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The Huck Institute for Life Sciences

# CHROMATIN AND DNA FUNCTION: RECURRING QUESTIONS AND EVOLVING ANSWERS

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Xi Wang



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The thesis of Xi Wang has been reviewed and approved* by the following:

Robert T. Simpson
Professor and Holder of the Verne M. Willaman
Chair in Biochemistry
Thesis Adviser and Chair of Committee

Jerry L. Workman
Paul Berg Professor of Biochemistry and Molecular
Biology

Song Tan
Assistant Professor in Biochemistry & Molecular
Biology

Andrew Henderson
Associate Professor of Veterinary Science

Hong Ma
Professor of Biology

Richard Frisque
Professor of Molecular Virology
Co-Director of the Huck Institute for Life Sciences

*Signatures are on file in the Graduate School.

# Abstract

In this thesis, *in vivo* analyses are presented to better understand the specific parameters by which gene transcription is regulated in the context of chromatin.

A novel DNase I probing assay is established and employed to detect both histone-DNA and non-histone-DNA interactions in living cells. By introducing a bovine pancreatic DNase I gene into yeast under control of a galactose responsive promoter, we mapped chromatin structure at nucleotide resolution in whole cells without isolation of nuclei. The validity and efficacy of the strategy are demonstrated by footprinting a labile repressor bound to its operator. Investigation of the inter-nucleosome linker regions in several types of repressed domains has revealed different degrees of protection in cells, relative to isolated nuclei. These different structural signatures likely reflect the *in vivo* chromatin architectures that result in different biological behaviors of these domains. Moreover, this strategy has been applied to map active promoters and suggested that TBP, and possible other transcription factors, are persisting at some, if not most, active promoters through multiple transcription cycles *in vivo*. This conclusion was supported by chromatin immunoprecipitation (ChIP) assays.

Unique chromatin structure characterizes cell type gene regions, including the **a** cell-specific gene domains in yeast. In this study, the componential and structural information of chromatin along the *MFA1* gene, one of the **a** cell-specific genes, was investigated comprehensively by employing multiple approaches. Employing minichromosome affinity purification (MAP) and electron microscopy (EM) techniques, we observed this domain as a highly compact higher order chromatin structure. By doing Western blot, ChIP, and knock-out assays, we detected the presence of Tup1p and Hho1p in this domain, and their possible roles have also been discussed.

# TABLE OF CONTENTS


**LIST OF FIGURES** ..... vii

**LIST OF TABLES** ..... viii

**LIST OF ABBREVIATIONS** ..... ix

**ACKNOWLEDGEMENTS** ..... x

**CHAPTER I INTRODUCTION** ..... 1

**1.1 Chromatin structure and transcription** ..... 3
1.1.1 Chromatin structure ..... 3
1.1.2 Chromatin structure and transcription ..... 5
1.1.3 Regulation of mating type specific genes in *Saccharomyces cerevisiae* ..... 6
1.1.4 Ssn6-Tup1 mediated gene repression ..... 10

**1.2 Methods for *in vivo* analysis of chromatin structure** ..... 20
1.2.1 Nuclease digestion of isolated nuclei ..... 20
1.2.2 Mapping chromatin structure by expressing enzymes in living cells ..... 22
1.2.3 Chromatin Immuno-precipitation (CHIP) ..... 25
1.2.4 Electron microscopy (EM) and chromatin ..... 27
1.2.5 Minichromosome affinity purification (MAP) ..... 30
1.2.6 Other methods ..... 32
1.2.7 Conclusion ..... 33

**CHAPTER II CHROMATIN STRUCTURE MAPPING IN *SACCHAROMYCES CEREVISIAE IN VIVO* WITH DNASE I** ..... 34

**Abstract** ..... 35

**2.1 Introduction** ..... 36

**2.2 Materials and methods** ..... 39
2.2.1 Plasmid construction ..... 39
2.2.2 Cell growth ..... 39
2.2.3 Nuclear and DNA preparation and analysis ..... 40
2.2.4 Southern blots ..... 41

**2.3 Results** ..... 43
2.3.1 DNase I expression *in vivo* ..... 43
2.3.2 DNase I footprinting a labile repressor *in vivo* ..... 46
2.3.3 Different nucleosome linker accessibilities in repressed domains *in vivo* ..... 48

**2.4 Discussion** ..... **52**

**Acknowledgements** ..... **70**

## CHAPTER III TATA BOX BINDING PROTEIN PERSISTS AT ACTIVE YEAST PROMOTERS THROUGH MULTIPLE TRANSCRIPTION CYCLES *IN VIVO* ..... 71

**3.1 Introduction** ..... **73**

**3.2 Materials and methods** ..... **78**
3.2.1 Yeast Strains and medium ..... 78
3.2.2 Nuclei and DNA preparation and analysis ..... 78
3.2.3 Chromatin immunoprecipitation (ChIP) ..... 80
3.2.4 Quantitative PCR ..... 81
3.2.5 Nuclei ChIP ..... 81

**3.3 Results** ..... **83**
3.3.1 Promoters of active genes are accessible and "nucleosome-free" ..... 83
3.3.2 TBP binds to promoters of different genes with the same occupancy level ..... 85
3.3.3 Differential TBP binding patterns between living cells and isolated nuclei ..... 89
3.3.4 TBP binds to a group of promoters with same occupancy level ..... 90

**3.4 Discussion** ..... **94**
3.4.1 TBP plays a different role in initiation and reinitiation ..... 94
3.4.2 A comparison between TBP binding patterns in living cells and isolated nuclei ..... 98

**Acknowledgements** ..... **120**

## CHAPTER IV THE ROLE OF HIGHER ORDER CHROMATIN STRUCTURE IN REPRESSION OF THE *MFA1* GENE IN A CELLS ..... 121

**4.1 Introduction** ..... **123**

**4.2 Materials and methods** ..... **127**
4.2.1 Yeast strains and the minichromosome ..... 127
4.2.2 Minichromosome affinity purification ..... 127
4.2.3 Western blot ..... 129
4.2.4 Electron microscopy (EM) ..... 130
4.2.5 Nuclei and DNA preparation and analysis ..... 130
4.2.6 Chromatin immunoprecipitation (ChIP) ..... 131
4.2.7 Quantitative PCR ..... 132

**4.3 Results** ..... **133**
4.3.1 Nucleosomes are positioned over the regions required for *MFA1* expression in $\alpha$ cells ..... 133
4.3.2 The MFA1-ALT minichromosome ..... 134
4.3.3 EM images of the MFA1-ALT minichromosome isolated from $\alpha$ cells ..... 136
4.3.4 Multiple copies of Tup1p associate with the repressed *MFA1* locus *in vivo* ..... 137
4.3.5 Chromatin structure of the *MFA1* locus in a *tup1* mutant strain ..... 139
4.3.6 Tup1p spreads over the entire *MFA1* chromatin domain ..... 140
4.3.7 Hho1p binds to the repressed *MFA1* locus in $\alpha$ cells ..... 141

**4.4 Discussion** ..... **143**

**Acknowledgements** ..... **166**

**CHAPTER V SPECULATION ON FUTURE STUDIES AND AIMS** ..... **167**

**5.1 Improvement of *in vivo* DNase I mapping** ..... **168**

**5.2 Further applications of MAP in exploring mechanisms of gene repression** ..... **170**
5.2.1 Is the compact chromatin structure specific for a cell-specific genes? ..... 170
5.2.3 The distribution of Ssn6p-Tup1p complex along repressed domains ..... 171
5.2.4 Deeper investigations of Hho1p function ..... 172

**SUMMARY** ..... **178**

**REFERENCES** ..... **184**

**APPENDIX** ..... **200**

# LIST OF FIGURES

Figure 2.1: Cytotoxicity of DNase I. ..... 57
Figure 2.2: DNase I expressed *in vivo* introduces nicks in and degrades plasmid DNA. ..... 58
Figure 2.3: Time course of DNase I degradation of DNA *in vivo*. ..... 60
Figure 2.4: DNase I footprinting of the genomic Mat$\alpha$2p/Mcm1p complex binding site in intact cells and isolated nuclei. ..... 62
Figure 2.5: Chromatin structure of the recombination enhancer *in vivo*. ..... 64
Figure 2.6: Chromatin structure of the *STE6* promoter *in vivo* ..... 66
Figure 2.7: Chromatin structure of a nucleosome adjacent to the E silencer at *HMRa in vivo*. ..... 68

Figure 3.1: Indirect end labeling mapping of chromatin structure of the promoter of several genes. ..... 102
Figure 3.2: Primer extension mapping of DNase I cutting sites around the promoter and coding region of the *PGK1* gene. ..... 104
Figure 3.3: Primer extension mapping of DNase I cutting sites around the promoter and coding region of the *YCL056C* gene. ..... 106
Figure 3.4: Chromatin immunoprecipitation for transcription factor binding. ..... 108
Figure 3.5: TBP occupancy of the *STE6* promoter and open reading frame (ORF) regions in living cells and isolated nuclei. ..... 110
Figure 3.6: TBP binds to the promoter of **a** cell-specific genes only in **a** cells. ..... 112
Figure 3.7: RNA polymerase II and TFIIH occupancy at selected promoters. ..... 114
Figure 3.8: Summary of the ChIP data. ..... 116
Figure 3.9: TBP and RNA polymerase II occupancy at promoters of selected histone genes. ..... 118

Figure 4.1: Chromatin structure of *MFA1* locus in $\alpha$ cells. ..... 148
Figure 4.2: Minichromosome construct. ..... 150
Figure 4.3: Primer extension mapping of the chromatin structure of the MFA1-ALT minichromosome ..... 152
Figure 4.4: Electron micrographs of MFA1-ALT minichromosomes isolated from $\alpha$ cells, negatively stained with uranyl acetate. ..... 154
Figure 4.5: Western blot analysis of the affinity-purified MFA1-ALT minichromosome probed with anti-Tup1p antibodies. ..... 156
Figure 4.6: Nucleosome mapping of *MFA1* in a *tup1* mutant strain. ..... 158
Figure 4.7: Chromatin immunoprecipitation assay for Tup1p binding. ..... 160
Figure 4.8: Hho1p binds to *MFA1* region in $\alpha$ cells. ..... 162
Figure 4.9: Model for repression of *MFA1* gene in $\alpha$ cells. ..... 164

Figure 5.1: The schematic of the experiment to determine the ratio between Mcm1p and Tup1p associated with the MFA1-ALT minichromosome. ..... 176

# LIST OF TABLES

Table 5.1: Effects on chromatin structure of Mcm1p binding at the *STE6* locus..... 175

Table A.1: Primers used in ChIP PCR reactions ..... 201

# LIST OF ABBREVIATIONS

ALT – ARS1/Lac-operator/TRP1
ARS – Autonomously Replicating Sequence
ATP – Adenosine Tri Phosphate
bp – base pair
BSA – Bovine Serum Albumin
ChIP – Chromatin ImmunoPrecipitation
DNA – DeoxyriboNucleic Acid
DNase I – Deoxyribonuclease I
dNTPs – deoxy Nucleoside Tri Phosphates
EDTA – Ethylene Diamine Tetraacetic Acid
EM – Electron Microscopy
GAL – GALactose (genes involved in the regulation of galactose metabolism)
HHF – Histone H Four
HHO – Histone H One
HHT – Histone H Three
MAP – Minichromosome Affinity Purification
mg – milligrams
ml – milliliters
MNase – micrococcal nuclease
nm – nanometers
NMR – Nuclear Magnetic Resonance
NP-40 – Nonidet 40
ORF – Open Reading Frame
PMSF – Phenyl Methyl Sulfonyl Fluoride
RNA – RiboNucleic Acid
SDS – Sodium Dodecyl Sulfate
SIR – Silent Information Regulator
SNF – Sucrose Non Fermentor
SSN – Suppressor of Snf1
SUC – SUCrose fermentation (invertase gene)
SWI – SWItch
TAF – TBP Associated Factor
TBP – TATA box Binding Protein
TF – Transcription factor
TRP – N-phosphoribosyl-anthranilate isomerase gene
UAS – Upstream Activation Sequence
U.V. – Ultra Violet
ug – micrograms
ul - microliters

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*Let your acquaintances be many,*
*But your advisors one in a thousand.*
*A faithful friend is a sure shelter,*
*Whoever finds one has found a rare treasure.*
*---Ecclesiasticus*

# Chapter I

# Introduction

The importance of chromatin had been appreciated for many years before the information regarding components and the structure of chromatin was known. For example, in 1944 (about one year before the acceptance of DNA as genetic material, nine years before the elucidation of double helix structure of DNA, and thirty years before the discovery of nucleosome), Erwin Schrödinger mentioned in his lecture *What is Life?* (Schrödinger, 1944): "the chromosome structures are at the same time instrumental in bringing about the development they foreshadow. They are law-code and executive power – or, to use another simile, they are architect's plan and builder's craft – in one."

Decades of intensive efforts have provided plenty of evidence for this statement and revealed that in eukaryotic cells, DNA transcription, replication, recombination and repair all take place in the context of chromatin (Jenuwein and Allis, 2001; Kornberg and Lorch, 1999; Workman and Kingston, 1998a). Therefore, exploration of structural and componential information of chromatin is crucial to the understanding of these DNA functions.

In the first section of this chapter, I will describe the current knowledge of chromatin structure and its role in transcription regulation. In addition, I will use the regulation of **a** cell-specific genes in yeast and the Ssn6-Tup1 complex mediated gene repression as examples. In the second section, I will briefly review the application, advantages, and disadvantages of several chromatin analyzing methods.

## 1.1 Chromatin structure and transcription

### 1.1.1 Chromatin structure

Now it is clear that chromatin is a dynamic complex of the nucleic acid with histones and other proteins. Nucleosome, the basic repeating unit of chromatin, contains nucleosome core particle and linker DNA that connects one core particle to the next in chromatin. A nucleosome core can be defined as a histone octamer, made up of two each of H2A, H2B, H3 and H4, with ~146 bp of DNA wound on the outside. The (H3)2(H4)2 tetramer lies at the center, and H2A-H2B dimmers stay at the ends of the DNA path. Each histone is organized into two domains: a central fold (histone fold) which constrains the DNA super-helix and contributes to the compact core of the nucleosome, and an unstructured amino-terminal tail which extends outside the core and provides a basis for interaction among nucleosomes and regulation (Luger et al., 1997).

In higher eukaryotic organisms, linker DNA between nucleosomes is associated with a histone termed linker histone (histone H1 or H5) (Vignali and Workman, 1998; Widom, 1998). In *Saccharomyces cerevisiae*, *HHO1* encodes a putative linker histone with very significant homology to histone H1 (Landsman, 1996; Ushinsky et al., 1997). While Hho1p has not been shown to affect global chromatin structure, nor has its deletion shown any detectable phenotype, it can form a stable ternary complex with a reconstituted core dinucleosome at a molar ratio of one *in vitro.* After micrococcal nuclease

digestion of chromatin the reconstituted nucleosomes showed a kinetic pause at ~168 bp, as do nucleosomes associated with histone H1 (Patterton et al., 1998; Ushinsky et al., 1997). It is reported *HHO1* and those genes encoding the core histones are highly transcribed during *S* phase in yeast, indicating that Hho1p possibly functions in a coordinated fashion with the core histones (Spellman et al., 1998). Recently, Freidkin et al (2001) presented that *HHO1* is both transcribed and translated in living yeast cells, the protein co-purifies with the core histones and that *HHO1* disruption does have a transcription effect on a subset of genes and that it is preferentially concentrated at the repeated sequences that encode rRNA. They also measured its relative stoichiometry to the core histones in the cell, finding that hho1p is in far fewer copies in the cell than core nucleosomes. All those evidence is consistent with Hho1p being a bona fide linker histone protein and performing its functions locally in yeast cells. However, much work is still needed to define the details of Hho1p's functions.

While people have observed that a chain of nucleosomes could be further packaged into 30-nm fibers with six nucleosomes per turn in a spiral or solenoid arrangement, it remains unclear how nucleosomal arrays twist and fold this chromatin fiber into such a defined higher order structure (Van Holde, 1989). Reversely, the 30-nm fiber could unfold to generate a template for transcription, in the form of an 11-nm fiber or beads on a string, by an unknown mechanism. Therefore, future studies would focus on elucidating

the higher-order conformation and conformational changes of chromatin under different physiological circumstances.

### 1.1.2 Chromatin structure and transcription

Chromatin plays an important role in the process of gene regulation in eukaryotic cells (Kornberg and Lorch, 1999). Even 60 years ago, it was found that a gene could be on or off without changing the sequence. After the concept of nucleosome has been given, *in vitro* competition experiments with histones and basal transcription (Knezetic et al., 1988; Knezetic and Luse, 1988; Lorch et al., 1987; Matsui, 1987; Workman et al., 1988; Workman and Kingston, 1992b; Workman and Roeder, 1987b) have shown that packaging promoters in nucleosomes prevents the initiation of transcription by bacterial and eukaryotic RNA polymerases. Later investigators found that the nucleosome can inhibit several processes that must occur for a eukaryotic gene to be appropriately regulated. These processes include: binding of activators to both enhancer and promoter regions; transcription initiation, elongation and termination (Clark and Felsenfeld, 1992; Felsenfeld, 1992; Felsenfeld et al., 1996; Studitsky et al., 1994; Workman and Kingston, 1998b).

These *in vitro* experiments were quickly followed by experiments, which demonstrated that nucleosome positioning and remodeling of chromatin structure *in vivo* also affect the transcription (Almer et al., 1986; Han et al., 1987; Han and Grunstein, 1988; Han et al., 1988; Kim et al., 1988; Morse et

al., 1987; Simpson et al., 1993). For example, after turning off histone synthesis by genetic means in yeast, and consequent nucleosome loss, transcription of all previous inactive genes tested can be turned on (Han and Grunstein, 1988). Recently, investigations on acetylation, methylation, ubiquitation and phosphorylation of histone tails lead to the "histone code" hypothesis, which predicts that such modifications will result in distinct "read out" of the genetic information, such as gene activation versus gene silencing (Jenuwein and Allis, 2001). Moreover, explorations on functions of ATP-dependent chromatin remodeling complexes suggest that disruption of nucleosomes is required for binding of RNA polymerase, transcription factors and activators (Hassan et al., 2001; Vignali et al., 2000).

Currently, it is well accepted that chromatin can affect transcription at different levels. These include the modifications of histones; the binding of nonhistone proteins such as activators, transcription factors, and repressors; positioning and remodeling of nucleosomes; higher order chromatin structures (interactions among nucleosomes); and the localization within the nucleus. Many detailed mechanisms still remain unclear. Among these are the mechanisms by which the constitutively active transcription of the house keeping genes is maintained, and the conformation and the conformational changes of local chromatin under different functional states.

### 1.1.3 Regulation of mating type specific genes in *Saccharomyces cerevisiae*

In eukaryotic organisms, the number of genes is in significant excess of the required gene products for any given cell under a particular set of circumstances. Therefore, some genes are turned on only in certain cell (tissue) types, at certain developmental stages, or in response to certain signals (e.g. nutrient, temperature, or hormone). Moreover, inappropriate expression of some of these genes will lead to diseases in human beings such as cancer and auto-immuno diseases. Among these are the mating type specific genes in *Saccharomyces cerevisiae*.

The yeast *Saccharomyces cerevisiae* is an ideal experimental organism. It is a microorganism that has a fast rate of growth, with a generation time of only ninety minutes under optimal conditions. Genetic methods have been developed that allow straightforward and generally easy manipulation of its genome. Any desired mutation can be incorporated into the *Saccharomyces cerevisiae* genome, allowing powerful genetic analyses to be performed. *Saccharomyces cerevisiae* shares many fundamental properties with other eukaryotes, including humans. Therefore, what is learned from studies of *Saccharomyces cerevisiae* is often directly relevant to issues in human biology.

*Saccharomyces cerevisiae* exists in three cell types: **a** and $\alpha$ and diploid **a**/$\alpha$ (Dolan and Fields, 1991; Herskowitz, 1989). The **a** or $\alpha$ type of a haploid cell is determined by the expression of master regulatory protein genes from the active mating type locus (MAT). In MAT$\alpha$ cells the MAT$\alpha$1 and MAT$\alpha$2 genes are expressed coding for the Mat$\alpha$1p and Mat$\alpha$2p proteins

respectively. Mat$\alpha$1p activates transcription of $\alpha$ cell specific genes and Mat$\alpha$2p represses transcription of **a** cell specific genes. In **a**/$\alpha$ diploids, where both an active MATa and MAT$\alpha$ locus are present, haploid specific genes are repressed by a hetero-dimer of Mat$\alpha$2p and a MATa product, Mata1p. In MATa cells, neither Mat$\alpha$1p nor Mat$\alpha$2p is present, so **a** cell specific genes can be expressed and no $\alpha$ cell specific genes are activated (Andrews and Herskowitz, 1990).

In MAT$\alpha$ cells the **a** cell specific genes are thought to be repressed by the formation of a complex of proteins at the $\alpha$2 operator, a nearly symmetric 31 bp sequence present approximately ~200 bp upstream of the seven **a** cell specific genes (Johnson and Herskowitz, 1985; Zhong et al., 1999). A homo-dimer of the Mat$\alpha$2p repressor binds to this operator in a cooperative manner with a homo-dimer of another protein, Mcm1p, a non-cell type specific MADS box protein (Acton et al., 1997; Mead et al., 2002). Mcm1p binds to the center of the operator while Mat$\alpha$2p binds to operator sequences flanking the Mcm1p binding site. Binding of Mat$\alpha$2p/Mcm1p to the $\alpha$2 operator establishes a repressive chromatin structure adjacent to the operator, in which nucleosomes are precisely positioned over essential promoter elements of the **a** cell specific genes and extend into the coding region of the genes (Ducker and Simpson, 2000; Patterton and Simpson, 1994; Roth et al., 1992; Shimizu et al., 1991; Simpson et al., 1993). Chromatin was implicated in the repression of the **a** cell specific genes in $\alpha$ cells by virtue of the absence of a nucleosomal array on the **a** cell specific genes in **a** cells. It was also shown

that nucleosomes are positioned less well defined on *STE6*, one of the **a** cell specific gene, in $\alpha$ cells expressing histone H4 with amino-tail mutations. Under these conditions, partial derepression of the **a** cell specific genes was also reported (Roth et al., 1992). It was initially proposed from these data that repression is established by Mat$\alpha$2p directly interacting with the tails of histone H4 positioning nucleosomes on essential promoter elements, and masking these elements from DNA binding trans-acting activator proteins and/or basal transcription factors. However, it was reported that at least two other proteins, Ssn6p (Schultz and Carlson, 1987) and Tup1p (Lemontt, 1980; Smith and Johnson, 2000), are also necessary for full repression of the **a** cell specific genes. Keleher et al (1992) have demonstrated that in *ssn6* knockout strains, the **a** cell specific genes are derepressed, even though the Mat$\alpha$2p/Mcm1p complex is bound at the $\alpha$2 operator. They also showed that the targeting of Ssn6p to a heterologous promoter via fusion to a LexA DNA binding domain, acts to repress transcription from the heterologous promoter in a Tup1 dependent fashion. Neither Tup1p nor Ssn6p show any DNA binding ability, instead the two proteins are thought to be recruited to the **a** cell specific genes promoter by Mat$\alpha$2p and bind to histone tails (Davie et al., 2002; Ducker and Simpson, 2000; Komachi et al., 1994; Smith and Johnson, 2000; Tzamarias and Struhl, 1994; Watson et al., 2000). Recently, our lab has shown that the Tup1p specifically associates with the repressed chromatin at a ratio of about two molecules per nucleosome along the promoter region and entire genomic coding region of *STE6* and *MFA1*, two of

the **a** cell specific genes, in α cells (this study and Ducker, 2001; Ducker and Simpson, 2000). Also, collaborating with Dr. Woodcock, we observed a highly organized secondary chromatin structure in these same repressive domains under EM (this study and Ducker, 2001). These studies clearly showed that there exists a special higher order chromatin structure along these repressed domains.

Future studies regarding regulation of these **a** cell-specific genes should focus on understanding in details the forces that hold these chromatin structures together. Many questions need to be answered. What is the methylation, acetylation, phosphorylation, and ubiquitation status of the nucleosomes within these domains, both in active and repressed states? What proteins other than Ssn6-Tup1 participate in the repression of these genes? Are these genes localized to certain places inside the nucleus when they are active or repressed? And, most interestingly, can we reassemble these structures from defined components *in vitro* and show that they have properties similar to those inferred from these above *in vivo* biochemical, biophysical, and/or genetic studies? Certainly, fully understanding the mechanisms by which the **a** cell-specific genes are activated or repressed will provide insights into the regulation of tissue specific genes or developmental stage specific genes in higher organisms and will also expand the understanding of several human diseases.

### 1.1.4 Ssn6-Tup1 mediated gene repression

In addition to activation, gene specific repression of transcription also plays a central role in gene regulation. A gene can be repressed through two pathways. First, a gene present in a cell type can be repressed because of the lack of necessary activators to activate this gene. The second pathway is termed “active repression”, which means a gene (or a set of genes) can be repressed even when the necessary activators are present in the cells. Various protein complexes, called repressors, are involved in the process of active repression. Repressors can repress selected genes through different ways: modifying histones, organizing specialized chromatin structures, interfering with activators, and/or the transcription machinery (Smith and Johnson, 2000).

Ssn6-Tup1 complex is a well documented repression complex. This repressor is composed of the Ssn6p (also called Cyc8p) and Tup1p proteins. Repression by Ssn6-Tup1 has several distinguishing features. First, this complex has an exceedingly efficient repression capacity. For example, the repression ratio (the ratio of the transcription level under active conditions to the transcription level under repression) can be as high as 1,000 times (Redd et al., 1996). Second, Ssn6-Tup1 complex can repress as many as 3% of the *Saccharomyces cerevisiae* genes (DeRisi et al., 1997). Third, the Ssn6-Tup1 repressor can cause strong repression when it is attracted to DNA at a variety of positions along the upstream control region of its target genes (Herschbach and Johnson, 1993; Keleher et al., 1988; Tzamarias and Struhl, 1995). Moreover, this complex can prevail against many kinds of activators and can

repress genes that are activated by several different activators working together. Finally, Ssn6p and Tup1p both belong to protein families that are evolutionarily conserved (Smith and Johnson, 2000 and references there in). Although the analyses are not yet complete, the available results reveal that many species contain repressors that resemble Ssn6p and Tup1p not only in sequence but also in function. Those species are yeasts (including *Saccharomyces cerevisiae, Candida albicans, and Schizosaccharomyces pombe*), worms, flies, and mammals (including mouse and human).

1.1.4.1 Biochemistry of Ssn6p and Tup1p

Tup1p is a 78kD protein with three functional domains (Ducker, 2001; Williams and Trumbly, 1990). The N-terminal domain mediates tetramerization of Tup1p and is necessary for the formation of a stable 4:1 complex between Tup1p and Ssn6p (Jabet et al., 2000; Tzamarias and Struhl, 1994; Tzamarias and Struhl, 1995; Varanasi et al., 1996; Williams and Trumbly, 1990). The C-terminal domain of Tup1p contains seven copies of the WD40 repeat motif (Zhang et al., 2002b). The WD40 repeat is a degenerate sequence ~40 amino acids in length and is present in many proteins with diverse functions (Komachi et al., 1994; Schultz and Carlson, 1987; Williams and Trumbly, 1990). For example, at least 77 *Saccharomyces cerevisiae* proteins, including Ste4p, Cdc4p, Cdc20p, and Mak11p contain WD40 repeats (Komachi et al., 1994; Schultz and Carlson, 1987; Williams and Trumbly, 1990). Moreover, a number of significant *Drosophila* proteins, including extra sex combs and groucho, contain the WD40 motif (Gutjahr et

al., 1995). *In vitro* studies have revealed that the WD40 repeat domain is involved in mediating protein-protein interactions, and that each of the seven repeats of Tup1p is necessary for the repression of different set of genes (Carrico and Zitomer, 1998; Zhang et al., 2002a). For example, the C-terminal WD40 repeats 1 and 2 of Tup1p have been shown to interact directly with Mat$\alpha$2p (Komachi and Johnson, 1997; Komachi et al., 1994). The central domain of Tup1p contains a defined repression domain (Tzamarias and Struhl, 1994) that interacts with the tails of histones H3 and H4, suggesting that there may be a connection between these two functions (Edmondson et al., 1996).

Ssn6p belongs to an evolutionarily conserved family of proteins, which is characterized by the 34 amino acid repeat sequence termed the tetratricopeptide motif (TPR) (Goebl and Yanagida, 1991). Forty two proteins in *Saccharomyces cerevisiae* contain the TPR repeat (Rhee et al., 1989). Ssn6p contains 10 tandomly repeated TPRs at its N-terminus that are required for full function of the protein (Schultz and Carlson, 1987; Schultz et al., 1990). Tzamarias and Struhl (1995) have demonstrated that the TPR repeats do not function as a single unit. Instead, different sets of the TPRs are necessary for repression of different genes by interacting with a number of different DNA-binding proteins (Tzamarias and Struhl, 1995). For example, the first three TPR repeats are sufficient for binding to Tup1p and to Mat$\alpha$2p and hence, for repression of mating type genes (Smith et al., 1995; Tzamarias and Struhl, 1994); Repeats 1 through 7 are necessary for

repression of hypoxia-induced genes (Tzamarias and Struhl, 1994); and all of the TPR repeats are required for repression of DNA damage-regulated genes (Tzamarias and Struhl, 1994). Notably, Schultz *et al*. (1990) have shown that the C-terminal region of Ssn6p has a high content of PEST residues (8% proline, 18% glutamate, and 25% serine and threonine), a characteristic feature of proteins with short metabolic half-lives (Rechsteiner and Rogers, 1996; Rogers et al., 1986).

Interestingly, both Tup1p and Ssn6p have been shown to be phosphorylated *in vivo* (Redd et al., 1997; Schultz et al., 1990). This post-translational modification has been suggested to be involved in the function of these proteins, although there is no direct evidence to support this hypothesis.

1.1.4.2 Genes controlled by Tup1p and/or Ssn6p

The deletion of either Tup1p or Ssn6p is not lethal to the yeast cells. However, compared to wild type cells, the Tup1p and/or Ssn6p mutants do exhibit a number of distinct phenotypes (Keleher et al., 1992; Wahi et al., 1998). These include flocculation, a loss of mating in $\alpha$-cells, slow growth, poor sporulation, the ability to take up thymidine from the media (which is where Tup1p gets its name – Thymidine Up-take Positive), and the loss of some aspects of the glucose-dependent regulatory circuit (from which Ssn6p gets its name – Suppressor of Sucrose Non-fermentor). A recent microarray analysis (DeRisi et al., 1997) suggested that these phenotypes were caused by the inappropriate expression of more than 150 yeast genes. Some of these

genes have been shown to be repressed by Tup1p and/or Ssn6p (Smith and Johnson, 2000; Wahi et al., 1998). These genes can be divided into different families. Each family of genes functions in a specific cellular process and has a common sequence specific DNA-binding protein that is responsible for the recruitment of Tup1p/Ssn6p, as neither Tup1p nor Ssn6p has the ability to interact with DNA on their own. These proteins are Crt1p (for the DNA damage-inducible genes) (Huang et al., 1998), Mig1p (for the glucose-repressible genes) (Treitel and Carlson, 1995), Rox1p (for the hypoxia-induced genes) (Balasubramanian et al., 1993), and Mat$\alpha$2p (for the **a** cell-specific genes) (Herschbach et al., 1994; Komachi and Johnson, 1997; Komachi et al., 1994; Smith et al., 1995). Notably, researchers have not found any similarity between any two of the proteins responsible for recruiting Tup1p and/or Ssn6p to specific genes (Smith and Johnson, 2000; Wahi et al., 1998). Those proteins are different from each other either in their DNA-binding motif or in any amino acid residues responsible for the interaction with the Tup1p/Ssn6p complex.

As described above, Tup1p contains seven copies of WD motif, and Ssn6p contains ten tandem arrays of TPR repeats (Smith and Johnson, 2000; Tzamarias and Struhl, 1995). In addition, the Ssn6-Tup1 complex is composed of four Tup1p molecules and one Ssn6p molecule (Varanasi et al., 1996) and adopts an elongated conformation (Redd et al., 1997; Varanasi et al., 1996). These features provide the Ssn6-Tup1 complex the flexibility to interact with diverse sets of DNA-binding proteins with different conformations

and under different conditions. For example, the WD40 motif of Tup1p and the TPR repeats mediate the interaction with Mat$\alpha$2p, whereas Mig1p and Rox1p seem to interact with Ssn6p but not directly with Tup1p (Tzamarias and Struhl, 1995). Furthermore, Mat$\alpha$2p can interact with any one of the TPR repeats in Ssn6p and any one of the four Tup1p in the same complex. Thus, the Ssn6-Tup1 complex can be oriented in many different ways when interacting with Mat$\alpha$2p, allowing significant flexibility in the way that it can bridge distances along DNA and interact with other proteins (Smith and Johnson, 2000).

1.1.4.3 Mechanism of the Ssn6-Tup1 mediated repression

Although studies have firmly established the importance of the Ssn6-Tup1 repressor in the repression of many genes, many questions regarding the precise mechanism by which the Ssn6-Tup1 repressor functions remain unanswered. Two general models, which are not mutually exclusive, have been advanced to explain how the Ssn6-Tup1 repressor might repress gene transcription once the repressor has been brought to the DNA. These models must be able to account for the general features of Ssn6-Tup1 repression mentioned above and it is proposed that the Ssn6-Tup1 complex can utilize multiple mechanisms to repress the transcription of any given gene.

**(1) Direct interference with activators and/or the transcription machinery.** Several studies support this model. First, the Ssn6-Tup1 complex could exert tight transcriptional repression while still allowing occupancy of a UAS by activators (Gavin et al., 2000; Keleher et al., 1992; Redd et al., 1997).

