatin immunoprecipitation has been used extensively in studies with a number of organisms to detect the *in vivo* binding of proteins to genomic DNA (reviewed in Kuo and Allis, 1999). This technique has also been useful for the detection of proteins that are in the vicinity of chromatin as part of large complexes. In such studies, investigators generally demonstrate that the preferential presence of a specific DNA sequence in the immunoprecipitate depends on prior treatment of cells with formaldehdye, on the presence of the (tagged) protein in the cells, and/or on the use of the anti-tag/protein antibody for immunoprecipitation. I was therefore surprised that I readily obtained preferential recovery of *DIT1* DNA in immunoprecipitates from cells that had not been treated with cross-linker. For comparison, I carried out similar ChIP experiments to assess the recovery of *GAL1* promoter DNA on immunoprecipitation of Gal4-containing chromatin from extracts of cells. The interaction of Gal4 with its target UAS in the *GAL1-10* intergenic region has been well studied (reviewed in Lohr *et al.*, 1995). In particular, I wished to compare recovery of *GAL1* DNA from cells that had not been treated with cross-linker to that from cells that had been treated with cross-linker.

For this experiment I transformed yeast cells with a plasmid allowing expression of an HA-tagged polypeptide containing the DNA-binding (DB) of Gal4. The same set of four primer pairs were used as described above and gave approximately equal amounts of amplified product

**Figure 7. Co-immunoprecipitation of *GAL1* DNA with Gal4(BD)-HA in chromatin from cells that had been treated or had not been treated with formaldehyde.**

Cells of strain YM4271 (*gal80Δgal4Δ*) were transformed with pASII, which encodes Gal4(BD)-HA. The relative efficiencies of co-immunoprecipitation of *GAL1* DNA with Gal4(BD)-HA from extracts of these cells that had not been treated with formaldehyde (panel A) and from extracts of cells that had been previously treated with formaldehyde (panel B) were compared. PCR was performed with two-fold serially diluted samples of DNA from whole cell extracts (lanes 1 to 3, 5 to 7, and 9 to 11) and of an aliquot of DNA recovered from the anti-HA chromatin immunoprecipitates (lanes 4, 8, and 12). The anti-HA antibody was omitted from the immunoprecipitations carried out with the samples of lane 12. The immunoprecipitates of the samples represented in lane 8 were subjected to more stringent washing conditions than those normally used (see Materials and Methods) prior to recovery of DNA. PCR primer pairs were used for the amplification of DNA in the promoter regions of the *GAL1*, *DIT1*, *IME1*, and *ACT1* genes. The PCR products were analysed on an agarose gel. The positions of the 400, 300, and 200 bp molecular markers are indicated at the left edge of the gel.


A Not treated with FA
WCE
IP
WCE
IP
WCE
IP
400
300
GAL1
DIT1
IME1
ACT1
1 2 3 4 5 6 7 8 9 10 11 12
B Treated with FA
400
300
GAL1
DIT1
IME1
ACT1
1 2 3 4 5 6 7 8 9 10 11 12
Anti-HA Ab
washes (IP)
+
Normal
+
Stringent
-
Normal

from the sheared chromatin in whole cells extracts (Fig. 7A and B, lanes 1-3, 5-7, and 9-11) with the exception that *GAL1* DNA was slightly underrepresented. The fragment containing the GAL1 promoter was preferentially amplified from immunoprecipitates prepared with anti-HA antibody (Fig. 7, lane 4). A much greater amount of *GAL1* DNA was recovered by PCR amplification of the immunoprecipitate prepared from cells that had not been treated with formaldehyde than from cells that had been treated with formaldehyde (compare Fig. 7A. lane 4 with Fig. 7B, lane 4). The enrichment for *GAL1* DNA in the immunoprecipitates was dependent on the use of the anti-HA antibody (Fig. 7, lanes 9 to 12).

