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Studies of Transcriptional Repression by the yeast $\alpha 2$ protein

by

Brenda Michele Herschbach

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Biophysics

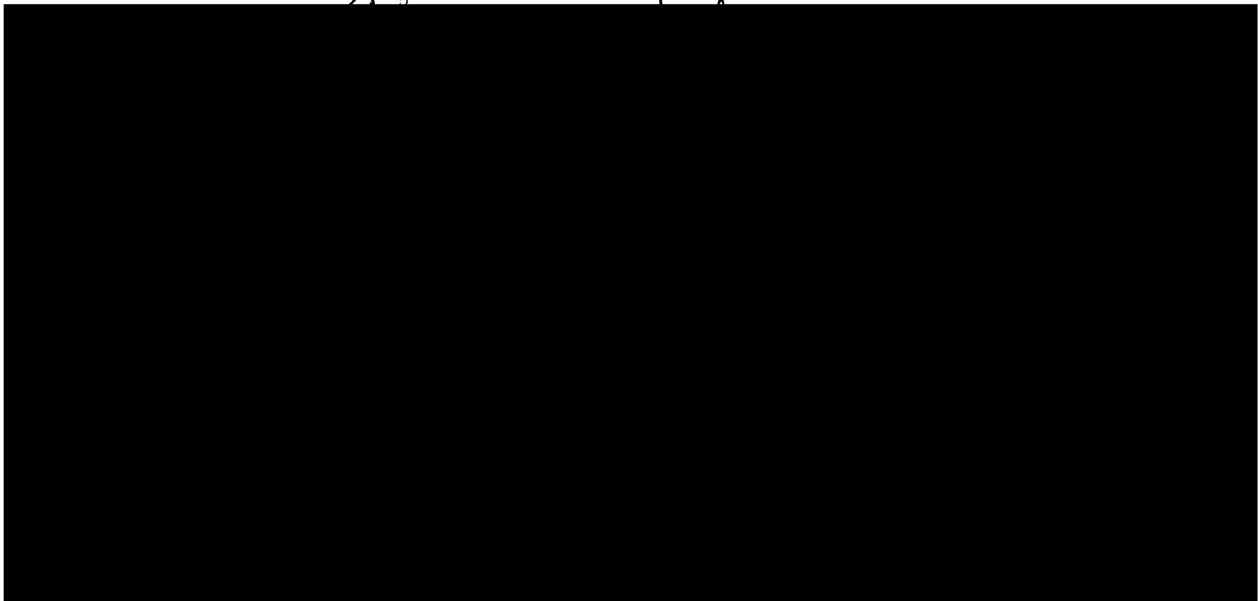
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Dedicated to Robert Dudley Herschbach (1907-1992)

He always told me that "life is an adventure" and that we must "try to learn something new every day". This simple, direct advice (often given in the context of some wonderful story) was invaluable in my graduate career, as I am sure that it will be in the rest of my life. Thank you, Grandad. I love you.

Acknowledgments

It is difficult to express the debt that I owe to the people who have encouraged me through the years in graduate school. Whether it was sharing their expertise in experimental procedures I was struggling to master, listening to my moans and groans of "will I survive graduate school and what will I do next" angst, or just showing me where the stapler is kept, their help was invaluable.

My advisor, Sandy Johnson, spent untold hours trying to help a rather unsophisticated, though eager, graduate student develop into a scientist. His was a tough job. I greatly appreciate all the time and energy he invested in me.

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I would also like to thank my family, for teaching me enthusiasm (in the extreme) and also for setting high standards for me as a scientist and, more importantly, as a human being. I thank Sophie (woof!), my constant companion (when she's in town) and great friend.

Finally, I thank Kevin Jarrell. For everything.

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Studies of Transcriptional Repression by the yeast $\alpha 2$ protein

Brenda Michele Herschbach

Abstract

This thesis describes my investigations into the molecular mechanism of transcriptional repression by the yeast $\alpha 2$ protein. The introductory chapter outlines a conceptual framework for the known mechanisms of transcriptional repression, arguing that for each step in the transcription initiation reaction, there probably exist repressors that can block it. The second chapter describes my studies of the role of chromatin structure in mediating $\alpha 2$ repression. I find that depleting histone H4 from yeast cells has only a modest effect on $\alpha 2$ repression, which suggests that chromatin structure is unlikely to be essential to $\alpha 2$ repression. Work presented in the third chapter asks whether or not $\alpha 2$ can repress transcription catalyzed by RNA polymerases I and III, in addition to its effect on RNA polymerase II transcription. I find that $\alpha 2$ can repress transcription by RNA polymerases I and II but not III. This result suggests that the target of $\alpha 2$ repression is likely to be some component of the general RNA polymerase II transcription machinery that is common with RNA polymerase I. In order to extend this result, I set up an *in vitro* transcription system and asked if $\alpha 2$ could direct transcriptional repression *in vitro*. The results presented in Chapter 4 show that $\alpha 2$ can repress transcription *in vitro*. The *in vitro* system allowed me to demonstrate that $\alpha 2$ represses the low level of *in vitro* transcription observed in the apparent absence of transcription activators. This observation strongly suggests that $\alpha 2$ represses transcription by interfering with the activity of one (or more) of the RNA polymerase II general transcription factors.

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Chapter 1

Introduction: Transcriptional repression in eukaryotes

INTRODUCTION

Thirty years ago, Jacob, Monod, and their colleagues developed the idea of gene repressors and operators (Jacob & Monod 1961). So compelling were the arguments and so powerful the model that, at first, negative regulation was invoked to explain nearly all examples of genetic control in prokaryotes. Only gradually were examples of positive genetic control fully acknowledged and incorporated into the general theory of prokaryotic gene regulation (Englesberg & Wilcox 1974).

In contrast, the studies of eukaryotic gene expression first emphasized positive control even though transcriptional repressors were among the first recognized eukaryotic gene regulatory proteins. Multicellular eukaryotic organisms employ hundreds of different cell types, each of which requires expression of a different collection of genes. The argument was presented that it would be much more efficient to turn the appropriate cell-type specific genes on in the proper cell type rather than to repress them in all other cell types (Alberts *et al.* 1983). Therefore, it was proposed that positive control mechanisms should predominate in higher organisms. The discovery in 1981 of transcriptional enhancers (Banerji *et al.* 1981) -- DNA sequences that can activate transcription when positioned thousands of basepairs upstream of the transcription start site-- supported this idea and also posed a fascinating series of mechanistic questions that attracted the attention of many molecular biologists. Finally, since most eukaryotic promoters require DNA-bound activator proteins to function *in vivo*, there was a natural reluctance to study repression, a process that disrupted a sequence of events that itself was only beginning to be understood.

It now appears that eukaryotic regulatory circuits may have evolved to maximize evolutionary flexibility rather that economy; negative regulatory mechanisms appear to be quite common in eukaryotes. In addition, it has now been shown that negatively acting DNA sequences-- variously termed silencers, operators, extinguishers, etc.-- can, like enhancers, control transcription from a distance. Finally, the recent advances in our

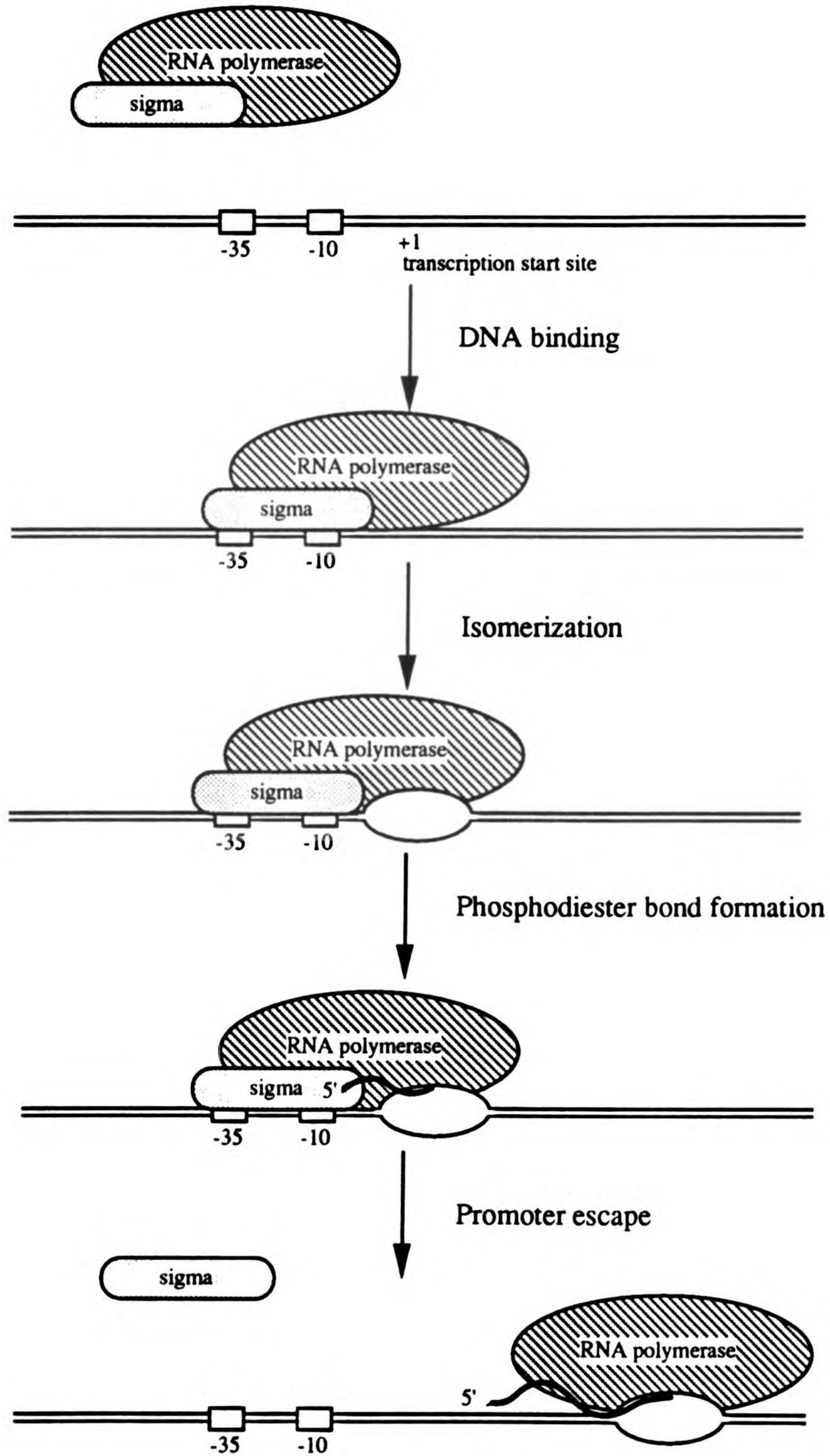
understanding of transcription initiation in eukaryotes provides appropriate background for a review of negative control mechanisms. Due to the space limitations of this review, we have generally given only a single example of each type of negative control discussed. Where possible, we have chosen examples where the biology behind the regulatory circuit is understood and insights as to the molecular mechanism have been uncovered. In other cases, our choice of example was arbitrary, and we apologize for the inevitable omissions. We begin by emphasizing an important lesson from prokaryotic examples of negative control: for every step in transcription initiation, there probably exists a repressor that can block it.

NEGATIVE REGULATION IN PROKARYOTES

The initiation of transcription in bacteria involves a series of discreet, ordered steps (Figure 1-1; see Chamberlin 1974, McClure 1985, Krummel & Chamberlin 1989). In the first step of prokaryotic transcription initiation, the RNA polymerase holoenzyme (RNA polymerase core enzyme plus a sigma factor that assists in promoter recognition) binds to the promoter. In the second step, the "closed" RNA polymerase-DNA complex isomerizes to an "open" form, a process that results in the unwinding of the DNA helix near the transcription start site. The first few phosphodiester bonds of the RNA transcript are formed in the third step of the reaction. The final step in prokaryotic transcription initiation is viewed as the escape of RNA polymerase from the promoter, with the concomitant release of the sigma factor.