For example, Gavin *et al*. (2000) have suggested that one of the mechanisms of Tup1p mediated repression of the **a**-cell specific genes is the stabilization of the Mat$\alpha$2p/Mcm1p complex. Therefore, the Ssn6-Tup1 repressor can block the Mcm1p mediated activation and the chromatin remodeling activity. It is also possible that Tup1p could interact with an activator directly once Tup1p is recruited to DNA and compromise the activator's ability to activate transcription. As yet, this hypothesis has not been proved directly. Second, Herschbach *et al.* (1994) and others (Redd et al., 1997) observed that recombinant Mat$\alpha$2p and yeast extracts prepared from strains over-expressing Ssn6p and Tup1p could repress transcription of a naked DNA reporter construct *in vitro*. However, the repression level was modest as compared to the repression *in vivo*. Finally, several genetic screens have identified genes whose products could affect repression by the Ssn6-Tup1 complex. These gene products include some components of the mediator/holoenzyme complex such as Srb7p, Ssn5p, Ssn2p, Ssn3p, Ssn8p, Gal11p, Rgr1p, Sin4p, and Rox3p (Carlson, 1997). Although the precise functions of these proteins are not clear, these proteins have been biochemically linked to the RNA polymerase II transcription machinery. In addition, genetic evidence suggests that the mediator/holoenzyme complex plays a role not only in transcriptional activation but also in repression (Carlson, 1997). However, there is no direct evidence for this argument so far.

**(2) Establishment of the local chromatin environment.** This model is supported by several lines of evidence. First, Ssn6-Tup1 complex repressed genes are associated with the establishment of chromatin domains in which nucleosomes are precisely positioned over essential promoter elements and transcription initiation sites (Cooper et al., 1994; Gavin and Simpson, 1997; Li and Reese, 2001; Roth, 1995). Deletion of *TUP1* resulted in derepression of the gene and disruption of the positioned nucleosomes (Cooper et al., 1994; Gavin and Simpson, 1997; Li and Reese, 2001). Second, Tup1p interacts with N-terminal tails of histones H3 and H4 *in vitro*. *In vivo*, deletion or mutation of these tails has been observed to partially relieve Ssn6-Tup1 complex mediated repression (Edmondson et al., 1996; Edmondson et al., 1998). Third, several HDACs can interact with Ssn6-Tup1 complex and affect the repression (Watson et al., 2000). Fourth, by employing multiple techniques, studies performed in our lab showed that the Ssn6-Tup1 complex spreads along the promoter region and the entire coding sequence of two **a** cell-specific genes, *STE6* and *MFA1*, in repressed status (this study and (Ducker, 2001; Ducker and Simpson, 2000).

In summary, all these evidence suggest that the Ssn6-Tup1 complex may employ multiple mechanisms to repress a diversity of genes, and it is possible that the Ssn6-Tup1 complex may employ different mechanisms to repress different genes (Davie et al., 2002).

1.1.4.4 Relieve of the Ssn6-Tup1 mediated repression

Most, if not all, the genes repressed by Ssn6-Tup1 complex must be derepressed under specific but distinct conditions. For example, the *GAL* genes and the *SUC2* gene are expressed in mediums using galactose as carbon source (Smith and Johnson, 2000); when the cells are challenged with moderate salt concentrations (400 mM NaCl), the osmotic stress response genes must be activated for cells to survive (Proft et al., 2001). Obviously, these genes can not be derepressed through inactivating the Ssn6-Tup1 complex, which will lead to expression of all the genes it controls. In stead, the relief of repression results from the disassociation (Crt1p), degradation (Mat$\alpha$2p), or exiting from nucleus (Mig1), of the sequence specific binding proteins that recruit Ssn6-Tup1 complex to certain DNA regions. Therefore, the Ssn6-Tup1 complex is released (Smith and Johnson, 2000). However, this view has been challenged by several recent investigations showing that in at least some cases, the Ssn6-Tup1 complex remains bound even though the target genes are derepressed (Papamichos-Chronakis et al., 2002; Proft and Struhl, 2002).

1.1.4.5 Future directions

Future studies will focus on (1) using MAP technique to confirm distribution of Ssn6-Tup1 complex along repressed **a** cell-specific genes ( and other Ssn6-Tup1 repressed genes) by employing immuno-EM technique, and other new techniques; (2) the role of phosphorylation of Ssn6p and Tup1p, since they are both phosphorylated proteins; and (3) the interaction between the Ssn6-Tup1 complex and other histone modifiers such as methylases.

## 1.2 Methods for *in vivo* analysis of chromatin structure

### 1.2.1 Nuclease digestion of isolated nuclei

Most agents used to assess chromatin structure do not permeate cells. Therefore, historically, the typical source of chromatin is within isolated nuclei. Typically, nuclei are isolated from living cells and subjected to digestions of nucleases. After the purification of DNA, the digestion sites can be analyzed by indirect end labeling or primer extension (for details, see Simpson, 1998). Both specific restriction endonucleases and non-specific nucleases, including DNase I, DNase II and MNase, have been used (Simpson, 1998). The use of restriction endonucleases to infer the presence of a nucleosome is relatively simpler and quantification of the cutting can be done. However, it is also less informative in that it cannot provide information about either the translational or the rotational position of a nucleosome. Moreover, convincing results can be obtained only when the restriction site is in or very close to the dyad of a tightly positioned nucleosome. Therefore, DNase (I and II) and MNase are more widely used for chromatin mapping. Two major classes of information, the positioning of nucleosomes and hypersensitive sites, have been obtained by digesting isolated nuclei with these nucleases.

A positioned nucleosome is located in a precise site relative to DNA sequence in all cells of a given population (Simpson, 1991). Nucleosome positioning can be detected by MNase (detecting the translational positions) and DNase I (detecting the rotational positions) in isolated nuclei.

Nucleosome positioning is determined by several mechanisms and has significant functional consequences (Simpson, 1991). For example, when an autonomously replicating sequence of a minichromosome was covered by a positioned nucleosome, the copy number of the minichromosome decreased dramatically (Simpson, 1990).

In chromatin, nucleosome-free regions known as nuclease hypersensitive sites represent the “open windows” that enhanced access of crucial *trans*-factors. These regions are typically two orders of magnitude more sensitive to DNase (I or II) and MNase than other regions in bulk chromatin (Elgin, 1988; Gross and Garrard, 1988). These regions are always associated with very important *cis*-acting DNA sequences such as enhancers, promoters, replication origins or other sites which are important features of DNA activities. Moreover, these hypersensitive sites can be used to predict the discovery of new classes of *cis*-acting DNA sequences, perhaps involved in the functional punctuation of chromatin domains (the boundary elements), chromosome condensation and decondensation, meiotic chromosome pairing, and other processes that remain to be discovered. However, the mechanisms leading to the formation, maintenance, and propagation of hypersensitive sites are poorly understood and represent challenging questions (Elgin, 1988; Gross and Garrard, 1988).

Clearly there are numerous parameters to consider before concluding that chromatin in an isolated nucleus represents native chromatin in a cell. For example, TBP binding has been reported to change drastically upon isolation

of nuclei (this study and Pfeifer and Riggs, 1991); it was reported that nucleoplasmin was readily lost upon isolation (Krohne and Franke, 1980); and histone H1, was also degraded very soon in isolated nuclei(Krohne and Franke, 1980). Even for nucleosome positioning, it has been reported that care should be taken to analyze the data (Lohr and Lopez, 1995). Finally, the swelling of chromatin in the low ionic strength digestion buffer makes it impossible to obtain information about higher order chromatin structure.

### 1.2.2 Mapping chromatin structure by expressing enzymes in living cells

This strategy bypasses the need to isolate nuclei. So far, several classes of enzymes have been expressed in living cells to probe chromatin structures. These are DNA methylases (Kladde et al., 1999b), DNase I (Wang and Simpson, 2001), and certain restriction endonucleases (Iyer and Struhl, 1995; Mai et al., 2000).

Several studies suggested that DNA methylases (MTase) can be expressed *in vivo* and test chromatin structure (Kladde et al., 1999b and references therein). More than 20 years ago, Pratt and Hattman suggested that MTase could be used to analyze protein-DNA interaction based on their observation that MTase modified linker DNA preferentially to DNA associated with histones in nucleosomes (Hattman et al., 1978; Pratt and Hattman, 1981). Gottschling (1992) and Singh and Klar (1992) also supported the use of MTase accessibility as a probe for chromatin structure from their independent studies on genes when repressed or expressed by using

expression of *E. coli dam* MTase in *Saccharomyces cerevisiae* cells. The original DAM MTase, which was used for the assay, recognizes sequences of 4 bp, GATC, which occur randomly every several hundred base pairs. Efforts made mainly in our lab have increased the number of potential target sequences greatly by extending this method of analysis to more promiscuous MTases, such as the SssI enzyme, recognizing CG, and the MCviPI enzyme, recognizing GC, (Kladde et al., 1999b). To map chromatin structure with this strategy, MTase is usually integrated into the genome under control of an inducible promoter and is expressed in living cells and modifies its target sites in chromatin under physiological conditions, and the accessibility of the enzyme to its cognate site reflects the local chromatin structure (Kladde et al., 1999b). Compared to other methods, this method has many advantages: the method eliminates the need for isolation of nuclei (they can be expressed *in vivo*) and does not impair cell viability when the modification level is low; it does not damage DNA; and it can detect both histone-DNA and nonhistone-DNA interactions. But this method also has disadvantages. For one, its resolution is not high enough. For example, the most widely used enzyme, M.SssI, modifies cytosine in the sequence CG. CG is underrepresented in many genomes, including *Saccharomyces cerevisiae,* where it is present once every ~35 bp. Another disadvantage is that there is endogenous MTase in many organisms; this greatly limits the application of this method (Kladde et al., 1999b).

DNase I was the first enzyme used to define the general nuclease sensitivity that distinguish active from inactive genes by Weintraub and Groudine (1976). Later on, Wu and Gilbert used this same nuclease for description of nuclease hypersensitive sites that signaled a regulated promoter in the active state (Wu and Gilbert, 1981). Most importantly, extension of the general rule that DNase I hypersensitivity marks the sites where the action is in chromatin has made hypersensitive sites synonymous with enhancers, promoters, replication origins, or other features of DNA activity (Elgin, 1988; Gross and Garrard, 1988). In an indirect-end label study of DNase I digestion of ~50 kilobase pairs of the left arm of yeast chromosome III, we have confirmed the correlation between these DNA elements and hypersensitivity to the nuclease (S. Ercan and R.T.S., unpublished observation). Moreover, digestion of core particle DNA with a periodicity of ~10 nucleotides leads to a distinctive pattern for rotationally positioned nucleosomes (Wolffe, 1998). Differential sensitivity of linker DNA allows DNase I mapping of nucleosome locations, although not with the precise resolution of micrococcal nuclease which cuts linker DNA almost exclusively (Simpson, 1998; Simpson, 1999). For these reasons, we elected to attempt to establish DNase I as an *in vivo* chromatin probe (Wang and Simpson, 2001). The advantages of this strategy include: (1) it can detect interactions between non-histone proteins and DNA very efficiently (chapter II and III); (2) as a non-specific endonuclease, DNase I can, in principle, detect protein binding on any DNA sequences; (3) there is no need to isolate nuclei.

However, there are also some inherent disadvantages: (1) since it detects chromatin structure in living cells, it is hard to distinguish whether the cutting is from direct or indirect effects (for example, the cutting of DNA may induce DNA damage reactions and DNA repair); (2) it is hard to distinguish whether the protection comes from real binding of proteins or just from the steric hindrance in the space due to the large size of DNase I; (3) it can provide only a hint about protein binding, but not definitive information as to what proteins are binding.

Several studies have also expressed specific restriction enzymes in living cells (Iyer and Struhl, 1995; Mai et al., 2000). This strategy can yield quantitative analysis and specific information. However, the sequence specificity and the strong cutting properties (which lead to rapid cell death) simultaneously limit its applications.

DNA repair enzymes, specifically photolyase, have been shown to be responsive to chromatin organization and are therefore suggested for *in vivo* tests of chromatin structure (Suter et al., 1999). However, as repair requires an initial insult to the DNA by UV light to create lesions, the utility of these enzymes for the study of normal cellular architecture is limited.

### 1.2.3 Chromatin Immuno-precipitation (CHIP)

ChIP is a valuable approach for analyzing the association of specific proteins with certain DNA regions in the context of chromatin. This approach uses formaldehyde fixation of cells, fragmentation of the nucleoprotein

complex by sonication or MNase digestion, and isolation of regions of DNA that are connected to a particular protein by immunoprecipitation using antibodies to that protein. After reversal of the crosslinking, the presence of certain DNA fragments in the pellet can be tested by slot blot or quantitative PCR (Hecht and Grunstein, 1999; Orlando, 2000).

Several advantages rapidly made this a popular approach (Hecht and Grunstein, 1999; Orlando, 2000; Orlando et al., 1997; Simpson, 1999). First, in principle, ChIP offers the ability to detect whether any given protein is associated "in time and space" with specific genomic regions. In particular, this method can analyze proteins that are not bound to DNA directly or that depend on other proteins for binding activity *in vivo*. Second, the macromolecular chromosomal structures in living material, such as tissue culture cells or embryos, can be fixed very efficiently and the chromatin is used as a substrate for immunoprecipitation. Third, antibodies directed against the protein of interest allow immuno selection of all genomic binding sites. Fourth, the crosslinking can be fully reversed and the DNA can be analyzed. Fifth, the DNA can be analyzed by quantitative PCR, which is rapid and sensitive, and which allows fine mapping of chromosomal proteins in regions as small as 300 bp. Sixth, by employing antibodies which specifically recognize histones with certain modifications ( for examples, see Deckert and Struhl, 2001; Litt et al., 2001a; Litt et al., 2001b; Lo et al., 2001), several studies have checked the status of histones in active versus inactive regions and have thus made great contributions to the "histone code" model

(Jenuwein and Allis, 2001; Strahl and Allis, 2000). Finally, the use of this approach along with DNA microarray (genome chip) technology (ChIP-chip) can principally identify all the *in vivo* DNA targets of any protein of interest (Ren et al., 2000; Simon et al., 2001; Wyrick et al., 1999). The availability (currently or in the near future) of complete sequence information of several genomes, including yeast, *Drosophila*, mouse, and human, will markedly increase the potential power of this type of analysis.

However, this approach also has several obvious limitations. First, in the ChIP assay, the immunoprecipitation step requires highly stringent conditions. Therefore, many factors may affect the final results by influencing the accessibility of the first and/or the secondary antibody. For example, too much crosslinking has been observed to mask histones. In addition, in some cases, the buffer conditions may not be compatible with certain antibodies. Moreover, certain antigen epitopes are more sensitive to formaldehyde. In this regard, polyclonal antibodies are better than monoclonal antibodies for ChIP assay. Second, this approach can not be used to detect unknown proteins binding on DNA regions of interest. Third, this approach may lead to overinterpretation in some cases in which the given protein binds to DNA transiently or is located very close to DNA.

### 1.2.4 Electron microscopy (EM) and chromatin

Obviously, the most direct way to explore a region of chromatin is to look and see. In this regard, EM is one of the few techniques available to obtain

information about the spatial relationships among arrays of nucleosomes and about other conformational issues that can not be approached directly by other techniques. Successful examples of the use of EM include the spectacular images of transcribing genes (Miller and Beatty, 1969), the ubiquitous “beads-on-string” nucleosomal arrangement of chromatin (Olins and Olins, 1974), the demonstration of the salt-dependent changes in the folding of nucleosomal chains (Thoma et al., 1979), and the determination of the sites and structures associated with RNA synthesis and processing (Raska et al., 1990; Spector, 1993). All these results were obtained by transmission EM, examining samples either *in situ* (fixed whole cell or nucleus) or *in vitro* (isolated components) samples (Woodcock and Horowitz, 1997; Woodcock and Horowitz, 1998). However, certain problems arise in the conventional EM method and introduce uncertainties concerning the degree to which the final images actually correspond to the original structures (Woodcock and Horowitz, 1997; Woodcock and Horowitz, 1998). First, transmission electron microscopy requires the specimens to withstand a high vacuum, to provide sufficient electron contrast, and to be thin enough to allow penetration of the electron beam (Woodcock and Horowitz, 1997). To fulfill these requirements, samples (cells, nuclei or isolated nuclear components) were fixed, hydrated, embedded in plastic media, cut to be thin enough to allow electron beam transmission, and stained with metals or metal salts. All these preparatory treatments are highly disruptive to biological materials. Second, the chromatin must usually be affixed to flat substrates, and this

inevitably affects the 3D conformation of the chromatin. Finally, the resolution of nucleosomes and linker DNA is too low for the EM to provide an unequivocal answer to the important question of how chains of nucleosomes compact in solution. Based on these considerations, many approaches to the imaging of isolated chromatin have been developed, designed to avoid these drawbacks (Bednar and Woodcock, 1999; Engel and Colliex, 1993; Muller and Engel, 2001). Among them is the cryo-EM (Bednar and Woodcock, 1999; Woodcock and Horowitz, 1997).

Cryo-EM is based on two principles (Woodcock and Horowitz, 1997). First, if cooled rapidly enough, water assumes a vitreous (glassy) state, instead of crystallizing into ice. Second, when temperatures are sufficiently low, samples can be placed in the high vacuum of the electron microscope without significant water loss or vacuum degradation, thanks to the low vapor pressure of vitreous water (Dubochet et al., 1988). Unfixed samples, which are small enough to be embedded in a thin aqueous film over a hole that can be rapidly frozen, can be imaged directly in this way, without staining with heavy metal. Thus, the native conformation can be retained (Dubochet et al., 1988). Further, this new technique can be used to examine changes occurring over time as small as milliseconds (Bednar and Woodcock, 1999; Berriman and Unwin, 1994). Therefore, the applications of cryo-EM include (1) imaging the 3D conformation of small particles of interest, such as viruses (Dubochet et al., 1988), (2) defining the complete 3D conformation of a unique individual polynucleosome by determining the orientation of

nucleosomes (Bednar et al., 1995) and the path of the linker DNA segments (Dubochet et al., 1992; Dustin et al., 1991). The samples can be *in vitro* reconstituted chromatin (Bednar and Woodcock, 1999; Woodcock and Horowitz, 1997); or MAP isolated *in vivo* packed chromatin (but it needs large amount of samples; see below) under different physiological states; (3) testing the effects of histone modifications or some non-histone proteins (such as H1, HMG proteins) on 3D conformation of chromatin, by using "random sequence" chromatin (Bednar and Woodcock, 1999; Woodcock and Horowitz, 1997); (4) monitoring specific short lived intermediate conformations or conformational changes of chromatin during processes such as transcription (Bednar and Woodcock, 1999).

The major problems of cryo-EM include: (1) the extreme sensitivity of the unfixed, unstained specimen to the electron beam, which makes low-dose imaging mandatory and leads to a very low contrast; (2) the quality of the specimen and preparation cannot be judged before taking images, which places a premium on the skills of the operator; (3) large of amount of samples are needed to get images. However, despite these considerations, cryo-EM is the technique of choice for obtaining 3D information about macromolecular assemblies and for examining rapid (~msec) conformational changes of such assemblies in solution. In addition, further technical developments now in progress will make cryo-EM less challenging.

### 1.2.5 Minichromosome affinity purification (MAP)

MAP is an approach to learning the composition, structure, and function of unique genes packaged as chromatin in different functional states. This approach is based on several principles. First, in yeast, there exist minichromosomes, the small extrachromosomal plasmids that can be stably maintained and packaged as chromatin in living yeast cells (Roth and Simpson, 1991). Second, after being integrated into a commonly used minichromosome, the ALT(Ducker and Simpson, 2000), a genomic domain behaves in the same way as its genome copy (this study; Cooper et al., 1994; Ducker and Simpson, 2000; Patterton and Simpson, 1994; Roth et al., 1990; Roth et al., 1992; Roth and Simpson, 1991; Shimizu et al., 1991). Third, the minichromosome can be isolated based on a protein-DNA affinity approach (for details see chapter IV and (Ducker, 2001; Ducker and Simpson, 2000).

The application of the MAP methodology to chromatin is still in its infancy, and there are numerous methodological questions to be answered and improvements to be made (Ducker and Simpson, 2000; Simpson et al., 2003). However, it is already providing useful new information concerning the histone modifications, the non-histone components and the 3-D conformation of chromatin of specific domains and promises to become a valuable complement to biophysical and molecular techniques (Ducker and Simpson, 2000; Simpson et al., 2003). The applications of MAP include (1) determining stoichiometry of proteins associated with particular gene sequences (Ducker and Simpson, 2000); (2) identifying proteins that leave no footprint on DNA and/or proteins whose absence is lethal; (3) identifying new proteins

associated with particular gene sequences (Ruan C. and Simpson RT. unpublished data); (4) by combining with EM, observing the higher order chromatin structure under different functional states (chapter IV and (Ducker, 2001).

### 1.2.6 Other methods

In addition to the methods mentioned above, there are several other methods used to analyze chromatin structure, each having advantages and disadvantages (Simpson, 1999; Zaret, 1999). These methods include the application of small chemical modifying agents of DNA that can pass through cell wall and membrane; the use of fluorescent proteins to determine the localization of chromosome segments in living cells; and *in situ* mapping, in which nonionic detergents or some antibiotics are used to permeabilize the cell membrane and/or cell wall sufficiently to allow the entry of enzymatic probes of chromatin such as DNase I and micrococcal nuclease.

It becomes more and more interesting to know whether the localization of a specific chromosome segment is related to its functions. Recently, a method has been developed to “track” the localization of certain chromosome segment of interest in living cells (Robinett et al., 1996). Two hundred and fifty six tandem repeats of the *Escherichia coli lac* operator sequence (~5 kb total) was inserted into a specific location of the yeast, plant or CHO-cell genomic sequence (Belmont and Straight, 1998; Kato and Lam, 2001; Kato and Lam, 2003; Robinett et al., 1996; Straight et al., 1996). A *lac I* repressor protein

fused to GFP can be expressed in the same cell, bind to the operator repeats, and indicate the localization of the tagged chromosome region in the living cell under fluorescent light-microscope. Resolution of this strategy can be increased by employing immuno-EM. Up to today, this strategy has been used in several studies to investigate the localization of important chromosome segments (Belmont and Straight, 1998; Kato and Lam, 2001; Kato and Lam, 2003; Kato et al., 2002; Robinett et al., 1996; Straight et al., 1996). One example is that telomeres occupy a perinuclear location in yeast cell nuclei (Gotta et al., 1996). However, the significance and the detailed mechanisms of such localizations remain unexplored.

### 1.2.7 Conclusion

In this section, I have briefly reviewed several commonly used and newly emerged methodologies for investigating chromatin structure *in vivo*. Each method has its inherent advantages and disadvantages, and in many cases, each provides complementary information regarding the components, structure and function of chromatin.

In this study, we focused mainly on the establishment and/or application of two of these methods: the *in vivo* DNase I mapping strategy (chapter II and III), and MAP (chapter IV).

# Chapter II

## Chromatin structure mapping in *Saccharomyces cerevisiae in vivo* with DNase I

## Abstract

Most methods for assessment of chromatin structure involve chemical or nuclease damage to DNA followed by analysis of distribution and susceptibility of cutting sites. The agents used generally do not permeate cells, making nuclear isolation mandatory. *In vivo* mapping strategies might allow detection of labile constituents and/or structures that are lost when chromatin is swollen in isolated nuclei at low ionic strengths. DNase I has been the most widely used enzyme to detect chromatin sites where DNA is active in transcription, replication or recombination. We have introduced the bovine DNase I gene into yeast under control of a galactose-responsive promoter. Expression of the nuclease leads to DNA degradation and cell death. Shorter exposure to the active enzyme allows mapping of chromatin structure in whole cells without isolation of nuclei. The validity and efficacy of the strategy are demonstrated by footprinting a labile repressor bound to its operator. Investigation of the inter-nucleosome linker regions in several types of repressed domains has revealed different degrees of protection in cells, relative to isolated nuclei.

## 2.1 Introduction

Eukaryotic DNA transcription, replication, recombination and repair all take place in the context of chromatin, the complex of the nucleic acid with histones and other proteins. Increasingly, the relevance of structural features of chromatin to these functions of DNA is being appreciated (reviewed in (Kornberg and Lorch, 1999; Workman and Kingston, 1998a). Analysis of chromatin structure is done mainly by determination of features of DNA structure using nucleases or chemicals that modify the nucleic acid. Patterns of modification are revealed by primer extension or indirect end-label methods and interpreted in the context of known chromatin structures, such as nucleosomes, or by comparison with *in vitro* complexes of DNA with particular proteins (Simpson, 1998; Simpson, 1999). Most chemical methods (ultraviolet light and psoralens are exceptions) and all nucleases require isolation of nuclei for their utilization. This requires time, creating the possibility that short half-life proteins may be absent in the analysis; this possibility is reality in usual analyses of the yeast cell type-specific repressor Mat$\alpha$2p (Murphy et al., 1993). In addition, the buffers often employed for nuclease digestion are low in ionic strength, leading to swelling of the chromatin. This raises the possibility that nucleoprotein structures normally present in the nucleus of a living cell may be distorted or disrupted at the time of analysis by nucleases.

To counter these potential problems, we have attempted to develop methods that assess chromatin structure in living yeast cells. Two different methyltransferases, recognizing cytosine in CG or GC sequences,

respectively, have been utilized (Kladde et al., 1999a; Kladde et al., 1999b; Xu et al., 1998). Both serve well, allowing survival of cells when expressed at low levels (one modification per ~500–2500 bp) while mapping both regulatory protein and histone interactions with DNA. Both suffer from the relative infrequency of their modification site in native yeast DNA and the long times necessary for achieving levels of modification suitable for mapping experiments. In search of a more general reagent for mapping chromatin, we have turned to the first reagent used for this purpose with isolated nuclei, bovine pancreatic DNase I.

DNase I was used in the first definition of the general nuclease sensitivity that distinguishes active from inactive genes by Weintraub and Groudine (Weintraub and Groudine, 1976). Wu and Gilbert used this same nuclease for description of nuclease hypersensitive sites that signaled a regulated promoter in the active state (Wu and Gilbert, 1981). Digestion of core particle DNA with a periodicity of ~10 nt leads to a distinctive pattern for rotationally positioned nucleosomes. Differential sensitivity of linker DNA allows DNase I mapping of nucleosome locations, although not with the precise resolution of micrococcal nuclease which cuts linker DNA almost exclusively (Simpson, 1998; Simpson, 1999). Most importantly, extension of the general rule that DNase I hypersensitivity marks the sites where the action is in chromatin has made hypersensitive sites synonymous with enhancers, promoters, replication origins or other features of DNA activity (Clark et al., 1993; Elgin, 1988; Gross and Garrard, 1988). In an indirect end-label study of DNase I digestion of ~50

kb of the left arm of yeast chromosome III, we have confirmed the correlation between these DNA elements and hypersensitivity to the nuclease (S.Ercan and R.T.Simpson, unpublished observation). For these reasons, we elected to attempt to establish DNase I as an *in vivo* chromatin probe.

Worrall and co-workers (Doherty et al., 1993; Worrall and Connolly, 1990) synthesized and expressed a number of variants of the predicted DNA sequence for the DNase I protein. The different proteins encoded by these DNAs varied in specific enzymatic activity. We have cloned the DNA sequence corresponding to the native protein plus a nuclear localization signal (NLS) into a shuttle expression vector. Fortuitous expression in bacteria required modification of the vector to eliminate this toxic activity. Expression in yeast under control of the GAL1 promoter was sufficiently controlled by dextrose repression to allow growth of transformed strains without differences from similar strains lacking the nuclease gene. When these strains were cultured in galactose, expression of the DNase I gene was lethal for long-term growth. Prior to cell death, chromatin mapping *in vivo* is possible using the nuclease to explore the DNase I susceptibility of the genome without perturbation of the normal nuclear environment of chromatin. In this report, in addition to documenting the methodology for use of DNase I as an *in vivo* probe for chromatin structure, we present evidence that suggests an unexpected higher order structure for some silenced or strongly repressed parts of the yeast chromosome.

## 2.2 Materials and methods

### 2.2.1 Plasmid construction

A synthetic DNA designed to code for the native form of bovine pancreatic DNase I and cloned in M13 was kindly provided by Dr A.F. Worrall (Worrall and Connolly, 1990). The SV40 NLS (ATG CCA AAG AAG AGA AAG GTT), flanked by an *Eco*RI site, was attached to the N-terminus using the polymerase chain reaction. In the same reaction, an *Escherichia coli lac*-UV5 constitutive promoter in antisense orientation and an *Xba*I site were added to the C-terminus of the coding sequence. The fragment was cloned into the polylinker of pYES2 (Invitrogen) (Sikorski and Hieter, 1989). This vector is a high copy number shuttle expression vector containing a polylinker between the *GAL1* promoter and the *CYC1* terminator, a *URA3* selection marker and 2μ origin of replication, as well as the ampicillin resistance gene and colE1 replication origin for selection/growth in *E.coli*. After growth in bacteria and confirmation of the construction by restriction endonuclease mapping and limited sequencing, the plasmid, pSUN-1, was transferred by electroporation to *Saccharomyces cerevisiae* YPH500 (*MAT*$\alpha$*, ade2-101, his3-*$\Delta$*200, leu2-1, lys2-801, trp1-*$\Delta$*63, ura3-52*) (Sikorski and Hieter, 1989). As a control, the parental plasmid, pYES2, was transformed into the same strain in parallel.

### 2.2.2 Cell growth

Standard yeast media, both rich (YPD) and synthetic medium lacking uracil [CSM-Ura (Bio 101), 0.67% yeast nitrogen base without amino acids

(Difco), and an appropriate carbon source (2% dextrose, 4% lactic acid or 2% galactose)] were used. Cells containing pSUN-1 or pYES2 were grown at 30°C to $OD_{600}$ 1.0 in CSM-Ura dextrose medium. Similar densities of cells were spread on CSM-Ura dextrose or CSM-Ura galactose plates to observe any effect of DNase I on cell viability. For chromatin structure studies in cells expressing DNase I, yeast cells transformed with pSUN-1 were grown at 30°C to $OD_{600}$ 0.8 in 10 ml CSM-Ura dextrose medium, changed to 10 ml CSM-Ura lactic acid medium and grown to $OD_{600}$ 1.0, and then switched to CSM-Ura galactose medium for periods of up to 12 h.

### 2.2.3 Nuclear and DNA preparation and analysis

Yeast nuclei were prepared as described from cells grown at 30°C to $OD_{600}$ of 1.0 in YPD (Roth et al., 1992). DNase I digestion, isolation of nuclear DNA and determination of the locations of DNase I cleavage sites by primer extension were all performed exactly as previously described (Weiss and Simpson, 1997). For isolation and analysis of DNA cut by DNase I *in vivo*, cells were harvested by centrifugation, broken by homogenization with glass beads in 100 mM Tris–HCl pH 8.0, 50 mM EDTA, 2% sodium dodecyl sulfate, and the DNA was extracted (Rose et al., 1990). Purification of DNA involved treatment with 100 ng/ml RNase A at 37°C for 1 h and then 100 ng/ml proteinase K in 2% Sarkosyl, 200 mM $NaClO_4$ at 50°C for 2 h. DNA was further purified by extractions with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation. The DNA was dissolved in 20 µl 0.1x

TE and passed through a 1 ml Sephadex G-50 spin column prior to analysis. Primer extension was carried out as previously described using the following primers: recombination enhancer (RE) region, B297 (Weiss and Simpson, 1997); *HMR***a** region, p14 (Ravindra et al., 1999); and *STE6* region, –373 to –352 (relative to transcription start site) 5'-CGTACCATTCCATTGGCTTTTC-3' (Shimizu et al., 1991). In Figures 2.5, 26, and 2.7, the schematic diagrams of nucleosome locations are based on previously published micrococcal nuclease primer extension maps (Ravindra et al., 1999; Shimizu et al., 1991; Weiss and Simpson, 1997) using the same primers and identical molecular weight standards to those employed in the present work. Analysis of cutting of the GAL control region of pSUN-1 from 0–12 h of induction of DNase I expression revealed similar cutting site patterns at all time points. Intensities of bands increased with time and shorter fragments became more prominent, as expected.

### 2.2.4 Southern blots

Yeast cells transformed with either pSUN-1 or pYES2 were grown sequentially in CSM-Ura with dextrose, lactic acid and galactose, as detailed above. At 0, 1 and 6 h after exposure to galactose, cells were harvested and DNA was recovered by the glass bead method (Rose et al., 1990). DNA from equal cell numbers (based on $A_{600nm}$) was subjected to electrophoretic separation on a 1.2% agarose gel, transferred to Hybond-NX membrane

(Amersham), crosslinked with UV light and hybridized as previously described (Ducker and Simpson, 2000). The pYES2 plasmid was random primer labeled with [$\alpha$-$^{32}$P] dATP for use as probe. Blots were exposed to film or a PhosphorImager screen and analyzed using Image Quant v.5.0 software (Molecular Dynamics).

## 2.3 Results

### 2.3.1 DNase I expression *in vivo*

Others have successfully cloned and expressed native DNase I in bacterial systems (Doherty et al., 1993; Worrall and Connolly, 1990). Our attempts to clone DNase I with a NLS into yeast shuttle expression vectors led to deletions or mutation of the active site histidyl residue (data not shown). Further analysis showed that these mutations occurred during bacterial manipulations. Apparently, expression from a surrogate promoter led to nuclease toxicity and selection for inactive mutants. To block DNase I expression in bacteria, we inserted a constitutively active *E.coli* promoter, *lac*-UV5, downstream of the DNase I gene in the opposite transcriptional orientation. Inhibition of nuclease expression may involve antisense RNA, physical interference with polymerase movement, or another unknown mechanism. In any case, this approach allowed cloning of the intact DNase I gene in bacteria.

Yeast containing the DNase I plasmid, pSUN-1, grew on dextrose-containing medium identically to controls bearing an empty vector (Figure 2.1). Plating the two strains on galactose led to striking differences in growth patterns. While the control strain, lacking DNase I, grew more slowly than it did on dextrose, colony numbers were similar. However, the strain containing the DNase I gene under *GAL1* control failed to form any colonies when expression of the gene was induced (Figure 2.1). Similarly, in suspension

cultures the strain carrying pSUN-1 did not grow in 12 h after a medium change from CSM-Ura dextrose to CSM-Ura galactose medium (data not shown). It is likely that DNase I killed cells in which it was expressed by degradation of genomic DNA.