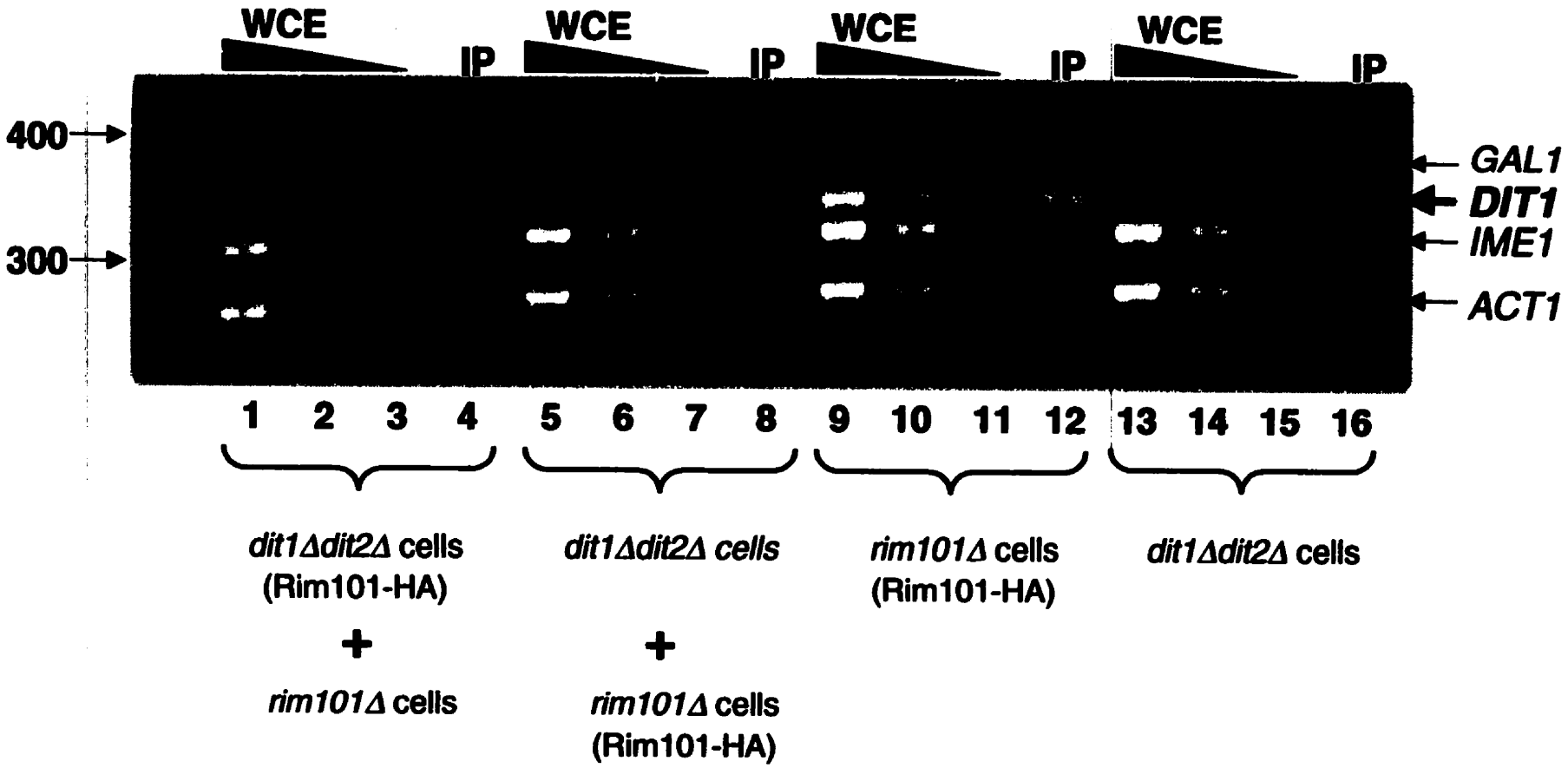
I next tested the effect of increasing the stringency of the washing conditions used for the immunoprecipitates. The washing buffer should remove DNA that is not cross-linked to protein by disrupting electrostatic protein-DNA interactions but not the antigen-antibody interaction nor the covalent bonds introduced by formaldehyde treatment. Although increasing the stringency of the washing conditions substantially reduced enrichment for *GAL1* DNA in the IP from non formaldehyde-treated cells, the amplified product was still above the level obtained with the immunoprecipitate from formaldehyde-treated cells (Fig 7A and B, lanes 8).

## 3.E Cellular Rim101-HA present in lysates can associate with chromatin and allow selective recovery of *DIT1* DNA in anti-HA immunoprecipitates

The co-immunoprecipitation of *DIT1* DNA with Rim101-HA in extracts of cells that had not been treated with formaldehyde raised the possibility that Rim101-HA could selectively associate with *DIT1*-containing chromatin fragments in cell lysates and that this interaction was of sufficient affinity to survive the immunoprecipitation and washing procedures. A major rationale for treatment of cells with cross-linker in ChIP experiments is to prevent dissociation of protein-DNA complexes once cells have been lysed. Another rationale, particularly in studies of dynamic protein-DNA complexes, is to ensure that stringent wash conditions can be used such that complexes resulting from redistribution of proteins in cell lysates are not measured. To test the possibility that Rim101-HA could redistribute from solution to DNA, or from one DNA target site to another, in cell lysates I carried out the following mixing experiment. I mixed cells that were deleted for the genomic *DIT1-DIT2* region and that expressed Rim101-HA with *rim101Δ DIT1 DIT2* cells that did not express Rim101-HA. My results clearly showed that