Where along this pathway do known bacterial repressors act? The cI repressor of the coliphage lambda (bound at the O_R1 and O_R2 operators) blocks the initial binding of RNA polymerase to the promoter (Hawley *et al.* 1985). The Arc repressor of bacteriophage P22 is thought to allow RNA polymerase binding but to prevent the transition from the closed to the open complex (Vershon *et al.* 1987). The *Escherichia coli* Gal repressor permits both RNA polymerase binding and isomerization of the RNA

Figure 1-1: Transcription Initiation by *E. coli* RNA Polymerase



polymerase-promoter complex, but blocks the formation of the first phosphodiester bond (Choy & Adhya 1992). Finally, Lee & Goldfarb (1991) have argued that the *E. coli* Lac repressor prevents promoter escape at the lacUV5 promoter.

Thus studies of negative regulation in prokaryotic systems have identified transcriptional repressors that act at each step in the transcription initiation process. Whereas our understanding of repression mechanisms in eukaryotic transcription is not nearly as well developed, we might anticipate the same outcome. Below, we discuss the transcription initiation process at eukaryotic promoters and review some examples of negative control mechanisms, with an emphasis on the steps at which they may act to block transcription.

NEGATIVE REGULATION IN EUKARYOTES

Transcription initiation is more complex in eukaryotes than in prokaryotes. Unlike prokaryotic cells, which utilize a single RNA polymerase to synthesize all RNA molecules, eukaryotic cells contain three distinct RNA polymerases, each of which transcribes a different set of genes (reviewed in Sentenac 1985); RNA polymerase I transcribes a single gene encoding the 35S ribosomal RNA precursor; RNA polymerase II transcribes protein-coding genes. RNA polymerase III transcribes small genes encoding functional RNAs (tRNAs, 5SrRNA, U6 snRNA, etc.). In this review, we limit our discussion to negative regulation of RNA polymerase II transcription.

RNA polymerase II consists of approximately 12 subunits, some of which are shared with RNA polymerases I and III (Sentenac 1985; Woychik *et al.* 1990; Carles *et al.* 1991). In contrast to bacterial RNA polymerases, eukaryotic RNA polymerase II cannot correctly initiate transcription on its own (Matsui *et al.* 1980). Several additional proteins, termed general transcription factors (see Table 1-1), must first assemble at the promoter to allow transcription by RNA polymerase II (reviewed in Zawel and Reinberg 1993).

Table 1-1: The General Transcription Factors of Eukaryotic RNA Polymerase II

Factor	Native mass	Polypeptide composition	Function
TFIID	>700 kD	TBP: 38 kD TAFs: 30-200 kD	Binds to TATA in first step of pre-initiation complex assembly
TFIIB	33 kD	33 kD	Binds to TFIID-TATA complex
TFIIF	220 kD	30 kD 74 kD	Recruits RNA polymerase IIa into the pre-initiation complex Role in transcriptional elongation
TFIIE	200 kD	34 kD 56 kD	Binds to DBPolIIaF complex Kinase homology
TFIIH	230 kD	90 kD 62 kD 43 kD 41 kD 35 kD	Binds to DBPolIIaFE complex CTD kinase activity associated with 62 kD subunit 90 kD subunit identical to *ERCC-3* DNA repair helicase
TFIIJ	unknown	unknown	Binds to DBPolIIaFEH complex

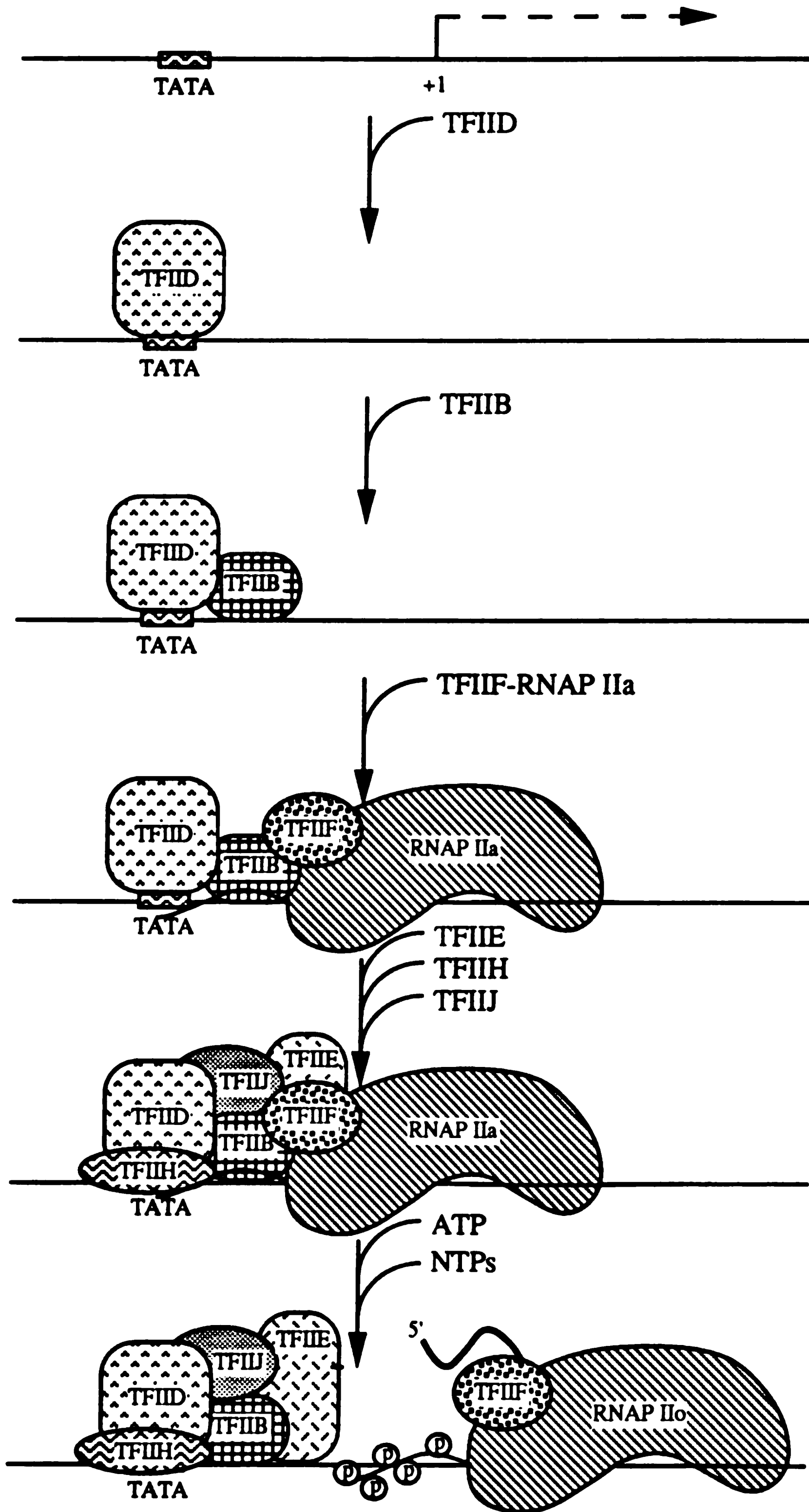
Figure 1-2 diagrams the initiation process at most eukaryotic promoters transcribed by RNA polymerase II. The first step involves binding of TFIID at the TATA element, typically located approximately 30 base pairs upstream of the transcription start site. In higher eukaryotes, TFIID consists of several polypeptides. The TATA Binding Protein (TBP) contacts DNA in the minor groove of the TATA element (D.K. Lee *et al.* 1991, Starr & Hawley 1991, Nikolov *et. al* 1992). The other components of TFIID, termed TBP Associated Factors (TAFs), somehow contribute specificity to the TFIID complex (for review, see Gill 1992, Sharp 1992, White & Jackson 1992, Rigby 1993) and may serve as the target for some transcriptional activator proteins (Hoey *et al.* 1993).

Once TFIID has bound to the TATA element, the other general transcription factors assemble onto the complex in a prescribed order. TFIIB joins first. RNA polymerase II is then delivered to the complex in association with TFIIF. TFIIE, TFIIH, and TFIIJ follow, thus completing assembly of the pre-initiation complex. (Another factor, TFIIA, may play a role in stabilizing assembling transcription complexes, perhaps by displacing an inhibitor associated with TFIID [for review, see Zawel and Reinberg 1993]. TFIIA is not required for *in vitro* transcription systems that use recombinant TBP produced in bacteria.)

Assembly of this multi-component pre-initiation complex is analogous in certain ways to RNA polymerase binding at prokaryotic promoters. Initiation of transcription still requires unwinding of the DNA over the initiation site, formation of the first phosphodiester bond of the RNA transcript, and escape of RNA polymerase II from the promoter. The details of these later steps are not understood for eukaryotic systems; however, a reasonable model can be assembled from collected observations.

Recent work has demonstrated that the largest subunit of the general transcription factor TFIIH has helicase activity (Schaeffer *et al.* 1993). This TFIIH helicase (also known as *ERCC-3*; Weeda *et al.* 1990) is inhibited by the same concentration of the

Figure 1-2: Transcription Initiation by Eukaryotic RNA Polymerase II



detergent Sarkosyl as blocks transcription *in vitro* (Schaeffer *et al.* 1993 and references therein). Furthermore, both the transcription initiation reaction and the TFIIH-catalyzed DNA unwinding require hydrolysis of the β-γ bond of ATP (Schaeffer *et al.* 1993 and references therein). Thus, it seems likely that the TFIIH helicase is responsible for unwinding the DNA helix over the start site during transcription initiation.

Promoter escape probably requires phosphorylation of RNA polymerase II. The C-terminal domain of the large subunit of RNA polymerase II contains, depending on the species, between 26 and 52 copies of a heptapeptide repeat bearing the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Intact RNA polymerase II isolated from cells exists in two forms: IIo, in which the C-terminal tail is highly phosphorylated, and IIa, an unphosphorylated form. It is the unphosphorylated form (IIa) that associates with TFIIF and incorporates into assembling transcription complexes (Lu *et al.* 1991, Chestnut & Dahmus 1992). However, elongating polymerases are highly phosphorylated in the C-terminal repeats, which suggests that phosphorylation occurs during the initiation process (Cadena & Dahmus 1987, Payne & Dahmus 1989, Laybourn & Dahmus 1990). Furthermore, the phosphorylated form of RNA polymerase II does not interact with TBP, though the non-phosphorylated form does (Usheva *et al.* 1992). Thus it seems likely that phosphorylation of the C-terminal tail stimulates release of RNA polymerase II from the pre-initiation complex.

In addition to the helicase activity associated with its large subunit, the general transcription factor TFIIH contains a kinase activity associated with its 62 kD subunit that is capable of phosphorylating the C-terminal tail of RNA polymerase II (Feaver *et al.* 1991, Lu *et al.* 1992, Serizawa *et al.* 1992). This activity is stimulated by TFIIE and by DNA containing a TATA box and a transcription start site. TFIIH can use either ATP or GTP as a phosphate donor when phosphoryating the C-terminal tail of RNA polymerase II. However, initiation of transcription by RNA polymerase II requires hydrolysis of ATP prior to formation of the first phosphodiester bond; GTP cannot be substituted for

ATP. These observations indicate that phosphorylation of the C-terminal tail is not the only step involving ATP hydrolysis in transcription initiation.

Once the pre-initiation complex has been formed, the DNA has been unwound, and RNA polymerase II has been phosphorylated, addition of nucleoside triphosphates (NTPs) allows elongation. Additional transcription factors have been described that affect elongation by promoting or hindering RNA polymerase II processivity (Rappaport *et al.* 1987, Reinberg & Roeder 1987, Flores *et al.* 1989, Price *et al.* 1989, Bengal *et al.* 1991).