Since DNase I preferentially cuts one strand of duplex DNA, a highly sensitive assay for its activity in living cells is to measure conversion of a plasmid from closed circular form to a nicked, relaxed form; a single DNase I cut per plasmid molecule is measured by this assay. We examined the topological state of the pSUN-1 plasmid in yeast at various times after transferring cells to galactose. At the time of medium change, 10% of the isolated plasmid DNA was nicked, probably due to damage during preparation. Within 1 h of growth in galactose, ~35% of plasmid DNA was in the nicked form and this fraction increased to ~45% after 6 h exposure (Figure 2.2A and B). There is a decrease in total plasmid DNA during expression of DNase I; this is likely due to cutting of the nuclease sensitive replication origin in the minichromosome. Control cells, either containing the DNase I plasmid and grown in dextrose or containing the plasmid backbone without the nuclease gene and grown in galactose, had a constant content of episomal DNA with ~10% nicked plasmid circles (data not shown). Examination of genomic DNA on native agarose gel electrophoresis revealed mostly high molecular weight material with some smearing to smaller fragment sizes, most >1500 bp. The amount of digestion was insufficient to generate a

nucleosomal ladder. Attempts to use samples digested *in vivo* for indirect end label mapping experiments failed due to the low levels of DNase I digestion.

The time course of DNase I cutting of plasmid DNA was examined over a 12 h period (Figure 2.3). Undigested or zero time samples were all high molecular weight. Patterns of cutting at 3 and 6 h were similar with a large amount of DNA of relatively high molecular weight and similar cutting sites. More material was present for cutting sites closer to the primer in the 6 h sample than the 3 h, as expected. After 12 h of DNase I expression, most of the plasmid DNA had been degraded to sizes <500 nt and some changes in cutting site patterns appeared. Clearly, the 3 and 6 h samples were in the range of single hit kinetics for fragments <1 kb in length, making these times appropriate for analysis of DNase I susceptibility. The 6 h sample gave a higher yield of fragments in the mapping size range; this expression time was therefore used for all the experiments presented below.

Primer extension analysis of the GAL control region DNA in the pSUN-1 plasmid showed that most of the nuclease cutting sites were similar to those cut in naked DNA by pure DNase I *in vitro* (data not shown). This finding, together with data presented below (Figure 2.7), demonstrates that chromatin mapping *in vivo* reflects expression of the bovine DNase I gene and not artefactually induced, unknown yeast nucleases. The three observations, nicking of the plasmid, plasmid DNA degradation, and the similarity of cutting sites on the plasmid to naked DNA, confirm DNase I expression under GAL

control and its activity in yeast cells. They validate use of DNase I expressed in cells as a tool for *in vivo* analysis of chromatin structure.

### 2.3.2 DNase I footprinting a labile repressor *in vivo*

In $\alpha$-cells, Mat$\alpha$2p binds as a homodimer together with a homodimer of Mcm1p to a 31 bp operator DNA sequence to repress expression of **a**-cell-specific genes (Johnson and Herskowitz, 1985; Keleher et al., 1988; Sauer et al., 1988). The repressor is one of the shortest-lived proteins in yeast, with a half-life of <5 min. Consequently, it is absent from its cognate binding sites in isolated nuclear preparations. It is likely that the protein is gone well before spheroplasting, a mandatory prerequisite for nuclear isolation, is finished (Hochstrasser et al., 1991). We previously mapped features of interactions of Mat$\alpha$2p with DNA by using a very rapid method for isolation of crude nuclei in a yeast strain that overproduced the protein and lacked several ubiquitination enzymes (Murphy et al., 1993). It was also possible to demonstrate Mat$\alpha$2p binding to its operator sequence in living cells using the *Sss*I methyltransferase; a single CpG site was blocked by interactions of one monomer with DNA (Kladde et al., 1996). The structure of the DNA binding domains of Mat$\alpha$2p and Mcm1p in a ternary complex with $\alpha$2 operator DNA is known at 2.25 Å resolution from X-ray crystal studies (Tan and Richmond, 1998). Taken together, the *in vitro* structural information and the *in vivo* difficulties in studying binding of Mat$\alpha$2p and Mcm1p to the operator made this system a good test of the possibility of mapping chromatin structures of regulatory protein complexes with DNA using DNase I expressed in living

cells. There are nine functional Mat$\alpha$2p/Mcm1p binding sites in the yeast genome (Johnson and Herskowitz, 1985; Zhong et al., 1999). Seven of these reside upstream of **a**-cell-specific genes and repress their expression when occupied by the protein complex in $\alpha$-cells. The other two are located in the recombination enhancer and help to control directionality of mating type interconversion (Haber, 1998b; Szeto and Broach, 1997; Wu and Haber, 1995). We have mapped chromatin structure of four of these Mat$\alpha$2p/Mcm1p binding sites *in vivo* using DNase I.

Figure 2.4 shows the DNase I cutting patterns at the recombination enhancer $\alpha$2#1 operator, located 29194–29224 bp from the left end of chromosome III. Naked DNA from this region was susceptible to DNase I at a number of sites throughout the $\alpha$2/MCM1 operator and its flanking sequences. DNase I cutting was severely restricted in both isolated nuclei and *in vivo* digested chromatin samples, relative to the protein-free DNA pattern. In isolated nuclei, where Mat$\alpha$2p is expected to be absent, four prominent nuclease-susceptible sites were present. Two sites flanked the operator and two were located within the operator. Mcm1p is known to bind to the 11 bp central region of the operator, while Mat$\alpha$2p binds to the 10 bp on either side of the central segment. The two susceptible sites in the operator in isolated nuclei likely resulted from Mcm1p-induced bending of the DNA with consequent widening of the minor groove (Tan and Richmond, 1998). DNase I is known to approach and cut DNA in the minor groove and the slightly bent, partially open minor groove resembles the geometry of DNA in complex with

the enzyme (Weston et al., 1992). These two sites were inaccessible when the analysis was performed with DNase I expressed *in vivo*. A large region between the two operator-flanking hypersensitive sites was blocked from digestion by the nuclease (Figure 2.4). Footprints for the $\alpha$2 operator in nuclei and *in vivo* for the recombination enhancer $\alpha$2#2 site and for the transcriptional repressor $\alpha$2 operators upstream of the *STE2* and *STE6* genes in $\alpha$-cells were essentially identical to those shown in Figure 2.4 (data not shown). In **a**-cells, which lack Mat$\alpha$2p, *in vivo* and nuclear footprints were indistinguishable and closely resembled the pattern observed for $\alpha$-cell nuclei. We presume that binding of Mat$\alpha$2p precludes nuclease access to operator DNA in $\alpha$-cells *in vivo*.

### 2.3.3 Different nucleosome linker accessibilities in repressed domains *in vivo*

In yeast chromatin, a striking example of DNase I susceptibility of a precisely positioned nucleosome was found near the $\alpha$2#1 operator in the recombination enhancer at 29403–29560 map units (m.u.) (Weiss and Simpson, 1997). In isolated nuclei, digestion was highest in linkers and at the ends of the core particle and diminished symmetrically to a low point at the center of the nucleosome. While these accessible linkers may characterize some yeast chromatin (see below), use of DNase I expressed in whole cells has revealed nucleosomes with protected linker chromatin in some repressed domains *in vivo*. One of these is the RE nucleosome at 29403–29560 m.u.

We confirmed the DNase I digestion pattern for this nucleosome in isolated nuclei. The linker and edges of the core particle were accessible to DNase I digestion. The lowest levels of cutting were in the center of the core particle (Figure 2.5). In contrast, for the sample cut by DNase I *in vivo*, the cutting site susceptibilities were almost reversed. Sites near the center of the nucleosome core particle were cut *in vivo* more frequently than sites near the ends of the nucleosomes and the intervening short linkers. This observation was confirmed for DNase I cuts in the other strand through this same region. Furthermore, the short linkers between several other pairs of positioned nucleosomes in the RE in $\alpha$-cells also were differentially digested by DNase I between isolated nuclei and *in vivo* samples (data not shown).

Another type of repressed domain with organized chromatin in $\alpha$-cells encompasses the **a**-cell-specific genes, specifically *STE6* (Roth et al., 1990; Shimizu et al., 1991). A characteristic feature of these chromatin domains is the presence of closely packed dinucleosomes with a short (<10 bp) linker. Longer linkers, in the range of 40–45 bp, connect these dinucleosomes to one another (Simpson et al., 1993). Both linkers of the first nucleosome, adjacent to the $\alpha$2 operator, were protected *in vivo* (Figure 2.6 and data not shown). Cutting in nuclei was observed at a single site in the short linker between the first and second nucleosomes. Within the second nucleosome, cutting in nuclei was concentrated towards the ends of the core particle while cutting *in vivo* was relatively greater towards the center of the nucleosome. Significant differences were observed between nuclear and *in vivo* nuclease sites in the

long linker following the second nucleosome (Figure 2.6). The strong nuclear site that marks the edge of the second nucleosome at the long linker was absent *in vivo* while the site at the other end of the linker was present in both samples. Three DNase I cutting sites were present for the long linker in the *in vivo* sample. In nuclei, one of these was also DNase I sensitive, the other two were weakly cut and two different sites were strongly cut. It is apparent that significant features of this linker region, in terms of proteins bound or packaging, are altered during the preparation of nuclei.

The silent mating type loci, *HMLα* and *HMR***a**, are perhaps the best-characterized repressed domains in the yeast genome (Ravindra et al., 1999; Weiss and Simpson, 1998). The chromatin structure of the regions between the E and I silencers has been determined by mapping with micrococcal nuclease. *HMR***a** is the smaller and simpler of the two loci, having 12 precisely positioned nucleosomes between the silencer elements. These are arranged as six pairs of closely packed dinucleosomes with ~20 bp linker between the pairs (Ravindra et al., 1999). Figure 2.7 shows the results of mapping DNase I cutting sites in nuclei and *in vivo* for the nucleosome that is closest to the E silencer. The linkers flanking the nucleosome were significantly more susceptible to DNase I than the central region of the core particle, both *in vivo* and in isolated nuclei. For both samples, cutting sites within the core particle were spaced at ~10 nt intervals. However, several strong cutting sites were present in nuclei at the E end of the nucleosome and in the silencer, but absent in whole cells. This observation suggests that certain proteins, e.g.

silent information regulator proteins (SIRs), involved in establishing or stabilizing the repressive domain, might protect this region in living cells.

## 2.4 Discussion

Aspects of chromatin structure can be inferred because of a distinctive component, DNA, which can be assayed by enzymes and chemicals that specifically probe its structure. Based on known features of the reagent and permutations of the nucleoprotein environment of the chromatin segment under study, we can infer features of chromatin structure such as presence or absence of nucleosomes, their organization and possible positioning, binding of regulatory proteins, unusual DNA geometries, etc. Almost all such studies are carried out in the context of isolated nuclei, since reagent access to the nuclear contents is blocked in most cases by the plasma membrane and/or cell wall. Here we have surmounted this limitation by controlled intracellular expression of a nuclease and targeting the enzyme to the nucleus, where it can map chromatin organization without disruption of cell structures.

The nuclease of choice is DNase I. Micrococcal nuclease, the other relatively non-specific enzyme used widely for chromatin structure studies, cuts linker regions preferentially and is therefore used for mapping nucleosome locations. It has been less useful in revealing features of interactions of regulatory proteins with DNA (Simpson, 1998). Of greater concern, however, is the fact that micrococcal nuclease is a general nuclease, with a strong preference for degrading single-stranded nucleic acids. Thus, it will likely be cytotoxic by extensive degradation of RNA. Such activity might

well kill cells before the desired levels of DNA cutting for a mapping experiment were achieved.

DNase I, on the other hand, has no ribonuclease activity, is active with micromolar concentrations of calcium or magnesium and makes single strand nicks in double-stranded DNA as its preferential mechanism of action. A crystal structure of the enzyme as a complex with a DNA substrate is available (Weston et al., 1992). This structure shows both the N- and C-termini of the enzyme to be on the opposite side of the protein from the active site; with this knowledge, we could modify the N-terminus of the enzyme with the NLS, confident that it would not alter DNase I enzymatic activity or specificity. DNase I is a good probe for chromatin structure, cutting within the nucleosome at ~10 nt intervals as well as cutting linker DNA. Additionally, DNase I has been the benchmark enzyme for defining sites of DNA function in chromatin, marking interactions of regulatory proteins with DNA, for over two decades (Elgin, 1988; Simpson, 1998; Weintraub and Groudine, 1976; Wolffe, 1998; Wu and Gilbert, 1981).

The nuclease was outfitted with an NLS and placed in yeast under GAL control. Culture of cells in galactose induced expression of DNase I leading to nicking and degradation of plasmid DNA. Cytotoxic effects of DNase I expression led to cell death, presumably due to damage to genomic DNA in excess of the capacity of repair systems. Nuclease activity footprinted binding of regulatory proteins and in a critical test detected the interactions of a labile repressor with its cognate binding site, interactions which are absent in

isolated nuclei. The nuclease susceptibility of the Mat$\alpha$2p/Mcm1p operator is completely consistent with the structure of the ternary complex of the DNA binding domains of the two proteins with DNA (Tan and Richmond, 1998) and the observation that, *in vitro*, full-length Mat$\alpha$2p is bulky enough to protect the entire operator from a nuclease (Sauer et al., 1988). While methyltransferases also provide the ability to detect binding of regulatory proteins in living cells, the resolution of studies using these enzymes is less than for DNase I, due to the relative infrequency of modification sites for the methyltransferases compared to susceptible sites for the nuclease (Kladde et al., 1996). Another limitation to use of some methyltransferases is the occurrence of endogenous cytosine methylation in many eukaryotic species. Use of DNase I expressed in whole cells as a general probe for chromatin structure thus offers many advantages over currently available technologies for investigation of interaction of regulatory proteins with DNA.

Features of chromatin structure involving histone DNA interactions, higher order structure, and arrangements of linker DNA and perhaps H1 histones are also amenable to study using *in vivo* expressed DNase I. A striking and unexpected set of observations about the organization of chromatin has emerged from this study. For several repressed yeast chromatin domains, differences in DNase I digestion patterns between samples digested with the enzyme *in vivo* and those analyzed with exogenous enzyme in isolated nuclei have been found. Unique features of the DNase I digestion pattern span a spectrum from minor changes in living cells versus isolated nuclei to

wholesale alterations in the relative nuclease susceptibilities of linkers and core particle nucleosome segments in the two situations. Higher order chromatin structure in larger eukaryotes is stabilized by the linker histone, H1 (Vignali and Workman, 1998; Widom, 1998). The yeast ortholog, Hho1p, differs from usual lysine rich histones by having two globular domains and lacking highly basic N- and C-terminal tails (Freidkin and Katcoff, 2001; Landsman, 1996; Patterton et al., 1998; Ushinsky et al., 1997). Given this composition, we anticipate that binding of Hho1p to DNA might be more labile than typical H1 histones. The possible role of Hho1p in the differences between chromatin features detected by DNase I in isolated nuclei and *in vivo* remains to be evaluated. Similarly, a contribution to the observed results of differential repair in particular chromatin regions cannot be dismissed out of hand. Contributions of endogenous nucleases to the results seem less likely and if present must reflect a yeast nuclease with similar properties to bovine pancreatic DNase I.

Each of the three types of repressed domains, RE (Weiss and Simpson, 1997), **a**-cell-specific genes (Simpson et al., 1993) or HM locus (Ravindra et al., 1999), has a distinctive organization of positioned nucleosomes. Chromatin structure of each is dependent on a corepressor, Tup1p or Sir3p, that has been shown to interact *in vitro* with the basic N-terminal regions of histones H3 and H4 (Edmondson et al., 1996; Hecht et al., 1995). For the two domains that depend on Tup1p for repression, RE and **a**-cell-specific genes, there are significant differences in linker susceptibility between chromatin in

cells and chromatin in isolated nuclei (Figure 2.5 and 2.6). Protection from DNase I cutting *in vivo* suggests that some degree of higher order structure and/or protection of linker regions by interaction of histone tails with the corepressor is present *in vivo*. In the case of the **a**-cell-specific genes, this suggestion is strongly supported by electron microscopic images of repressed chromatin domains in isolated minichromosomes. A portion of the repressed *STE6* gene exists as a loop or hairpin of highly condensed chromatin (Ducker, 2001).

Somewhat surprising was the observation that the chromatin structure of the *HMR***a** locus was similar in nuclei and *in vivo*. The simple pattern with linker susceptibility greater than that in the center of the core particle is presumed to be characteristic of extended or zigzag strings of nucleosomes. Since the HM loci are the prime examples of silencing thought to be based in chromatin architecture, one anticipated some indication of higher order structure for these elements. Instead, the sole indication of additional protein interactions is protection of sensitive sites near the nucleosome–E silencer border *in vivo*. Direct investigation of the extent of interaction of the HM domains with corepressors and their morphology when isolated and visualized in the electron microscope is of high interest.

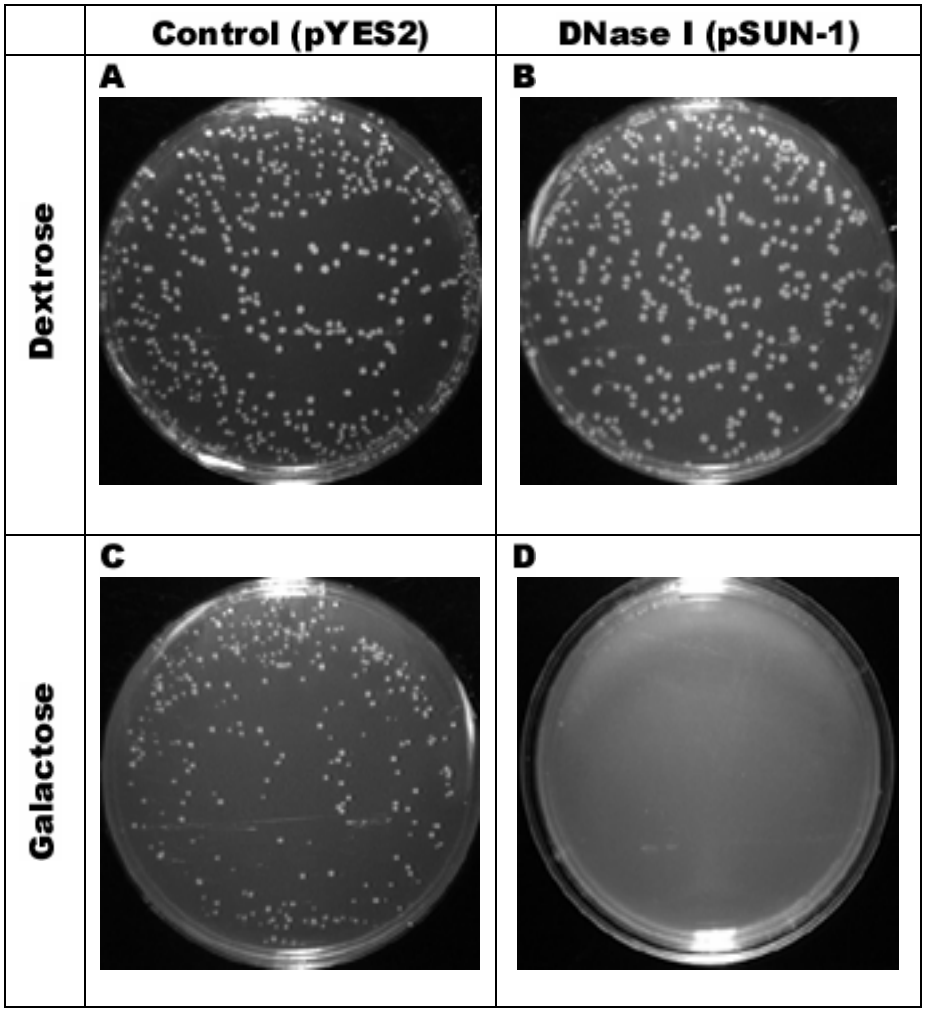



Figure 2.1: Cytotoxicity of DNase I. Equal numbers of control cells containing the vector, pYES2, alone (A,C) or cells containing the DNase I plasmid, pSUN-1, (B,D) were plated on SD-Ura medium containing dextrose (A,B) or galactose (C,D). Expression of DNase I under control of a galactose responsive promoter is toxic to yeast (D).

Figure 2.2: DNase I expressed *in vivo* introduces nicks in and degrades plasmid DNA. Southern blot (A) and PhosphorImager quantification (B) of pSUN-1 plasmid DNA during 6 hours of growth in galactose. Results are shown for duplicate independent transformants in (A) and averaged in (B). Samples from cells expressing DNase I under control of a galactose responsive promoter were removed for analysis after 0, 1 and 6 hours of incubation, as indicated. Migration positions of supercoiled (S) and nicked (N) plasmid DNA are indicated. Standards in (A) are plasmid DNA cut with *PvuII* (P) – no sites; *Eco*RI (E) or *Xba*I (X) – one site. The major species changes from supercoiled to nicked plasmid DNA and the total plasmid DNA present decreases during the incubation.

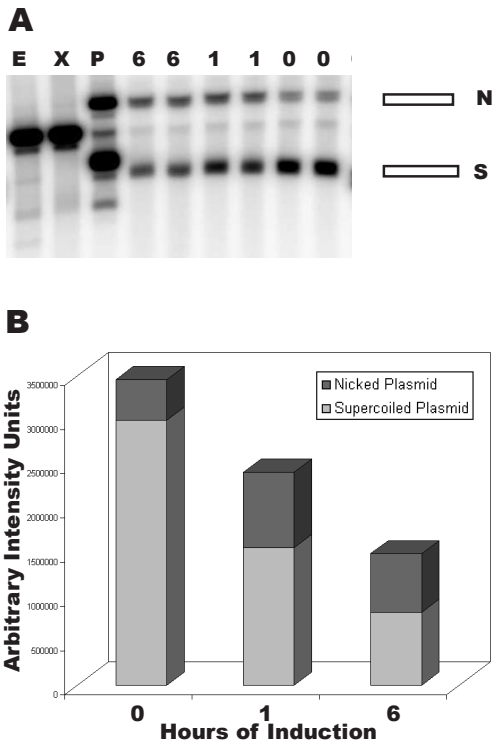

A
E X P 6 6 1 1 0 0
N
S
B
Nicked Plasmid
Supercoiled Plasmid
Arbitrary Intensity Units
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
1
6
Hours of Induction

Figure 2.3: Time course of DNase I degradation of DNA *in vivo*. Primer extension mapping of cutting sites in the pSUN-1 plasmid after induction of expression of DNase I for 0, 3, 6 and 12 h, as indicated. Patterns are compared with those from digestion of protein-free DNA with DNase I *in vitro*, as indicated. The primer was located 222 bp upstream of the *GAL1* promoter TATA box and the region mapped extended to the beginning of the transcriptionally active DNase I gene. Numbers to the left of the gel are coordinates relative to the start of the DNase I coding sequence. The rectangular box denotes the position of the TATA box.

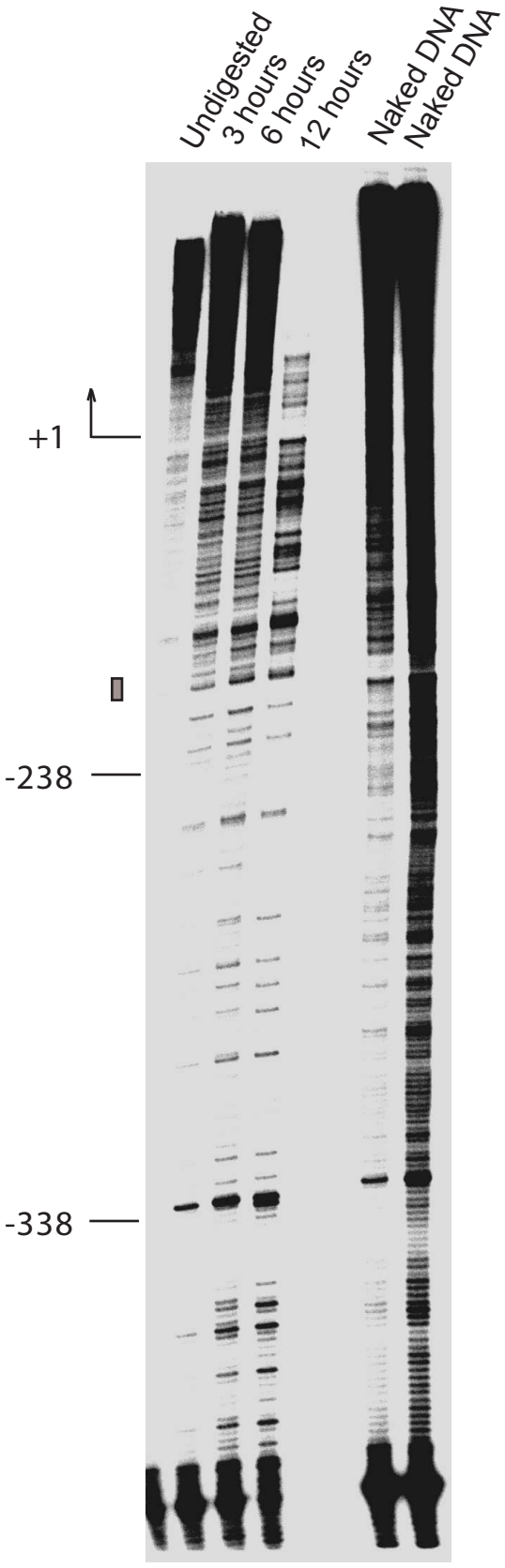

Undigested
3 hours
6 hours
12 hours
Naked DNA
Naked DNA
+1
-238
-338

Figure 2.4: DNase I footprinting of the genomic Mat$\alpha$2p/Mcm1p complex binding site in intact cells and isolated nuclei. (A) Primer extension mapping of DNase I cutting sites around the $\alpha$2#1 operator (chromosome III, 29194 to 29224 mu). Lane 1: protein free DNA digested with DNase I *in vitro*. Lane 2: DNA from $\alpha$-cells containing pSUN-1 grown in dextrose where the DNase I gene is repressed. Lane 3: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in galactose where the DNase I gene is expressed. Lanes 4,5: DNA from $\alpha$-cell nuclei isolated from wild type cells and digested with two concentrations of DNase I *in vitro*. (B) Densitometric scans of data in lanes 1 (DNA), 3 (*In vivo*) and 4 (Nuclei) of (A). The rectangles indicate the location of the $\alpha$2 operator.

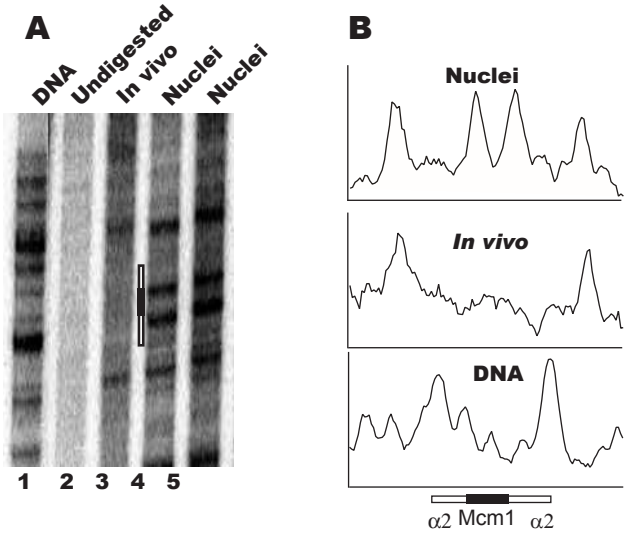

A
DNA
Undigested
In vivo
Nuclei
Nuclei
1 2 3 4 5
B
Nuclei
In vivo
DNA
α2 Mcm1 α2

Figure 2.5: Chromatin structure of the recombination enhancer *in vivo*. (A) Primer extension analysis of DNase I cutting sites in the second nucleosome of the RE domain at 29403 to 29560 mu of chromosome III, near the $\alpha$2#1 operator. Features of chromatin structure previously determined by analysis of MNase cutting sites (16) are shown to the left of the autoradiogram. Ellipses mark inferred positions of nucleosomes. Open rectangles indicate linkers. The filled box marks the location of the $\alpha$2 operator. Lane 1: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in galactose where the DNase I gene is expressed. Lanes 2,3,4: DNA from $\alpha$-cell nuclei isolated from wild type cells and digested with three concentrations of DNase I *in vitro*. Lane 5: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in dextrose where the DNase I gene is repressed. Lane 6: protein free DNA digested with DNase I *in vitro*. (B) Densitometric scans of data in lanes 6 (DNA), 1 (*in vivo*) and 3 (Nuclei) of (A). Features of chromatin structure previously determined by analysis of MNase cutting sites are shown below the scans.

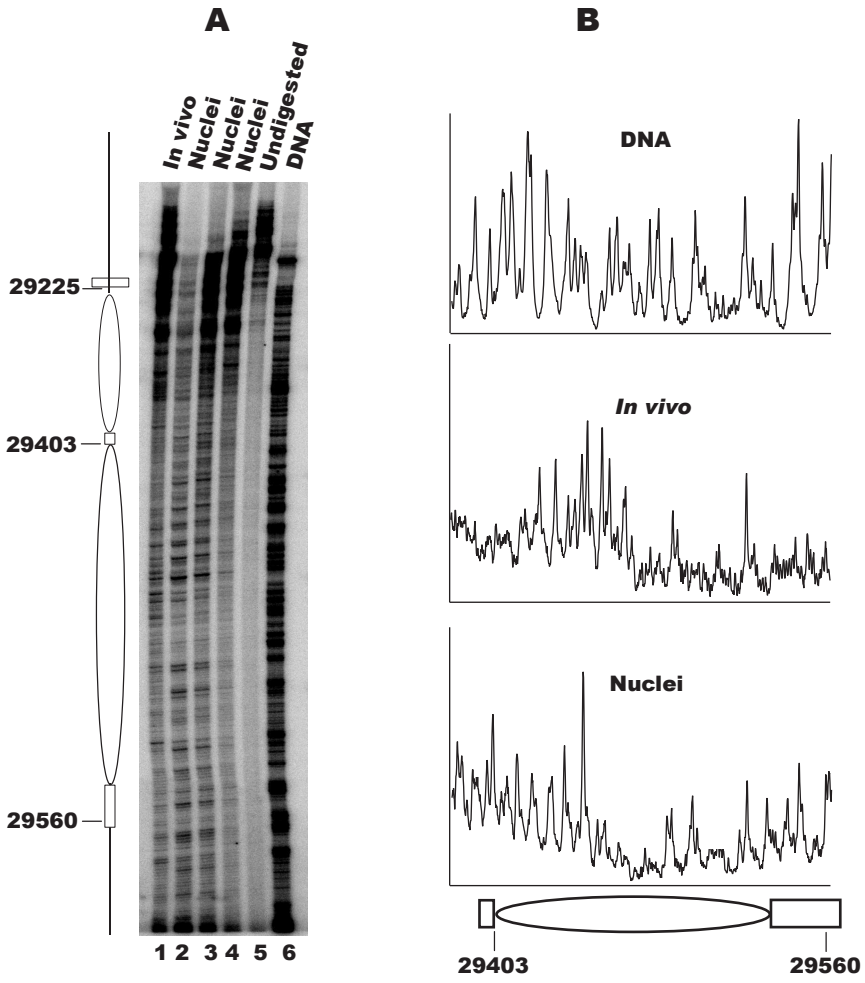

A
B
In vivo
Nuclei
Nuclei
Nuclei
Undigested
DNA
29225
29403
29560
1 2 3 4 5 6
DNA
In vivo
Nuclei
29403
29560

Figure 2.6: Chromatin structure of the *STE6* promoter *in vivo*. (A) Primer extension analysis of DNase I cutting sites in the first two nucleosomes of the *STE6* domain adjacent to the $\alpha$2 operator. Features of chromatin structure previously determined by analysis of MNase cutting sites (18) are shown to the left of the autoradiogram. Ellipses mark inferred positions of nucleosomes. Open rectangles indicate linkers, either short (S) or long (L). The filled box marks the location of the $\alpha$2 operator. Lane 1: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in dextrose where the DNase I gene is repressed. Lane 2: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in galactose where the DNase I gene is expressed. Lanes 3,4,5: DNA from $\alpha$-cell nuclei isolated from wild type cells and digested with three concentrations of DNase I *in vitro*. (B) Densitometric scans of data in lanes 2 (*In vivo*) and 3 (Nuclei) of (A). Features of chromatin structure previously determined by analysis of MNase cutting sites are shown below the scans.