**Figure 8. Cellular Rim101-HA present in lysates can associate with chromatin and allow selective recovery of *DIT1* DNA in anti-HA immunoprecipitates.**

Cells that had not been treated with formaldehdye were mixed prior to lysis as follows. The DNA analysed in lanes 1 to 4 was prepared from a mixture of *dit1Δ dit2Δ* cells that contained pWL42, which encodes Rim101-HA, and *rim101Δ* cells. The DNA analysed in lanes 1 to 4 was prepared from a mixture of *dit1Δ dit2Δ* cells and *rim101Δ* cells that contained pWL41, which encodes Rim101-HA. The DNA analysed in the samples of lanes 9 to 12 was from pWL41-containing *rim101Δ* cells only. The DNA analysed in the samples of lanes 13 to 16 was from *dit1Δ ditΔ* cells only. PCR was performed with two-fold serially diluted samples of DNA from whole cell extracts (lanes 1 to 3, 5 to 7, 9 to 11, and 13 to 15) and of an aliquot of DNA recovered from the anti-HA chromatin immunoprecipitates (lanes 4, 8, 12, and 16).


WCE
IP
WCE
IP
WCE
IP
WCE
IP
400
300
GAL1
DIT1
IME1
ACT1
1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
dit1Δdit2Δ cells
(Rim101-HA)
+
rim101Δ cells
dit1Δdit2Δ cells
+
rim101Δ cells
(Rim101-HA)
rim101Δ cells
(Rim101-HA)
dit1Δdit2Δ cells

Rim101-HA that was present in *dit1Δ dit2Δ* cells could bind to the *DIT1-DIT2* chromatin provided by cells that did not express Rim101-HA after the cells had been mixed and lysed (Fig. 8, lane 1-4). Control experiments showed that *DIT1* DNA was selectively amplified from the immunoprecipitate prepared from the lysate of a mixture of *dit1Δ dit2Δ* cells and *rim101Δ DIT1 DIT2* cells that expressed Rim101-HA prior to lysis (Fig. 8, lane 5-8) and from the immunoprecipitate from *rim101Δ DIT1 DIT2* cells that expressed Rim101-HA (Fig. 8, lane 9-12). As expected, there was no amplification of *DIT1* DNA in either chromatin present in the whole cell extract or in the immunoprecipitate prepared from *dit1Δ dit2Δ* cells that expressed Rim101-HA (Fig. 8, lane 13-16).

Thus, previous results have to be interpreted with caution. If DNA can be co-immunoprecipitated with a protein in the absence of an *in vivo* cross-linker, this may be due to rearrangements after cells are lysed. The resulting protein-DNA interaction may not be physiologically relevant. In such cases, other corroborating evidence is required to establish the significance of the protein-DNA interaction.

# Discussion and Future Directions

## 4.A Rim101 and protein X contribute to repression through NRE42, a bipartite operator.

As outlined in the introduction, *RIM101* was identified in our lab as a gene required for $NRE^{DIT}$-mediated repression. Rim101 contains three zinc fingers which are highly similar to those of PacC, a transcriptional regulator of *Aspergillus nidulans*. The greatest degree of identity is in those regions of the fingers predicted to contact DNA. We took advantage of the fact that the consensus target site for PacC had been defined to search for such a site in $NRE^{DIT}$. Indeed, a high-affinity PacC target site is present in the downstream region of NRE42 (Tilburn *et al.*, 1995; J. Tanny, M.Sc. thesis, 1998b). We proposed that Rim101 binds to this site, which we refer to as the $PacC^{DIT}$ site, and recruits the Ssn6-Tup1 repression complex to $NRE^{DIT}$. Experiments carried out by J. Tanny and C. Commisso, former members of our lab, demonstrated that Rim101 binds specifically to regions of $NRE^{DIT}$ that span the $PacC^{DIT}$ site.

In his M.Sc. thesis, Jason Tanny demonstrated that NRE44 (nt -504 to -464) retains most, if not all, of the repression activity of NRE76 (nt -537 to -462) and presented data that suggest that the NRE contains two sub-elements (J. Tanny, M.Sc. thesis, 1998b). In my thesis work, I have confirmed that $NRE^{DIT}$ is bipartite and I have delimited the two sub-sites by examining the ability of various fragments to act as operators. I first confirmed that NRE42-mediated repression is comparable to NRE76-mediated repression in terms of effectiveness and Rim101-dependence. Although DNA fragments that spanned various portions of NRE42 had very little operator function, multimers of some of these fragments mediated significant repression. In my studies I have shown that multimerisation of NRE22D, a DNA fragment that spans the downstream portion of NRE42 and includes the $PacC^{DIT}$ site, creates a Rim101-dpendent operator that is as effective as NRE42 in repressing transcription. Multimerisation of NRE25, a DNA fragment that spans the upstream portion of NRE42, also creates an operator that decreases expression of the *CYC1-lacZ* reporter gene. The 3xNRE25 operator, which mediates repression in a Rim101-independent manner, is not as efficient as the 3xNRE22D operator. The operator function of both 3xNRE22D and 3xNRE25 depends on Tup1, suggesting that at least in part the