Another difference between eukaryotic and prokaryotic transcription is that many, perhaps all, eukaryotic genes are expressed *in vivo* at very low levels (or not at all) unless stimulated by one or more transcriptional activators. Typically, such activators recognize specific DNA elements located upstream (sometimes several thousand base pairs) of the transcription start site. Activators stimulate either the rate of transcription complex assembly, or the fraction of functional complexes that assemble at a promoter in a given amount of time. Although the mechanisms of transcriptional activation are not understood in detail, at least some activators appear to act early in the pathway for assembly of the pre-initiation complex, perhaps stimulating DNA binding by TFIID, or helping to recruit TFIIB (Stringer *et al.* 1990, Horikoshi *et al.* 1991, Lee *et al.* 1991, Lin & Green 1991, Lin *et al.* 1991, Stringer *et al.* 1991, Sundseth & Hansen 1992).

In principle, negative regulators of eukaryotic gene expression could inhibit transcription by interfering with any step in the transcription initiation pathway. From the breadth of repression mechanisms observed in prokaryotic systems, we anticipate the discovery of eukaryotic repressors working at most, if not all, of these steps. Some eukaryotic repressors might block transcriptional activation. Activator function could be affected at many levels, such as nuclear localization, DNA binding, or ability to stimulate transcription once bound to DNA. Other negative regulators might affect the general transcription machinery itself, preventing formation of a functional pre-initiation

complex. For example, repressors might occlude promoter DNA from the transcription apparatus. Alternatively, negative regulators could block association of one of the general transcription factors or of RNA polymerase II with the assembling pre-initiation complex. Some negative regulators might act late in the initiation pathway, perhaps interfering with the kinase activity of TFIIH and thus preventing the escape of RNA polymerase II from the promoter. Such late-acting repressors would be useful at genes requiring rapid induction in response to environmental stimuli.

We have arranged the following discussion of eukaryotic transcriptional repression into sections that correspond to the steps of the initiation process at which negative regulators could, in theory, act (see Figure 1-3). For each step, we describe one or two examples that illustrate the principle. We have chosen, where possible, to describe examples in which the biological relevance of the regulatory circuit is understood. In those cases where clear-cut examples of a proposed repression mechanism are lacking, we have speculated as to the relevance of published instances of negative regulation. It is beyond the scope of this review to provide a catalog of all known transcriptional repressors; again, we apologize for any blatant omissions.

Repressors that interfere with transcriptional activators

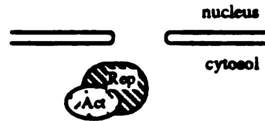
First, we discuss examples of repressors that interfere with the ability of transcriptional activators to stimulate transcription. Negative regulators use many different mechanisms to block activator function.

INTERFERENCE WITH ACTIVATOR NUCLEAR LOCALIZATION. One of the earliest steps at which a repressor could interfere with the activity of a transcriptional activator is the transport of the activator from the cytoplasm into the nucleus. The IκB family of transcriptional inhibitors exemplifies this idea. IκBs block the nuclear import of members of the Rel family of transcriptional activators. The Rel family includes factors

Figure 1-3: Mechanisms of transcriptional repression in eukaryotes:

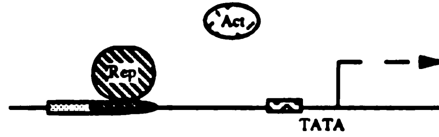
Repressors that interfere with transcriptional activators

Interference with activator nuclear localization

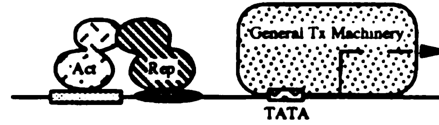


Interference with assembly of multisubunit activators

Interference with activator DNA binding

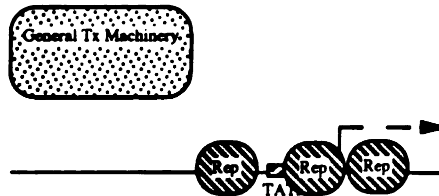


Interference with activity of DNA-bound activators

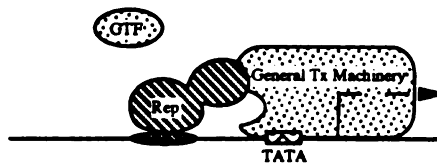


Repressors that interfere with the general transcription machinery

Interference with access of general transcription machinery to the DNA



Interference with pre-initiation complex assembly



DNA Silencing

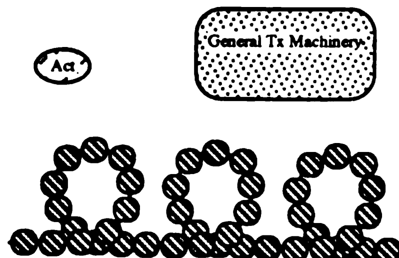


Figure 1-3

Mechanisms of transcriptional repression in eukaryotes. A schematic view of some of the molecular mechanisms described in the text.

responsible for regulation of immune function and inflammation response genes in humans (NFκB; Ghosh *et al.* 1990, Kieran *et al.* 1990, Nolan *et al.* 1991), oncogenesis in chickens (v-*rel* and c-*rel*; Ballard *et al.* 1990, Bull *et al.* 1990), and determination of dorsoventral axis polarity in fruit flies (*dorsal*; Steward, 1987). These proteins are related through an amino-terminal domain of approximately 300 amino acids, called the Rel homology domain. Their carboxy terminal domains are highly divergent.

The Rel homology domain contains sequences important for DNA binding, nuclear localization, and oligomerization. Rel domains interact with each other. NFκB, for example, is a heterodimer of two Rel proteins, p50 and p65. p50 can also homodimerize to activate transcription of a different set of genes as the positive regulatory factor KBF-1 (Kieran *et. al.* 1990). Different IκB proteins can interfere with the activities of different sets of Rel dimers (Zabel & Bauerle 1990, Davis *et al.* 1991, Haskill *et al.* 1991, Geisler *et al.* 1992, Inoue *et al.* 1992a, Kerr *et al.* 1992, Kidd 1992, Tewari *et al.* 1992).

How do IκB proteins prevent nuclear import of the Rel dimers? IκBs asociate with a region of the Rel homology domain that contains the highly conserved Rel nuclear localization sequence (Beg *et al.* 1992). Presumably, interaction with an IκB masks the Rel nuclear localization signal and thus prevents nuclear import (Nolan *et al.* 1991, Beg *et al.* 1992, Inoue *et al.* 1992b). Given the amino acid similarity among the Rel proteins, it might be anticipated that the IκB proteins would also be related to one another. Several IκB genes have recently been cloned (human: *Mad-3*, Haskill *et al.* 1991; bcl-3, Kerr *et al.* 1992; mouse: IκBγ, Inoue *et al.* 1992a; rat: RI/IF-1, Tewari *et al.* 1992; chicken: pp40, Davis *et al.* 1991; fruit fly: cactus, Geisler *et al.* 1992, Kidd 1992). Each has five to eight copies of a 32 amino acid motif known as the ankyrin repeat. Ankyrin repeats are found in proteins with highly diverse functions, including putative integral membrane proteins, viral host-range factors, and multisubunit transcription factors (for review, see Bennet, 1992; Blank *et al.* 1992). The ankyrin motifs in IκB proteins mediate interaction

with the Rel homology domain (Inoue *et al.* 1992b). Presumably, multiple interactions along the Rel/ankyrin interaction surface provide the specificity that dictates which IκB inhibits which type of Rel dimer.

In addition to masking Rel factor nuclear localization sequences, IκBs are capable of disrupting complexes of Rel factors bound to DNA (Zabel & Bauerle 1990). It is not clear that this activity is relevant *in vivo*, however, since IκBs are not known to enter the nucleus.

If IκB factors prevent transcriptional stimulation of genes controlled by Rel activators by sequestering Rel factors in the cytoplasm, how is this association reversed? The activity of IκB proteins is regulated by phosphorylation. Different IκBs seem to be inactivated by different treatments *in vitro* (Gosh & Baltimore 1990, Link *et al.* 1992). Presumably, different phosphorylation cascades in the cell inactivate different IκBs. If only a subset of IκBs are inactivated in response to a particular physiological signal, only the appropriate Rel activators will be released to stimulate transcription.

INTERFERENCE WITH THE ASSEMBLY OF MULTISUBUNIT ACTIVATORS. Many transcriptional activators consist of more than one polypeptide subunit. Some repressors work by competing for association with one of the activator subunits, thereby preventing the formation of a functional activator.

Members of the basic-region-helix-loop-helix (bHLH) family of transcriptional activators bind to DNA as dimeric (or higher-order oligomeric) complexes (Murre *et al* 1989b). The HLH domain, a conserved region of hydrophobic amino acids predicted to form two amphipathic helices separated by a loop, mediates oligomerization (Murre *et al.* 1989ab, Voronova & Baltimore 1990); the basic region contributes to DNA sequence recognition (Lassar *et al.* 1989, Davis *et al.* 1990, Voronova & Baltimore 1990; Ferre-D'Amare *et al.* 1993).

In the fruit fly *Drosophila melanogaster*, bHLH proteins play an essential role in the development of the peripheral nervous system. The body of the adult fly is lined with mechano- and chemo-sensory organs termed sensilla. The precursor cells that give rise to these sensilla develop during the late larval and early pupal stages from undifferentiated epithelial sheets that also give rise to ordinary epidermal cells (see Hartenstein & Posakony 1989). At some point, undifferentiated cells undergo the developmental decision to become either a sensillum precursor or an epidermal precursor. The *daughterless* (*da*) gene and the *achaete* (*ac* or T5), *scute* (*sc* or T4), and *asense* (*ase* or T8) genes of the *achaete-scute* complex (*AS-C*) are thought to encode subunits of transcriptional activators involved in the decision to develop into a sensillum precursor. Loss-of-function mutations in these genes result in the loss of sensory organs (García-Bellido & Santamaria 1978, Caudy *et al.* 1988a, Dambly-Chaudiére *et al.* 1988, Cline 1989, Romani *et al.* 1989). Overexpression leads to development of ectopic sensilla (García-Alonso & García-Bellido 1986, Campuzano *et al.* 1986).

The *da* gene and the three *AS-C* genes each encode a bHLH protein (Villares & Cabrera 1987, Alonso & Cabrera 1988, Caudy *et al.* 1988b, Murre *et al.* 1989a). These proteins interact with each other *in vitro* and probably *in vivo* to form heterodimeric complexes that bind to DNA and presumably stimulate transcription of sensillum-specific genes (Dambly-Chaudiére *et al.* 1988, Van Doren *et al.* 1991).

The same genes that are activated in sensory organ precursors must be kept silent in epidermal precursors. Two genes, *extramachrochaetae* (*emc*) and *hairy* (*h*), are known to suppress sensory organ development. Thus, *emc* and *h* are, formally, negative regulators of the genes activated by *da* and *AS-C*. Furthermore, the phenotype conferred by mutations in *emc* or *h* is sensitive to the wild-type dosage of *da* and *AS-C* (Moscoso del Prado & García-Bellido 1984). Such dosage-sensitive relationships suggest that the proteins encoded by these genes might physically interact, an intriguing possibility given their opposing developmental roles.

The amino acid sequence of the *emc* gene product suggests a model to explain these observations (Ellis *et al.* 1990, Garrell & Modolell 1990). The *emc* protein contains an HLH dimerization motif but lacks a nearby basic region necessary for DNA binding. This observation suggests that *emc* might be able to heterodimerize with other bHLH proteins (in this case, with *da* and/or the *AS-C* proteins), thus creating complexes that cannot bind to DNA and therefore do not activate transcription (Van Doren *et al.* 1991). By interacting with *da* or *AS-C* proteins, *emc* would prevent their association with each other and would therefore block formation of the transcriptional activators required for development of sensory organ precursors.