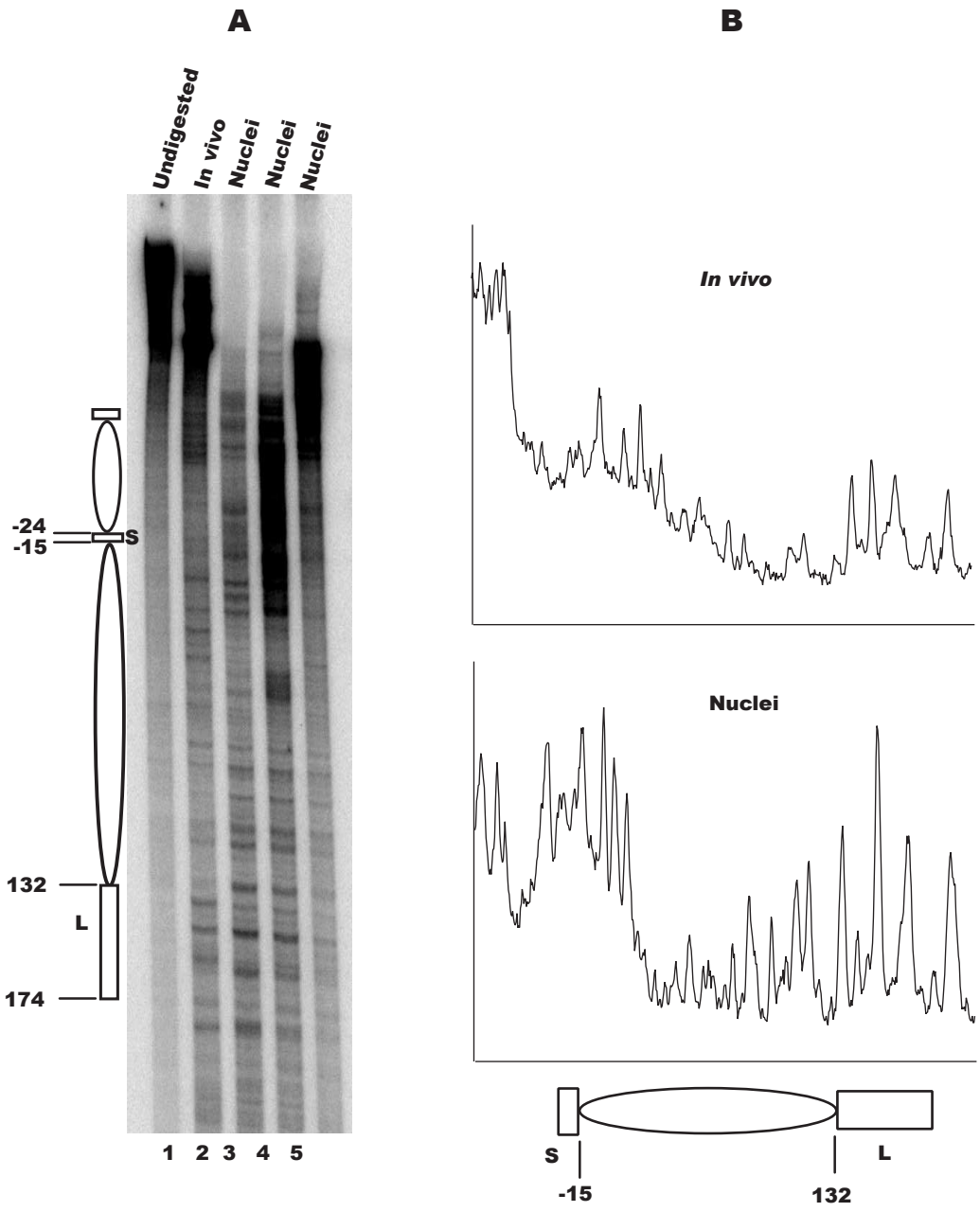
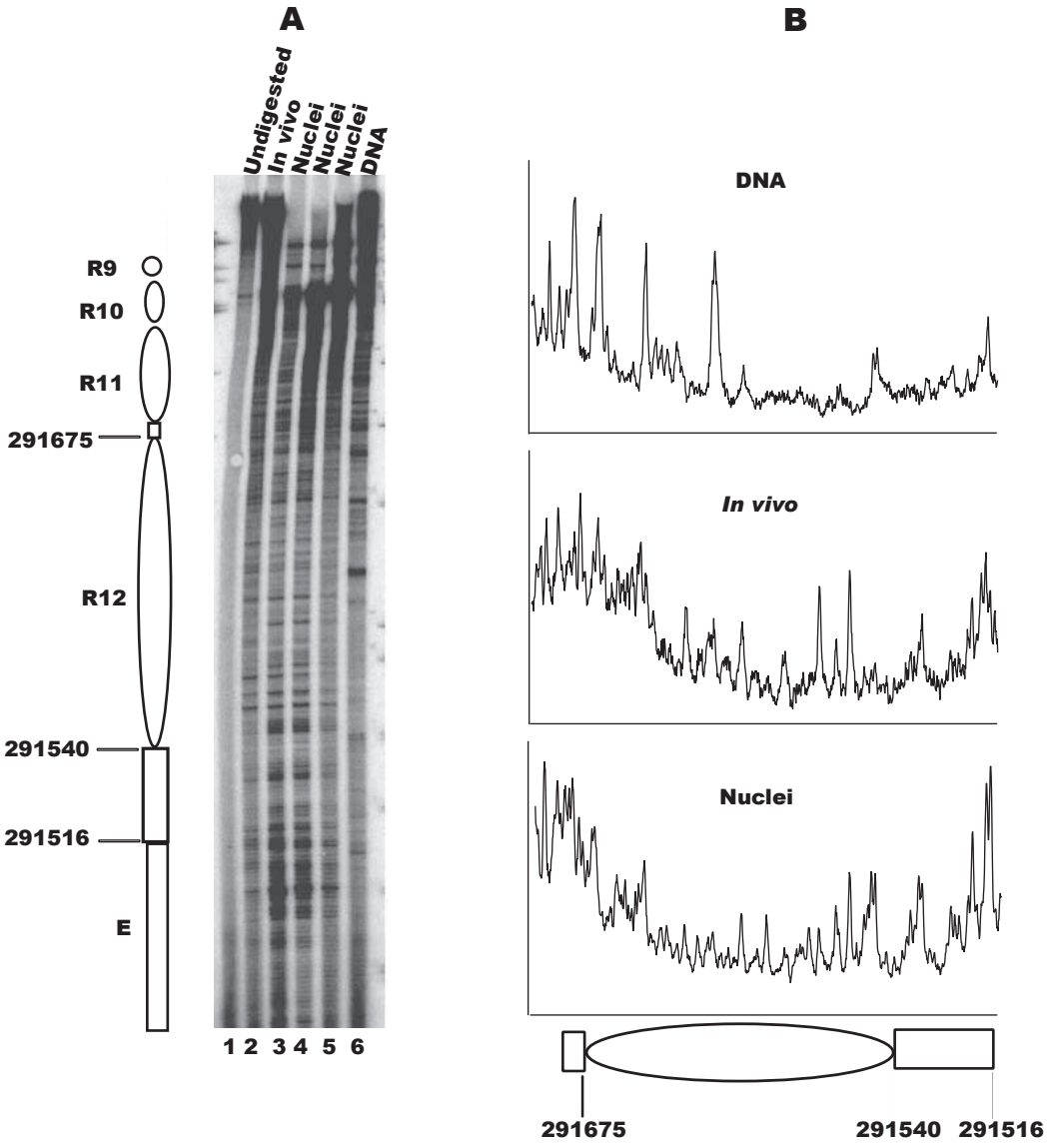

A
B
Undigested
In vivo
Nuclei
Nuclei
Nuclei
-24
-15
S
132
L
174
1 2 3 4 5
In vivo
Nuclei
S
-15
132
L

Figure 2.7: Chromatin structure of a nucleosome adjacent to the E silencer at *HMRa in vivo*. (A) Primer extension analysis of DNase I cutting sites in the first nucleosome of the *HMR* domain adjacent to the E silencer. Features of chromatin structure previously determined by analysis of MNase cutting sites (17) are shown to the left of the autoradiogram. Ellipses mark inferred positions of nucleosomes. Open rectangles indicate linkers. The filled box marks the location of the E silencer. Lane 1: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in dextrose where the DNase I gene is repressed. Lane 2: DNA from $\alpha$-cells containing pSUN-1 grown for 6 hours in galactose where the DNase I gene is expressed. Lanes 3,4,5: DNA from $\alpha$-cell nuclei isolated from wild type cells and digested with three concentrations of DNase I *in vitro*. Lane 6: protein free DNA digested with DNase I *in vitro*. (B) Densitometric scans of data for nucleosome R12 in lanes 6 (DNA), 2 (*in vivo*) and 3 (Nuclei) of (A). Features of chromatin structure previously determined by analysis of MNase cutting sites are shown below the scans. The linker to the right is adjacent to the E silencer.


A
B
Undigested
In vivo
Nuclei
Nuclei
Nuclei
DNA
R9
R10
R11
291675
R12
291540
291516
E
1 2 3 4 5 6
DNA
In vivo
Nuclei
291675
291540
291516

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# Chapter III

## TATA box binding protein persists at active yeast promoters through multiple transcription cycles *in vivo*

## Abstract

TATA box binding protein (TBP) plays a pivotal role in the initiation of RNA polymerase II mediated transcription. However, it is unclear whether TBP remains bound to the TATA box after the initiation, during subsequent transcription cycles. We have used a controlled, DNase I gene to investigate chromatin and promoter structure in living yeast cells. We find that a region around the TATA box in the promoter of both high and low rate genes is protected against DNase I. Moreover, chromatin immunoprecipitation assays reveal that two sets of coordinately regulated genes have approximately equal amounts of TBP associated with their promoters, irrespective of their transcription level. In contrast, occupancy by RNA polymerase II correlates with transcription frequency. Our results, in addition to the observations that the promoters of active genes are nucleosome free, suggest that TBP may occupy the promoter region of active genes through multiple rounds of transcription, and that binding of TBP to DNA is not a rate limiting step in the activation of transcription reinitiation *in vivo*.

## 3.1 Introduction

In eukaryotic cells, most of the protein-encoding genes must be repetitively transcribed by RNA polymerase II to perform housekeeping functions and to respond to sustained inductive signals. The transcription level of those genes is affected by the rate of two critical steps: initiation during induction, and reinitiation in subsequent transcription cycles (Hahn, 1998; Szentirmay et al., 1998). Initiation and reinitiation both require various transcription factors plus RNA polymerase II itself to form a complex called, in jargon, "polymerase machinery" on the promoter (Hahn, 1998). However, a number of observations suggest that initiation and reinitiation might occur through different pathways (Hahn, 1998; Hawley and Roeder, 1987). First, initiation and reinitiation are regulated by different activators (Hahn, 1998; Szentirmay et al., 1998). For example, GAL4-AH mainly speeds up initiation, HSF only affects the reinitiation step, and Gal4-VP16 can work on both steps (Sandaltzopoulos and Becker, 1998; Yudkovsky et al., 2000). Second, sarkosyl at certain concentrations can block reinitiation but not initiation (Hawley and Roeder, 1987). Finally, several *in vitro* studies have shown that rates of reinitiation at some promoters are several fold higher than those of initiation (Jiang and Gralla, 1993; Yean and Gralla, 1997). Thus, controls of initiation and reinitiation must be considered separately in assessing regulation of promoter activity.

TBP, one of the essential general transcription factors, plays a pivotal role in the initiation step both *in vitro* and *in vivo* (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Lee and Young, 2000; Pugh, 2000; Xiao et al., 1995; Yean and Gralla, 1997). Either associated with TAFs as TFIID or in isolation (Kuras et al., 2000; Li et al., 2000), TBP is one of the first components of the polymerase machinery to be recruited to the promoter (Lee and Young, 1998; Pugh, 2000; Zawel et al., 1995). Once TBP has become bound to a promoter, the rest of the polymerase machinery assembles quite rapidly (Lee and Young, 2000; Pugh, 2000; Stargell and Struhl, 1996; Zawel et al., 1995). *In vivo*, recruitment has also been inferred from the results of "activator bypass" experiments in which fusion of TBP to a sequence-specific DNA binding domain generates high levels of activator-independent gene expression (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao et al., 1995). In living yeast cells, moreover, chromatin immunoprecipitation (ChIP) experiments revealed that TBP is physically associated with promoters of several genes under activated, but not repressed, conditions (Kuras and Struhl, 1999; Li et al., 1999). Thus, *in vitro* and *in vivo* evidence imply that recruitment of TBP to the TATA element is an important step in initiation.

Although *in vitro* studies firmly establish the importance of TBP in reinitiation (Hahn, 1998; Yudkovsky et al., 2000), many issues regarding the precise mechanism by which TBP functions in this step remain unexplored. One key question is whether TBP remains bound to the promoter through

multiple rounds of transcription or whether it needs to be recruited anew for every cycle of transcription. Several *in vitro* studies have been performed to address this issue, but the conclusions of these studies are inconsistent. Employing *Drosophila* extracts, Kadonaga suggested that TBP was dissociated completely from the promoter following each round of RNA polymerase II transcription (Kadonaga, 1990). However, several other studies favored an alternative conclusion, that TBP remained associated with the promoter through multiple rounds of transcription. For example, Zawel et al. (1995) showed that once bound, TBP can remain promoter bound through multiple transcription cycles. The multiple techniques they used include a defined reconstituted transcription system, transcription of templates attached to solid supports coupled to western blotting, and template competition assays. These results are consistent with several previous studies that utilized fractionated HeLa extracts (Hawley and Roeder, 1987; Van Dyke et al., 1988; Van Dyke et al., 1989; Van Dyke and Sawadogo, 1990). More recently, a report using an immobilized-template assay and yeast extracts suggested that, following each transcription cycle, TBP and some other transcription factors were left at the promoter in a complex so-called “scaffold” complex (Yudkovsky et al., 2000). The scaffold is competent to support reinitiation of the next transcription cycles. In addition, Hoopes et al. (1998) observed that equivalent occupancy of two TATA boxes by TBP resulted in different levels of transcription. This lends additional support to the hypothesis

that TBP persists at the promoter through multiple cycles of transcription. However, this *in vitro* based hypothesis has not been tested in living cells.

While it is difficult to provide *in vivo* evidence for the proposal that TBP persists at the promoter during reinitiation transcription cycles, if this hypothesis is believable, one expectation would be that equal amounts of TBP would be present on the promoters of genes with different transcription rates. In this study, for the sake of brevity only, we arbitrarily defined the genes with a transcription rate higher than 80 mRNA per hour (http://web.wi.mit.edu/young/expression/) as “high rate genes”, and the genes with a transcription rate lower than 15 mRNA per hour (http://web.wi.mit.edu/young/expression/) as “low rate genes”, respectively (Holstege et al., 1998). We have investigated this expectation, employing several techniques. We examined chromatin structure upstream of a number of different genes using DNase I expressed in living *Saccharomyces cerevisiae,* and found that for all the genes we tested, there was a protected region around the TATA box. Roughly similar levels and extents of protection were present for genes that were transcribed at widely different rates. We also analyzed TBP binding to the promoters of two sets of genes by a modified ChIP assay. These sets of genes were coordinately regulated but had different expression levels. We found that the promoter regions around the TATA box of these genes in each group are associated with TBP in approximately equal amounts, irrespective of the transcriptional level. In contrast, the amount of RNA polymerase II associated with the promoter was

roughly proportional to the transcriptional level. Taken together, we conclude that many, if not all, transcriptionally competent genes have a complex of transcription factors bound at their promoter, unrelated to their actual level of transcription. In contrast, polymerase binding at the promoter correlates with the frequency of transcription. Therefore, a rate-limiting step in transcriptional control must lie between these two facets of the process.

## 3.2 Materials and methods

### 3.2.1 Yeast Strains and medium

The wild type yeast strains used in all experiments are YPH499 (MAT**a** *ade2-101 ura3-52 his3-200 leu2-1 trp1-63 lys2-1*) and YPH500 (MAT$\alpha$ *ade2-101 ura3-52 his3-200 leu2-1 trp1-63 lys2-1*), which are isogenic except at the MAT locus. Standard yeast media, both rich (YPD) and synthetic medium lacking uracil [CSM-Ura (Bio 101), 0.67% yeast nitrogen base without amino acids (Difco), and an appropriate carbon source (2% dextrose, 4% lactic acid or 2% galactose)] were used.

### 3.2.2 Nuclei and DNA preparation and analysis

Nuclei preparation was carried out essentially as described (Weiss and Simpson, 1997). Briefly, yeast from a 1-liter culture grown to an optical density of about 1.0 at 600nm was harvested and digested with Zymolyase 100T (Seikagaku). Nuclei were purified by differential centrifugation and resuspended in digestion buffer (10mM HEPES, pH 7.5, 0.5mM $MgCl_2$, 0.05mM $CaCl_2$) and incubated with 0, 2, and 4 units/ml MNase (Worthington) or 0, 0.05, and 0.1 units/ml DNase I (Worthington) for 10 minutes at 37 °C. The digestions were terminated by the addition of EDTA, and the DNA was purified by RNase A and proteinase K digestion and phenol/chloroform extraction. The DNA pellet was resuspended in 0.1×TE buffer.

*In vivo* DNase I digestion was performed as described previously (Wang and Simpson, 2001) with a minor modification. Briefly, the expression of DNase I was induced by switching the carbon source to galactose for 6 hours. The cells were harvested by centrifugation, broken by homogenization with glass beads in 100 mM Tris–HCl pH 8.0, 50 mM EDTA, 2% sodium dodecyl sulfate, and the DNA was extracted. $NH_4OAc$ was added and precipitation was allowed to proceed on ice for 2 hours. Purification of DNA involved treatment with 100 ng/ml RNase A at 37°C for 1 hour and then 100 ng/ml proteinase K in 2% Sarkosyl, and 200 mM $NaClO_4$ at 50°C for 2 hours. DNA was further purified by extractions with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation. The DNA was dissolved in 0.1x TE (pH 8.0) prior to analysis.

For low-resolution mapping of nucleosomes by indirect end labeling, the purified DNA was subjected to a secondary digestion by *EcoR I*, then electrophoresed in 1.4% agarose gels in 1×TAE buffer, and transferred to Hybond-NX membrane (Amersham) and crosslinked with UV light. The specific DNA sequences were detected by hybridization with a random-primer labeled probe directed toward the end of the *EcoR I* site. For high-resolution mapping, multiple rounds of *Taq* DNA polymerase-based primer extension was carried out from a $^{32}$P-end-labeled primer, and the products were then resolved on a 6% polyacrylamide (19:1), 50% urea gel. Images were captured

on a PhosphorImager screen. The image was then scanned and analyzed with Image Quant v.5.0 software (Molecular Dynamics).

### 3.2.3 Chromatin immunoprecipitation (ChIP)

Chromatin-containing extracts were prepared as previously described (Hecht and Grunstein, 1999) with minor modifications. Extracts were prepared from 200-ml cultures at a density of about 1.0 at 600nm. Cells were fixed in 3% formaldehyde and were disrupted with glass beads and transferred to a 15 ml centrifuge tube (the final volume was adjusted to 2 ml). The chromatin-containing extract was sonicated to yield an average DNA size of 300 bp (the majority of the fragments were approximately 300 bp long, but a very small fraction of the fragments were as small as 50 bp or as large as 500 bp). Sonication conditions were 40% output, 90% duty cycle, fifteen 12-second cycles with a Branson Sonifier 450. The chromatin size was confirmed for each input sample by running 10% of the DNA on a 2% agarose gel. The sonicated extract was subsequently clarified by centrifugation.

The antibodies used in the immunoprecipitation step are: polyclonal antibody against TBP (provided by J. Reese), polyclonal antibody against Kin28 subunit of TFIIH (Covance), monoclonal antibody (8WG16) against Rpb1 subunit of RNA polymerase II (Covance), polyclonal antibody against TFIIB (provided by S. Hahn), and polyclonal antibody against TFG2 subunit of TFIIF (provided by S. Hahn).

### 3.2.4 Quantitative PCR

All primers were designed to be 19- to 25-mers, with a $T_m$ of approximately 60°C. Primer sequences are shown in Appendix. The PCR conditions were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 28 cycles. A 5-minute 94°C step prior to the cycles and a 5-minute 72°C extension following completion of the cycles were added. Several dilutions of each sample were used for PCR. For the input DNA, the initial dilution series was from 1/4,000 to 1/100; for the immunoprecipitated DNA, the initial dilution series was from 1/20 to 1/5. Only one titration of input and immunoprecipitated DNA was shown in the figures, except Figure 3.6B, to conserve space. The PCR products were detected by UV illumination of an ethidium bromide stained 2% agarose gel and analyzed with Image Quant v.5.0 software (Molecular Dynamics).

### 3.2.5 Nuclei ChIP

Nuclei were isolated as above, and the quality of the chromatin structure was checked by digestion of a portion of the isolated nuclei with micrococcal nuclease and gel electrophoresis of the resulting DNA fragments (data not shown). The remaining nuclei were subjected to ChIP analysis. The isolated nuclei were crosslinked with varying concentrations of formaldehyde. Sonicated, soluble chromatin of an average size of 0.3 kb was immunoprecipitated with the TBP antibodies or TFIIH (kin28) antibodies as

above. The amount of *STE6* promoter and ORF DNA present in the pellet was determined by quantitative PCR as described above.

## 3.3 Results

### 3.3.1 Promoters of active genes are accessible and "nucleosome-free"

Transcription of genes occurs in the context of chromatin, thought to pose an obstacle to recruitment of TBP and other trans-acting factors to DNA (Workman and Kingston, 1998a). Many *in vitro* studies have shown that a "kinetic competition" takes place between TBP and histones: packaging promoter DNA in nucleosomes impedes accessibility of TBP to template DNA, whereas prior binding of TBP or other factors to the promoter blocks nucleosome assembly on important cis-acting DNA elements (Abmayr et al., 1988; Adams and Workman, 1993; Almouzni et al., 1990; Imbalzano et al., 1994; Svaren and Horz, 1997; Workman and Kingston, 1992a; Workman and Roeder, 1987a; Workman et al., 1991). Therefore, the promoter regions of most, if not all, active genes are nucleosome-free. We expect that if TBP persists at the promoter through multiple transcription cycles, such an "active" chromatin structure will be observed on promoters of not only high rate genes but also low rate genes.

One of the most important experimental features of active promoter chromatin structure is that these nucleosome-free regions, in isolated chromatin or nuclei, were more sensitive to nucleases (such as MNase and DNase I) than bulk chromatin (Elgin, 1988; Gross and Garrard, 1988). Previous studies demonstrated that gene-specific increases in nuclease susceptibility for those sensitive regions were associated with activated

transcription (Gavin and Simpson, 1997; Simpson et al., 1993; Svaren and Horz, 1997). However, these studies focused on a small number of inducible genes controlled by specific transcriptional regulators. As a more general evaluation of the relationship between hypersensitivity to nucleases and transcription, we have now analyzed sensitivity to nucleases at several constitutively active promoters.

For all the promoters tested, we found a strong correlation between sensitivity to nucleases and transcription, regardless of whether the transcription frequency was high or low. In contrast, repressed promoters are blocked from nuclease digestion. As shown in Figure 3.1, for example, the promoter of *MFA1* is occluded by a positioned nucleosome and therefore is resistant to micrococcal nuclease (MNase) in $\alpha$ cells where the gene is repressed, whereas the promoter is sensitive to MNase in **a** cells where the gene is active. The same change has been previously observed for other **a** cell-specific genes (Simpson et al., 1993; Teng et al., 2001). Additionally, promoter regions of other inducible genes have been reported to become sensitive to MNase upon activation (Almer et al., 1986; Fedor and Kornberg, 1989).

Next, we checked the sensitivity to nucleases of more unrelated promoters. Sensitivity was observed not only for the promoters of high transcriptional rate genes such as *MFA1* and *PGK1*, but also for low rate genes such as *STP1*, *MRPL28* (Figure 3.1 and data not shown). Notably, a separate study (Ercan and Simpson, in revision) revealed a DNase I

hypersensitive site upstream of most of the 28 ORFs in a ~50 kb region of yeast chromosome III in isolated nuclei, further supporting the correlation between transcription competence and the lack of a nucleosome over the promoter region.

Although we favor the idea that the nucleosome-free characteristic of promoters in isolated chromatin derives from TBP binding during initiation and is maintained by continuous presence of TBP and possibly other transcription factors (see Discussion), we did not see the footprint of TBP or other transcription factors on promoters when we did high resolution (single base pair level) mapping of isolated swollen nuclei (Figure 3.2, 3.3 and data not shown). Actually, in isolated nuclei, such a footprint has been observed in only a very few cases (Chen et al., 1994). We suggest that this may be due to possible dissociation of TBP and other transcription factors during isolation of nuclei (see Discussion). Therefore, we used methodology previously developed by us to analyze chromatin structure of multiple genomic loci by inducing expression of DNase I in living yeast cells (Wang and Simpson, 2001).

### 3.3.2 TBP binds to promoters of different genes with the same occupancy level

To better understand the interaction between trans-acting factors and DNA in the native chromatin environment inside living cells, we have expressed the gene for bovine DNase I under GAL control in *Saccharomyces*

*cerevisiae* (Wang and Simpson, 2001). While yeast cells eventually die when the nuclease is active, they survive for hours, long enough to let us get snapshots of the chromatin structure of particular loci.

DNase I has been the gold standard for chromatin structure studies since the seminal studies of Carl Wu (Wu, 1980). We have mapped the DNase I cutting pattern of the 31 bp $\alpha$2 operator in different situations. The central 11 bp is bound by a homo-dimer of Mcm1 protein in **a** and $\alpha$ cells while the flanking 10 bp on each side is bound by Mat$\alpha$2 protein in $\alpha$ cells (Keleher et al., 1989). Previously we have shown that the 31 bp region is protected in $\alpha$ cells, whereas in nuclei isolated from $\alpha$ cells, only the central 11 bp is protected from DNase I digestion (Wang and Simpson, 2001). The cutting pattern for this region is identical in wild type **a** cells and **a** cell nuclei (Wang and Simpson, 2001). These observations agree well with earlier studies suggesting that only Mcm1p remains bound at the operator in isolated nuclei (Murphy et al., 1993).

These data demonstrated the efficacy of the *in vivo* chromatin mapping strategy and prompted us to study the binding of TBP and other transcription factors in living cells. Using primer extension, we mapped DNase I cutting sites around the promoter region and the beginning of the coding region of several genes that are transcribed by RNA polymerase II. These genes represent mechanistically distinct classes of genes and the transcription rate (mRNA per hour) of these genes spans a more than a 1300 fold range (http://web.wi.mit.edu/young/expression/). Surprisingly, for every gene

analyzed, there is a protected region surrounding the TATA box in living cells but not in isolated nuclei or protein free DNA samples (Figure 3.2, 3.3, and data not shown). Two examples are shown in Figures 3.2 and 3.3.

Figure 3.2 shows the DNase I cutting patterns at the promoter and coding regions of *PGK1*, a highly active gene with a transcription rate of 110 transcripts per hour. Naked DNA from this region was susceptible to DNase I at a number of sites throughout the promoter and coding region. Cutting in isolated nuclei (lanes 4, 5, and 6) has a similar, but not identical, digestion pattern to that of naked DNA (lanes 7 and 8). This is in agreement with other studies showing that the promoter and coding regions of active genes are hypersensitive to nuclease in isolated nuclei (Elgin, 1988; Gross and Garrard, 1988). In contrast, in a region surrounding the TATA box (from ~10 bp upstream to ~60 bp downstream), DNase I cutting was severely restricted in the *in vivo* digested chromatin samples (lanes 2 and 3), relative to the digestion pattern in both naked DNA and isolated nuclei. Beyond this region, including the coding region, a similar cutting pattern was observed for all three samples. Notably, a site ~ 10 bp upstream of the TATA box is hypersensitive to DNase I *in vivo*; this site may mark the edge of the binding site of the transcription complex. The protection at the promoter seems to arise from binding of TBP and other transcription factors.

Figure 3.3 shows the DNase I cutting patterns at the promoter and coding regions of the *YCL056C* gene, with a transcription rate of only 3.5 transcripts per hour. Again, cleavage of isolated nuclei (lanes 3 and 4) and naked DNA

(lanes 5 and 6) yielded complex patterns of cutting through the region. Interestingly, sequences in the promoter region and the beginning of the coding region of this gene were also protected from DNase I in living cells (lane 2). Again, a hypersensitive site which is ~ 10 bp upstream of the TATA box was observed in the *in vivo* digestion sample. These results are in agreement with the *in vitro* result (Yudkovsky et al., 2000) suggesting that TBP and other factors form a scaffold complex and remain bound to promoters through multiple cycles of transcription. These experiments also imply that elements or structures present on promoters in living cells may be lost during nuclear isolation or chromatin preparation, as suggested by previous investigations (Pfeifer and Riggs, 1991; Zaret, 1999).

To investigate what proteins might be associated with promoters, we used chromatin immunoprecipitation assays. Formaldehyde fixed chromatin was sonicated to reduce DNA length to <500 bp (Figure 3.6A) and immunoprecipitated with specific antisera. After reversing the crosslinking, DNA was purified, and the presence of a particular DNA sequence was detected by polymerase chain reaction amplification. TBP, TFIIB, TFIIF, TFIIH, and RNA polymerase II were found to be associated with the promoter of both of the genes mapped above (Figure 3.4). As a negative control, none of the above factors was found to bind to the upstream activation site (UAS) of the *STE3* gene, which lacks a TATA element. These results suggest that the protection seen *in vivo* reflects binding of these factors.

### 3.3.3 Differential TBP binding patterns between living cells and isolated nuclei

Our data showed that in isolated nuclei the TATA box is hypersensitive to DNase I, and, in contrast, this region is protected in living cells. An appealing notion, therefore, is that some structures are disrupted during nuclei purification. In support of this idea is evidence that *in vitro*, TBP can protect its cognate sites from nucleases, either by itself or in association with TFIIA or TFIIB. However, these results are not sufficient to prove directly that these DNA footprinting differences are due to the differential binding conditions of TBP in living intact cells versus isolated nuclei.

To further determine whether or not TBP binds to DNA in isolated nuclei in the same way as in living cells, ChIP was carried out to monitor binding of TBP and RNA polymerase II to the *STE6* promoter and to a region in the *STE6* ORF. The region of the *STE6* ORF is located more than 1.5 kilobases (kb) from the promoter (Figure 3.5A).

First, we examined the *STE6* promoter and ORF occupancy by TBP and RNA polymerase II in whole cells. Figure 3.5B shows that nearly ten times more of the *STE6* promoter was present relative to the *STE6* ORF DNA in TBP immunoprecipitates. In contrast, RNA polymerase II was found associated with both promoter and ORF DNA at similar levels. This observation is consistent with previous studies (Kuras and Struhl, 1999; Li et al., 1999; Pokholok et al., 2002).

We next investigated the association of TBP in isolated nuclei (see Materials and Methods). It was striking that in isolated nuclei, we could not detect enrichment of TBP at the *STE6* promoter DNA relative to the ORF DNA. Instead, TBP occupies the *STE6* promoter and ORF regions at roughly similar occupancy level (Figure 3.5C). Several lines of evidence make the ChIP results convincing. First, we explored the possibility that the crosslinking efficiency of TBP to DNA in isolated nuclei could be further optimized by exposing nuclei to formaldehyde at varying concentrations and by performing PCR reactions under different conditions – neither of which produced changes in ChIP results (data not shown). Furthermore, by comparing with the input DNA controls, we concluded that the IP DNA for both promoter and ORF regions was still in the linear range in which PCR signals are proportional to the amounts of the template DNA added to the reactions (Figure 3.5C). Finally, the results obtained with another transcriptional factor, TFIIH, were similar to those obtained with TBP. In whole cells, TFIIH was found bound to the *STE6* promoter at much higher occupancy levels relative to the ORF region; whereas in isolated nuclei, TFIIH was found associated with the *STE6* promoter and ORF regions at similar occupancy levels (data not shown). These observations strongly suggest that we have quantitatively measured TBP occupancy, and that during isolation of nuclei, the properties of TBP binding to DNA changed drastically.

### 3.3.4 TBP binds to a group of promoters with same occupancy level

In different regulatory situations, TBP can adopt different conformations and exist in complexes with different proteins such as Mot1p, NC2, or various TAFs (Geisberg et al., 2001; Geisberg et al., 2002; Lee et al., 1998; Lee and Young, 1998; Pugh, 2000). To ensure a constant context, we set out to analyze TBP occupancy level at a group of promoters that met three criteria: (1), the genes should be coordinately activated by well defined regulators; (2), the promoters of these genes should contain the same TATA sequence (Butler and Kadonaga, 2002; Smale, 2001); (3), these genes should be transcribed at different rates. The **a** cell-type specific genes are good candidates. They are all repressed by Mat$\alpha$2 protein, Mcm1p and other proteins such as Tup1p and Ssn6p in $\alpha$ cells (Elble and Tye, 1991; Herskowitz, 1989). In **a** cells, they are constitutively activated by *MCM1* and *STE12* gene products (Herskowitz, 1989). The promoters of *MFA2*, *MFA1*, *STE6*, and *BAR1* all contain the TATAAA sequence. The transcription rate of these genes spans a more than 25 fold range (Holstege et al., 1998). *MFA2*, for example, can produce 282 transcripts per hour, whereas *STE6* and *BAR1* only make ~ 10 mRNA molecules per hour. Taken together, the fact that the **a** cell-specific genes have a broad transcription rate range and the same regulatory pathway makes them good subjects for evaluating whether the same amount of TBP binds to genes with different transcriptional rates.

In order to evaluate quantitatively the relationship between the TBP occupancy level and transcription rate, we again adopted the ChIP assay and quantitative PCR. To make sure that the ChIP data produced quantitive

results, we confirmed that the amount of PCR product was proportional to the amount of template DNA added to the reaction for every pair of primers. Figure 3.6B shows the experimental data using primers spanning the promoter region of the *MFA1* gene. Over a ten-fold range of input DNA, the amount of the PCR product was proportional to the amount of template DNA. DNA added to the reactions was adjusted so that the PCR product was in the linear range.

We first analyzed TBP occupancy at different promoters in cells with different mating types: **a** or $\alpha$. We analyzed the *PGK1* gene, which is constitutively active both in **a** and $\alpha$ cells, four **a** cell-specific genes, and, as a control, three regions which lack a promoter (Figure 3.6). One of these three controls is a region within the *STE6* open reading frame, and the other two are the UAS regions of the *STE3* gene and the *SUC2* gene. The results indicate that roughly comparable levels of TBP were bound to the *PGK1* promoter in both **a** cells and $\alpha$ cells, whereas for the **a** cell-specific gene promoters, TBP binding was undetectable (as low as the background level) in $\alpha$ cells (Figure 3.6D) but was high in **a** cells (Figure 3.6C). Only a weak signal was detected for the *STE6* open reading frame (ORF) fragment and the UAS of *STE3* and *SUC2*, all of which lack a promoter (Figure 3.6C).