two sites contribute to a common mechanism of repression. I note, however, that it has not yet been demonstrated that Tup1 is recruited to NRE$^{DIT}$ and it remains possible that the role of Tup1 is indirect.

Based on my results and those of others in our lab, I present the following model for NRE-mediated repression (Figure 9). This model is similar to that presented by Jason Tanny (M.Sc. thesis, 1998b). First, our data provide good support for the idea that Rim101 binds to a region spanning the PacC$^{DIT}$ site (nt -484 to -477 site). The importance of the PacC$^{DIT}$ site has been confirmed by the observation that a mutation in this site in the context of NRE30 or NRE76 reduces Rim101-mediated repression about 50-fold (J. Tanny, M.Sc. thesis, 1998). I suggest that Rim101-binding to the PacC$^{DIT}$ site is required for protein X to bind to an adjacent upstream site that is present within NRE25 (nt -505 to -481). My comparison of the effectiveness of multimers of various fragments to mediate repression is consistent with the idea that the binding sites for Rim101 and protein X are partially overlapping. I found that 2xNRE22U, which extends from nt-505 to -484, is a less efficient operator than was 2xNRE25, which extends from nt-505 to -481 and contains half of the PacC$^{DIT}$ site (Fig. 2 and data not shown). Moreover, the multimerised NRE25 is a more efficient operator in the absence of Rim101, implying that in the presence of a partial PacC$^{DIT}$ site Rim101 interferes with the effectiveness of protein X-mediated repression. Jason Tanny (M.Sc. thesis, 1998b) reached the same conclusion based on the observation that a mutation in a multimer of NRE30 (nt -493 to -464) enhances Rim101-independent repression.

My data indicate that the region from -486 to -464 (NRE22D), which spans the PacC$^{DIT}$ site, suffices for Rim101-dependent repression when present in multiple copies. However, repression mediated by multimers of the fragment from nt -505 to -477 (NRE23), which also includes the PacC$^{DIT}$ site but no downstream bases, has no Rim101-dependence (see Fig. 2). Thus the minimal PacC$^{DIT}$ site may not be sufficient for recruitment of Rim101.

I propose that Rim101 and protein X act synergistically in the context of NRE42 to recruit the Ssn6-Tup1 co-repressor complex. The ability of Rim101 and protein X to act independently to promote Ssn6-Tup1-dependent repression appears to require the presence of multimers of their proposed binding sites. I speculate that Rim101 has greater affinity for its target site than protein X has for its target site. It is possible that Rim101 and protein X form a heterodimer in solution and that the Rim101 monomer is responsible for recruiting the heterodimer to DNA. I suggest that once Rim101 has contacted its target site, protein X is able

to bind to DNA. I speculate that this interaction may depend on some rearrangement of the monomers in the heterodimer to allow co-operative and high affinity binding. Alternatively a Rim101-dependent conformational change in protein X may allow protein X to bind to DNA with high affinity, in part displacing Rim101. The Rim101-protein X-DNA complex then provides a platform for docking the Ssn6-Tup1 complex which directs repression of gene expression.

It is also possible that Rim101 and protein X cannot bind simultaneously to their adjacent sites. In this case, Rim101 would be responsible for delivering protein X to its target site (or vice versa). Once protein X is bound to its target site it might cause dissociation of Rim101. In this case, the observation that 3xNRE25 serves as a more efficient operator in the absence of Rim101 than in its presence (Fig. 3C) can be explained by suggesting that if Rim101 is present but cannot bind to DNA, it interferes with Protein X's ability to bind to DNA.

## 4.B Does Rim101 bind to NRE$^{DIT}$ *in vivo*?

One of the goals of my thesis research was to test for direct, *in vivo* binding of Rim101 to the genomic NRE$^{DIT}$ site. Both Jason Tanny and Cosimo Commisso have demonstrated that the portion of Rim101 that contains the zinc fingers binds to NRE30 and NRE22D *in vitro* (see above). Cosimo Commisso has also shown that a fusion protein consisting of the zinc-finger region of Rim101 fused to the activation domain of Gal4 activates *in vivo* expression of an NRE22D-containing reporter gene.