Members of other families of transcriptional regulatory proteins also form functional activators by complexing with themselves or with members of their protein family. By assembling transcriptional activators from multiple polypeptide subunits, each of which can be used in more than one activator, cells can elaborate complex transcriptional regulatory circuits with a limited number of proteins. This mechanism allows efficient generation of many different activators from a small number of cellular components and provides a convenient step at which repressors can regulate transcription (see, for example, Descomber & Schibler 1991; Nakabeppu & Nathans 1991; Ron & Habener 1992). Furthermore, a repressor that works by heterodimerization could in principle inactivate a whole family of transcriptional activators.

INTERFERENCE WITH ACTIVATOR DNA BINDING. The *emc* protein described in the previous section represses transcription by interacting directly with individual subunits of the *da/AS-C* activators, thus forming complexes that are incapable of binding DNA. Other transcriptional repressors interfere at a later step, competing with a functional activator for access to the same DNA sequences.

For example, DNA binding sites for the *Krüppel* (*Kr*) repressor protein of *Drosophila* often overlap binding sites for transcriptional activator proteins (Stanojevic *et*

al. 1989, Small *et al.* 1991, Zuo *et al.* 1991). A well studied example of this is found in the stripe 2 element of the *even-skipped* (*eve*) promoter. *eve* encodes a homeodomain protein that is first detected during embryonic nuclear cleavage cycle 12, when it is distributed uniformly in all nuclei. By cycle 14, *eve* has disappeared from both poles of the embryo. Twenty to thirty minutes later, *eve* expression is restricted to a series of seven transverse stripes along the length of the embryo. Each stripe is about 5-6 nuclei wide (Frasch & Levine 1987). Promoter fusion experiments have revealed that independent regulatory regions upstream of the *eve* promoter direct expression in individual stripes (Goto *et al.* 1989, Harding *et al.* 1989).

In addition to *Kr* binding sites, the stripe 2 regulatory element of the *eve* promoter contains several binding sites for the gap genes *hunchback* (*hb*) and *giant* (*gt*) and for the maternal morphogen *bicoid* (*bcd*) (Stanojevic *et al.* 1989, Small *et al.* 1991). *bcd* and *hb* activate transcription of genes linked to the stripe 2 element. *gt* and *Kr* act as repressors (Frasch & Levine 1987, Small *et al.* 1991). *gt*, probably in cooperation with other factors, determines the anterior boundary of stripe 2. *Kr* is responsible for shutting off *eve* expression at the posterior boundary. Virtually all of the *bcd* and *hb* binding sites overlap with, or are closely linked to, a *Kr* or *gt* recognition sequence (Stanojevic *et al.* 1989, Small *et al.* 1991). Significantly, DNA binding studies have demonstrated that *bcd* and *Kr* cannot co-occupy closely linked sites (Small *et al.* 1991). Apparently, *Kr* sets the posterior limit on *eve* expression by competing with activators for access to DNA. Because mutations in a single *bcd* binding site can have dramatic effects on *eve* expression in stripe 2, it seems likely that *Kr* could effectively shut off the stripe 2 element by interfering with just one activator site (Small *et al.* 1992).

One important aspect of this mode of repression is that repressor and activator sites must be tightly linked. For example, *Kr* bound to its sites in the stripe 2 element does not interfere with transcriptional activators bound in the stripe 3 element, about 1.5 kb away (Goto *et al.* 1989, Harding *et al.* 1989). This sort of short-range repression

mechanism, which allows for complex promoters constructed of several autonomous modules, might also occur in mammalian promoters, where regulatory regions often include interdigitated activation and repression elements (see, for example, Maniatis *et al.* 1987).

INTERFERENCE WITH THE ACTIVITY OF DNA-BOUND ACTIVATORS. Even after a transcriptional activator has been successfully imported into the nucleus, with its subunits properly assembled, and has bound to DNA, repressors can interfere with its ability to stimulate transcription.

In many promoters, repressor binding sites are adjacent to, though not overlapping with, binding sites for transcriptional activators. Repressors and activators can often occupy their sites simultaneously. In these cases, a repressor could block activator function by directly contacting the adjacent, DNA bound activator and masking the protein domain responsible for transcriptional stimulation.

Expression of the mammalian c-*myc* gene is turned off in terminally differentiated plasma cells. The identification of a derepressed c-*myc* allele in murine plasmacytomas indicates that inappropriate expression of c-*myc* probably contributes to tumor development (Kakkis *et al.* 1988). A repressor, myc-PRF, binds to a DNA site upstream of the c-*myc* promoter and shuts off c-*myc* transcription (Kakkis *et ai*l 1987, 1989). Notably, myc-PRF is absent in cell lines that represent early stages of B-cell development when c-*myc* is still expressed. This correlation suggests that myc-PRF is responsible for repression of c- *myc in vivo* in terminally differentiated plasma cells. The binding site for myc-PRF in the c-*myc* promoter is located immediately adjacent to a binding site for the widely expressed transcriptional activator myc-CF1 (Kakkis *et al.* 1987, Riggs *et al.* 1991). Studies of DNA-bound complexes indicate that myc-PRF and myc-CF1 can simultaneously occupy the same promoter; moreover, they physically interact (Kakkis *et al.* 1989). It seems plausible that myc-PRF represses c-*myc* expression in terminally

differentiated B cells by binding next to and, through direct physical interaction, masking the activation surface on myc-CF1.

Repressors need not bind adjacent DNA sequences in order to interact with and mask activating regions on transcriptional activators. Proteins bound at distant sites can interact by looping out the intervening DNA (Ptashne 1986, Choy & Adhya 1992, Schleif 1992). Some repressors complex with DNA-bound activators but do not themselves bind DNA at all. The Gal80 protein of the yeast *Saccharomyces cerevisiae* is perhaps the best-studied example of this. The Gal4 activator stimulates transcription of genes required for galactose metabolism in yeast (the *GAL* genes; reviewed in Johnston 1987). Gal4 recognizes a 17 bp DNA element found in several copies upstream of the *GAL* genes, from which it activates transcription when galactose is present in (and glucose is absent from) the growth medium. Gal80 interacts with the C-terminal region of Gal4, which also contains an acidic activation domain (S.A. Johnston *et al.* 1987, Lu *et al.* 1987, Ma & Ptashne 1987ab, Salmeron *et al.* 1990). Presumably, association with Gal80 masks the nearby activation region of Gal4, thereby blocking transcriptional stimulation by Gal4.

Studies both *in vivo* and *in vitro* have revealed that Gal80 represses Gal4 activity even though Gal4 is bound to DNA (Giniger *et al.* 1985, Lohr & Hopper 1985, Lue *et al.* 1987). Furthermore, even during galactose induction, Gal80 remains associated with Gal4 (Chasman & Kornberg 1990, Leuther & Johnston 1992). It has been proposed that a conformational change, which may involve phosphorylation of Gal4 (Mylin *et al.* 1989, 1990, Parthun & Jaehning 1992), allows exposure of the Gal4 activating region despite the continued association of Gal80. This seems a particularly efficient way for a repressor to respond to environmental signal; since the complex never dissociates, the repressor need not relocate the activator to reestablish repression.

Repressors that interfere with the assembly of the general transcription machinery

We have discussed above examples of negative regulation by interference with activator proteins. While effective against individual activators, these repression mechanisms share a disadvantage: most eukaryotic genes respond to several different transcriptional activators. Full repression of such genes by activator interference would require a dedicated repressor for each different activator protein. A more efficient approach to repressing genes controlled by multiple activators would be to interfere directly with the assembly of the general transcription machinery.

INTERFERENCE WITH ACCESS OF THE GENERAL TRANSCRIPTION MACHINERY TO THE DNA. Perhaps the first recognized transcriptional repressor in eukaryotes, the simian virus 40 T antigen (SV40 Tag), represses transcription by occluding promoter DNA from the general transcription machinery (Hansen *et al.* 1981). Tag accumulates in the early stages of SV40 lytic growth. After reaching a threshold concentration, Tag both stimulates SV40 replication and represses transcription of the viral early genes (reviewed in Tjian 1981). Tag binds, probably as a tetramer, to three adjacent sites within the initiation region of the SV40 early promoter (Tjian 1978, Shalloway *et al.* 1980, Hansen *et al.* 1981). Although it is not clear exactly which components of the transcription machinery are excluded from the DNA when Tag is bound, the locations of the Tag binding sites suggest that RNA polymerase II, and possibly also TFIID, are likely to be affected.

Other negative regulators may also function by occluding promoter elements from components of the transcription machinery (see, for example, Ohkuma *et al.* 1990, Kaufman & Rio 1991, Rijcke *et al.* 1992).

The histone proteins can act as transcriptional repressors of eukaryotic genes, probably by preventing TFIID access to the DNA. The DNA of eukaryotic organisms is wrapped around octamers of histone proteins to form complexes termed nucleosomes. It has long been postulated that such packaging would interfere with the ability of DNA-

binding proteins to recognize their sites. In particular, it has been proposed that packaged promoter DNA would be inaccessible to TFIID until the nucleosomes were removed. It has further been suggested that one role of transcriptional activator proteins might be to clear the promoter DNA of inhibitory nucleosomes, thereby allowing access of TFIID to the TATA box. Consistent with this idea, Grunstein and colleagues have observed that nucleosomes can be depleted from yeast cells in which histone genes have been put under the control of a heterologous, experimentally regulatable promoter (Han & Grunstein 1988, Han *et al.* 1988). Such nucleosome depletion induces transcription of many yeast genes (Han & Grunstein 1988, Han *et al.* 1988, Durrin *et al.* 1990). Furthermore, even genes whose upstream activator binding sites had been removed are expressed when histones are depleted (Han & Grunstein 1988, Han *et al.* 1988). These results suggest that, in the absence of histones, transcriptional activators are no longer required for expression of these yeast genes.

Studies of transcription *in vitro* also support the idea that nucleosomal structures might repress transcription by interfering with the assembly of the general transcription factors at the promoter (Knezetic & Luse 1986, Matsui 1987, Wasylyk & Chambon 1979, 1980). Incubation of TFIID with the template DNA prior to nucleosome assembly prevents nucleosomal inhibition, which suggests that it is TFIID binding that is inhibited by the presence of histone complexes. (Matsui 1987, Workman & Roeder 1987, Knezetic *et al.* 1988).

Specifically positioned nucleosomes have been observed at some promoters and have been proposed be involved in transcriptional regulation (see, for example, Almer *et al.* 1986, Benezra *et al.* 1986, Pérez-Ortín *et al.* 1987, Matallana *et al.* 1992). According to this idea, some gene repressor proteins might block transcription from a target promoter by directing the formation of a positioned nucleosome over the TATA box (Roth *et al.* 1990, 1992, Shimizu *et al.* 1991) (see below).

INTERFERENCE WITH PRE-INITIATION COMPLEX ASSEMBLY. Even when promoter DNA is accessible to the general transcription machinery, negative regulators could effectively repress transcription by interfering with proper assembly of any one of the general transcription factors into the pre-initiation complex.

The *Drosophila* homeodomain protein *even-skipped* (*eve*) is an example of a eukaryotic transcriptional repressor that interferes with the assembly of a functional pre-initiation complex (Johnson & Krasnow 1992). *eve* is one of a large family of homeodomain proteins that control the early development of the *Drosophila* embryo (reviewed in Hayashi and Scott 1990).

Experiments carried out *in vitro* have revealed that *eve* represses transcription from promoters containing homeodomain-binding sites upstream of the TATA box (Biggin & Tjian 1989, Johnson & Krasnow 1992). Since transcription in these experiments apparently initiates without an activator protein, *eve* must be acting directly on components of the general transcription machinery. *eve* does not affect the kinetics of transcription initiation, but rather reduces the probability that a functional pre-initiation complex will assemble at the promoter (Johnson & Krasnow 1992). Additionally, pre-initiation complexes become resistant to *eve* repression early in their assembly pathway, which indicates that *eve* affects one of the first steps in the formation of pre-initiation complexes (Johnson & Krasnow 1992). Although the precise step has not yet been identified, DNA binding by TFIID or recruitment of TFIIB seem to be likely possibilities.