Since the few **a** cell-specific genes are expressed at different rates, we assessed whether the occupancy level of TBP of these promoters correlated with their transcriptional rate. By measuring the IP efficiency with quantitative PCR, we compared the occupancy level of TBP at these promoters in **a** cells

where these genes are active. The level was approximately the same for all the genes tested, although their transcription rates span a more than 25 fold range (Figure 3.8). These results suggest that TBP persists at promoters through multiple transcription cycles.

To determine if other transcription factors and RNA polymerase II itself also remain at the promoters through multiple rounds of transcription, we used the crosslinking assay to measure the association of TFIIH and RNA polymerase II with these promoters. As shown in Figure 3.7B and Figure 3.8, for all the **a** cell-specific gene promoters tested, TFIIH had a similar binding level, like TBP. However, there was a correlation between transcriptional rate and promoter occupancy for RNA polymerase II (Figure 3.7A and 3.8). These results are consistent with a previous *in vitro* study (Yudkovsky et al., 2000), and imply that the rate-limiting step of reinitiation may be between TBP recruitment and the binding of RNA polymerase II.

To test this idea further, we turned to four arginine-rich histone genes, *HHT1, HHT2, HHF1* and *HHF2*. In the yeast genome, *HHT1* is paired with *HHF1* and *HHT2* is paired with *HHF2*. Each pair of genes is divergently transcribed from a shared activation region. As shown in Figure 3.9, TBP occupancy level is approximately the same at the promoters of these genes. In contrast, RNA polymerase II occupancy level is roughly proportional to the transcription rates of these genes. This observation reinforces the conclusion that TBP stays at the promoter region of active genes through multiple cycles of transcription.

## 3.4 Discussion

We have analyzed the *in vivo* TBP occupancy at promoters of active genes with different transcription frequency. The hypothesis is that persistence of TBP at competent promoters should make protection against DNase I digestion and occupancy level the same for high and low transcriptional rate genes. We find that the promoter regions of both groups of genes are nucleosome free and a region around the TATA box in the promoter of both high and low rate genes is protected against DNase I *in vivo*. The occupancy level of TBP was same for the promoters of two groups of similarly regulated genes, despite the fact that the transcription frequency of the genes in either group is different from each other. In contrast, the occupancy level of RNA polymerase II correlated well with the transcription frequency. These findings support a model that after initiation of the first cycle of transcription, TBP persists at the promoters of active genes through multiple cycles of transcription.

### 3.4.1 TBP plays a different role in initiation and reinitiation

Initiation and reinitiation are two important steps in the process of transcription. Initiation controls the on or off status of a gene. Once a gene is activated, most of the subsequent RNA synthesis probably results from reinitiation rather than de novo initiation events because the rate of reinitiation is much faster than the rate of initiation (Yean and Gralla, 1997; Yudkovsky et

al., 2000). In living eukaryotic cells, genes are transcribed at tremendously different levels. However, little is known about the mechanisms that control the frequency of reinitiation at different promoters (Hahn, 1998). In fact, in contrast to the numerous investigations regarding initiation (Kuras and Struhl, 1999; Li et al., 1999; Pugh, 2000), only few *in vitro* investigations (Kadonaga, 1990; Van Dyke et al., 1988; Van Dyke et al., 1989; Yudkovsky et al., 2000; Zawel et al., 1995), and no *in vivo* studies, have been performed to date to test whether the same mechanism is applied to initiation and reinitiation. Here, we provide *in vivo* evidence that TBP plays different roles in initiation and reinitiation.

Previous studies have established that activation of genes is accompanied by the recruitment of TBP to the promoters (Kuras and Struhl, 1999; Li et al., 1999). Our data support this idea by showing that the promoter regions of **a** cell-specific genes are occluded by positioned nucleosomes in $\alpha$ cells. In **a** cells, where these genes are transcribed, these nucleosomes are disrupted and TBP occupancy can be easily detected (Figure 3.1 and 3.6).

We investigated the role TBP plays in regulating reinitiation *in vivo*. *In vitro* studies have demonstrated that TBP can bind stably to its DNA target and remain bound at the promoter through multiple transcription cycles (Van Dyke et al., 1988; Van Dyke et al., 1989; Yudkovsky et al., 2000; Zawel et al., 1995). All of these suggest the model that TBP persists at the promoter of active genes, and that the binding of TBP is not the rate limiting step of reinitiation. Therefore, we might expect to find similar levels of TBP

occupancy at the promoters of high rate genes and low rate genes. Indeed, our results are consistent with this hypothesis.

Our results (Figure 3.1, 3.2, 3.3; and Ercan and Simpson, in revision), together with a large body of earlier work (Adams and Workman, 1993; Workman and Kingston, 1998a; Workman and Roeder, 1987a), indicated that the promoter regions of active genes, both high and low rate genes, are nucleosome-free. It is well accepted that transcription occurs in the context of chromatin and in most, if not all, cases, active transcription requires that the promoter region is nucleosome-free (Adams and Workman, 1993; Simpson et al., 1993; Svaren and Horz, 1997; Workman and Kingston, 1998a; Workman and Roeder, 1987a). If TBP binds to the promoters of active genes through multiple cycles of transcription, then the promoters of these genes may be free of nucleosomes. Indeed, a number of studies have suggested that the normal nucleosomal array is disrupted in the promoter regions of active genes (Adams and Workman, 1993; Workman and Kingston, 1998a; Workman and Roeder, 1987a). First, as mentioned above, active promoters are hypersensitive to nucleases in isolated nuclei (Elgin, 1988; Gross and Garrard, 1988). Second, neither the tails nor the histone fold domains of the core histones can be cross-linked to the heat shock gene promoters, which are bound by TBP (Nacheva et al., 1989; Tsukiyama et al., 1994). Finally, while inducible genes can be derepressed upon nucleosome loss even under non-inducing conditions (Han and Grunstein, 1988), a genome wide analysis revealed that expression of 75% of already induced genes in yeast did not

change when nucleosome content was reduced by deleting histone H4 (Wyrick et al., 1999).

Although it has been well accepted that for inducible genes, recruitment of TBP to the promoter is mediated by activators and/or chromatin remodeling complexes (Becker and Horz, 2002; Jenuwein and Allis, 2001), the mechanism of TBP recruitment remains unknown for constitutively active genes. The most likely and simplest model is that during DNA replication, TBP binds to the promoter and prevents the assembly of nucleosomes around this region, as is the case for some other transcription factors (Fedor and Kornberg, 1989). *In vitro* studies showed that prebinding of TBP to promoters prior to nucleosome assembly can prevent nucleosome-mediated repression of transcription (Abmayr et al., 1988; Almouzni et al., 1990; Workman and Roeder, 1987a; Workman et al., 1991).

Using *in vivo* DNase I digestion, we observed roughly similar extents and levels of protection at the promoters of both high and low transcriptional rate genes. Notably, the primer extension experiments to map these two classes of promoters were performed using the same DNA samples. That is, those promoter regions were all mapped from the same *in vivo* DNase I digested DNA sample, and the similarity we observed among promoters was reproducible in multiple experiments. This indicates that some proteins bind to the promoter region at all times. We conclude that TBP, and possibly other factors, remain bound at the promoters after each cycle of transcription.

Using a ChIP assay, we quantitatively measured TBP occupancy level at the promoters of four **a** cell-specific genes. These genes are thought to be regulated by the same mechanism and the TATA sequences are the same for their promoters, so accessibility of the antibody to TBP should not be affected by conformation or different factors associating with TBP. Although the transcription rate of these genes spans more than a 25 fold range, the TBP occupancy level is same for their promoters. Similarly, equivalent TBP occupancy is observed among promoters of four histone genes. This strongly supports the conclusion that TBP remains on promoters through multiple cycles of transcription.

We suggest that TBP, possibly together with other transcription factors, binds to the promoter of active genes upon induction by activators or during replication. Then, unless receiving dissociating signals, TBP, perhaps together with some other factors, will form a postinitiation complex on the promoter to facilitate further cycles of transcription.

### 3.4.2 A comparison between TBP binding patterns in living cells and isolated nuclei

Nuclease digestion of isolated nuclei has been used for many decades to map chromatin structure (Simpson, 1998). Using this method, nucleosomes have been shown to be positioned on certain DNA sequences and such positioning has important functional consequences (Simpson, 1991; Simpson, 1998). For example, when an autonomously replicating sequence of a

minichromosome was covered by a positioned nucleosome, the copy number of the minichromosome decreased dramatically (Simpson, 1990). However, for non-histone binding studies, it has been a concern that artifacts may be produced during the process of nuclei preparation, these artifacts are caused either by the dissociation or degradation of proteins, by buffer conditions different from physiological conditions, or by disruption of some nuclear structure (Kornberg et al., 1989; Zaret, 1999). One example is the binding of Mat$\alpha$2p, a short half-life protein (Murphy et al., 1993). The footprint of this protein is lost in isolated nuclei (Murphy et al., 1993); however, Mat$\alpha$2p can protect binding sites *in situ* or in living cells against UV light, methylase and DNase I (Gavin et al., 2000; Kladde et al., 1996; Murphy et al., 1993; Wang and Simpson, 2001; Wu et al., 1998). A striking depletion of TBP bound to the Xa promoter within nuclei isolated from human cells compared to that within permeabilized cells has also been reported (Pfeifer and Riggs, 1991). It has been shown in several cases that the TATA box is protected from modification by UV light in living cells; however, in isolated nuclei, these sites are hypersensitive to nucleases (Elgin, 1988; Gross and Garrard, 1988). While these results imply that TBP dissociates from its target sites in isolated nuclei, interpretation is complicated by the differing DNA sequence specificities and other characteristics of these modifiers and nucleases (Van Dyke and Dervan, 1983; Zaret, 1999).

In this study, we compared the DNase I digestion pattern at several active promoters in samples from isolated nuclei and also from *in vivo* samples.

Strikingly, for the promoters of all the genes we tested, differences in DNase I digestion patterns between samples digested with the enzyme *in vivo* and those analyzed with an exogenous enzyme in isolated nuclei have been observed. *In vivo*, the TATA box and a region surrounding the TATA box within each gene are protected from DNase I; in isolated nuclei, however, such regions are hypersensitive to nucleases compared to the bulk chromatin (Figure 3.1, 3.2, 3.3, and data not shown). Considering that *in vitro* binding assays showed that TBP, either in purified form or associated with TFIIA and/or TFIIB, can protect the binding site from DNase I (Sawadogo and Roeder, 1985; Van Dyke et al., 1989), we suggest that TBP and other factors were dissociated from their cognate sites upon isolation of chromatin. This idea is further supported by the ChIP assay showing that redistribution of TBP and other general transcriptional factors occurs upon nuclei isolation (Figure 3.5 and data not shown). As such, our results favor (although they do not prove) the idea that TBP and other general transcriptional factors bind to active promoters specifically in living cells; in isolated nuclei, however, TBP and other general transcriptional factors bind to DNA elements nonspecifically, transiently and weakly.

Possible explanations for these observed differences between *in vivo* and isolated chromatin samples are as follows. First, it could be proposed that TBP, like Mat$\alpha$2p, has been degraded during the process of nuclei preparation. This idea is contradicted by the observation that, in many cases, TBP can be easily detected in nuclear extracts (Yudkovsky et al., 2000). A

second explanation might be that TBP dissociates from the promoter due to the hypotonic buffer used in the nuclei preparation process. Although it is difficult to exclude this second possibility, it is clearly inconsistent with the fact that TBP can bind to its target DNA in various conditions, and that the affinity of TBP for the TATA box is very high ($K_D$ ~ 1nM) (Pugh, 2000).

We favor a third possibility. This explanation assumes that TBP is binding to the promoter in the context of some nuclear structure. Existence of a filamentous structure in the nucleus of many cell types has been supported by numerous experimental approaches (Nickerson et al., 1997; Wan et al., 1999). Some observations have implied that active genes are attached to these filamentous structures so that mRNA transcription occurs in hundreds to thousands of discrete foci in the nucleus (Cockell and Gasser, 1999; Szentirmay and Sawadogo, 2000). TBP, the competent promoters, and perhaps other transcription factors, might associate in the context of nuclear compartments. Upon nuclear isolation, disruption of these nuclear compartments might lead to loss of TBP footprinting and the binding specificity. Such disruption may be caused by the loss of cytoskeletal architecture, the diffusion of nucleoplasm from the nuclei, or the altered ionic environment (Kornberg et al., 1989; Zaret, 1999).

Therefore, our results strongly suggest that care should be taken in concluding that chromatin in the isolated nucleus represents native chromatin in the living cell. In addition, perhaps the term “isolated chromatin” should be used when referring to isolated nuclei.

Figure 3.1: Indirect end labeling mapping of chromatin structure of the promoter of several genes. Nuclei isolated from wild type **a** or $\alpha$ cells were digested with increasing amounts of micrococcal nuclease at 37ºC for 10 minutes. The DNA was purified, digested with *EcoR I*, and analyzed as described in the Materials and Methods section. Lane 1, the undigested control. Lanes 2, 3, and 4, DNA from nuclei isolated from wild type **a** cells and digested with three concentrations of DNase I *in vitro*. Lanes 5, 6, and 7, DNA from nuclei isolated from wild type $\alpha$ cells and digested with three concentrations of DNase I *in vitro*. The closed circles mark the positioned nucleosomes in $\alpha$ cells. The gray boxes indicate the TATA box of different genes: T1 for *MFA1* gene, T2 for *MRPL28* gene, T3 for *STP1* gene, and T4 for *SPP41* gene. F stands for the full length fragment.

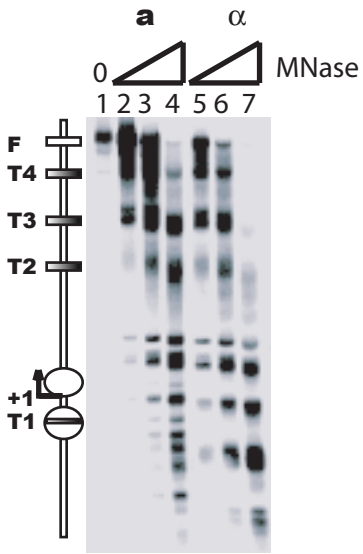

a
α
0
MNase
1 2 3 4 5 6 7
F
T4
T3
T2
+1
T1

Figure 3.2: Primer extension mapping of DNase I cutting sites around the promoter and coding region of the *PGK1* gene. Lane 1, DNA from **a** cells containing pSUN-1 grown in dextrose where the DNase I gene is repressed. Lanes 2 and 3, two independent DNA samples from **a** cells containing pSUN-1 grown for 6 h in galactose where the DNase I gene is expressed. Lanes 4, 5, and 6, DNA from nuclei isolated from wild-type **a** cells and digested with three concentrations of DNase I *in vitro*. Lanes 7 and 8, protein-free DNA digested with two concentrations of DNase I *in vitro*. The band marked with "*" is a primer extension artifact occurring in nondigested DNA sample and should be ignored.

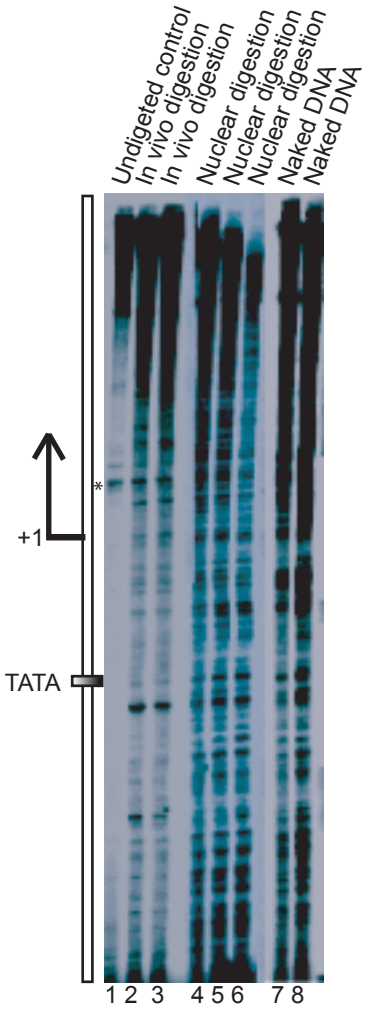

Undigeted control
In vivo digestion
In vivo digestion
Nuclear digestion
Nuclear digestion
Nuclear digestion
Naked DNA
Naked DNA
*
+1
TATA
1 2 3 4 5 6 7 8

Figure 3.3: Primer extension mapping of DNase I cutting sites around the promoter and coding region of the *YCL056C* gene. Lane 1, DNA from **a** cells containing pSUN-1 grown in dextrose where the DNase I gene is repressed. Lane 2, DNA from **a** cells containing pSUN-1 grown for 6 h in galactose where the DNase I gene is expressed. Lanes 3 and 4, DNA from nuclei isolated from wild-type **a** cells and digested with two concentrations of DNase I *in vitro*. Lanes 5 and 6, protein-free DNA digested with two concentrations of DNase I *in vitro*.

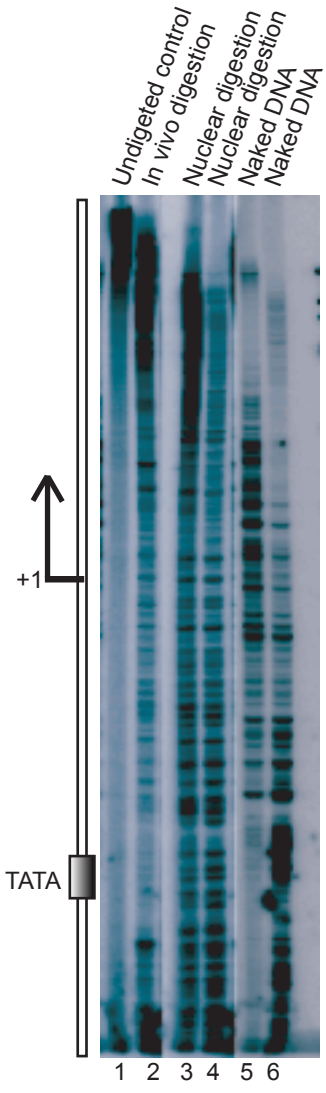

Undigeted control
In vivo digestion
Nuclear digestion
Nuclear digestion
Naked DNA
Naked DNA
+1
TATA
1 2 3 4 5 6

Figure 3.4: Chromatin immunoprecipitation for transcription factor binding. Sonicated chromatin was prepared from the formaldehyde-fixed wild type cells. Immunoprecipitation was carried out using antibodies to different transcription factors. Immunoprecipitated and input DNA were amplified by PCR using primers specific for *PGK1* promoter, *YCL056C* promoter, and the UAS region of the *STE3* gene where no promoter sequence has been found. The PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. The experiments were repeated twice with similar results.

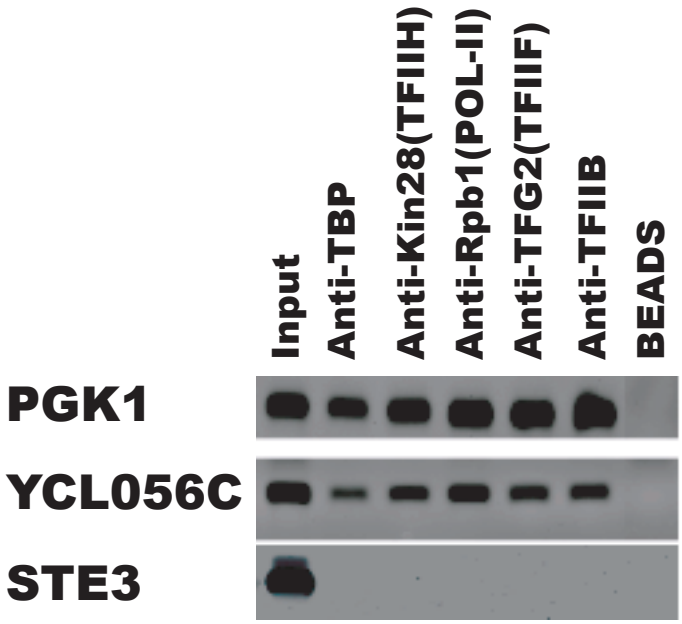
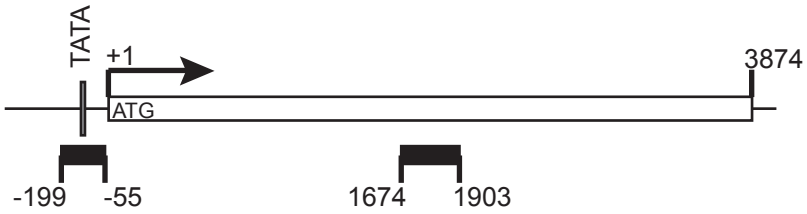
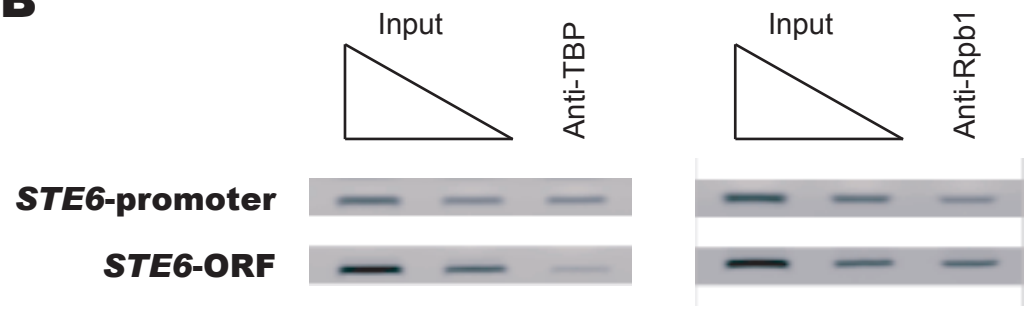

Input
Anti-TBP
Anti-Kin28(TFIIH)
Anti-Rpb1(POL-II)
Anti-TFG2(TFIIF)
Anti-TFIIB
BEADS
PGK1
YCL056C
STE3

Figure 3.5: TBP occupancy of the *STE6* promoter and open reading frame (ORF) regions in living cells and isolated nuclei. **(A)** Schematic representation of the *STE6* gene and probes for ChIP PCR. TATA element (TATA) is shown by the gray box. The open box depicts the *STE6* coding sequence which is 3873 bp long. The translation initiation site (ATG) is presented. Thick lines represent the regions amplified by PCR in the ChIP experiments and the numbers show the positions of these probes relative to *STE6* translation initiation site (ATG). **(B)** ChIP assay of TBP and RNA polymerase II occupancy level at *STE6* promoter and ORF regions in living **a** cells. The sonicated DNA was immunoprecipitated by using antibodies against TBP or Rpb1p, a subunit of RNA polymerase II. **(C)** ChIP assay of TBP occupancy level at the *STE6* promoter and ORF regions in nuclei isolated from wild type **a** cells. The sonicated DNA was immunoprecipitated by using antibodies against TBP.

**A**


TATA
+1
3874
ATG
-199
-55
1674
1903

**B**


Input
Anti-TBP
Input
Anti-Rpb1
STE6-promoter
STE6-ORF

**C**

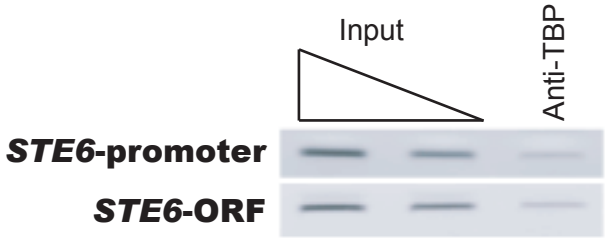
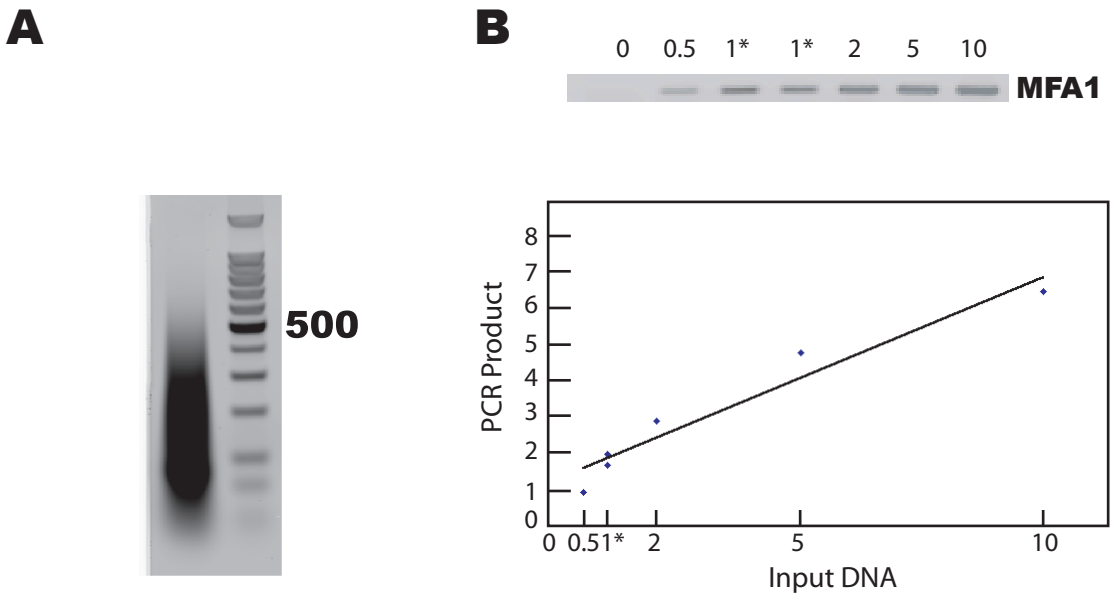

Input
Anti-TBP
STE6-promoter
STE6-ORF

Figure 3.6: TBP binds to the promoter of **a** cell-specific genes only in **a** cells. (**A**) Sonicated DNA is less than 500 bp. An ethidium stained gel of the sonicated DNA used for a typical ChIP experiment is shown. (**B**) Dependence of PCR product yield on amount of input chromatin template. PCR reactions were performed with primers for the *MFA1* promoter using increasing amount of chromatin DNA template. The amounts of PCR product obtained versus input chromatin DNA substrate are plotted below. All PCR reactions shown in (C) and (D) in this Figure and Figure 3.7 were performed by using an amount of DNA yielding a product within the linear response range. 1* means the 1/2000 dilution of the input DNA. (**C**) PCR was performed by using primers for the indicated promoters and chromatin DNA derived from wild type **a** cells. ChIP was done with anti-TBP antibody. (**D**) PCR was performed by using primers for the indicated promoters and chromatin DNA derived from wild type $\alpha$ cells. ChIP was done with anti-TBP antibody.


A
500
B
0 0.5 1* 1* 2 5 10
MFA1
PCR Product
0 1 2 3 4 5 6 7 8
0 0.51* 2 5 10
Input DNA

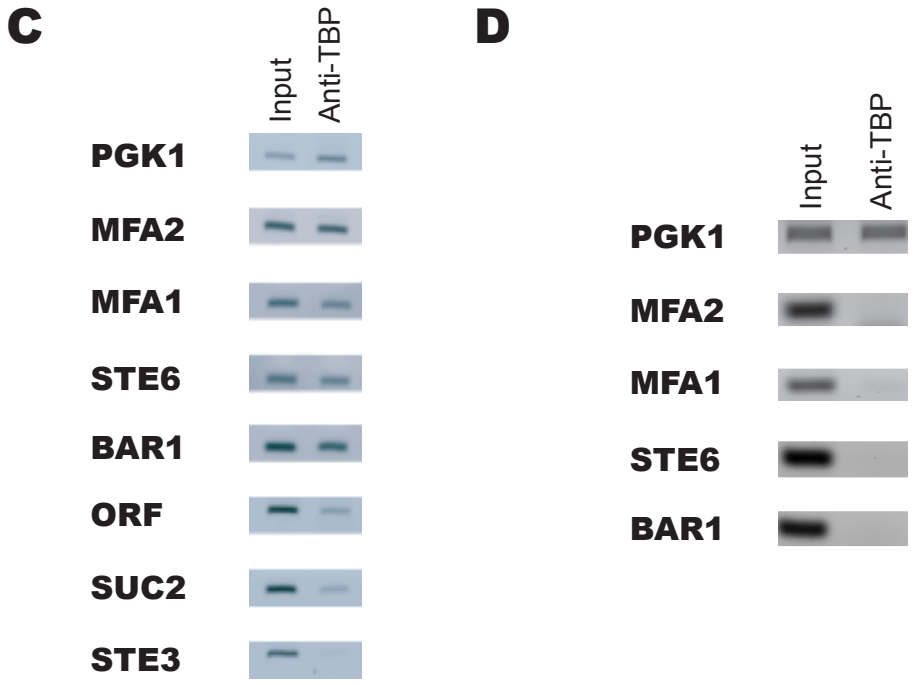

C
Input
Anti-TBP
PGK1
MFA2
MFA1
STE6
BAR1
ORF
SUC2
STE3
D
Input
Anti-TBP
PGK1
MFA2
MFA1
STE6
BAR1

Figure 3.7: RNA polymerase II and TFIIH occupancy at selected promoters. Crosslinked chromatin DNA prepared from wild type **a** cells was immunoprecipitated with antibody to Rpb1, one subunit of RNA polymerase II (**A**) or Kin28, one subunit of TFIIH (**B**). PCR products corresponding to those indicated promoters were generated from total chromatin or immunoprecipitated DNA.

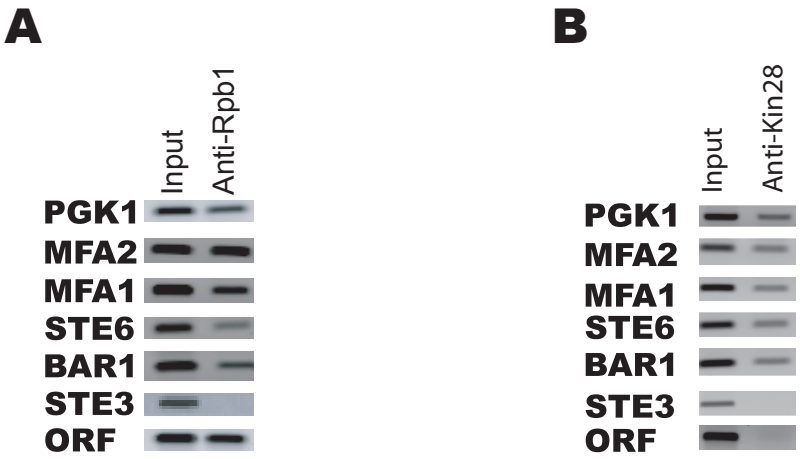

A
Input
Anti-Rpb1
PGK1
MFA2
MFA1
STE6
BAR1
STE3
ORF
B
Input
Anti-Kin28
PGK1
MFA2
MFA1
STE6
BAR1
STE3
ORF

Figure 3.8: Summary of the ChIP data. All the numbers are averaged from at least two independent assays. The transcriptional frequency numbers are from Young's database (http://web.wi.mit.edu/young/expression/). *, for each promoter, the relative occupancy level is indicated in terms of the percent of the observed occupancy level at the *STE6* promoter, which is arbitrarily defined as 100. ^, for each promoter, the relative occupancy level is indicated in terms of the percent of the observed occupancy level at the *MFA2* promoter, which is arbitrarily defined as 100.