I used an *in vivo* formaldehyde cross-linking and chromatin immunoprecipitation technique in an attempt to demonstrate that Rim101 is present at the genomic NRE$^{DIT}$ in mitotic cells. I found that *DIT1* DNA could not be detected co-immunoprecipitating with Rim101-HA-containing chromatin if cells had been treated with formaldehyde; however in the absence of formaldehyde treatment of cells, I found significant enrichment for *DIT1* DNA in the Rim101-containing immunoprecipitates of chromatin (see Figure 5). This observation was puzzling. In particular, the dependence of enrichment of a DNA fragment in a chromatin immunoprecipitate on prior treatment of cells with formaldehyde has often been considered as a control in such experiments. Dependency on cross-linking is taken to be an indication that the protein is actually at the site *in vivo* rather than binding to the site after the cells have been lysed. For comparison, I

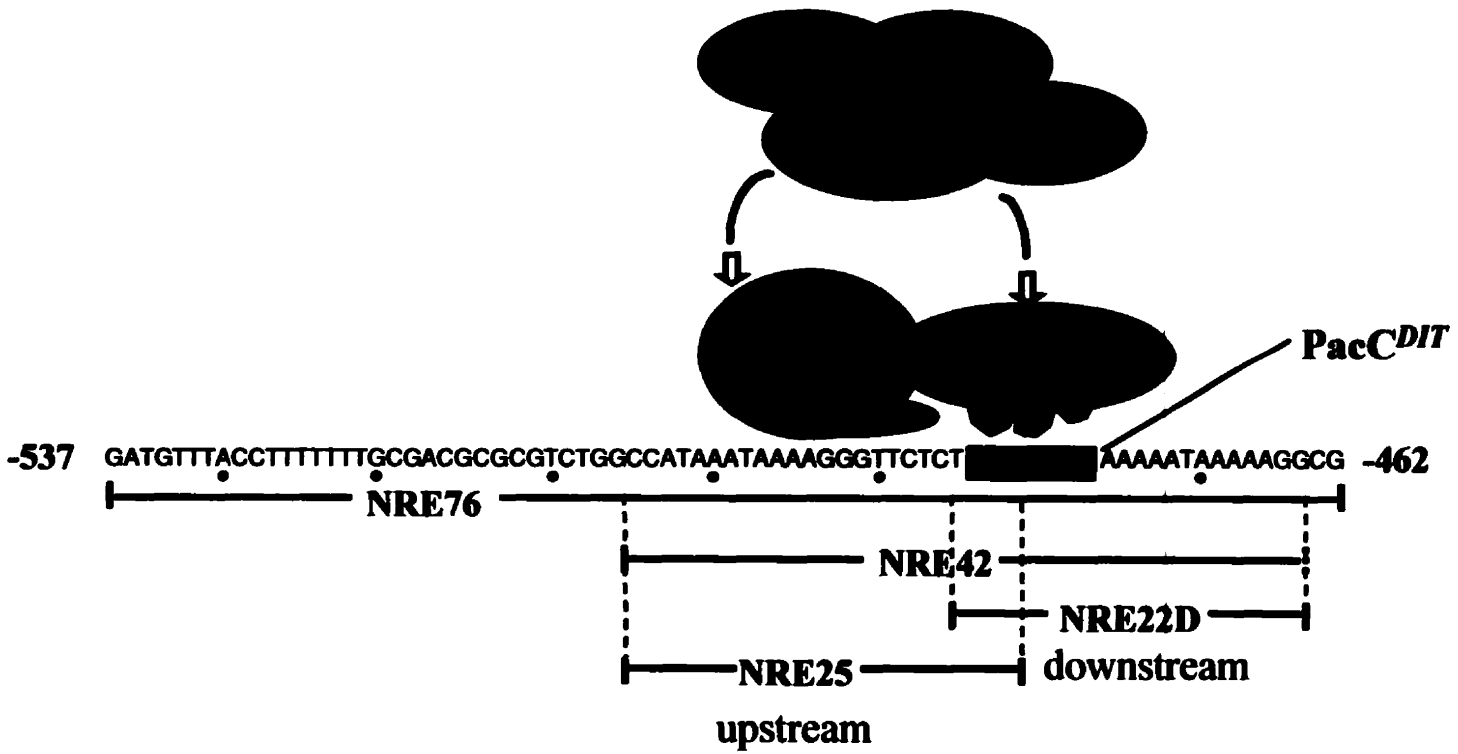



**Figure 9. An updated model for NRE-mediated repression.**

In our present model for NRE-mediated repression, Rim101 and Protein X contribute to full repression through NRE42 by recruiting the Ssn6-Tup1 co-repressor complex. Rim101 binds to the PacC$^{DIT}$ site but also requires bases downstream of this site for optimum repression. In the absence of Rim101, the binding site for protein X may partially overlap with the PacC$^{DIT}$ site. When both proteins are present in a cell, they may interact and bind to NRE42 as a heterodimer (please see text for further details).