The multiple steps required to assemble the transcriptional machinery at a promoter provide many opportunities for negative regulation. Certainly, other examples of transcriptional repressors that interfere with the general transcription machinery will be forthcoming. One likely candidate is the *S. cerevisiae* repressor Ssn6/Tup1 (Keleher *et al.* 1992). Ssn6/Tup1 is involved in transcriptional repression of several diverse sets of yeast genes, including **a**-specific, haploid-specific, and glucose-repressible genes (Carlson *et al.* 1984, Trumbly 1986, Mukai *et al.* 1991, Keleher *et al.* 1992). The

Ssn6/Tup1 complex is believed to be recruited to the promoters it represses by interaction with other proteins that bind to DNA (Keleher *et al.* 1992). Ssn6/Tup1 repression is equally effective against transcription catalyzed by RNA polymerases I and II, but not against transcription catalyzed by RNA polymerase III (Herschbach & Johnson 1993). This result suggests that the repressor interacts with some component common to the RNA polymerase I and II transcriptional machines. Since the activation systems used by these two RNA polymerases are not interchangeable (Butlin & Quincy 1991), it seems likely that Ssn6/Tup1 represses transcription not by blocking transcriptional activation, but rather by interfering with the activity of some component of the general transcriptional machinery that is similar for (or shared by) RNA polymerases I and II. While the target of Ssn6/Tup1 repression has not yet been identified, recent work indicates that the pre-initiation complexes of the three eukaryotic RNA polymerases have more in common than was originally expected (Mann *et al.* 1987, Woychik *et al.* 1990, Carles *et al.* 1991, Buratowski & Zhou 1992, Dequard-Chablat *et al.* 1991, Colbert & Hahn 1992, Gill 1992, López-De-León 1992, Sharp 1992, White & Jackson 1992, Rigby 1993).

INTERFERENCE WITH LATE STEPS IN INITIATION. Although negative regulators that block transcription initiation after assembly of the pre-initiation complex have not yet been identified, it is possible to predict several steps at which such regulation might occur. Repressors might interfere with unwinding of the DNA helix over the initiation site, or with the phosphorylation of the C-terminal domain of RNA polymerase II. Studies of the *hsp70* heat shock promoter in *Drosophila* have revealed that RNA polymerase II is bound at the promoter, and has synthesized the first few phosphodiester bonds, when the gene is transcriptionally inactive (Gilmour & Lis 1986, Rougive & Lis 1988). Might this "engaged" polymerase be prevented from escaping the promoter by a late-acting repressor that blocks the transition from initiation to elongation? Mutational analysis of

the *hsp70* promoter has indicated that sequences upstream of the *hsp70* TATA contribute to the formation of engaged RNA polymerase complexes (Lee *et al.* 1992). At least one transcriptional regulator, the GAGA factor, binds to DNA within this upstream region. One possibility is that the GAGA factor, normally a transcriptional activator, represses transcription of this promoter by interacting so strongly with the general transcription machinery that it prevents promoter escape. Such a late-acting mechanism might make sense for promoters whose rapid induction is required for survival in stressful environmental conditions.

Some eukaryotic repressors probably interfere with more than one step in the transcription initiation reaction

It is important to point out the possibility that individual negative regulators might be able to repress transcription by more than one of the mechanisms outlined here (see, for example, Appel & Sakonju 1993). For example, as described above, the *Drosophila Kr* protein probably represses *eve* transcription at the posterior boundary of stripe 2 by competing with activators for access to DNA. However, there is also evidence that *Kr* can interfere with the activity of some activators even when their DNA binding sites do not overlap (Licht *et al.* 1990, Zuo *et al.* 1991). In fact, in at least one case, *Kr* can prevent transcriptional stimulation by an activator without itself binding to DNA at all (Zuo *et al.* 1991). It seems likely that direct interaction between *Kr* and these activators masks the activation surface and thereby prevents transcriptional stimulation.

Similarly, the *eve* repressor described above blocks transcription from the *Ubx* promoter by interfering with assembly of the pre-initiation complex. However, *eve*, whose DNA binding specificity overlaps that of other homeodomain proteins, can also repress transcription by competing with homeodomain activators for access to DNA sites (Han *et al.* 1989). At some promoters, *eve* may simultaneously use both mechanisms, ensuring tight repression by blocking both activator binding and functional assembly of

any pre-initiation complexes that may begin to form at the promoter despite the absence of an activator.

The Ssn6/Tup1 repressor may also use more than one mechanism to repress transcription. Simpson and colleagues have described a positioned nucleosome that forms adjacent to the DNA binding site involved in Ssn6/Tup1 repression of the **a**-specific genes in yeast, and have suggested that such a structure could contribute to transcriptional repression by Ssn6/Tup1 by obscuring neighboring DNA sequences important for expression of the downstream gene (Roth *et al.* 1990, 1992, Shimizu *et al.* 1991). Studies of a glucose-repressed gene also show a correlation between Ssn6/Tup1 repression and the presence of a positioned nucleosome in the initiation region (Pérez-Ortín *et al.* 1987, Matallana *et al.* 1992). Thus, occlusion of promoter DNA by a positioned nucleosome might contribute to transcriptional repression by Ssn6/Tup1.

It seems likely that many negative regulators can interfere with transcription initiation by more than one mechanism, and can thereby ensure highly efficient gene repression.

Position effects and DNA silencing

Thus far, we have discussed mechanisms of negative regulation that shut off transcription at individual promoters. We now turn to larger scale repression mechanisms, by which whole regions of DNA become refractory to transcription. This phenomenon is often referred to as transcriptional silencing or position effects, since it was first observed that gene expression can vary depending on chromosomal location. Silencing probably results from the folding of nucleosomal DNA into a form of especially compacted chromatin which obscures the DNA from the transcription machinery. In addition to being transcriptionally inert, silenced DNA often replicates very late, suggesting that both RNA and DNA polymerases have restricted access to silenced sequences.

Perhaps the most dramatic example of transcriptional silencing is found in female mammals, where one of the two X chromosomes is inactivated in every cell (Lyon 1961, reviewed in Grant & Chapman 1988, Rastan & Brown 1990). Female mammals have two X chromosomes, while males have one X and one Y. Presumably because a double dose of X information would be deleterious, female cells permanently silence one of the two X chromosomes, chosen at random. Once an X has been inactivated, this state is stably maintained and faithfully inherited in all subsequent cell divisions.

The molecular mechanism of X-inactivation is not understood. Nor is it understood how the silenced state is faithfully inherited. Initiation of inactivation requires the presence of an X inactivation center (XIC in humans; Xic in mice) in *cis*. Recently, a gene that maps to the XIC/Xic has been cloned from humans (XIST) and mice (Xist) (Brown *et al.* 1992, Brockdorff *et al.* 1992) XIST/Xist is expressed only from the inactive X. Furthermore, the XIST/Xist RNA lacks any conserved open reading frame and is localized in the nucleus rather than with the cytoplasmic translational machinery. These observations suggest that the XIST/Xist gene product encodes a functional RNA, although the role this RNA molecule plays in X-inactivation is not understood.

Maintenance of the silent state of the inactivated X chromosome involves methylation of cytosine (C) residues, primarily at CpG sites (Hockey *et al.* 1989, Singer-Sam *et al.* 1990, Norris *et al.* 1991). Such methylation may help prevent re-activation of the inactivated chromosome by interfering, directly or indirectly, with DNA binding by transcriptional activators (Watt & Molloy 1988, Peifer *et al.* 1990ab, Boyes and Bird 1991, Pfeifer & Riggs 1991). This two-tier system of X-inactivation presumably ensures complete transcriptional silencing.

The use of compacted chromatin forms to turn off transcription of blocks of genes appears to be universal in eukaryotes. Both *Drosophila* and *S. cerevisiae* display transcriptional position effects, wherein the expression of a gene is affected by its

chromosomal location. Examination of this phenomenon has revealed that some regions of fly and yeast chromosomes are refractory to transcription. As with the inactivated X, these transcriptionally silent regions replicate late and are packaged into complex chromatin structures (for reviews, see Henikoff 1990, Rivier & Rine 1992, Sandell & Zakian 1992).

Studies of X-ray induced chromosomal translocations in *Drosophila* have revealed that the compacted chromatin structures associated with silenced sequences can "spread" along DNA. That is, genes that are normally expressed can be silenced if they are translocated near a region of compacted chromatin. Moreover, the expression of such translocated genes is often variable: some cells express the gene; other cells do not, indicating that the compacted chromatin structures have spread to different boundary points in different cells. This phenomenon, known as position effect variegation, is particularly striking when the translocated gene encodes an eye pigmentation protein. In such cases, the *Drosophila* eye contains clusters of pigmented and unpigmented cells. The existence of these clusters indicates that, once the extent of chromatin spreading has been set, it is stably inherited during subsequent cell divisions. Furthermore, there is a stochastic component to the decision; exactly which cells express the pigment gene and which do not varies from eye to eye. Although the molecular basis for position effect variegation is not yet understood, the proteins and DNA sequences involved in transcriptional silencing in *Drosophila* and yeast are beginning to be characterized (reviewed in Henikoff 1990, Rivier & Rine 1992, Sandell & Zakian 1992).

Genomic Imprinting

The chromatin-mediated repression mechanisms described above apparently allow cells to maintain developmental decisions by permanently inactivating regions of the genome. Other mechanisms for long-term gene inactivation probably exist. In particular, the phenomenon of genomic imprinting, wherein the expression of a

mammalian gene depends on whether it was inherited from the mother or the father, seems not to involve large-scale changes in chromatin structure (Sasaki *et al.* 1992). Rather, methylation of CpG dinucleotides seems to be involved in the imprinting process (reviewed in Bird 1993). Because mammalian cells contain a maintenance methylase that acts only on hemimethylated CpG sequences, DNA methylation patterns can be faithfully inherited upon DNA replication (reviewed in Razin *et al.* 1984). Furthermore, the expressed and unexpressed copies of an imprinted gene are often differentially methylated (Bartolomei *et al.* 1991, Chaillet *et al.* 1991, Sasaki *et al.* 1992, Ferguson-Smith *et al.* 1993).

The maternal and paternal copies of the mouse *Igf2r* gene, which encodes a receptor for insulin-like growth factor, are differentially expressed (only the maternally-derived copy is active), and are also differentially methylated. Two clusters of CpG sites in the *Igf2r* gene, one covering the gene promoter and one located within a downstream intron, display different methylation patterns depending on their parental origin (Stöger *et al.* 1993). Only the paternal, transcriptionally inactive, copy of the promoter CpG sequence is methylated. For at least one other imprinted gene, the *H19* gene, methylation of CpG dinucleotides correlates with gene inactivity (Bartolomei *et al.* 1991). However, both the *H19* CpG sequences and the *Igf2r* promoter CpG sites are unmethylated in the sperm and therefore cannot be the original imprinting signal (Ferguson-Smith *et al.* 1993, Stöger *et al.* 1993). Rather, this methylation is thought to be involved in maintenance of the imprinted state.

The intronic CpG sequence in the *Igf2r* gene, on the other hand, is methylated on the maternal, transcriptionally active copy of the gene (Stöger *et al.* 1993). Furthermore, this methylation is observed in the oocyte and may actually serve as the original imprinting signal. The molecular mechanism by which one copy of an imprinted gene is transcriptionally repressed while the other copy is transcriptionally active is not understood.