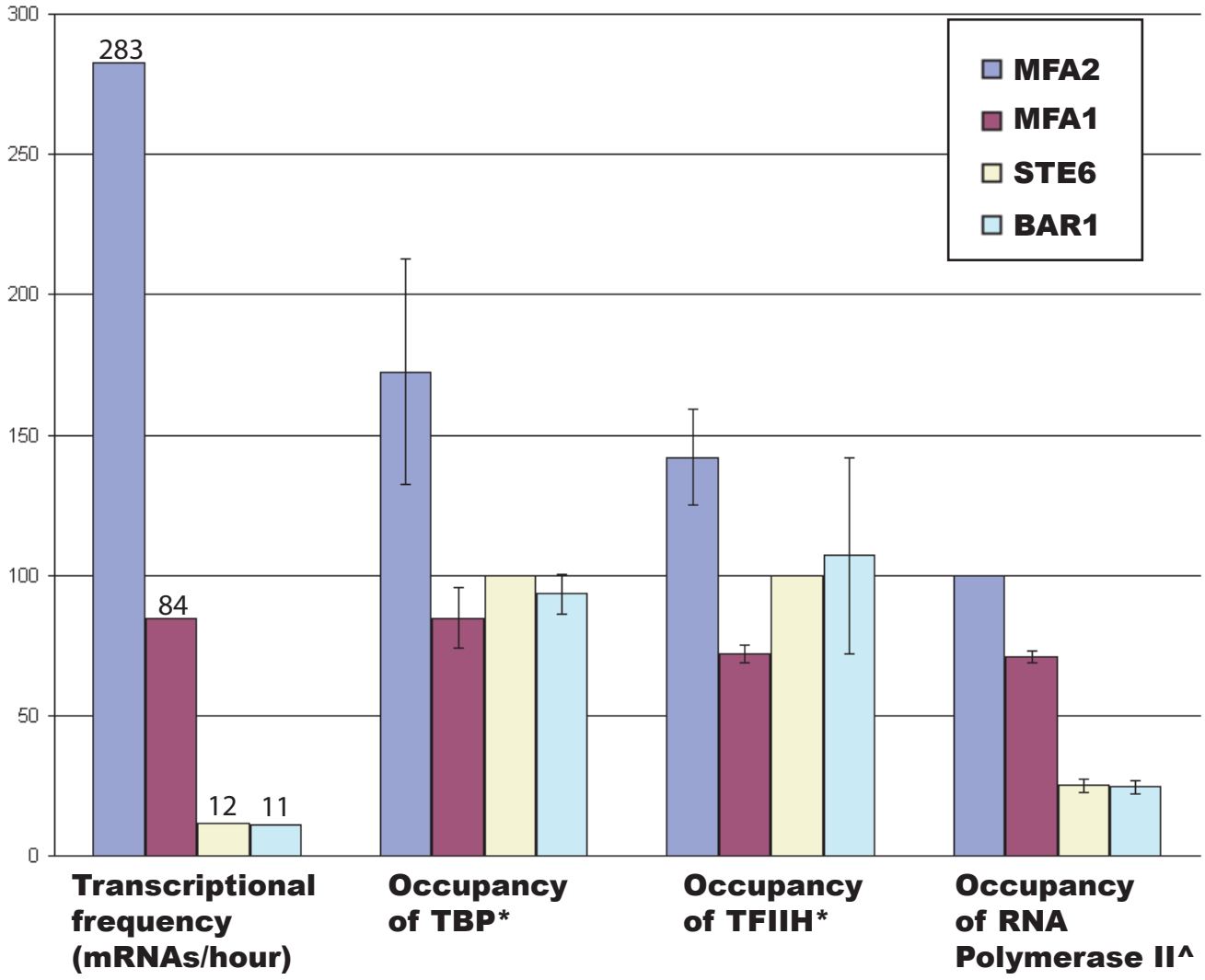
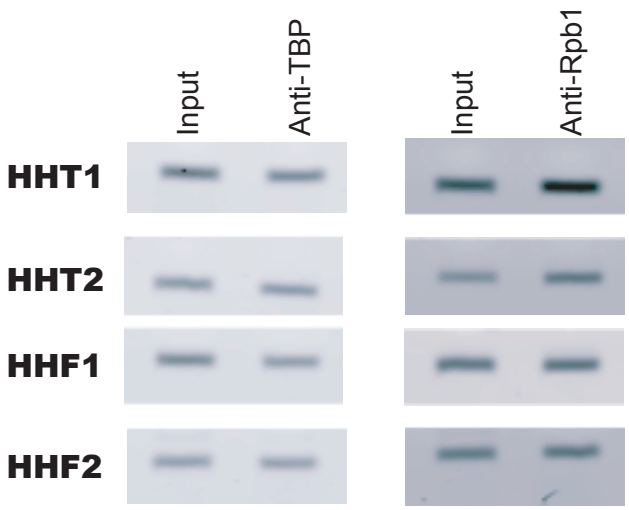
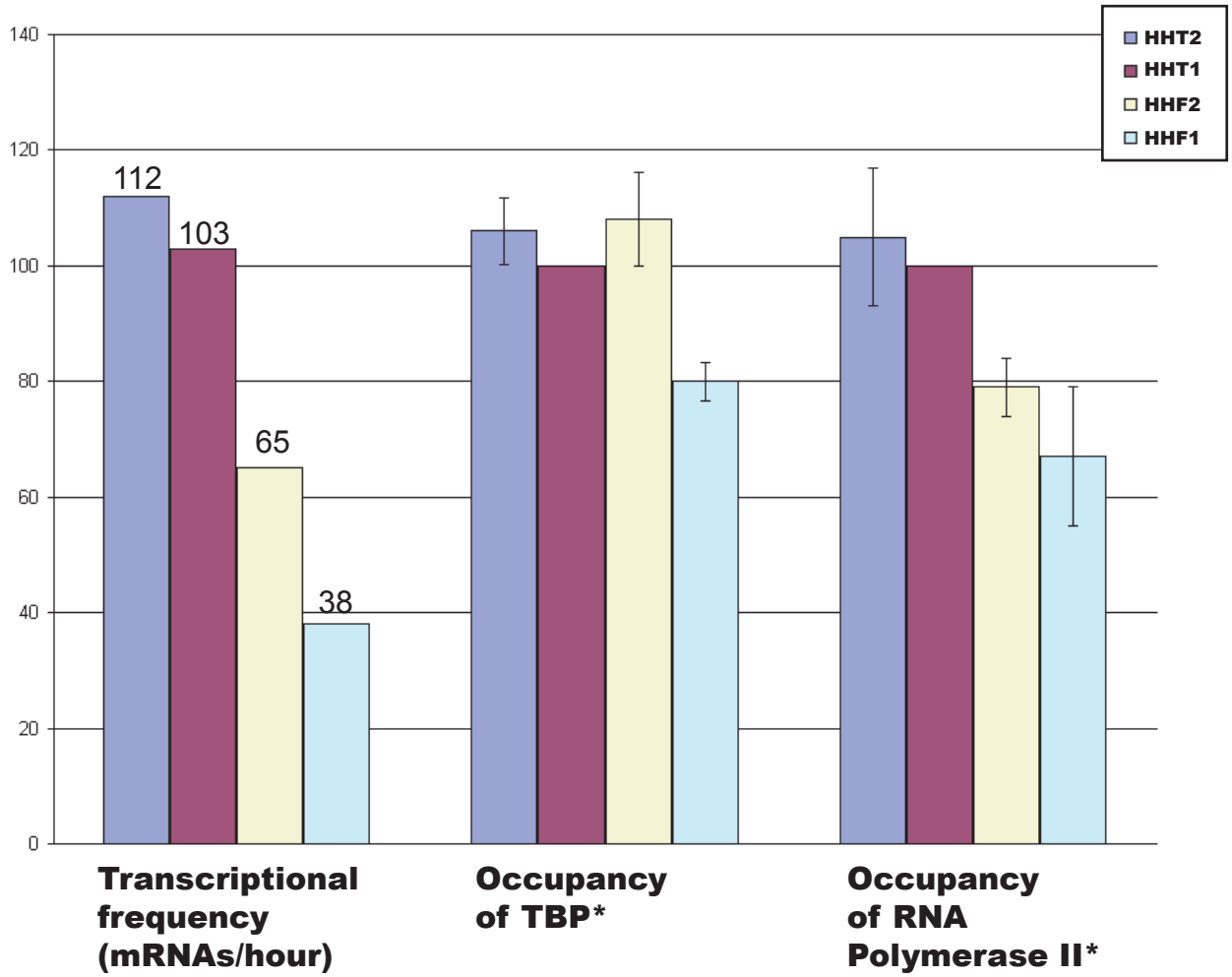

300
250
200
150
100
50
0
283
84
12
11
MFA2
MFA1
STE6
BAR1
Transcriptional frequency (mRNAs/hour)
Occupancy of TBP*
Occupancy of TFIIH*
Occupancy of RNA Polymerase II^

Figure 3.9: TBP and RNA polymerase II occupancy at promoters of selected histone genes. (**A**) Crosslinked chromatin DNA prepared from wild type **a** cells was immunoprecipitated with antibody to TBP or Rpb1, one subunit of RNA polymerase II. (**B**). Summary of the ChIP data. All the numbers are averaged from at least two independent assays. The transcriptional frequency numbers are from Young's database (http://web.wi.mit.edu/young/expression/). *, for each promoter, the relative occupancy level is indicated in terms of the percent of the observed occupancy level at the *HHT1* promoter, which is arbitrarily defined as 100.

**A**


Input
Anti-TBP
Input
Anti-Rpb1
HHT1
HHT2
HHF1
HHF2

**B**


HHT2
HHT1
HHF2
HHF1
140
120
100
80
60
40
20
0
112
103
65
38
Transcriptional frequency (mRNAs/hour)
Occupancy of TBP*
Occupancy of RNA Polymerase II*

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# Chapter IV

## The role of higher order chromatin structure in repression of the *MFA1* gene in $\alpha$ cells

## Abstract

It is generally accepted that packaging DNA into chromatin plays a crucial role in the constitutive repression of gene transcription in eukaryotic cells. In *Saccharomyces cerevisiae*, the repression of the **a** cell-specific genes is associated with the organization of a chromatin domain in which an array of nucleosomes is precisely positioned over the essential promoter elements and the entire coding region of these genes. In a previous study (Ducker, 2001), the minichromosome affinity purification (MAP) technique has been improved to allow the isolation of a unique genomic locus as *in vivo* packaged chromatin and facilitate the observation of higher-order structure of this locus under EM. By using this strategy, the images of higher-order chromatin structure of the *STE6* gene have been obtained. These images clearly showed that when the gene was repressed, the nucleosomes associated with the gene adopted a highly ordered, compact “hairpin” conformation. Here, we checked the higher order chromatin structure of the *MFA1* gene using the same strategy. A tightly compact conformation has been observed. Using western blot and chromatin immunoprecipitation assays, we confirmed that Tup1p is associated with this repressed region and spreads along the entire repressed *MFA1* locus. Interestingly, we found that Hho1p also binds to this region, indicating that Hho1p plays a structural role in this region and may facilitate the bending of the linker DNA between these two positioned nucleosomes.

## 4.1 Introduction

It has been widely accepted that modulation of chromatin structure has a pivotal influence on the regulation of gene expression in eukaryotes. The functional consequence of chromatin packaging, in general, is to restrict access of DNA to a variety of DNA-binding proteins that regulate gene activity. Thus, transcription activation is often accompanied by the alteration of chromatin so that its DNA sequences become more transparent to the transcriptional apparatus. These local changes of the chromatin structure are often mediated by ATP-dependent nucleosome-remodeling complexes and histone tail modifiers, both of which can be brought to DNA by the activators. On the other hand, different proteins, named repressors, make chromatin structure less transparent and help repress transcription (Kornberg and Lorch, 1999; Workman and Kingston, 1998a).

Chromatin is composed of repeating units termed nucleosomes (see Chapter 1). Many *in vitro* biochemistry studies have shown that the packaging of promoter DNA into nucleosomes impedes accessibility of general transcription factors and/or activators to template DNA, whereas prior binding of these factors to the promoter blocks nucleosome assembly on important cis-acting DNA elements (Abmayr et al., 1988; Adams and Workman, 1993; Almouzni et al., 1990; Imbalzano et al., 1994; Svaren and Horz, 1997; Workman and Kingston, 1992a; Workman and Roeder, 1987a; Workman et al., 1991). *In vivo*, nucleosomes exert a similar inhibitory effect upon

transcription. For example, in yeast, activation of several previously—repressed genes was accompanied by nucleosome loss, as a result of turning off histone synthesis by genetic means (Han and Grunstein, 1988; Han et al., 1988; Kim et al., 1988). These and other observations have led to the conclusion that nucleosomes serve as general repressors for transcription, and that a "kinetic competition" takes place between transcription activators and general transcription factors and core nucleosomes.

In evaluating repression of gene expression, accumulating observations suggest a mechanism beyond single nucleosome—based promoter extinction. For example, numerous studies have found that many silencing regions do appear to have a highly regular nucleosome array and decreased acetylation of histone tails (Kornberg and Lorch, 1999; Ravindra et al., 1999; Weiss and Simpson, 1998). Conversely, many active genes in vertebrates can reside in large chromosomal domains, characterized by elevated accessibility to DNase I and increased acetylation of histone tails (Bulger et al., 2002; Hebbes et al., 1994). Furthermore, recent investigations have revealed a correlation between transcription activity and phosphorylation of histone tails, which has been long correlated with chromosome condensation (Wei et al., 1998). Finally, gene silencing by heterochromatin seems likely to depend on its higher order chromatin structure (Kornberg and Lorch, 1999).

However, the pattern of coiling a chain of nucleosomes in a thicker fiber remains uncertain, and, thus, information about the higher order chromatin

structure beyond the nucleosome is crucial for understanding chromatin function.

The current methodology for assaying the chromatin structure relies mainly on the accessibility of the DNA in chromatin to nucleases or modifying enzymes in isolated nuclei or in living cells (Simpson, 1998; Simpson, 1999). However, instead of giving direct information, these techniques provide only a hint of the three-dimensional (3D) chromatin structures in the nucleus. Among the few techniques available to investigate the 3D conformation of the nuclear chromatin architecture, electron microscopy (EM) can produce images which provide information of not only the spatial relationships among arrays of nucleosomes but also on the effect of associated proteins on these arrays (Woodcock and Dimitrov, 2001; Woodcock and Horowitz, 1997; Woodcock and Horowitz, 1998). Furthermore, in conjunction with biochemical and biophysical data, EM can provide a way to establish the significance of the 3D structure of chromatin in the regulation of DNA transcription, repair, recombination, and replication (Woodcock, 1989). However, in most cases, EM has only focused on examining overall chromatin structure *in situ*, because of the difficulty identifying a particular region of the genome (Van Holde, 1989).

The MAP methodology (see chapter 1) is the first technique available to isolate a specific gene, the *STE6* gene, as chromatin (Ducker, 2001). Combining this technology with negative staining, a highly ordered hairpin conformation has been observed to be associated with the repressed *STE6*

gene (Ducker, 2001). However, it is still uncertain whether such a structure is unique to the *STE6* gene, or whether it can also be observed for other gene loci.

Here we describe a comprehensive analysis of the chromatin structure of the *MFA1* region in a repressed state. A combination of high-resolution and low-resolution micrococcal nuclease (MNase) sensitivity mapping studies clearly demonstrates that in $\alpha$ cells, an array of positioned nucleosomes covers the promoter and extends into the coding sequence. Using MAP and EM technology, we found that this nucleosome array can form a special higher order structure in living cells. The possibility of the participation of the Ssn6-Tup1 complex and Hho1p is also discussed.

## 4.2 Materials and methods

### 4.2.1 Yeast strains and the minichromosome

The yeast strains YPH499 (MAT**a** ade2-101 ura3-52 his3-200 leu2-1 trp1-63 lys2-1), YPH500 (MAT$\alpha$ ade2-101 ura3-52 his3-200 leu2-1 trp1-63 lys2-1), and YPH500 $\Delta$TUP1 (MAT$\alpha$ ade2-101 ura3-52 his3-200 leu2-1 trp1-63 lys2-1 tup1::ura3) were used in this study.

As shown in figure 4.2, the MFA1-ALT minichromosome was created by inserting a 914 bp fragment containing the *MFA1* coding sequence from -401 to +513 (the start site of the ORF is set as +1) into the ALT minichromosome, as described previously (Ducker and Simpson, 2000).

### 4.2.2 Minichromosome affinity purification

The minichromosomes were isolated as described previously (Ducker and Simpson, 2000). Briefly, yeast cells carrying the minichromosomes were harvested by centrifugation at an $OD_{600}$ of 1.0 - 1.5. The cells were treated with Zymolyase 100T (Seikagaku) and spheroplast formation was determined microscopically. Washed spheroplasts were gently resuspended in 10 ml of minichromosome binding buffer (MBB) [20 mM HEPES, pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.1% Tween20] plus protease inhibitors [1 mM PMSF, 10 ug/ml A-protinin, 2 ug/ml Leupeptin, 2 ug/ml Pepstatin A] and chilled on ice for 15 minutes. The chilled spheroplasts were lysed in a Thomas® glass homogenizer and Teflon motor driven pestle with approximately 8 strokes.

The resulting lysates were held on ice for 2 - 4 hours with occasional agitation to allow the minichromosomes to be released from the nuclei. The lysates were then clarified by centrifugation in a Sorvall SS-34 rotor at 40,000 g for 20 minutes, at 4° C. The supernatants were subjected to the *lac I-Z* affinity chromatography column prepared as described previously (Ducker and Simpson, 2000).

Prior to starting the affinity chromatography, 10 ml of MBB was run over the column at full gravity speed (the bed volume of the chitin beads is 1 ml and the dimension of the column s 1 cm, so that the flow speed is around 1 ml/minute) to ensure proper buffer equilibration. The yeast supernatants containing the minichromosomes were mixed in batch with the chitin-*lacI-Z* matrix in MBB for 1 hour at 4° C. The columns were then packed by running the slurry into the columns at full gravity speed. Each column was washed three times with 10 ml MBB at full gravity speed, and then the minichromosomes were eluted from the columns in 5 ml of MBB containing 300 mM NaCl and 1 mM IPTG, at full gravity speed. After concentrating, the minichromosome samples were divided into small aliquots and saved in -80°C.

For DNA analysis, nucleic acid was purified from samples taken throughout the isolation by treatment with 100 ug/ml RNase A at 37° C for 2 hour, followed by 50 ug/ml proteinase K at 50° C for 30 minutes. The DNA samples were phenol:chloroform extracted two times and ethanol precipitated.

For protein analysis, portions of the denatured minichromosome samples were directly loaded onto SDS-PAGE gel.

For electron microscopy analysis, the isolated minichromosomes were centrifuged in a 15-40% sucrose gradient containing 10 mM HEPES, pH 8.0; 50 mM NaCl; 0.2 mM EDTA at 4° C for 14 hours at 30,000 RPM in an SW41Ti rotor. Peak fractions from the gradient were dialyzed into the same buffer (without the sucrose) and imaged.

### 4.2.3 Western blot

Protein samples were electrophoresed on 10% SDS-polyacrylamide gels followed by electrotransfer to Hybond ECL membranes (Amersham Life Sciences, Inc.). The membranes were incubated in phosphate buffered saline plus 0.1% Tween20 (PBST) containing 5% powdered milk (w/v) on a shaking platform at room temperature for 1 hour. The membranes were washed 3 times in PBST for 10 minutes each at room temperature. Membranes were then incubated with anti-Tup1p antibodies (provided by J. Reese) or anti-hho1p antibodies (provided by H. Patterton) in PBST on a shaking platform for 1 to 2 hours at room temperature. The membranes were washed 3 times in PBST for 10 minutes each at room temperature. Anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Life Sciences, Inc.) were then incubated with the membranes in PBST on a shaking platform for 1 hour at room temperature. The blots were washed 3 times in PBST for 10 minutes each at room temperature. Blots were

developed with PICO super signal western development reagents (Pierce Biotechnology Inc.) and exposed to Fuji XAR film.

### 4.2.4 Electron microscopy (EM)

Methods for sample preparation including fixing, grid adhesion and staining can be found in Woodcock and Horowitz (1998).

### 4.2.5 Nuclei and DNA preparation and analysis

Nuclei preparation was carried out essentially as described by Weiss and Simpson (Weiss and Simpson, 1997). Briefly, yeast from a 1-liter culture grown to an optical density of about 1.0 at 600nm was harvested and digested with Zymolyase 100T (Seikagaku). Nuclei were purified by differential centrifugation and resuspended in digestion buffer (10mM HEPES, pH 7.5; 0.5mM $MgCl_2$; 0.05mM $CaCl_2$) and incubated with 0, 2, and 4 units/ml MNase (Worthington) for 10 minutes at 37 °C. The digestions were terminated by the addition of EDTA, and the DNA was purified by RNase A and proteinase K digestion and phenol/chloroform extraction. The DNA pellet was resuspended in 0.1XTE buffer.

For low-resolution mapping of nucleosomes by indirect end labeling, the purified DNA was subjected to a secondary digestion by *EcoR I*. DNA was then electrophoresed in 1.4% agarose gels in 1×TAE buffer, and transferred to Hybond-NX membrane (Amersham) and crosslinked with UV light. The specific DNA sequences were detected by hybridizing with a random-primer

labeled probe directed toward the end of the *EcoR I* site. For high-resolution mapping, multiple rounds of *Taq* DNA polymerase-based primer extension was carried out from a $^{32}$P-end-labeled primer, and the products were then resolved on a 6% polyacrylamide (19:1), 50% urea gel. Images were captured on a PhosphorImager screen. The image was then scanned and analyzed with Image Quant v.5.0 software (Molecular Dynamics).

### 4.2.6 Chromatin immunoprecipitation (ChIP)

Chromatin-containing extracts were prepared as previously described (Hecht and Grunstein, 1999) with minor modifications. Extracts were prepared from 200-ml cultures at a density of about 1.0 at 600nm. Cells were fixed in 3% formaldehyde and were disrupted with glass beads and transferred to a 15 ml centrifuge tube (the final volume was adjusted to 2 ml). The chromatin-containing extract was sonicated to yield an average DNA size of 300 bp (the majority of the fragments were approximately 300 bp long, but a small percentage of the fragments were as small as 50 bp or as large as 500 bp). Sonication conditions were 40% output, 90% duty cycle, fifteen 12-second cycles with a Branson Sonifier 450. The chromatin size was confirmed for each input sample by running 10% of the DNA on a 2% agarose gel. The sonicated extract was subsequently clarified by centrifugation.

The antibodies used in the immunoprecipitation step were: polyclonal antibody against Tup1p (provided by J. Reese), and polyclonal antibody against Hho1p (provided by H. Patterton) (Patterton et al., 1998).

### 4.2.7 Quantitative PCR

All primers were designed to be 19- to 25-mers, with a $T_m$ of approximately 60°C. Primer sequences are shown in Appendix. The PCR conditions were as follows: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 28 cycles. A 5-minute 94°C step prior to the cycles and a 5-minute 72°C extension following completion of the cycles were added. Several dilutions of each sample were used for PCR. For the input DNA, the initial dilution series was from 1/4,000 to 1/100; for the immunoprecipitated DNA, the initial dilution series was from 1/20 to 1/5. Only one titration of input and immunoprecipitated DNA was shown in the figures to conserve space. The PCR products were detected by UV illumination of an ethidium bromide stained 2% agarose gel and analyzed with Image Quant v.5.0 software (Molecular Dynamics).

## 4.3 Results

### 4.3.1 Nucleosomes are positioned over the regions required for *MFA1* expression in $\alpha$ cells

Yeast exists in two haploid cell types, a and $\alpha$ (Herskowitz, 1989). Seven genes, termed **a** cell-specific genes, are specifically expressed only in the **a** mating type yeast cells (Zhong et al., 1999). In $\alpha$ cells, MAT$\alpha$2p cooperates with Mcm1p to repress these **a** cell type-specific genes through binding to the $\alpha$2 operator, a 32 base-pairs (bp) long DNA sequence, which is located about 200 bp upstream from the start site of the open reading frame (ORF) of these genes. The binding of MAT$\alpha$2p and Mcm1p to the $\alpha$2 operator establishes an organized chromatin domain with a well-defined nucleosomal array. This chromatin domain begins ~15 bp downstream of the $\alpha$2 operator, extends through the coding region, and ends abruptly 30 to 70 bp downstream of the termination codon of the genes, thus forming a discrete domain (Ganter et al., 1993; Roth et al., 1992; Simpson et al., 1993; Teng et al., 2001). However, the mechanism of its termination remains uncertain.

In contrast, this highly organized chromatin appears to be disrupted in **a** cells, suggesting that it is required for, or a result of, repression of these genes in $\alpha$ cells. This idea was further supported by the observation that in $\alpha$ cells, mutations of the N-terminal tail of histone H4 resulted in both the disruption of chromatin and derepression of the **a** cell-specific genes (Roth et al., 1992).

*The MFA1* gene, one of the **a** cell-specific genes, encodes one of the **a** mating factors in **a** cells and is repressed in $\alpha$ cells. The open reading frame of this gene is only 111 bp long. In $\alpha$ cells, both low resolution and high resolution mapping of micrococcal nuclease cutting sites showed two 140-150 bp protected regions. Both regions are protected from micrococcal nuclease digestion and are flanked by nuclease-hypersensitive sites (Figure 3.1 and Figure 4.1). These results indicate that there are two precisely positioned nucleosomes abutting the $\alpha$2 operator (Figure 3.1, 4.1, and Y.Tsukagoshi and R.T.Simpson, unpublished data). One is positioned over the promoter region, with the TATA box lying at the center of this nucleosome; the other extends into the coding sequence and ends ~35 bp downstream of the termination codon of the *MFA1* gene. The length of the linker DNA between these two nucleosomes is ~40 bp long (Figure 4.1). In **a** cells, these two nucleosomes are imprecisely located, as expected (Figure 3.1 and Y.Tsukagoshi and R.T.Simpson, unpublished data).

### 4.3.2 The MFA1-ALT minichromosome

To observe the higher order chromatin structure of the repressed *MFA1* locus and to analyze non-histone proteins associating with *MFA1*, we created a minichromosome, termed MFA1-ALT minichromosome. The minichromosome is composed of the ALT backbone (Ducker and Simpson, 2000) and a 914 bp fragment containing the *MFA1* coding sequence and flanking DNA inserted into HSR B (Figure 4.2). In order to ensure the

inclusion of all regulatory elements, the "*MFA1* insert" in the MFA1-ALT minichromosome is comprised of extensive sequences both upstream (401 bp) and downstream (402 bp) of the coding region of the gene.

Quantitive analyses revealed that 40-60% of the 2371 bp MFA1-ALT minichromosome was released from the nuclei. Of the material loaded onto the Lac I-Z affinity column, more than 90% was retained and more than 90% of the minichromosomes were recovered in the eluate fraction (data not shown).

Functional and structural characterization of the MFA1-ALT minichromosome revealed that the *MFA1* gene fragment in the MFA1-ALT minichromosome contains all of the necessary regulatory sequences for the proper repression of this gene and for the organization of the characteristic chromatin structure observed in the genomic copy of this gene. First, northern analysis of mRNA isolated from strains carrying the MFA1-ALT minichromosome showed that the construct was transcribed in **a** cells and greatly repressed in $\alpha$ cells (data not shown). Therefore, *MFA*1 on the minichromosome seems to behave in the same way as the genomic copy of the gene does. Second, primer extension mapping of micrococcal nuclease digests of MFA1-ALT minichromosome in nuclei isolated from $\alpha$-cells showed two precisely-positioned nucleosomes abutting the $\alpha$2 operator (Figure 4.3), identical to the genomic locus (Figure 4.1). Finally, the copy number of this minichromosome is determined to be 25 copies per cell (data not shown). These results indicate (1) the minichromosome copy of the *MFA1* gene

accurately reflected the features of the genomic copy of this gene; and (2) the proteins necessary for repression and organization of the chromatin structure, such as Mat$\alpha$2p, Mcm1p, Tup1p, and Ssn6p, are not limiting, under these conditions (Ducker and Simpson, 2000).

### 4.3.3 EM images of the MFA1-ALT minichromosome isolated from $\alpha$ cells

There exists considerable evidence suggesting that repression of the **a**-cell specific genes in *Saccharomyces cerevisiae* is associated with the organization of a chromatin domain in which nucleosomes are precisely positioned over essential promoter elements and over the entire coding region of the gene (Cooper et al., 1994; Ducker and Simpson, 2000; Ganter et al., 1993; Patterton and Simpson, 1994; Roth et al., 1992; Shimizu et al., 1991; Simpson et al., 1993). However, it remains unclear whether (and how) these nucleosomes interact with each other and form higher order structure. In a recent seminal work (Ducker, 2001), affinity-purified minichromosomes were employed to investigate the 3D chromatin architecture of *STE6* gene in both transcriptionally active and repressed states. The results of this work showed that the minichromosomes isolated from **a** cells appeared as a "beads on a string" motif of evenly spaced nucleosomes. In contrast, minichromosomes isolated from $\alpha$ cells had a region in which the 10 nm fiber is interrupted by a more compact conformation of nucleosomes. In many cases, this compact region of the minichromosome adopted a doubled-over

or hairpin structure. Based on these observations, we concluded that these positioned nucleosomes form special hairpin-like higher-order chromatin structures in the repressed *STE6* locus.

To provide further insight into this issue, by collaborating with Dr. Chris Woodcock, we isolated the MFA1-ALT minichromosome from $\alpha$ cells and observed its chromatin structure under EM. By doing positive staining, a higher density region which is different from the backbone of the minichromosome was observed for many of the isolated MFA1-ALT minichromosomes (data not shown). Under negative staining conditions, a compact structure was again observed, which appeared to contain two tightly associated nucleosomes (Figure 4.4). When considered together with the SALT10 images we obtained previously (Ducker, 2001), we concluded that the structure is a "tip of the hairpin without a stem."

### 4.3.4 Multiple copies of Tup1p associate with the repressed *MFA1* locus *in vivo*

Another advantage of the MAP methodology is that it facilitates the characterization of non-histone proteins associated with certain gene loci. The best example of such application is the investigation of the role of Tup1p in the repression of the *STE6* gene, in the aforementioned work (Ducker, 2001; Ducker and Simpson, 2000).

As one of the best investigated co-repressors so far, Tup1p can form a tetramer by itself or by forming a complex with Ssn6p (see chapter 1 for

review). It can repress genes by three mechanisms which are not mutually exclusive(Smith and Johnson, 2000): (1) by blocking the activator; (2) by interfering with the general transcription factors; and (3) by forming a repressive chromatin structure by interacting with histone tails. The third hypothesis was supported by several observations. First, the repression domain of Tup1p has been demonstrated to interact directly with the N-terminal tails of histone H3 and H4 (Edmondson et al., 1996). Second, Tup1p is essential for the maintaining the deacetylation status of histone tails, which plays an important role in repression (Ducker, 2001; Edmondson et al., 1998; Watson et al., 2000). Third, TUP1 deletion induces a disorganization of the chromatin of several repressed loci (Cooper et al., 1994; Gavin et al., 2000; Gavin and Simpson, 1997; Weiss and Simpson, 1997).

As mentioned above, an investigation performed in our lab has shown that MAP can provide a powerful tool to investigate how Tup1p plays a role in gene repression. In this work, several yeast minichromosomes containing varying lengths of the *STE6* gene including flanking control regions were constructed. Tup1p was found to bind to these minichromosomes in $\alpha$ cells (Ducker and Simpson, 2000). Furthermore, these observations revealed that Tup1p associated with the repressed *STE6* gene at a level stoichiometric with nucleosomes, or, more quantitively, at a ratio of 2-2.4 molecules of Tup1p per nucleosome. Further, this work showed that Tup1p did not bind to the minichromosome backbone (the ALT), or to the minichromosome containing *STE6* in **a** cells (Ducker and Simpson, 2000).

Here, we used the same strategy to conduct a Tup1p stoichiometry analysis along the repressed *MFA1* locus. Figure 4.5A shows that Tup1p does bind to the MFA1-ALT minichromosome isolated from $\alpha$ cells (lane 5). Notably, the Tup1p antibody used in this study detects the Tup1p signal from a crude whole cell extract from wild type yeast cells (lane 1), but not from the *tup1* deletion cells (lane 2). Furthermore, the western signal for the recombinant Tup1p expressed from *E. coli* is proportional to the amount of the proteins loaded onto the gel (Figure5A, lanes 3 and 4; Figure 4.5B, lanes 2 to 6).

As shown in Figure 4.5B, a representative isolated MFA1-ALT minichromosome sample and a graded set of standards generated with recombinant Tup1p expressed in *E. coli* were loaded on 10% SDS-PAGE gel, transferred to an ECL membrane, and subjected to western blot analysis. Densitometry of the blot (Figure 4.5C) shows a ratio of ~7.7 Tup1p molecules per MFA1-ALT minichromosome isolated from $\alpha$ cells. Statistical analysis of two replicates of this experiment shows 7.7$\pm$1.3 copies of Tup1p per MFA1-ALT minichromosome. These results strongly support the hypothesis that there are two Tup1p tetramers associating with the repressed *MFA1* locus *in vivo*.

### 4.3.5 Chromatin structure of the *MFA1* locus in a *tup1* mutant strain

Next, we analyzed the requirement for Tup1p in the establishment of the chromatin structure of the repressed *MFA1* locus. The chromatin structure of

the *MFA1* locus in a *tup1* mutant stain (Weiss and Simpson, 1997) was mapped using MNase. In the absence of Tup1p, the highly organized chromatin structure of the *MFA1* locus, with an array of two nucleosomes, in wild type $\alpha$ cells disappeared (Figure. 4.6A). Notably, the MNase digestion patterns outside the *MFA1* locus are identical between wild type and mutant strains (Figure 4.6A).

Furthermore, we showed that deletion of the *TUP1* gene resulted in derepression of the *MFA1* gene (Figure 4.6B). Thus, like other **a** cell-specific genes (Cooper et al., 1994; Roth et al., 1992; Simpson et al., 1993), repression of *MFA1* requires Tup1p. This is also true for **a** and $\alpha$ strains (Figure 3.1).

### 4.3.6 Tup1p spreads over the entire *MFA1* chromatin domain

To further test if Tup1p associates with the regulatory region and the coding region of the genomic copy of the *MFA1* gene, chromatin immunoprecipitation (ChIP) was performed (see chapter 3 for the details of this approach). Tup1p antibodies were used to immunoprecipitate formaldehyde-cross-linked, sonicated chromatin from wild type **a** and $\alpha$ cells. After reversal of the crosslinks, the precipitated DNA was visualized by quantitive PCR (Figure 4.7). Each PCR-amplified fragment is around 200 bp long and was identified based on the position of the center of each fragment relative to the start site of the ORF of the *MFA1* gene. Figure 4.7C shows the *MFA1* fragments amplified from **a** cell immunoprecipitated material. Comparing this signal to the input for this cell type shows uniform background

amplification from the immunoprecipitated material. Figure 4.7A shows the *MFA1* fragments amplified from the $\alpha$ cell immunoprecipitated material. By comparing the signal from **a** cells to $\alpha$ cells, it is clear that all the fragments between the $\alpha$2 operator and the 3' end of the *MFA1* gene are preferitially precipitated from $\alpha$ cells, indicating that Tup1p spreads along the entire chromatin domain of the gene. No PCR product is obtained from the IP DNA when primers outside the *MFA1* gene (-0.50, and +0.55) are used. These results show that Tup1p spreads unidirectionally from the $\alpha$2 operator to the 3' end of the gene, corresponding exactly to the direction and scope of positioned nucleosomes in the *MFA1* chromatin domain (Figure 4.1 and Y.Tsukagoshi and R.T.Simpson, unpublished data).

A control for amplification from both cell types is also shown in Figure 4.7. The *SUC2* gene, a sucrose catabolism gene, is repressed by Tup1p in the presence of glucose (Gavin and Simpson, 1997). It should be repressed in both cell types in this experiment, and therefore should be associated with Tup1p in both cell types. As expected, the ChIP data shows roughly equal amplification from the *SUC2* locus immunoprecipitated with Tup1p antibodies from both **a** and $\alpha$ cells.

### 4.3.7 Hho1p binds to the repressed *MFA1* locus in $\alpha$ cells

Another possible candidate protein to hold the compact chromatin structure together is Hho1p, the putative linker histone in yeast (Freidkin and Katcoff, 2001; Landsman, 1996; Patterton et al., 1998; Ushinsky et al., 1997;

Zlatanova, 1997). Like the linker histones in other species, Hho1p may be primarily a structural protein and contribute to folding of the nucleosome filament into the next higher level of structure in special loci.