investigated the binding of Gal4(BD)-HA at the *GAL1* promoter. In this case, I could detect the co-immunoprecipitation of *GAL1* DNA in the presence and absence of formaldehyde treatment of cells. Because *GAL1* DNA remained in the immunoprecipitate prepared from non-cross-linked cells even after stringent washes; it is formally possible that the enrichment for *GAL1* DNA detected in the formaldehyde linked samples was from protein-DNA complexes that had not been cross-linked (see Figure 7). Finally, I detected rearrangement in lysates of cells that had not been treated with formaldehyde with Rim101 binding to *DIT1* DNA *in vitro* that it had not been bound to *in vivo* (see Figure 8).

There are a number of reasons why cross-linking/chromatin immunoprecipitation may not have allowed detection of the putative *in vivo* binding of Rim101-HA to the *DIT1-DIT2* intergenic region. Since formaldehyde is a mild denaturant, there is always a concern that treatment with formaldehyde might lead to the partial unfolding of the protein and/or the epitope (reviewed in Orlando, 2000). Clearly the epitope can survive formaldehyde treatment: *GAL1* DNA could be immunoprecipitated with Gal4(BD)-HA in the presence of formaldehyde. In the case of Rim101-HA, Rim101 itself may have been denatured upon formaldehyde treatment, preventing it from binding or making appropriate contacts with DNA. To check that Rim101-HA could be immunoprecipitated in the presence of formaldehyde linking, I compared (via Western blotting) extracts of cells treated and not treated with formaldehyde. Although the Western blots were somewhat messy, it appeared that a protein of the right size was immunoprecipitated even if cells were treated with formaldehyde. However, only a small amount of the protein could be immunoprecipitated (i.e. comparing WCE and IP lanes) and it is possible that this was the fraction that had not been cross-linked. Thus, the original problem may have been that Rim101 was sensitive to formaldehyde and in its presence it could not bind to DNA. A direct comparison of UV and formaldehyde cross-linking of two *Drosophila melanogastor* transcription factors, Zeste and Eve, to the *Ultarbithorax* promoter has been carried out. These experiments have revealed that while cross-linking with UV allows the detection of binding of both Zeste and Eve at high affinity sites, *in vivo* formaldehyde linking can only detect binding of Zeste, and not Eve, to its cognate sites. Purified transcription factors, Zeste and Eve, were also cross-linked to DNA fragments *in vitro*. Again, Zeste bound to cognate DNA sites ~50-100 times more efficiently than Eve (Toth and Biggin, 2000). These results are not unusual, for pioneering studies on the efficiency of formaldehyde linking have revealed that

certain proteins cannot be linked to their cognate sites *in vitro* (Solomon and Varshavsky, 1985). If Rim101-HA is sensitive to formaldehyde, another cross-linker, such as UV, could be used. However, formaldehyde cross-links protein to DNA with much more efficiency than UV light (Toth and Biggin, 2000). Given that Gal4(BD)-HA could be cross-linked to DNA with formaldehyde, but that this signal was rather weak, cross-linking Rim101-HA to DNA with UV may not be sufficient.

Another problem might have been the HA epitope. Although HA- and Myc-tagged proteins have been successfully used in chromatin immunoprecipitation (e.g. Aparicio *et al.*, 1997; Tanaka *et al.*, 1997; Koranda *et al.*, 2000), there are indications that polyclonal antibodies to native proteins will produce better results. A direct comparison between chromatin immunoprecipitation with a monoclonal anti-HA antibody and a polyclonal antibody against Mif2 revealed significantly better immunoprecipitation of *CEN* DNA with the α-Mif2 polyclonal antibody. Mif2 is believed to form part of the centromere-kinetochore complex and associates with yeast *CEN* DNA or segregation elements (Meluh and Koshland, 1997).