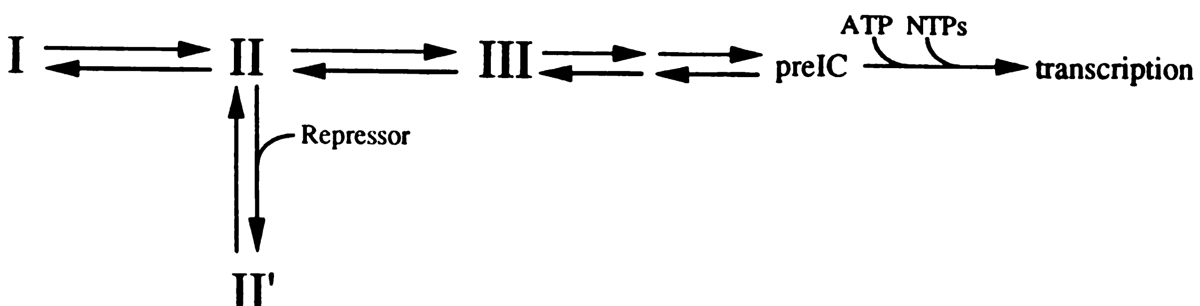
Global repression by inactivating a component of the general transcription machinery

When eukaryotic cells enter mitosis, transcription by all three RNA polymerases is shut down, presumably to allow easier separation of segregating chromosomes (Prescott & Bender 1962, Fink & Turnock 1977, L.H. Johnston *et al.* 1987, White *et al.* 1987). Recently, it was shown that one of the general transcription factors of RNA polymerase III, TFIIIB, is inactivated during mitosis, probably by phosphorylation (Hartl *et al.* 1993, J. Gottesfeld, V. Wolf, D. Forbes, and P. Hartl, personal communication). Since TFIIIB is required for initiation of RNA polymerase III transcription (Kassavetis *et al.* 1990), inactivation of this factor represses RNA polymerase III transcription during the mitotic phase of the cell cycle. The TATA binding protein and its RNA polymerase III-specific TAFs are essential components of TFIIIB. At least one component of TFIIIB that is phosphorylated in a mitotic extract is the same size as a previously identified RNA polymerase III-specific TAF (J. Gottesfeld, V. Wolf, D. Forbes, and P. Hartl, personal communication). Perhaps inactivation of TBP/TAF complexes by phosphorylation could serve as a global repression mechanism to repress transcription by all three nuclear RNA polymerases during mitosis.

CONCLUSIONS AND PERSPECTIVES

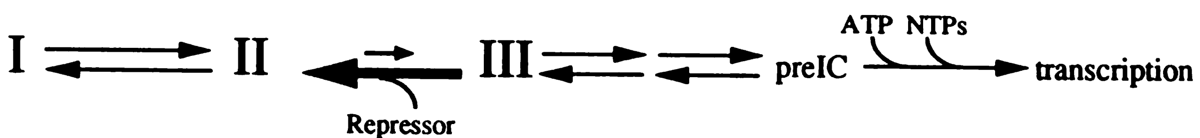
In this review, we have proposed that for each step in the pathway to transcription initiation there exists a repressor that blocks it, and have described several examples of transcriptional repressors that are known to affect one or more steps in the transcription initiation pathway. The transcription initiation reaction can be considered to be a linear series of equilibrium reactions. According to this view, negative regulators could reduce the overall level of transcription either by shifting the position of any individual equilibrium or by offering alternate, non-productive reaction paths (see Figure 1-4). However, studies of transcription reactions *in vitro* have suggested that a linear reaction

Transcription initiation as a linear series of equilibrium reactions:



Modes of repression

A. Shifting an equilibrium



B. Offering an alternate, nonproductive reaction path

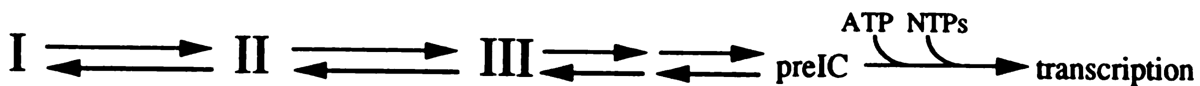


Figure 1-4

Transcription initiation as a linear series of equilibrium reactions. In this view, negative regulators could repress transcription either by shifting the position of any individual equilibrium, or by shunting assembling complexes off onto alternate, non-productive reaction paths.

path may not be the most appropriate model for the transcription initiation reaction. Only a small fraction of the available DNA templates are active in typical *in vitro* transcription reactions, though most templates are bound in protein complexes (Hawley & Roeder 1987, Horikoshi *et al.* 1988, Van Dyke *et al.* 1988, Kadonaga 1990, Maldonado *et al* 1990). This observation suggests that the majority of assembling transcription complexes have branched off onto non-productive reaction pathways. Thus, the transcription initiation reaction path may naturally contain branchpoints at which assembling transcription complexes partition between productive and non-productive forms (see Herschlag & Johnson 1993). Negative regulators of transcription might therefore repress transcription simply by influencing the number of assembling pre-initiation complexes that continue on the productive path versus those that branch off onto naturally available non-productive pathways.

Whether negative regulators shift equilibria along a linear reaction pathway or influence partitioning ratios in a branched pathway, it is clear that there are many steps at which they can affect the initiation of transcription. In this review, we have described some examples of molecular mechanisms used by transcriptional repressors. Further research will not only clarify the details of these mechanisms but will undoubtedly also uncover new tactics used by repressors to block transcription initiation.

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Chapter 2

Depletion of nucleosomes from yeast cells has only a modest effect on $\alpha 2$ repression

The DNA of eukaryotic organisms is wrapped around octamers of histone proteins to form complexes termed nucleosomes. It has long been postulated that the nucleosomal structure, or chromatin structure, of eukaryotic DNA might play a role in transcriptional regulation (for recent reviews, see Felsenfeld 1992; Kornberg and Lorch 1992). In particular, it has been proposed that wrapping DNA around histone octamers might "hide" the wrapped sequences from the cellular transcription machinery. It thus seemed possible that some transcriptional repressors might inhibit transcription by directing the formation of nucleosomes that occlude essential promoter information. We investigated this possibility for the yeast α2 repressor by asking if depleting histone H4 from yeast cells has any effect of the ability of α2 to repress transcription.

The yeast α2 protein is a cell-type-specific transcriptional repressor (present in α cells but absent from **a** cells) that binds to DNA cooperatively with the cell-type-ubiquitous Mcm1 protein and blocks the expression of genes required for a cell fate, the **a**-specific genes (Wilson and Herskowiz, 1984; Johnson and Herskowitz 1985; Keleher *et al.* 1988, 1989; Passmore *et al.* 1988, 1989; Ammerer 1990) In addition, insertion of an α2/Mcm1 binding site upstream of other RNA polymerase II-transcribed genes can bring those genes under negative regulation by α2 (Johnson and Herskowitz 1985). We report that depletion of nucleosomes from yeast cells has only a modest effect on α2 repression either of a *CYC1::LacZ* fusion gene put under the control of an upstream α2/Mcm1 operator, or of an endogenous **a**-specific gene.

Histone proteins in yeast are encoded by two redundant genes (Smith and Murray 1983). Grunstein and coworkers have constructed yeast strains in which both endogenous histone H4 genes, *HHF1* and *HHF2*, have been disrupted and a single copy of *HHF2*, now under the control of the experimentally regulatable *GAL1* promoter, is present on an episomal plasmid (Kayne *et al.* 1988, Kim *et al.* 1988). These strains grow well on galactose medium, as the *GAL1* promoter is active and the *HHF2* gene is expressed (Kim *et al.* 1988). When cells are shifted to glucose medium, however,

transcription of the *HHF2* gene is shut down. Approximately half of the nucleosomal structures in the cells are lost and the cells arrest in G2, with a block in chromosomal segregation (Kim *et al.* 1988). We utilized these strains to investigate the role of chromatin structure in α2 repression.

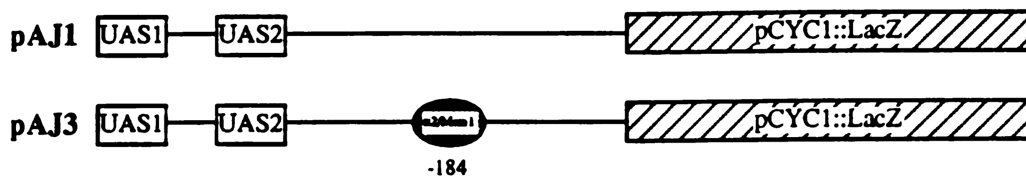
Figure 2-1A shows the constructs we used to investigate the effects of histone H4 depletion on α2 repression. These constructs contain the promoter of the yeast *CYC1* gene fused to the coding region of the LacZ gene from *E. Coli* (Guarente and Mason 1983; Guarente *et al.* 1984). An α2/Mcm1 operator inserted upstream of the transcriptional start site brings the expression of these *CYC1::LacZ* fusions under negative control by α2 (Johnson and Herskowitz 1985). Expression of these constructs was monitored by assaying ß-galactosidase activity (Miller 1972) in liquid cultures of yeast cells grown to mid-log phase in galactose medium and then either maintained in galactose medium or shifted into glucose medium.

The results presented in Figure 2-1B show that expression of the *CYC1::LacZ* fusion gene is not significantly altered, in **a** cells or in α cells, when cells are shifted from galactose medium (*HHF2* is expressed) to glucose medium (*HHF2* is not expressed) if the *HHF2* gene is under the control of its own promoter (lines 3-6). When expression of the *HHF2* gene is controlled by the *GAL1* promoter (lines 7-10), expression of the *CYC1::LacZ* fusion genes lacking upstream α2/Mcm1 operators is slightly reduced, perhaps reflecting the general poor growth of the cells, in both **a** and α cells after the shift from galactose medium to glucose medium (lines 7 and 9). Expression of the *CYC1::LacZ* fusion genes that contain an upstream α2/Mcm1 operator is also reduced in **a** cells (line 8), but is increased slightly in α cells (line 10) when cells are shifted from galactose medium to glucose medium. Thus, we observe a slight decrease (4-fold) in the magnitude of α2 repression (comparing the expression levels in α cells of constructs that do and do not contain an upstream α2/Mcm1 operator) when α cells carrying the *HHF2* gene under the control of the *GAL1* promoter are shifted from galactose medium to

Figure 2-1

A.

reporter constructs containing upstream activating sequences:



B.

line	H4 promoter	cell type	reporter	ß-galactosidase units when grown on galactose media	fold rep'n	ß-galactosidase units when grown on glucose media	fold rep'n
1.	H4	a	none	0.13		0.09	
2.	H4	α	none	0.20		0.09	
3.	H4	a	pAJ1	2394		1661	
4.	H4	a	pAJ3	2041		2084	
5.	H4	α	pAJ1	2122		1489	
6.	H4	α	pAJ3	32	**66X**	11	**135X**
7.	Gal	a	pAJ1	2088		873	
8.	Gal	a	pAJ3	2471		1326	
9.	Gal	α	pAJ1	430		245	
10.	Gal	α	pAJ3	4	**108X**	10	**25X**

Figure 2-1. Depletion of nucleosomes from yeast cells has only a modest effect on α2 repression of the *CYC1::LacZ* fusion gene.

A. Constructs used to assay α2 repression of the *CYC1::LacZ* fusion gene.

B. ß-galactosidase assays in liquid culture. **a** and α cells transformed with the indicated *CYC1::LacZ* reporter construct were grown to mid-log phase on galactose μεδιυμ and then either maintained in galactose μεδιυμ or shifted into glucose μεδιυμ for 2-3 doubling times. ß-galactosidase assays were performed as described (Miller 1972) except that yeast cells were permeabilized with 0.05% chloroform and 0.0025% SDS. Numbers presented represent averages of at least three independent transformants.

glucose medium. However, there is still significant repression (25 fold) by α2 in α cells on glucose medium (compare lines 9 and 10). Thus, depletion of histone H4 from yeast cells has only a modest effect on α2 repression of the *CYC1::LacZ* fusion gene.