To test this idea, we first did a Western blot to check if Hho1p binds to the MFA1-ALT minichromosome. As shown in Figure 8A, Hho1p was detected in the MAP-isolated MFA1-ALT minichromosome sample, but not in the ALT minichromosome sample. This indicates that Hho1p binds to the *MFA1* locus specifically.

Next we confirmed this conclusion by doing a ChIP assay. In $\alpha$ cells, Hho1p was found to bind to the *MFA1* locus, But not to the PGK1 locus (Figure 8B). In contrast, no Hho1p signal was detected on the *MFA1* locus in **a** cells (Figure 8C). As a control, Hho1p was found to bind to the *rDNA* repeating sequences, which is consistent with a previous report (Freidkin and Katcoff, 2001).

As shown in Figure 8D and 8E, Hho1p formed crosslinks in $\alpha$ cell, with highest efficiency to the region (-300 bp to +200) where the nucleosomes are positioned. No crosslink was observed at sequences upstream of the $\alpha$2 operator, or downstream the termination site of the *MFA1* codon. These results indicate that Hho1p binds to the repressed *MFA1* region and may play a role in the establishment of the highly organized chromatin structure.

## 4.4 Discussion

In *Saccharomyces cerevisiae*, transcriptionally inert regions of DNA form silenced domains. Mapping studies showed that arrays of precisely-positioned nucleosomes are associated with these domains (Ravindra et al., 1999; Simpson et al., 1993; Weiss and Simpson, 1997; Weiss and Simpson, 1998).

The size of these silenced domains varies. A silenced domain can be very large and may contain many genes. Some examples include telomeres and silenced mating type loci (Ravindra et al., 1999; Simpson et al., 1993; Weiss and Simpson, 1997; Weiss and Simpson, 1998). On the other hand, a silenced domain can be formed at the single-gene level. For example, a repressed domain can be formed along one of the **a** cell-specific gene loci in $\alpha$ cells (Cooper et al., 1994; Ducker and Simpson, 2000; Ganter et al., 1993; Patterton and Simpson, 1994; Roth et al., 1992; Shimizu et al., 1991; Simpson et al., 1993). These domains contain a well-organized nucleosomal array, which begins at the promoter, extends into the coding sequence, and ends just 30-70 bp downstream of the termination codon, without affecting either upstream or downstream regions (Simpson et al., 1993). However, whether and how these nucleosomes interact with each other remains unclear.

To address the correlation between higher order chromatin structure and gene repression, the MAP method has been developed to isolate a unique

gene locus as in vivo packed chromatin (Ducker and Simpson, 2000). By looking at the higher-order chromatin structure of MAP-isolated minichromosomes, a previous study (Ducker, 2001) in our lab found that the repressed *STE6* gene has a compact, "hairpin" like conformation. This structure contains two stacks of nucleosomes side by side. In this study, we reported the higher-order chromatin structure of the repressed *MFA1* locus under EM. Measurements of the structure in Figure 4.9 show that it is 10 nm wide and 20 nm long (Figure. 4.9). There is enough room to fit two nucleosomes side by side. These studies clearly show that in $\alpha$ cells, the nucleosomes associated with these **a** cell-specific genes adopt a highly ordered conformation, which is different from surrounding regions.

What forces then, would hold this structure together so tightly? We prefer the view that the Ssn6p/Tup1p complex bridges, or strengthens, the interactions among Mat$\alpha$2p, histones, and possibly, other proteins, based on the following characteristics. First, Tup1p and Ssn6p have been shown to bind directly to Mat$\alpha$2p (Smith and Johnson, 2000), the N-terminal tails of histone H3 and H4 (Edmondson et al., 1996; Huang et al., 1997), and histone deacetylases (HDACs) (Davie et al., 2002; Edmondson et al., 1998; Watson et al., 2000), *in vitro* and/or *in vivo*. Second, Tup1p can form a repressive chromatin structure by being artificially recruited (Tzamarias and Struhl, 1994). Third, a *TUP1* deletion leads to derepression of **a** cell-specific genes and disruption of the well organized chromatin structure (this study;(Cooper et al., 1994; Gavin et al., 2000). Fourth, by itself or in association with Ssn6p,

Tup1p can form a tetramer (Smith and Johnson, 2000; Varanasi et al., 1996), providing the advantage of being a connecter. Fifth, the number of Tup1p molecules interacting with repressed **a** cell-specific gene regions is approximately proportional to the positioned nucleosomes (this study and (Ducker, 2001), indicating that Tup1p is spreading along the entire chromatin domains. Finally, Tup1p was observed under EM to be present in the cavity of the "hairpin" structure of the *STE6* domain (Ducker, 2001).

In this study, we also assessed the stoichiometry of Tup1p with the nucleosomes of the repressed MFA1 gene. The results showed that Tup1p is associated with the MFA1 nucleosomes in a ration of (2N+4):N, where N is the number of nucleosomes along this region. This is consistent with our previous STE6 data. The extra Tup1p tetramer may contact the Mat$\alpha$2p dimer and bridge the interaction between Mat$\alpha$2p and histones (Figure 4.9).

Interestingly, our data strongly indicate that Hho1p plays a structural role in the *MFA1* locus. The structural role of linker histone has been described in other species (Shen et al., 1995; Widom, 1998). Whether or not Hho1p is the linker histone in yeast has been an elusive problem for many years (Landsman, 1996; Patterton et al., 1998; Ushinsky et al., 1997). Linker histones in other species have a central globular region and long N- and C-terminal basic tails. However, Hho1p has two globular domains, connected by a 42 amino acid long, lysine-rich domain. Hho1p also has N- and C-terminal basic tails, but the tails are shorter (Landsman, 1996). Here we confirmed by doing a western blot that Hho1p binds to the repressed *MFA1* (Figure 4.8A).

Moreover, ChIP assays show that Hho1p distribution is limited to the repressed chromatin region (Figure 4.8D and 4.8E). The binding of Hho1p would help to decide the sequence whereby the repressed **a** cell-specific gene domains are bent in half. Notably, all the **a** cell-specific genes have an even number of positioned nucleosomes associated with them when they are repressed. Also, the linker DNA between the two nucleosomes where the bend occurs has a unique micrococcal nuclease cutting pattern that differs from other linkers in the rest of the repressed domain (Figure 4.1 and Y.Tsukagoshi and R.T.Simpson, unpublished data). This strongly implies that there are some proteins binding on the linker regions of these domains. These two globular regions might be just what are needed to bind two nucleosomes at the "bend" site or the end of the hairpin, with the linker between them being determined by steric considerations and the length of peptide available between the two globular domains. In this regard, the globular region of mammalian H1 is thought to bind DNA at the entry/exit points from its path around the histone octamer in the nucleosomal core particle. Future studies will focus on the presence of Hho1p on these regions and the details of its structural role (see chapter V).

In summary, we have presented a model for the chromatin structure of the repressed *MFA1* locus as follows (Figure 4.9). The Mat$\alpha$2p/Mcm1p hetero-tetramer initiates the binding of Ssn6-Tup1 complex to this region. The Ssn6-Tup1 complex further recruits HDACs and positions the nucleosomes by interacting with the hypo-acetylated N-terminal tails of histone H3 and H4

(Bone and Roth, 2001; Watson et al., 2000). The recruitment of HDACs also ensures a more folded status of the chromatin (Annunziato and Hansen, 2000). Moreover, the binding of Hho1p ensures the proper bending of the linker DNA region and makes the compact chromatin structure more stable. It is possible that Hho1p does stabilize the end of the compact chromatin structure but Tup1p crosslinking of the terminal nucleosomes positions relative to each other suffices for the overall organization of the repressed domain.

This model provides a plausible explanation of how the **a** cell-specific genes can be repressed efficiently in $\alpha$ cells. Because of the extremely rapid dynamics of histone acetylation and deacetylation, in which a reversal of targeted acetylation occurs within 1.5 min (Katan-Khaykovich and Struhl, 2002), as well as the high average histone acetylation level in yeast (Waterborg, 2000), constant maintenance of histone deacetylation in chromatin is likely to be a critical requirement for transcription repression. The sequestration of the tails and recruitment of HDACs by Ssn6-Tup1 complex, in addition to the compact conformation, would prevent any modification of these tails that could lead to derepression of the gene. Furthermore, these results provide insight into the mechanism of how tissue-specific genes are regulated in higher organisms.

Figure 4.1: Chromatin structure of *MFA1* locus in $\alpha$ cells. (A) Schematic representation of the chromatin organization of the repressed *MFA1* locus in $\alpha$ cells. The positions of nucleosomes (ellipses), the $\alpha$2 operator (filled gray box), and the TATA box (open box) are shown. The figure was not drawn to scale. (B) and (C) Chromatin in nuclei isolated from wild type **a** and $\alpha$ yeast cells was digested with increasing amounts of micrococcal nuclease and subjected to primer extension analysis. N is naked DNA digested by micrococcal nuclease as a control for sequence specificity of the enzyme. The $\alpha$2 operator, the TATA box, and the start site of the MFA1 coding sequence are shown on the left of each gel. The inferred positions of nucleosomes in $\alpha$ cells are shown by ellipses with assigned numbers on the right of each gel.

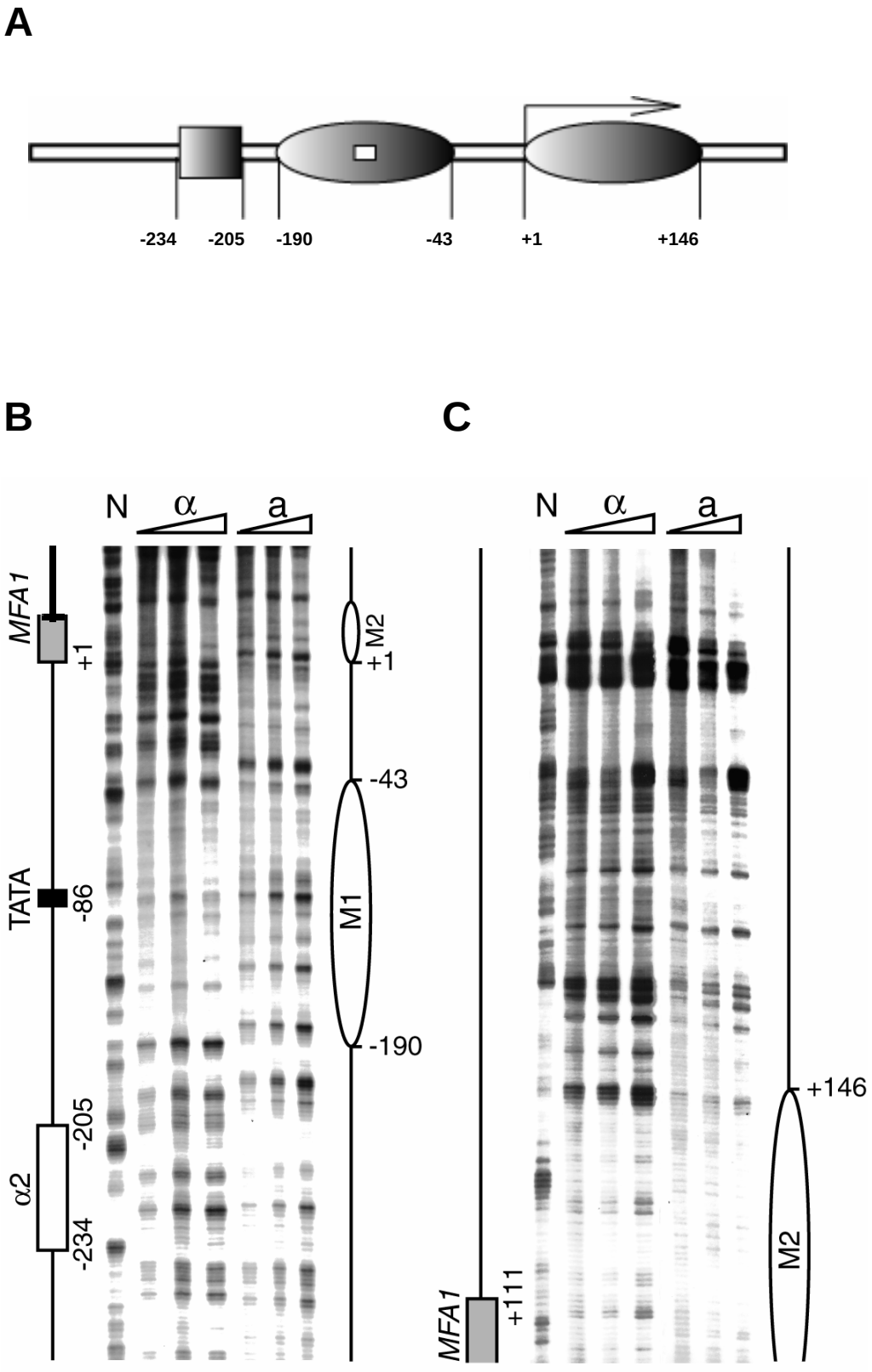

A
-234
-205
-190
-43
+1
+146
B
N
α
a
MFA1
+1
TATA
-86
α2
-205
-234
M2
+1
-43
M1
-190
C
N
α
a
MFA1
+111
+146
M2

Figure 4.2: Minichromosome construct. In the center is the unaltered TRP1/ARS1 minichromosome, showing the positions of the nucleosomes and nuclease-hypersensitive sites. The arrow represents the direction of transcription of the *TRP1* gene. In the expanded box at the bottom is a blow-up of the ARS region of the minichromosome showing the placement of the *lac* operator. Bases in bold are those shared between the B2 element and the *lac* operator. Expanded at the top is the *MFA1* insert for the minichromosome used in this study. The fragment was cloned into HSR B at the *EcoRI* site. Indicated are the $\alpha$2 operator and the translation start site of *MFA1* gene.

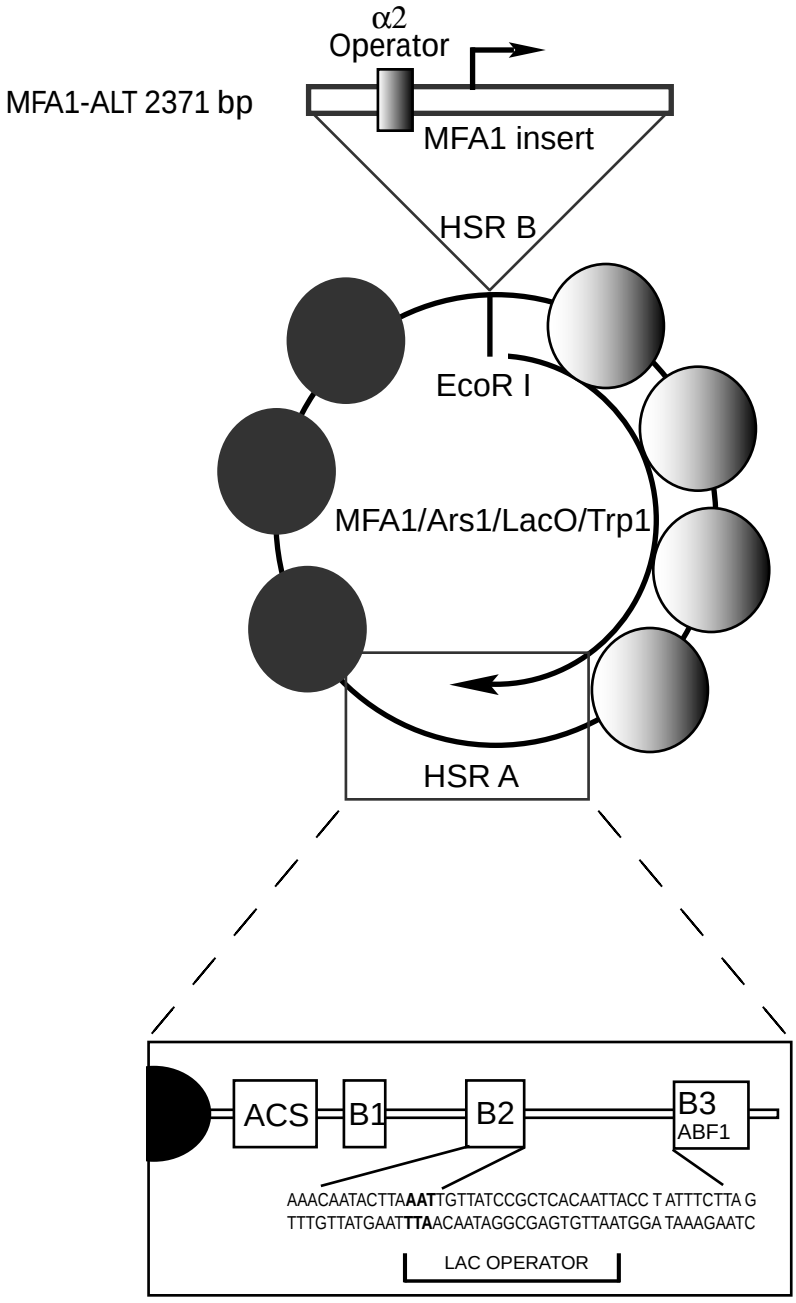

α2
Operator
MFA1-ALT 2371 bp
MFA1 insert
HSR B
EcoR I
MFA1/Ars1/LacO/Trp1
HSR A
ACS
B1
B2
B3
ABF1
AAACAATACTTAAATTGTTATCCGCTCACAATTACC T ATTTCTTA G
TTTGTTATGAATTTAACAATAGGCGAGTGTTAATGGA TAAAGAATC
LAC OPERATOR

Figure 4.3: Primer extension mapping of the chromatin structure of the MFA1-ALT minichromosome. The cleavage patterns were obtained by MNase digestion of MFA1-ALT minichromosome chromatin, MAP isolated from **a** and $\alpha$ cells. Lanes 1, 2, and 3 are DNA from nuclei isolated from $\alpha$ cells carrying the MFA1-ALT minichromosome and digested with three concentrations of MNase. Lanes 4, 5, and 6 are DNA from nuclei isolated from **a** cells carrying the MFA1-ALT minichromosome and digested with three concentrations of MNase. Lane 7 (0) is the undigested control. Lane 8 (D) exhibits the protein-free DNA digested with MNase *in vitro*. M is the marker DNA fragments corresponding to 726, 713, 553, 500, 427, 413, 311, 249, 200, 151, and 140 nucleotides from *HinfI* digest of ΦX174 RF DNA. The $\alpha$2 operator consensus sequence is shown by a filled gray box, and the ellipses correspond to inferred positions of nucleosomes in $\alpha$ cells. Numbers on the left side correspond to their distance from the A residue of the initiation codon for the *MFA1* gene.

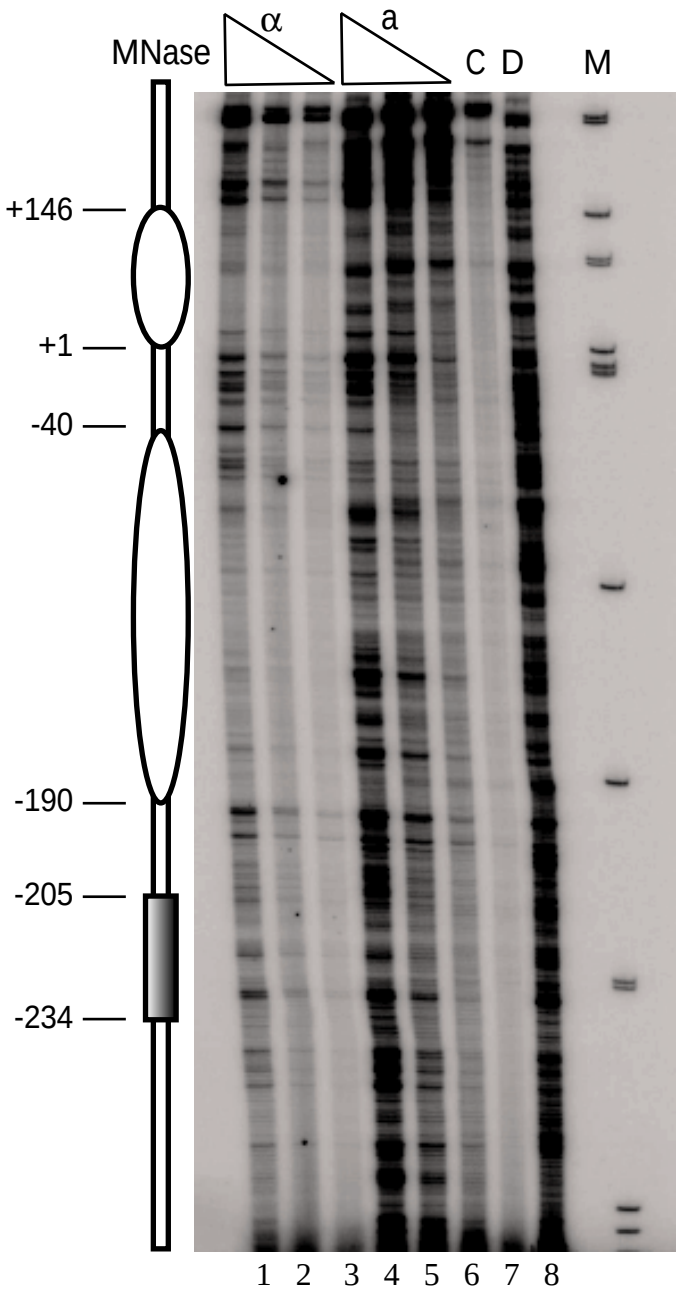

MNase
α
a
C D
M
+146
+1
-40
-190
-205
-234
1 2 3 4 5 6 7 8

Figure 4.4: Electron micrographs of MFA1-ALT minichromosomes isolated from $\alpha$ cells, negatively stained with uranyl acetate. The arrowheads indicate the putative region of the minichromosome showing the compact conformation of the *MFA1* nucleosomes.

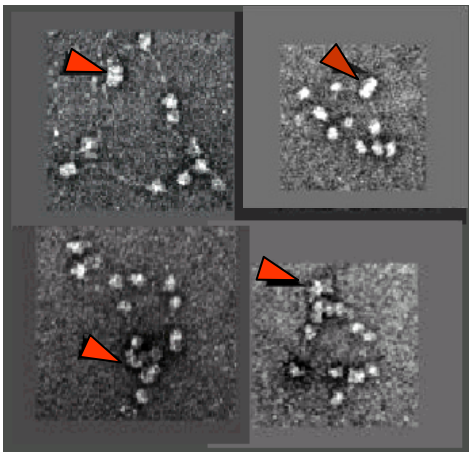
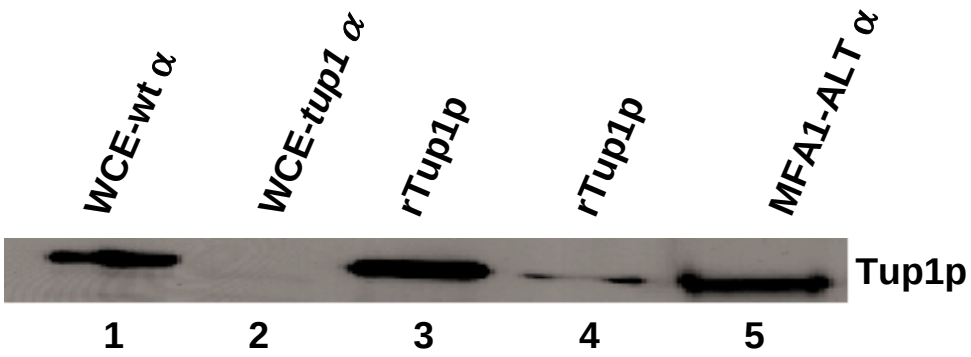
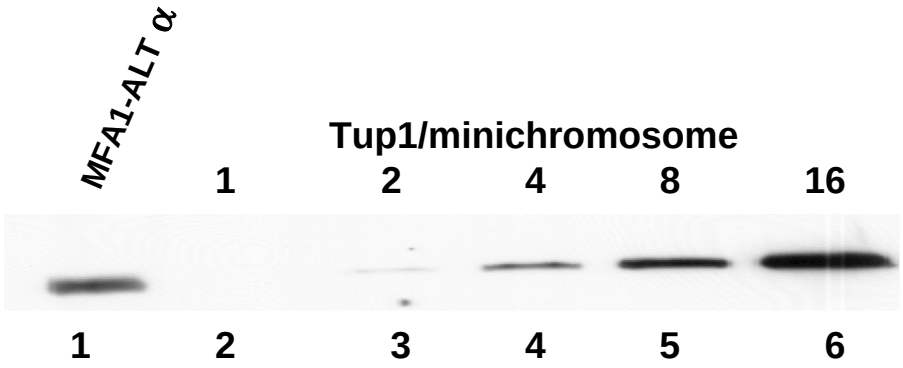


Figure 4.5: Western blot analysis of the affinity-purified MFA1-ALT minichromosome probed with anti-Tup1p antibodies. (A) Lane 1 shows the whole cell extract from wild type $\alpha$ cells. Lane 2 contains the whole cell extract from *tup1* mutant $\alpha$ cells. Lanes 3 and 4 are two titrations of *E. coli* expressed recombinant Tup1p. Lane 5 is MFA1-ALT minichromosome isolated from wild type $\alpha$ cells. (B) Lane 1 is MFA1-ALT minichromosome isolated from wild type $\alpha$ cells. Lanes 2-6 are a titration series of *E. coli* expressed recombinant Tup1p. Each lane in the titration series represents the indicated molar ratio of rTup1p to the MFA1-ALT minichromosome. (C) Densitometry of the Western blot analysis shows 7.7 copies of Tup1p molecules per MFA1-ALT minichromosome. Statistical analysis of two replicates of this experiment shows 7.7$\pm$1.3 copies of Tup1p per MFA1-ALT minichromosome. For the graph, the signal for lane 5 (8 Tup1p molecules per MFA1-ALT minichromosome) was arbitrarily defined as 100.

A


WCE-wt α
WCE-tup1 α
rTup1p
rTup1p
MFA1-ALT α
Tup1p
1
2
3
4
5

B


MFA1-ALT α
Tup1/minichromosome
1
2
4
8
16
1
2
3
4
5
6

C

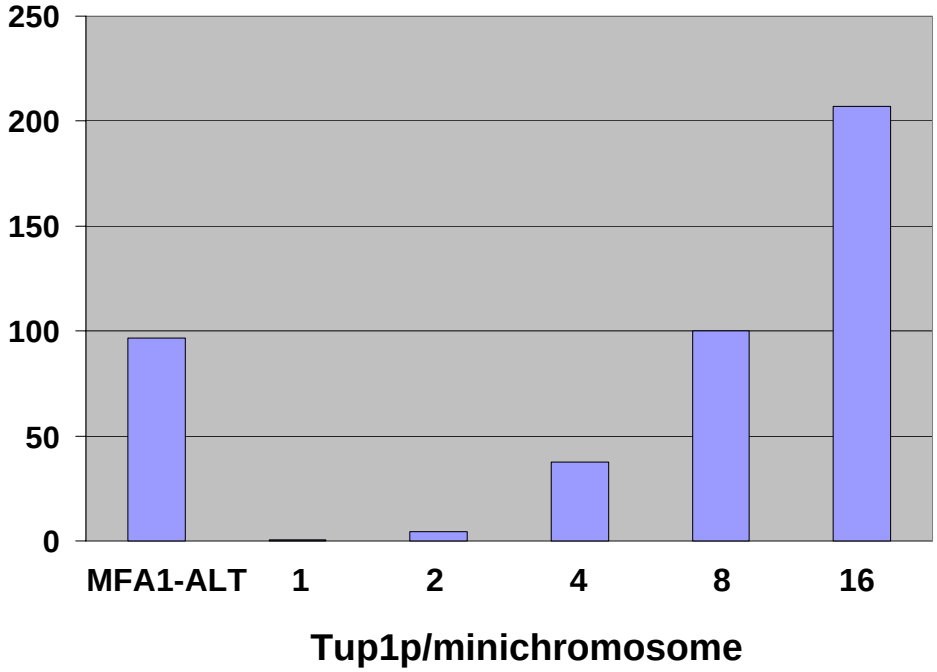
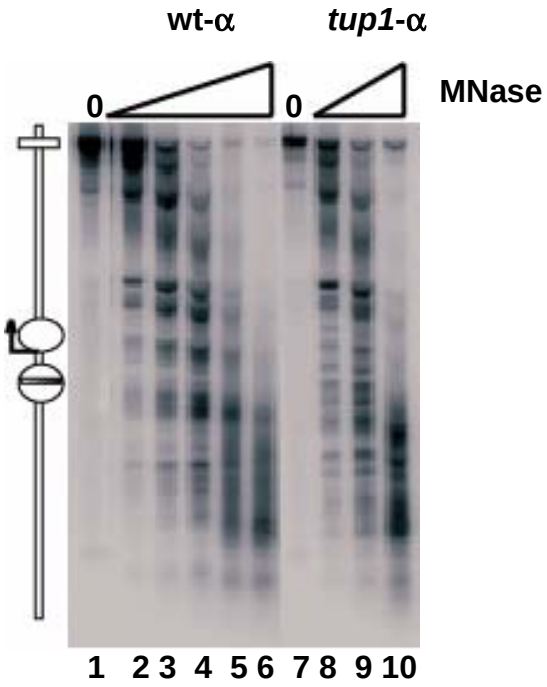

250
200
150
100
50
0
MFA1-ALT
1
2
4
8
16
Tup1p/minichromosome

Figure 4.6: Nucleosome mapping of *MFA1* in a *tup1* mutant strain. (A) Indirect end-labeling mapping of the chromatin structure of the *MFA1* locus. Chromatin in nuclei isolated from either wild type $\alpha$ cells (lanes 1-6) or *tup1* mutant $\alpha$ cells (lanes 7-10) was digested with increasing amounts of MNase. The purified MNase-cleaved DNA was subsequently digested to completion with *EcoRI* and electrophoresed on a 1.4% agarose gel, transferred to a membrane and probed with an [$\alpha$-$^{32}$P]dATP random primer-labeled fragment. This fragment is from (-312) to (+201) which includes the ORF of *MFA1*. The inferred positions of the TATA box (the filled gray box), the start site and the direction of the open reading frame (the arrow), the nucleosomes (filled ovals), and the full length fragment (the open box), are shown to the left of the gels. (B) Analysis of *MFA1* mRNA levels in wild type **a** and $\alpha$ strains and in *tup1* mutant **a** and $\alpha$ strains. *SCR1* is a loading control.

**A**




**B**

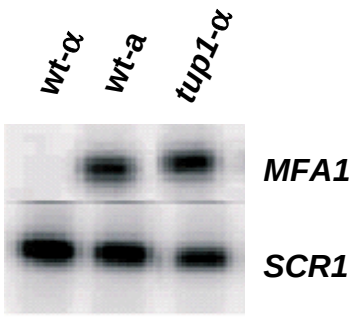


Figure 4.7: Chromatin immunoprecipitation assay for Tup1p binding. Sonicated chromatin was prepared from formaldehyde-fixed wild type $\alpha$ (A) and **a** (C) cells. Immunoprecipitations were carried out using polyclonal antibodies to Tup1p. The location of the PCR primer sets is given in kilobases with the starting ATG as a reference (0.0 kb). As a control, a fragment of the UAS of the *SUC2* gene was also amplified. All PCR primer sets were designed to generate ~200 bp products. The $\alpha$2 operator spans positions from -234 to -205. The bar graphs of the densitometry represent the signals from $\alpha$ cells (B) and **a** cells (D). For the graph, four independent experiments were averaged and the error bars are shown. Quantitative PCR products from one representative experiment are shown in (A) for $\alpha$ cells and (C) for **a** cells. The Tup1p occupancy for the -0.20 kb fragment from $\alpha$ cells was arbitrarily defined as 100. The signals of $\alpha$ cells and **a** cells are normalized based on the signals of the *SUC2* fragment from these two cell types.