Both Rim101-HA and Gal4(BD)-HA were expressed from high copy plasmids (2μ plasmids); their high expression levels may explain their binding to cognate DNA sites in the absence of formaldehyde linking. The $UAS_G$ promoter element of *GAL1* contains four Gal4 binding sites; experiments suggest that high induction of *GAL1 in vivo* is achieved by the co-operative binding of Gal4 molecules to these sites (Giniger and Ptashne, 1988). One of the ways that *GAL* genes are regulated is through the availability of Gal4; glucose represses expression of *GAL4* and this reduced availability of Gal4 is probably sufficient to abolish Gal4 DNA-binding. Gal4 has a dimerisation domain close to its C-terminus (and close to the DNA Binding Domain); by allowing for dimerisation and perhaps making oligomeric contacts possible, this domain is believed to contribute to the co-operative binding of Gal4 (reviewed in Lohr *et al.*, 1995). Given the strong correlation between DNA binding and Gal4 levels, it is possible that expressing Gal4(BD)-HA from a multicopy plasmid produced very strong co-operative binding at the *GAL1* promoter. In this manner, *GAL1* DNA could be immunoprecipitated even in the absence of formaldehyde linking. With Rim101, it may have been simply that the excess amount of Rim101 contributed to increased occupancy of the $PacC^{DIT}$ site. We believe that there is only one Rim101 binding site within $NRE^{DIT}$, and PacC, Rim101's homologue in *Aspergillus nidulans* appears to bind as a single molecule to its cognate site (Espeso *et al.*, 1997). Perhaps

physiological levels of Rim101 and Gal4 but more efficient immunoprecipitation with polyclonal antibodies against the proteins may allow a reduction in "background" levels (i.e. abolish recovery of DNA in the non cross-linked samples). Of course if Rim101 is sensitive to formaldehyde treatment and even polyclonal antibodies cannot detect the protein, even with modifications, this method may be inadequate. We believe that other experiments in our lab (see above) still support the model that Rim101 directly binds to the NRE.

## 4.C Is Rim101 an activator or repressor of transcription?

*RIM101* was originally isolated as a protein that contributes to the activation of *IME1*, a key regulator of early sporulation-specific gene expression (Su and Mitchell, 1993a and 1993b). Although Rim101 is referred to as an activator of *IME1*, there is no evidence that it acts directly through the *IME1* promoter. A poor PacC consensus site is present 1100 bases upstream of the *IME1* transcription start site. I have tested two fragments spanning this site for their ability to act *in vivo* as an activation element or an operator element (data not shown). Neither single nor multiple copies of either fragment mediated repression. Although both fragments acted as UASs, this effect was *RIM101*-independent (data not shown). It remains possible that Rim101 acts through sites yet to be identified in the 2 kb promoter region of *IME1*. As it has been pointed out in the introduction, attempts to demonstrate that Rim101 is an activator or a repressor of gene expression by the use of chimeric proteins have been unsuccessful. At the present time there is no direct evidence that Rim101 can act on its own as an activator or repressor of gene expression.

In contrast to our conclusion that Rim101 is a transcriptional repressor that acts through the PacC$^{DIT}$ site, Bogengruber *et al.* (1998) suggested that Rim101 is an activator of the *DIT1* and *DIT2* genes. By random mutagenesis of the 926 bp intergenic region of the *DIT1* and *DIT2* genes, Bogengruber *et al.* (1998) identified two point mutations that allowed this sequence to promote expression of a reporter gene in mitotic cells. Both mutations mapped within the site that we refer to as PacC$^{DIT}$. Although these investigators concur with our conclusion that the PacC$^{DIT}$ site is a major negative regulatory element, they concluded that the short form of Rim101 is an activator of expression of the *DIT1* and *DIT2* genes and that Rim101 does not require the PacC$^{DIT}$ site for this function. It is difficult to reconcile these very distinct findings.

It is possible that the different approaches used in the two studies account in part for the differing conclusions.

We have shown that the *DIT1-DIT2* regulatory region is complex. NRE76, which serves as an operator it mitotic cells, acts in conjunction with two other regions to promote expression during sporulation (Friesen *et al.*, 1997). Additionally, the study of Friesen *et al.* (1997) showed that there is redundancy in sites that mediate Tup1-dependent repression within the *DIT1-DIT2* intergenic region. Thus it is not surprising that the effects observed by Bogengruber and colleagues (1998) in the context of the entire intergenic region were much less (10- to 20-fold) than those observed in our studies (~500-fold). Indeed, it is the complexity of the *DIT1-DIT2* regulatory region that led us to study elements individually.

Brogengruber et al. (1998) also reported that the *DIT1-DIT2* intergenic region would only activate expression of a reporter gene in mitotic cells if the cells were expressing a truncated version of Rim101. Because Rim101 processing occurs under all growth conditions and it is the proteolytically cleaved form that predominates under regular growth conditions (Li and Mitchell, 1997), it is difficult to understand why expression of the reporter gene was minimal in both *RIM101* and *rim101Δ* strains but increased in a strain that expressed only the short form of Rim101.