We also investigated the effect of nucleosome depletion on α2 repression of the *MFA2* gene, an endogenous **a**-specific gene naturally regulated by α2. In these experiments, expression of the *MFA2* gene was monitored by northern analysis of RNA samples prepared from strains grown to mid-log phase in galactose medium and then either maintained in galactose medium or shifted into glucose medium. The results presented in Figure 2-2A show that the *MFA2* gene is expressed in **a** cells and not expressed in α cells regardless of which promoter controls the *HHF2* gene and on which medium the cells were growing. An ethidium-bromide stained gel of the same RNA samples, shown in Figure 2-2B, shows that similar amounts of RNA were present in all lanes. Thus, we conclude that depletion of nucleosomes from yeast cells does not significantly reduce the effectiveness of α2 repression.

It is important for the interpretation of these results to note that shifting cells whose *HHF2* gene is under the control of the *GAL1* promoter into glucose medium results in a loss of only about half of the chromosomal nucleosomes (Kim *et al.* 1988). It is possible that α2 repression involves the formation of a "superstable" nucleosome that is not disrupted when H4 is depleted. In fact, Simpson and colleagues have reported the observation of a statistically positioned nucleosome adjacent to α2/Mcm1 operators in α cells but not in **a** cells (Roth *et al.* 1990; Shimizu *et al.* 1991). It is possible that some interaction between the repression apparatus assembled at the operator and nucleosomal components would firmly position a nucleosome and also contribute to its stability. However, Roth *et al.* (1992) have shown that mutations in the N-terminus of histone H4 that disrupt the positioning of the nucleosome adjacent to the α2/Mcm1 operator decrease α2 repression only two to three fold. This observation, combined with our result that depletion of H4 from cells reduces α2 repression only 4-fold, suggests that, while

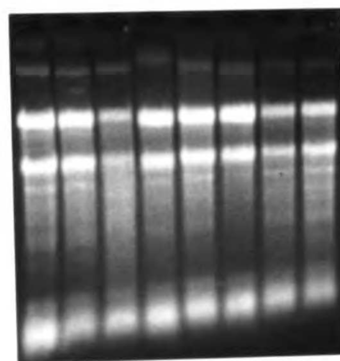
A.

H4	H4	Gal	Gal	Gal	Gal	H4	H4	H4 promoter
Gal	Gal	Glu	Gal	Gal	Glu	Glu	Glu	growth media
a	α	**a**	**a**	α	α	α	**a**	cell type

MFA2-

1 2 3 4 5 6 7 8

B.



1 2 3 4 5 6 7 8

Figure 2-2

Figure 2-2. Northern analysis of the *MFA2* transcript in **a** and α cells depleted for histone H4

A. Northern analysis of the *MFA2* transcript. RNA was prepared from **a** and α cells grown to mid-log phase in galactose medium and then either maintained in galactose medium or shifted into glucose medium for 2-3 doubling times. To prepare RNA, cells were vortexed with glass beads in a 1:1 mixture of buffer A (500 mM NaCl, 200 mM Tris-HCl pH 7.4, 10 mM EDTA pH 7.5, 1% SDS) and phenol-chloroform. Isolated RNA was incubated with 10 U of RQ1 RNase-free DNase I (Promega) per ml in 40 mM Tris-HCl pH7.5, 10 mM NaCl, 6 mM $MgCl_2$. DNase I was removed by phenol extraction. RNA was precipitated with 3V of ethanol and resuspended in water to a final concentration of 2-4 mg/ml. RNA samples were electrophoresed on 1% agarose gels containing 17% formaldehyde. RNA was transferred to a nylon membrane by capillary transfer in 20 X SSC and fixed by cross-linking with UV light (Church and Gilbert 1984). The *MFA2* transcript was detected by hybridization for at least 8 hours with a DNA fragment containing the *MFA2* gene that had been labeled with ^{32}P by nick translation as described (Maniatis *et al* 1982) and had been denatured by boiling for 5 minutes.

B. Ethidium-stained gel of RNA samples. Samples were prepared as described above, electrophoresed on a 1% agarose gel containing 17% formaldehyde, and stained with ethidium bromide for visualization of the RNA.

chromatin structure may contribute to α2 repression, it is probably not essential. One interesting possibility is that a positioned nucleosome adjacent to the α2 site contributes to α2 repression not by excluding some transcription factor from the DNA, but rather by complexing the DNA between the α2/Mcm1 operator and the transcription start site and thereby effectively bringing the repression apparatus closer to its target in the general transcription machinery.

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Chapter 3

The yeast α2 protein can repress transcription by RNA polymerases I and II but not III

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The Yeast α2 Protein Can Repress Transcription by RNA Polymerases I and II but Not III†

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The α2 protein of the yeast *Saccharomyces cerevisiae* normally represses a set of cell-type-specific genes (the a-specific genes) that are transcribed by RNA polymerase II. In this study, we determined whether α2 can affect transcription by other RNA polymerases. We find that α2 can repress transcription by RNA polymerase I but not by RNA polymerase III. Additional experiments indicate that α2 represses RNA polymerase I transcription through the same pathway that it uses to repress RNA polymerase II transcription. These results implicate conserved components of the transcription machinery as mediators of α2 repression and exclude several alternate models.

The α2 protein represses transcription of **a**-specific genes in *Saccharomyces cerevisiae* cells of α mating type by binding to DNA cooperatively with Mcm1, a protein that is found in both **a** and α cells (2, 47, 48, 80). The α2/Mcm1 complex recognizes a 32-bp operator found upstream of each **a**-specific gene. In the absence of α2 (in **a** cells), Mcm1 can bind alone to the operator and stimulate gene transcription (2, 5, 47, 53). Binding of α2 and Mcm1 together (in α cells) marks genes for repression. This repression requires at least two additional proteins, Ssn6 and Tup1 (32, 49, 57, 74, 90). Ssn6 and Tup1 are involved in transcriptional repression of diverse sets of genes, including **a**-specific, haploid-specific, and glucose-repressible genes (16, 49, 74, 105). Keleher et al. (49) have proposed that the Ssn6/Tup1 complex functions as a general repressor, targeted to specific genes by interaction with DNA-bound complexes. By this model, interaction with α2 and/or Mcm1 recruits the Ssn6/Tup1 complex to the promoters of **a**-specific genes.

The molecular mechanism by which a repression complex interferes with transcription is not understood. All of the genes naturally repressed by α2 are transcribed by RNA polymerase II. Previous work has demonstrated that insertion of the α2/Mcm1 operator upstream of other class II genes (*CYC1::lacZ TRP1 URA3 HIS3*) brings those promoters under negative control by α2 (39a, 43, 47, 52a, 85). To gain insight into the mechanism of α2 repression, we examined whether α2 could repress transcription by RNA polymerase I and RNA polymerase III. Transcription by each of these polymerases shares some characteristics with RNA polymerase II transcription, and each differs in interesting ways.

Most of the components of the RNA polymerase I and RNA polymerase III transcription machines are distinct from those involved in RNA polymerase II transcription. The yeast polymerases themselves each consist of 12 to 14 subunits (14, 93). Regions of amino acid sequence similarity identify the largest and second-largest subunits of RNA polymerases I, II, and III as homologs of each other and of the β′ and β subunits, respectively, of the *Escherichia coli* enzyme (1, 42, 70, 101, 112). Five of the smaller peptides are shared by RNA polymerases I, II, and III (14, 110). Two others are common to RNA polymerases I and III (25, 65). All of the other subunits appear to be used by only one polymerase.

RNA polymerases are recruited to the genes they transcribe by interaction with basal complexes assembled at promoters. Although most basal factors are specific to one polymerase, recent work has shown that some of the RNA polymerase II factors are used by the other polymerases. Specifically, the TATA-binding protein (TBP or TFIID) is required for transcription by all three polymerases (22, 23, 58, 66, 92, 99, 107). A TFIIB homolog involved in RNA polymerase III transcription has also been identified (10, 21, 59). Apparently, there are several common components within the different RNA polymerase complexes.

Promoter organization and regulatory mechanisms reveal further differences among the three RNA polymerases. RNA polymerase I transcribes the 35S rRNA gene, encoding a multifunctional transcript that is eventually processed into the 18S, 5.8S, and 25S rRNAs found in intact ribosomes. Transcription, RNA processing, and ribosome assembly all take place in the nucleolus, a specialized structure organized around actively transcribing 35S rRNA genes (references 45 and 83 and references therein). The organization of the 35S rRNA promoter is similar to that of class II genes. Essential promoter information is contained in DNA between positions −150 and +10 relative to the transcription start (11, 18, 50, 75). Transcription can be stimulated, to different degrees depending on the strain background, by an enhancer element located more than 2 kb upstream (29, 30, 44). This enhancer does not stimulate transcription by RNA polymerase II (12). Conversely, 35S rRNA transcription is not stimulated by class II activation elements (89). Apparently, despite similar organization of class I and class II promoters, activation mechanisms are not conserved.

RNA polymerase III transcribes small genes encoding functional RNAs (tRNAs, 5S rRNA, U6 small nuclear RNA [snRNA], etc.). The structural organization of most class III promoters is strikingly different from that of class II genes. Sequences within the gene, termed A box and B box, are required for transcription of most class III genes. Promoters of class III genes were originally termed intragenic because the coding sequence alone is often capable of directing

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† Dedicated to the memory of Eric Ritland (1964 to 1985).

correct product synthesis (6, 55, 87, 94; reviewed in reference 33). Recent work has indicated that in addition to the A box and B box, DNA up to −45 from the transcription start site is complexed by the RNA polymerase III transcription machinery (4, 40, 41, 46). Specific sequence elements within this upstream region do not usually affect RNA polymerase III transcription. However, the DNA must be available to the transcription machinery. This is evidenced by the finding that insertion of protein binding sites within 45 bp of the transcription start site of class III genes can interfere with their transcription (26, 67, 95, 102). When the same sites are moved farther away (beyond −45), inhibition is relieved. Thus, a more accurate description of class III promoters includes the gene-internal A box and B box and also an initiation region within 45 bp of the transcription start site.

Few class III genes are affected by DNA beyond these limits. Unlike class I and class II genes, class III genes in general do not respond to upstream regulators. One interesting exception to this rule is the U6 snRNA gene. DNA upstream of the RNA polymerase III-transcribed U6 snRNA gene contains several elements found in the promoter of the RNA polymerase II-transcribed U2 snRNA gene (8, 13, 24, 54, 106). These upstream elements are essential for full transcription of the U6 snRNA gene and can be substituted with elements from class II promoters (54, 58). Furthermore, in vertebrates and plants, canonical class III transcription elements are not required for RNA polymerase III transcription of the U6 snRNA gene. A gene-internal A-box homology is dispensable for transcription; a B-box homology is missing altogether (13, 24, 106). By contrast, the yeast U6 gene contains both upstream class II-like elements and a B-box homology, located downstream of the end of the transcription unit, that is essential for transcription (8, 9). Thus, the distinction between class II and class III promoter elements is not always clear.

Amino acid sequence comparisons of the largest subunits of yeast RNA polymerases I, II, and III indicate that RNA polymerases II and III are more closely related to each other than to RNA polymerase I (71). Perhaps, then, it is not so surprising to discover hybrid genes, with elements of both class II and class III promoters. There even exist examples of genes whose promoters direct transcription by both RNA polymerase II and RNA polymerase III (19, 68).

In this work, we have investigated the ability of the yeast α2 protein to repress transcription by RNA polymerases I and III. We find that α2 can repress RNA polymerase I transcription from an α2/Mcm1 operator positioned 200 bp upstream of the transcriptional start site. This repression requires the Ssn6 and Tup1 proteins, indicating that α2 uses the same pathway to repress transcription by RNA polymerases I and II. In contrast, α2 does not affect RNA polymerase III transcription.