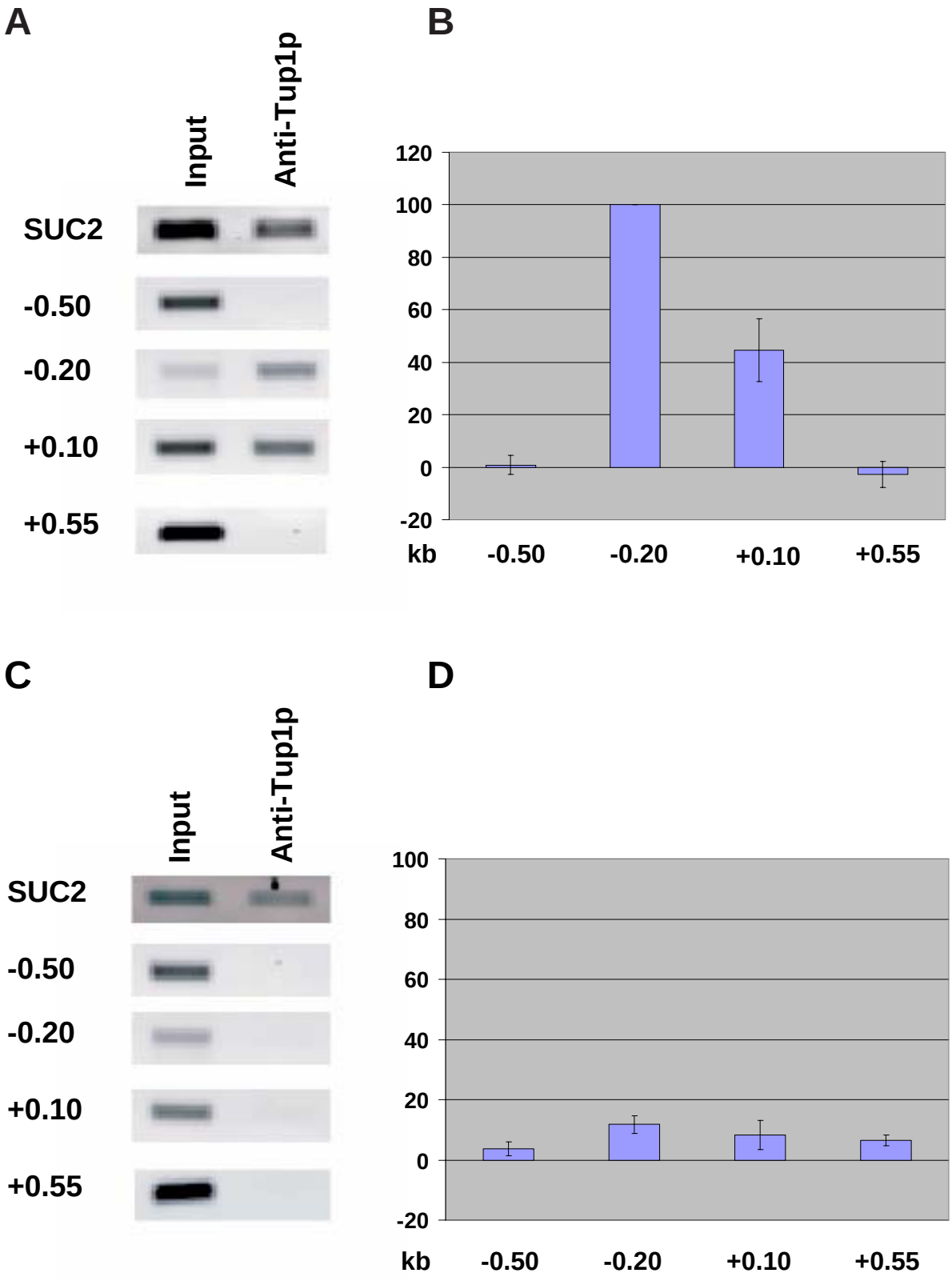

A
Input
Anti-Tup1p
SUC2
-0.50
-0.20
+0.10
+0.55
B
120
100
80
60
40
20
0
-20
kb
-0.50
-0.20
+0.10
+0.55
C
Input
Anti-Tup1p
SUC2
-0.50
-0.20
+0.10
+0.55
D
100
80
60
40
20
0
-20
kb
-0.50
-0.20
+0.10
+0.55

Figure 4.8: Hho1p binds to *MFA1* region in $\alpha$ cells. (A) Western blot analysis of affinity-purified MFA1-ALT minichromosome probed with anti-Hho1p antibodies. Lane 1 contains MFA1-ALT minichromosome isolated from $\alpha$ cells. Lane2 is ALT minichromosome isolated from $\alpha$ cells. (B), (C), (D), and (E) show chromatin immunoprecipitation assays for Hho1p binding. Sonicated chromatin was prepared from the formaldehyde-fixed wild type $\alpha$ (B, D and E) and **a** (C) cells. Immunoprecipitations were carried out using polyclonal antibodies to Hho1p. Two fragments covering the promoter region of *PGK1* gene and a part of the rDNA sequence (Freidkin and Katcoff, 2001) were used as controls in (B) and (C), respectively. In (D) and (E), the location of the PCR primer sets is given in kilobases with the starting ATG as a reference (0.0 kb). The $\alpha$2 operator spans positions -234 to -205. The bar graphs of the densitometry represent the signals from $\alpha$ cells (E). For the graph, three independent experiments were averaged and the error bars are shown. Quantitative PCR products from one representative experiment are shown in (D). All PCR primer sets shown in this figure were designed to generate ~200 bp products. The Hho1p occupancy for the -0.15 kb fragment from $\alpha$ cells was arbitrarily defined as 100.

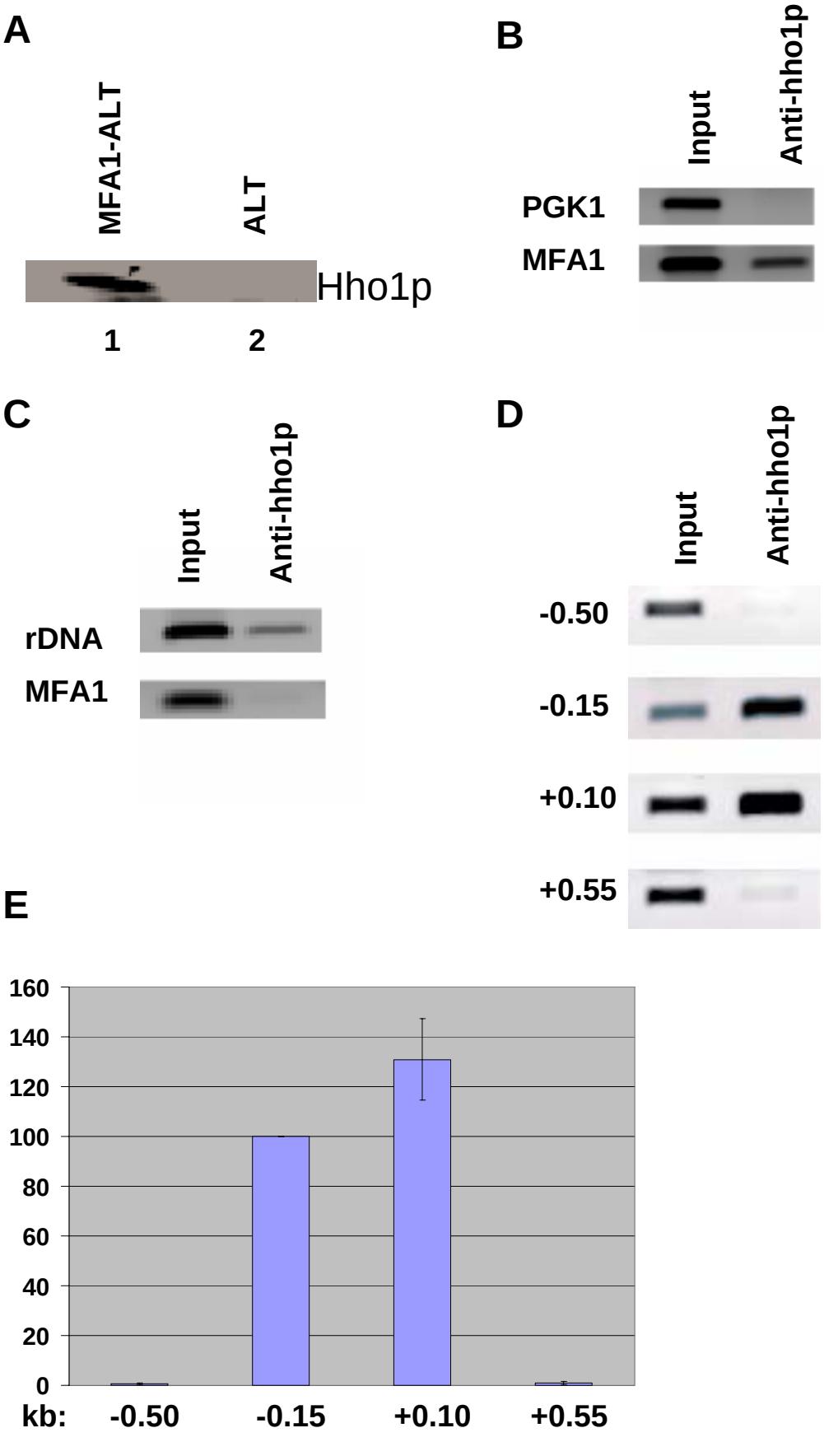
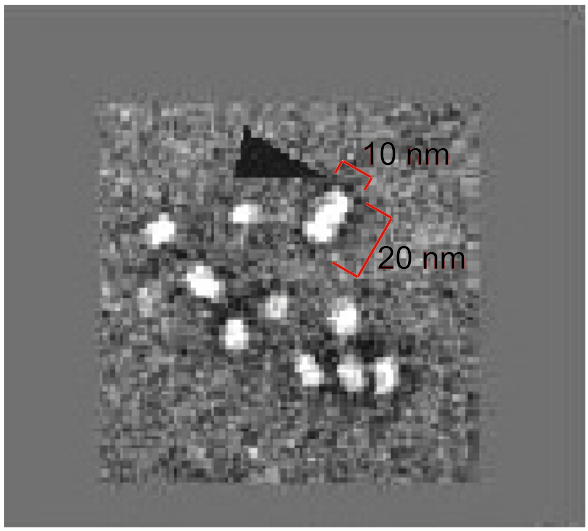
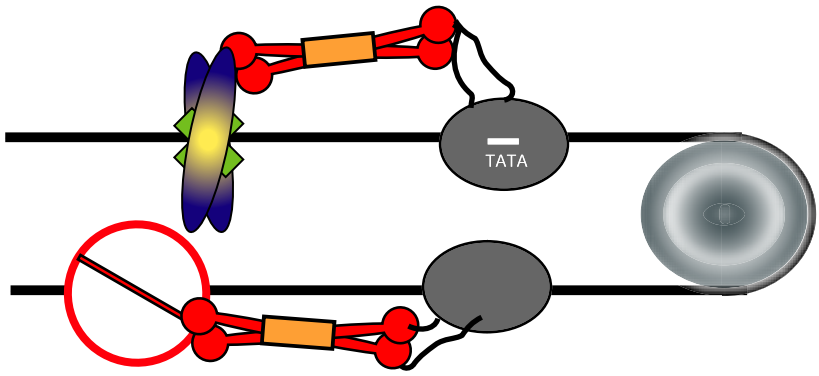

A
MFA1-ALT
ALT
Hho1p
1
2
B
Input
Anti-hho1p
PGK1
MFA1
C
Input
Anti-hho1p
rDNA
MFA1
D
Input
Anti-hho1p
-0.50
-0.15
+0.10
+0.55
E
160
140
120
100
80
60
40
20
0
kb:
-0.50
-0.15
+0.10
+0.55

Figure 4.9: Model for repression of *MFA1* gene in $\alpha$ cells.Tup1 is recruited to the *MFA1* gene by MAT$\alpha$2p and interacts with the H3/H4 tails forming a scaffold, which extends from the $\alpha$2 operator to the 3' end of the gene. Hho1p binds to the long linker between the two well-positioned nucleosomes. All the interactions contribute to the formation of the compact "tip of the hairpin without a stem" chromatin structure of the region. The figure was not drawn to scale and only two of the four histone N-terminal tails are drawn.




**The Players**

Mcm1p
MATa2p
Ssn6p
Tup1p
Hho1p
Stop code
Nucleosome
H3/H4Tail

## Acknowledgements

Yuko Tsukagoshi performed the primer extension mapping experiments shown in figure 4.1. We thank Dr. Chris Woodcock for EM work, Joe Reese and Hugh Patterton for antibodies, Christopher J. Graham for reading the manuscript, Charles E. Ducker and John D. Diller for technique help. We thank members of the labs of Drs. Robert Simpson, Jerry Workman, Joe Reese, and Song Tan for many valuable discussions. This work is supported by a grant from NIGMS to RTS.

# Chapter V

## Speculation on future studies and aims

## 5.1 Improvement of *in vivo* DNase I mapping

We have shown that *in vivo* DNase I mapping is a promising tool to investigate chromatin structure (chapter II) and the interaction between DNA and non-histone proteins (chapter II and III) in living yeast cells. However, some technical problems still exist. First, the digestion level is too low for indirect end labeling to check cutting patterns. Thus, the primer extension is obligatory. Current protocol requires induction time of at least 4-6 hours. Second, prior to galactose induction of nuclease expression, yeast cells need to grow in medium containing lactic acid as a carbon source, to relieve the repression from dextrose. In this medium, yeast cells grow very slowly (the double time is ~ 31 hours!).

To address these problems and increase the sensitivity of this strategy, we and our collaborator (Dr. Mike Kladde, Texas A&M University) will employ the following strategies. It was realized that the extremely low quantities of the Gal4p protein is rate-limiting for maximal induction of expression of genes driven by GAL promoters (Mylin et al., 1990; Schultz et al., 1987), especially when the desired GAL-promoter-gene fusion construct is carried on a high copy number plasmid (Mylin et al., 1990; Schultz et al., 1987), e.g. in the case of this study. Hence, increasing Gal4p amount would increase expression of target genes (Mylin et al., 1990; Mylin and Hopper, 1997; Sil et al., 2000). Our attempts using a vector bearing GAL10-promoter-Gal4p (Sil et al., 2000) failed (data not shown) for unknown reasons. However, it is still worthwhile to

express DNase I in a cell strain bearing the GAL10-promoter-Gal4p cassette in the genome (Mylin and Hopper, 1997).

Since DNase I is a foreign protein in yeast cells, it may be degraded rapidly by proteases, so expressing DNase I in protease deficient strains (Emr, 1990; Jones, 1991) may benefit our studies, and hence increase the sensitivity of this strategy.

In addition, an alternative strategy other than the galactose inducible system can be employed to clone and express DNase I. A more tractable system is based on regulatory elements of the xenobiotic *E. coli Tn-10*-specified tetracycline-resistance operon, through which the tetracycline repressor (*tet*R) negatively regulates transcription of several resistance-mediating genes (Hillen et al., 1983; Hillen et al., 1984; Klock et al., 1985). Presence of tetracycline related compounds releases *tet*R from its binding sites (*tet*R operators) located within the promoter region of the operon and derepresses transcription of target genes (Hillen et al., 1983; Hillen et al., 1984; Klock et al., 1985). This system has been applied in yeast (Dingermann et al., 1992), plant (Gatz et al., 1991; Gatz and Quail, 1988; Weinmann et al., 1994) and mammalian cells (Gossen and Bujard, 1992; Gossen and Bujard, 2002; Shockett et al., 1995). Superiority of this system is based on these observations: (1) specificity of *tet*R for its operator sequence (Hillen et al., 1983; Hillen et al., 1984; Klock et al., 1985); (2) high affinity of tetracycline for *tet*R (Takahashi et al., 1986); (3) well-studied chemical and

physiological properties of tetracycline; and (4) the absence of requirements for switching medium or temperature.

## 5.2 Further applications of MAP in exploring mechanisms of gene repression

MAP has been proved to be the technique of choice for exploring structure and composition of chromatin packaged *in vivo* under different functional states. Future studies will focus on the following areas.

### 5.2.1 Is the compact chromatin structure specific for a cell-specific genes?

We observed that for two of the repressed **a** cell-specific gene domains, the *STE6* domain (Ducker, 2001) and the *MFA1* domain (this study), there is a highly ordered, compact, "hairpin" like chromatin structure. We propose that Ssn6p-Tup1p bridges the nucleosomes and that Hho1p also plays a role in stabilizing this structure. It would be informative to employ the same strategy in looking at the higher chromatin structure of the following constructs.

- **a** cell-specific genes with a mutation of the Mcm1p binding site (GG:CC). This is a mutant which shows many interesting characteristics (table 5.1). This construct may provide information about the role of (1) Ssn6p-Tup1p; (2) Mat$\alpha$2p- Mcm1p; (3) histone acetylation; and (4) transcription, in the formation and/or

maintenance of the compact chromatin structures of the repressed **a** cell-specific gene domains.

- Other Ssn6p-Tup1p controlled genes, such as *RNR2* and *SUC2*. It has been shown that different activators and/or repressors can induce different acetylation states (Davie et al., 2002; Deckert and Struhl, 2001). Furthermore, the distribution of Tup1p is different between the **a** cell-specific genes and DNA damage response genes (Davie et al., 2002). Finally, the damage response genes have basal level of transcription, in contrast to the **a** cell-specific genes in $\alpha$ cells. Therefore, these constructs would tell us about (1) whether Ssn6p-Tup1p associates with these genes in the same way as with **a** cell-specific genes; (2) whether different repressors initiate different chromatin structure in the sense of higher order conformation; and (3) what the effect of basal transcription may be on the formation and maintenance of certain higher order chromatin structure; and (4) different roles of Ssn6p and Tup1p in different context.
- Other repressed genes not controlled by Ssn6p-Tup1p, such as *PHO5*. These constructs may further elucidate the relationships between Ssn6p-Tup1p and special higher order chromatin structure.

### 5.2.3 The distribution of Ssn6p-Tup1p complex along repressed domains

We proposed that the Ssn6p-Tup1p complex spreads along the entire repressed **a** cell-specific gene domains (Ducker, 2001). To confirm this conclusion, we plan to perform following experiments. The first strategy, explained in Figure 5.1, is a revised quantification method, which bypasses the requirement for quantifying the amount of minichromosome DNA. We have obtained purified recombinant Mcm1p from Dr. Song Tan and an antibody to the protein is commercially available. The only potential problem with this strategy is the possibility that Mcm1p may also bind to regions on the MFA1-ALT other than the $\alpha$2 operator. This possibility can be tested by using ALT as a control.

Another technique of choice is immuno-gold EM, which employs antibodies coupled to electron-dense material (about the technique, see (Woodcock, 1989). This technique will show directly the distribution of the Ssn6p-Tup1p complex along the isolated minichromosomes.

### 5.2.4 Deeper investigations of Hho1p function

Interestingly, we have observed that Hho1p is associated with the repressed *MFA1* locus in $\alpha$ cells. Hho1p is very unusual for an H1 histone. Instead of having a central globular region and long N- and C-terminal basic tails, the yeast protein has two globular domains, connected by a 42 amino acid long, lysine rich domain, and has shorter basic amino- and carboxyl-terminal tails. The globular region of mammalian H1 is thought to bind DNA at the entry/exit points from its path around the histone octamer in the chromatin

core particle (Vignali and Workman, 1998). Two globular regions might be just what is needed to bind two nucleosomes at the end of a hairpin, with the linker between them being determined by steric considerations and the length of peptide available between the globular domains. To further elucidate the functions of Hho1p, much work on this subject lies ahead.

- The *hho1* deletion and its effects;

  We will perform detailed analysis of micrococcal nuclease cutting of the long linker in the *MFA1* gene chromatin in wild type and *hho1* deletion strains of yeast. Since it is possible that like many elements in yeast structure determinants are redundant, we will also assess the structure of the long linker in a *tup1* deletion strain and a double mutant, *hho1/tup1,* or: in a GG:CC *mfa1* mutant strain and a double mutant, *hho1/*GG:CC. We anticipate that the distinctive cutting pattern in the *MFA1* linker will be lost in the absence of Hho1p. We attribute inactivity of the gene in the mutant to redundancy of organization of repressive chromatin structure.

- Immuno-EM staining;

  This will be done as described (Frado et al., 1983).

- Where else does Hho1p bind?

  There are around 2,000 molecules of Hho1p in each yeast cell. Therefore, Hho1p must bind to regions other than the *MFA1* domain. First, we will do western blots to check whether Hho1p associates with minichromosomes containing other **a** cell-specific genes and

repressed genes mentioned above. The presence of Hho1p in the genomic copy of these loci will be confirmed by ChIP. Finally, to determine more regions where Hho1p functions, we would carry out genome-wide location analysis for Hho1p using a method that combines the micro-array technique and ChIP together (ChIP-chip). This method has been used successfully to identify genomic binding sites for many other DNA associated factors (Ren et al., 2000; Simon et al., 2001; Wyrick et al., 1999). The facility for gene and interenic microarray is available at our university.

A. Sequence of the Mat$\alpha$2-Mcm1 operator in wild type *STE6* locus and the GG:CC mutation:

| | Mat$\alpha$2 site A | Mcm1 site | Mat$\alpha$2 site B |
|---|---|---|---|
| | CATGTAATTACCTAATAGGGAAATTTACACG | | |
| GG:CC: | | GG CC | |

B. Characteristics of wild type and GG:CC mutant constructs:

| | GG:CC $\alpha$ | Wild type $\alpha$ | Wild type **a** |
|---|---|---|---|
| Transcription[1,2] | - | - | + |
| Positioned nucleosomes[1] | +* | + | - |
| Mcm1p[2] | - | + | + |
| MAT$\alpha$2p[2] | - | + | - |
| Ssn6p-Tup1p[2] | - | + | - |
| Acetylation level of histone tails[2] | High | Low | High |
| Hairpin[2] | ? | + | - |

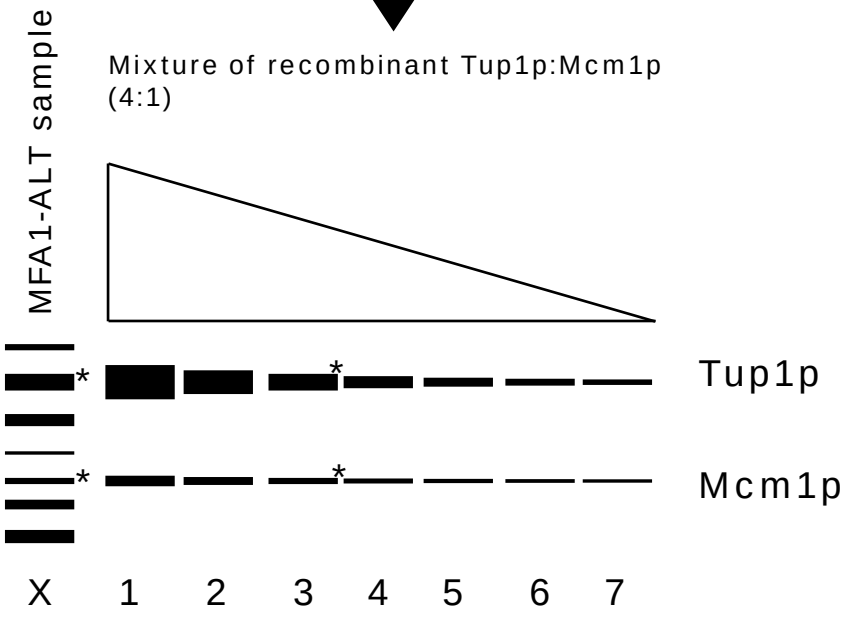
*: the positioning of nucleosomes is different from that in wild type $\alpha$ cells[1];
References:
[1]: (Gavin et al., 2000);
[2]: (Ducker, 2001).

Table 5.1: Effects on chromatin structure of Mcm1p binding at the *STE6* locus.

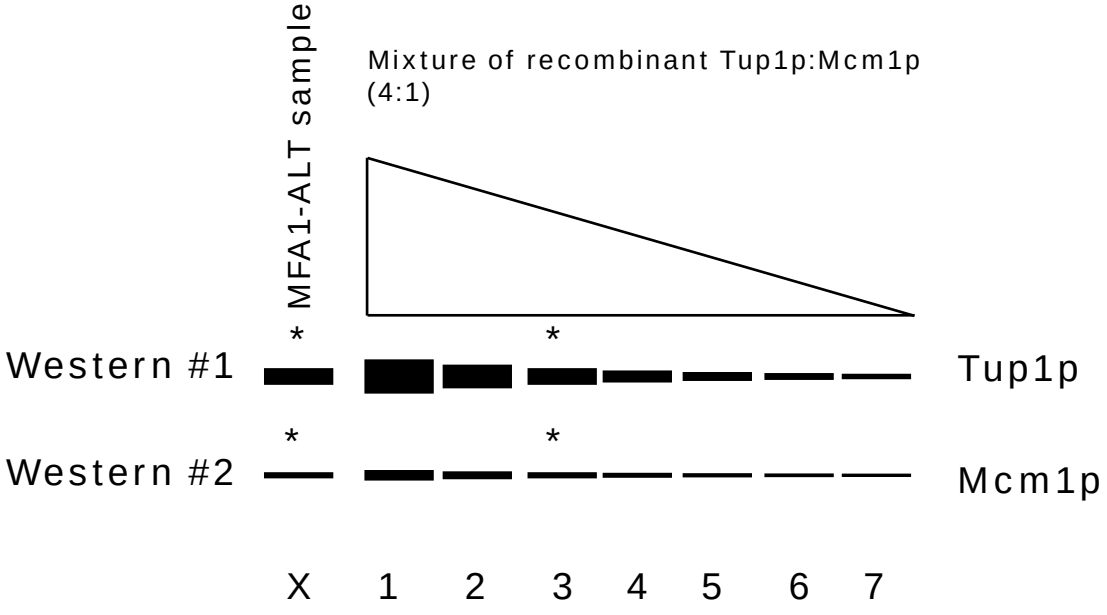
Figure 5.1: The schematic of the experiment to determine the ratio between Mcm1p and Tup1p associated with the MFA1-ALT minichromosome. This experiment is based on the fact that two molecules of Mcm1p bind to one copy of the *MFA1* region in the MFA1-ALT minichromosome.

1. Mix recombinant Tup1p and Mcm1p at a molar ratio of 4:1;

2. Load the isolated MFA1-ALT sample and a series of dilutions of the mixture on SDS-PAGE gel;




3. Check the stoichiometry between Tup1p and Mcm1p by doing Western using antibodies against Tup1p and Mcm1p sequentially.



# Summary

In eukaryotic cells, transcription, replication, recombination, and other functions of DNA all take place in the context of chromatin, the complex of the nucleic acid with histones and other proteins. Increasingly, the relevance of structural features of chromatin to these functions of DNA is appreciated. However, detailed knowledge and experimental criteria for chromatin organization beyond the nucleosome are still needed to fully understand the regulation of these DNA processes. To address these issues, the work described in this thesis was focused on several newly developed methods of analyzing active or repressed chromatin structures involving non-histone proteins and higher-order nucleosomal interactions *in vivo*.

In the first part (chapter II and III), we described the establishment and application of a new *in vivo* chromatin structure mapping strategy. Previously, we developed the methylase probing assay (Kladde et al., 1999b), a new methodology for the analysis of chromatin structure. This method allows detecting both histone-DNA and non histone-DNA interactions in living yeast cells. However, this method has two disadvantages that greatly affect its application. One is the sequence specificity of these methylases, which limits it resolution. The other one is the fact that many species have endogenous methylases. Here, we extend this strategy to DNase I, a nonspecific nuclease. DNase I has been the most widely used enzyme to detect

chromatin sites where DNA is active in transcription, replication or recombination. The cloning and expression of bovine pancreatic DNase I in yeast cells provides a powerful tool in chromatin structure mapping. Utilizing this sensitive and high-resolution assay, we detected a labile repressor binding to its cognate sites *in vivo.* These data demonstrated the validity and efficacy of this strategy. Investigation of the inter-nucleosome linker regions in several types of repressed domains has revealed different degrees of protection in cells, relative to isolated nuclei. Our data clearly showed that the HMR locus is less compact than repressed **a** cell-specific genes and the recombination enhancer. This observation correlates well with our EM images of these loci. Furthermore, the relatively less compact chromatin structure of HMR may be necessary for karyoskeleton interaction and this would explain the seeming paradox of a chromatin structure that precludes transcription yet is perfectly appropriate for recombination or transposon integration (Haber, 1998a; Haber, 1998b; Zou et al., 1996; Zou and Voytas, 1997; Zou et al., 1995).

Using this strategy, we further investigated the working mechanisms by which TBP regulates transcription *in vivo*. In contrast to the results obtained from previous studies, which suggested that promoters of active genes are hypersensitive to nucleases in isolated chromatin, we found that in living cells, these sites are protected from DNase I relative to surrounding regions. ChIP assays confirmed this conclusion. Then, we used ChIP and quantitive PCR to

investigate two sets of genes that are coordinately regulated: four **a** cell-specific genes (*MFA2, MFA1, STE6* and *BAR1),* and four arginine-rich histone genes (*HHT1, HHT2, HHF1* and *HHF2*). We found that approximately equal amounts of TBP are associated with the promoters of these genes in each group, irrespective of the transcription level. In contrast, the amount of RNA polymerase II associated with gene promoters is roughly proportional to the transcription level. Our results, in addition to the suggestions that the promoters of active genes are nucleosome free, suggest that TBP may occupy the promoter region of active genes through multiple rounds of transcription, and that binding of TBP to DNA is not a rate limiting step in the activation of transcription reinitiation in living cells.

In the second part (chapter IV), we described a comprehensive investigation of the repressed *MFA1* domain *in vivo* using multiple methods, including the newly developed MAP technique. The MAP protocol provides an opportunity to directly investigate the formation of higher order chromatin structure at any unique gene. Through EM imaging using negative staining for the MAP isolated MFA1-ALT minichromosome, we have observed a unique higher order chromatin structure associated with the repressed *MFA1* locus. This structure explains the fact that this gene is never depressed throughout the life of the MAT$\alpha$ cells. Western blot and ChIP assays also suggest that this structure appears to involve both histones and non-histone proteins that would hold the structure together. We confirmed that the Ssn6-Tup1 complex

plays an important role in the repression of *MFA1* gene. Interestingly, we also found for the first time that Hho1p is binding to a repressed locus *in vivo*.

In the third part (chapter V), we outlined the future studies and aims that are suggested by our preliminary data. We will improve the DNase I *in vivo* mapping strategy by increasing sensitivity. And obtaining EM images of more regions will clearly show the involvement of higher order chromatin structure in the repression of genes.

In summary, the research described in this thesis extends the previous studies by disclosing the unique features of the transcription regulation in living yeast cells. Our data provide evidence that in living cells where the situation is far more complicated than in simple, purified biochemical systems, the chromatin may function and be targeted in a different mode. While we have not yet determined the exact nature of the relationship between the chromatin function and structure, direction for future studies can be proposed based on our results. In regard to technology development, application of *in vivo* DNase I mapping and the MAP technique to the analysis of chromatin structure in living cells makes them very powerful strategies for the study of multiple fields. This approach should not be limited only to the study of transcription. For example, the idea of expressing DNase I in living cells is intriguing not only because of the application of mapping protein-DNA interaction. It also can be used to induce DNA damage and far reaching cell

death. It is noteworthy that the expression of DNase I will eventually induce cell death without disrupting the cell membrane and/or cell wall (data not shown). This may provide a new pathway to induce apoptosis. Finally, both the *in vivo* DNase I mapping strategy and the MAP methodology can be applied to investigate similar projects in higher organisms.

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# Appendix

Table A.1: Primers used in ChIP PCR reactions

| Name | Sequence |
|---|---|
| PGK1-W* | 5'-CAAGGGGGTGGTTTAGTTTAGTAGAACC-3' |
| PGK1-C* | 5'-CCTTCAAGTCCAAATCTTGGACAGAC-3' |
| YCL056C-W | 5'-CAAGCAGCGAACTTACACCACTCC-3' |
| YCL056C-C | 5'-CAAAGTATGGGACAAGCATTTCGCCC-3' |
| STE3-W | 5'-CTTTTCAAAAGACTTCTGCCC-3' |
| STE3-C | 5'-CACCACCAGAAGCGTTCTGGC-3' |
| STE6-TATA-W | 5'-AATAGGGAAATTTACACGCTGC-3' |
| STE6-TATA-C | 5'-GTGAACGTAACAACGGGAGATAG-3' |
| STE6-ORF-W | 5'-CCAATTGAGAGTTAAAACTTTCCAC–3' |
| STE6-ORF-C | 5'-CATATTGACGCATGAGTTGAGCC-3' |
| MFA2-W | 5'-CGAGAGGAAAAAGCTGTTGCATTAC-3' |
| MFA2-C | 5'-CCTTCTGAGTGGCTTGTGTGGA-3' |
| MFA1-W | 5'-CAAACGAGTGTGTAATTACCC-3' |
| MFA1-C | 5'- CCCTCTCATTAATTCATTTCTGGC-3' |
| BAR1-W | 5'-CGACAATAACATGTATACACAGCC-3' |
| BAR1-C | 5'-CGAACCACTAGAATTAAATCACGC-3' |
| SUC2-W | 5'-CCATTATGAGGGCTTCCATTATTC-3' |
| SUC2-C | 5'-ATCACATTCCTCGTCAGTTTTTCC-3' |
| HHT1-W | 5'-CCACGGCTCCTTGTTGAAATAC-3' |
| HHT1-C | 5'-CAGTGGACTTTCTTGCTGTTTGC-3' |
| HHT2-W | 5'-ATTGTTTTCTTGGGGCTTTACC-3' |
| HHT2-C | 5'-CGTTGCTTCTTGTGACCGC-3' |
| HHF1-W | 5'-ACGCTTGGCACCACCTTTAC-3' |
| HHF1-C | 5'-ATTGGTTGTGGAAAAGGTCTAA-3' |
| HHF2-W | 5'-CGTGTTTGTGCGTATGTAGTTAT-3' |
| HHF2-C | 5'-TAGACCTTTACCACCTTTACCTC-3' |
| MFA1 locus: | |
| (-0.50)-W | 5’-CAAAGATGCTGTACCGTTCAC-3’ |
| (-0.50)-C | 5’-CTTATGCCACGTTGCACACTATC-3’ |
| (-0.20)-W | 5’-CTGTTTCAGTGTTCAGAAAAAAGGC-3’ |
| (-0.20)-C | 5’-CCCTCTCATTAATTCATTTCTGGC-3’ |
| (-0.15)-W | 5’-CTACTGCTACGGTTGGCCCATAC-3’ |
| (-0.15)-C | 5’-CCCTCTCATTAATTCATTTCTGGC-3’ |
| (+0.10)-W | 5’-CGAATAGAAATGCAACCATCTACC-3’ |
| (+0.10)-C | 5’-CTAAAAAGAAATATTACGAACAAAC-3’ |
| (+0.55)-W | 5’-CATCGGCTTCGACCTCCTCCTTATC-3’ |
| (+0.55)-C | 5’-CGTCAATGGACAGACGACAATTAAC-3’ |

*: W: Watson Strand, C: Crick Strand.

# *VITA*

## Xi Wang

**Education:**

1994-1997 M.S. In Biophysics, Peking University, P. R. China.

1989-1994 B.M. In Medicine, Beijing Medical University (Medical School of Peking University), P. R. China.

**Publications:**

Wang, X., and Simpson, R. T. (2003) Involvement of higher order chromatin structure in repression of *MFA1* gene in yeast cells. (In preparation)

Wang, X., and Simpson, R. T. (2003) TATA box binding protein persists at active promoters *in vivo*. (Submitted)

Wang, X., and Simpson, R. T. (2001) Chromatin structure mapping in *Saccharomyces cerevisiae in vivo* with DNase I. *Nucleic Acids Res.* 29:1943-1950.

**Presentations**:

2002 *International Yeast Genetics and Molecular Biology Meeting.* Title: *In vivo* analysis of TFIID binding to DNA. (Poster)

2001 *Mid-Atlantic Yeast Genetic Meeting.* Title: Chromatin structure mapping in *Saccharomyces cerevisiae in vivo* with DNase I. (Poster)