## 4.D Future Directions

Discovering the identity of Protein X, defining its binding site, and determining if it interacts with Rim101, Ssn6 and/or Tup1 are long term goals of this project. Two proteins, Zap1 and Rim20, have been reported to interact with Rim101. The C-terminal region of Rim101 interacts with Rim20, a protein that is required for the proteolytic cleavage of Rim101 (Xu and Mitchell, 2000 – Yeast Meeting). The yeast Bro1 protein, which is the yeast protein with greatest similarity with Rim101, has 21% identity with the PalA protein of *Aspergillus nidulans*. Although the significance of this is not clear, because PalA is required for the proteolytic cleavage of PacC (Negrete-Urtasun *et al.*, 1997) and because Rim20 is required for proteolytic cleavage of Rim101, it is unlikely that Rim20 is protein X.

The Zap1-Rim101 interaction was identified in a genome-wide yeast-two-hybrid screen (Uetz *et al.*, 2000). Zap1, which is important in zinc homeostasis, is a transcriptional activator

required for the high level expression of a number of zinc-responsive genes (reviewed in Bird *et al.*, 2000b). The C-terminal region of Zap1 contains five zinc fingers of the Cys2-His2 type which are responsible for the interaction of Zap with the 11-bp zinc-responsive element (Zhao *et al.*, 1998; Bird *et al.*, 2000a). Because zinc regulation appears to be normal in a *tup1* mutant background, it is unlikely that Zap1 acts via Tup1 (Bird *et al.*, 2000b). Moreover, Cosimo Commisso found that mutation of *ZAP1* had no effect on the ability of 3xNRE25, 3xNRE22D, or NRE42 to act as operators (C. Commisso, personal communication). Therefore, Zap1 does not appear to be protein X.

There are several approaches that could be used to identify protein X. As a genetic approach, I could screen for mutants that allow expression of the *CYC1-lacZ* reporter gene that is present in pLGn3xNRE25. A distinct genetic approach is suggested by the following unpublished observations from our laboratory. NRE22D can serve as a UAS for Rim101$^{\text{zinc fingers}}$-Gal4$^{AD}$, but NRE42 cannot. However, mutation of bases in the upstream portion of NRE42 allow Rim101$^{\text{zinc fingers}}$-Gal4$^{AD}$ to activate expression of a reporter gene containing this mutated version of NRE42 as a UAS. We hypothesise that the ability of this mutated sequence to serve as a UAS reflects its inability to bind protein X. We infer that in the presence of protein X either Rim101$^{\text{zinc fingers}}$-Gal4$^{AD}$ can not bind to its site in NRE42 or the bound protein is no longer able to activate. Thus, we could screen for mutants that allow the Rim101$^{\text{zinc fingers}}$-Gal4$^{AD}$ fusion protein to activate expression of an NRE42-*lacZ* reporter gene. Biochemical approaches , based on the expectation that protein X interacts with Rim101, include affinity chromatography with Rim101 as ligand and immunoprecipitation of Rim101-containing complexes from yeast cells. Once protein X is identified, its target site can be defined and the ability Rim101 and protein X to co-occupy their sites in NRE42 can be assessed.

In addition to determining whether Rim101 and/or protein X interact with Ssn6 or Tup1 *in vitro*, it would be worthwhile to test for the presence of Ssn6 and/or Tup1 at NRE$^{DIT}$ with the use of a cross-linking / chromatin immunoprecipitation approach with antibodies against Tup1 (Ducker and Simpson, 2000; Wu *et al.*, 2001; Cassidy-Stone and Johnson, 2000). The results of my ChIP experiments with Rim101-HA suggest that further ChIP experiments should be approached with caution. However, if protein X can be cross-linked to DNA *in vivo*, then it might be possible to detect Tup1 if it is present in a repression complex at NRE$^{DIT}$.

It should be possible to gain insight into the mechanism of Rim101-mediated repression at $NRE^{DIT}$ by probing the nucleosomal structure and acetylation state of histones in this region. Such probing could be done in wild-type cells, in *rim101Δ* cells, and in *tup1Δ* cells. Nucleosomal positioning and hypoacetylated histones have both been linked to Tup1-mediated repression (see Introduction). If this mode of repression is important an $NRE^{DIT}$, one would expect to observe nucleosomal phasing and decreased acetylation of histones in wild type but not in *tup1Δ* cells. Since we believe that both Rim101 and Protein X can bring in the Ssn6-Tup1 complex, there may not be a difference between wild type and *rim101Δ* cells with respect to nucleosomal structure. It would also be interesting to compare the status of histone modifications at $NRE^{DIT}$ in vegetatively growing cells and in sporulating cells. Finally, the mechanism by which $NRE^{DIT}$-mediated repression is abrogated during sporulation and the mechanism by which expression of the *DIT1* and *DIT2* genes is activated remain to be elucidated.

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