MATERIALS AND METHODS

Yeast strains. The wild-type **a** and α strains used in this study are isogenic except at the *MAT* locus. Wild-type **a** cells are strain EG123 (*MAT***a** *suc2Δ trp1 leu2 ura3 his4*); wild-type α cells are strain 246-1-1 (98, 104). The *ssn6Δ9* deletion and the *Δtup1::LEU2* disruption were introduced into this strain background by Keleher et al. (49).

Strains NOY396 (*MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1*) and NOY446 (*MATα rpa135::LEU2 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1* pNOY102 [a high-copy-number plasmid carrying *pGAL7*-35S rDNA and *URA3*]) were provided by Masayasu Nomura (78).

SNR6 disruption strains YHM1 (*MAT***a**) and YHM2 [*MATα snr6::LEU2 ade2 his3-Δ200 leu2-Δ1 lys2-801*(am) *trp1-Δ63 ura3-52* YCp50-*SNR6* gene] were provided by Hiten Madhani (62). These strains were transformed to tryptophan prototrophy with the *SNR6*-containing plasmid pBH46 (see below). Plating on 5-fluoro-orotic acid expelled YCp50-*SNR6*. Retransformation to uracil prototrophy with YCp50-U6-5′Sp (62) and subsequent loss of pBH46 created BHY32 (*MAT***a**) and BHY33 (*MATα*). YCp50-U6-5′Sp contains *SNR6*5′Sp. In this construct, the 5′ stem-loop of *S. cerevisiae SNR6* has been substituted with that from *Schizosaccharomyces pombe*. This allele supports wild-type growth in *S. cerevisiae* but gives a truncated transcript (99 nucleotides instead of 112 nucleotides).

Plasmids and plasmid constructions. Plasmids pBH74 and pBH75 were made by subcloning a *Bam*HI-*Cla*I fragment containing the 35S rRNA::T7 gene fusion (without and with the rRNA enhancer, respectively) from YCprR8 or YCprR10 (30) into pRS315 (97). pBH101 and pBH104 were made by dropping a 37-bp oligonucleotide containing the α2/Mcm1 operator (5′CATGTAATTACCTAATAGGGAAATTTACA CGCTCGAG) into the *Sma*I sites of pBH75 and pBH74, respectively.

Plasmids pBH97 and pBH109 were constructed in two steps. First, the *CYC1::lacZ* fusion gene from pRY-32 (identical to pLG669 but with a *Bgl*II linker, 5′CGAGATCTG, in the *Sma*I site at −312 [37]) was cloned on a *Bgl*II-*Sca*I fragment into pRS314 (97) cut with *Bam*HI and *Sca*I. The resulting plasmid, pBH73, was linearized with *Sal*I and end filled with Klenow enzyme. Fragments containing the *SNR6* gene with or without the α2/Mcm1 site were isolated by *Eco*RI-*Sal*I digestion of pBH53 or pBH46, respectively. pBH46 has the *SNR6* gene and 120 bp of upstream DNA on an *Eco*RI-*Sal*I fragment from pCH6-120 (David Brow) in pSE358. pBH53 is pBH46 with the 36-bp α2/Mcm1 operator (5′TCGACATGTAATTACCTAATAGGGAAATTTACA CGC) in the *Sal*I site. The *SNR6*-containing fragments isolated from these plasmids were end filled with Klenow enzyme and ligated with the end-filled pBH73 fragment to make pBH109 (which has the α2/Mcm1 site) and pBH97 (which has no α2/Mcm1 site).

To make plasmids pBH132 and pBH143, the *SUP3*(am) gene was first cloned into pUC18 on a 137-bp *Bam*HI fragment from mWJ64 (88). The resulting plasmid, pBH23, was then digested with *Sal*I and *Eco*RI; the *SUP3*(am)-containing fragment was ligated into pSE358 (precursor to pUN10 [31]) to create pBH124. The *SUP3*(am) gene was then subcloned into pRS315 or pBH47 on a *Sac*I-*Xba*I fragment, creating pBH132 and pBH143, respectively. pBH47 is pRS315 with the 36-bp α2/Mcm1 operator in the *Sal*I site. Plasmids pBH148 and pBH149 were made by deleting an 18-bp *Hin*dII-*Pst*I fragment from the polylinkers of pBH132 and pBH143, respectively. After simultaneous digestion with both enzymes, overhanging ends were blunted with T4 DNA polymerase and plasmids were religated. pBH155 was made by dropping two additional α2/Mcm1 operators into the *Xho*I site of pBH143. Plasmids pRB55 and pRB58 were provided by Jasper Rine (88).

RNA extraction. RNA was prepared by vortexing yeast cells with glass beads in a mixture of buffer A (500 mM NaCl, 200 mM Tris-HCl [pH 7.4], 10 mM EDTA [pH 7.5], 1% sodium dodecyl sulfate [SDS]) and phenol-chloroform. Isolated RNA was incubated with 10 U of RQ1 RNase-free DNase I (Promega) per ml in 40 mM Tris-HCl (pH 7.5)–10 mM NaCl–6 mM $MgCl_2$. DNase I was removed by phenol extraction. RNA was precipitated with 3 volumes of ethanol

and resuspended in water to a final concentration of 2 to 5 mg/ml. All solutions were treated with diethyl pyrocarbonate to inactivate RNase.

Northern (RNA) blot analysis. RNA was prepared as described above. Samples were electrophoresed on 1% agarose gels containing 17% formaldehyde. RNA was transferred to a nylon membrane by capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and fixed by cross-linking with UV light (20). T7-tagged transcripts from the 35S rRNA::T7 promoter were detected with an antisense riboprobe generated by SP6 transcription of *Eco*RI-digested pSP-T7+ (44) in the presence of radioactive UTP by using the Riboprobe Gemini II system from Promega. Filters were hybridized and washed as described previously (27). *MCM1* control transcripts were detected by at least 8 h of hybridization at 37°C with a 19-nucleotide oligonucleotide (5'CAGCGCGCCTGCCGGTACC) radioactively labeled by phosphorylation in the presence of [γ-^{32}P]ATP. Hybridization and wash solutions were as described by Church and Gilbert (20).

Primer extension analysis. RNA was prepared as described above. Primer extensions were done by the method of McKnight and Kingsbury (69). RNA (10 to 50 μg) was denatured at 95°C for 3 min in the presence of 10^5 to 10^6 cpm of radioactively labeled primer in 250 mM KCl–0.2× Tris-EDTA. The primer used was U6D (5'AAAACGAAATAAA TCTCTTT), which hybridizes to sequences at the 3' end of the *SNR6* and *SNR65'*Sp transcripts (8). Incubation at 42°C for 1 h allowed the primer to anneal to the RNA; then 2.5 volumes of reverse transcription mix (70 mM Tris-HCl [pH 8.0], 7 mM $MgCl_2$, 0.14 mg of actinomycin D per ml, 14 mM dithiothreitol, 0.35 mM EDTA [pH 8.0], 0.35 mM deoxynucleoside triphosphates, 2 U of avian myeloblastosis virus reverse transcriptase per ml) was added. Samples were incubated at 42°C for 30 min to allow extension. Extended products were precipitated with 0.86 volume of 5 M ammonium acetate and 5 volumes of ethanol. Products were separated by electrophoresis through an 8% acrylamide–urea gel (64). All solutions were treated with diethyl pyrocarbonate to inactivate RNase.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Miller (72) except that yeast cells to be assayed were permeabilized with chloroform and SDS. Cells were grown to mid-log phase (optical density at 600 nm [OD_{600}] of 0.3 to 1.0) in selective medium supplemented with 2% glycerol. Approximately 10^7 cells were pelleted in a microcentrifuge and resuspended in 1 ml of Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). The OD_{600} of the resuspended culture was recorded. Cultures were diluted 2.5- to 20-fold, depending on their expected activity level. Cells were then permeabilized by incubation for 5 min at room temperature in the presence of 0.0025% SDS and 5% $CHCl_3$. β-Galactosidase activity was measured by addition of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to a final concentration of 0.8 mg/ml. Color was allowed to develop for less than 5 h. Reactions were stopped by addition of 5/12 volume of 1 M Na_2CO_3 to raise the pH and thereby inactivate the enzyme. Debris was pelleted in a microcentrifuge for 4 min. The OD_{420} of the supernatant was recorded. β-Galactosidase units were calculated by using the following formula: units = $1{,}000(OD_{420})/t$ (s) · vol (ml) · OD_{600}. Numbers represent averages of three independent isolates.

Invertase assays. Cells were grown to mid-log phase (OD_{600} of 0.3 to 1.0) in selective medium supplemented with 2% glucose. Approximately 10^7 cells were pelleted, resuspended in selective medium containing 0.1% glucose, and grown at 30°C with aeration for 2.5 to 3.5 h. Cells were pelleted again and resuspended in water. Secreted invertase, located in the periplasmic space, was assayed essentially as described by Goldstein and Lampen (34). Cells (10^5) were incubated with 12.5 μmol of sucrose in 100 mM sodium acetate (pH 4.9) prewarmed to 65°C. After 10 min at 30°C, reactions were stopped by addition of 1 volume of 0.5 M KPO_4 (pH 7.0) and immediate boiling for 3 min. Glucose released by the enzymatic hydrolysis of sucrose was detected after the reactions had cooled to room temperature. Five volumes of solution C (84 μg of glucose oxidase per ml, 10 μg of peroxidase per ml, 300 μg of *o*-dianisidine per ml, 38.25% glycerol, 9 mM KPO_4) was added. After less than 20 min at room temperature, color reactions were stopped by addition of 1.25 volumes of 6 N HCl. Developed color was read at 540 nm. Units of invertase are given in micrograms of glucose released per minute of hydrolysis per minute of color reaction per OD_{600} unit of cells. Values given represent averages of at least three independent isolates.

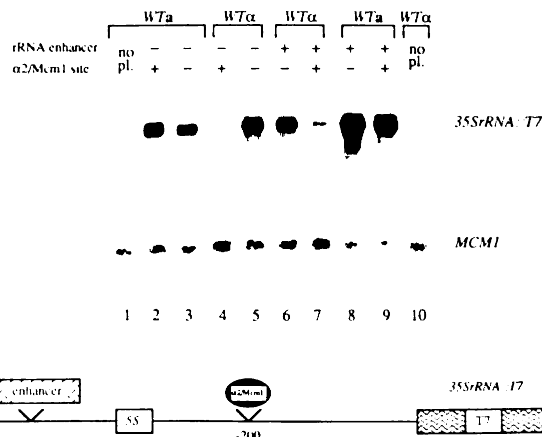


FIG. 1. Repression by α2 of RNA polymerase I transcription of the 35S rRNA::T7 promoter. RNA was isolated from wild-type (*WT*) *MAT***a** and *MAT*α strains transformed with no plasmid (no pl.; lanes 1 and 10), pBH104 (lanes 2 and 4), pBH74 (lanes 3 and 5), pBH75 (lanes 6 and 8), or pBH101 (lanes 7 and 9). The relevant features of these plasmids are indicated above the lanes. Samples were electrophoresed and blotted. The upper panel shows hybridization of the filter with a ^{32}P-labeled riboprobe that hybridizes to the T7 DNA marking the 35S rRNA::T7 test promoter constructs; the lower panel shows the same blot stripped and rehybridized with an oligonucleotide probe directed to the *MCM1* gene.

RESULTS

α2 represses RNA polymerase I transcription. In wild-type yeast cells, RNA polymerase I is responsible for transcription of a single gene, 35S rRNA. To investigate the ability of α2 to repress RNA polymerase I transcription, we used a plasmid-borne 35S rRNA minigene marked with 300 bp of phage T7 DNA (30). This allowed us to distinguish our test promoter transcripts from endogenous 35S rRNA transcripts. α2 represses its authentic, RNA polymerase II-transcribed target genes from an operator located 100 to 200 bp upstream of the transcription start site (43, 108, 109). By analogy, we inserted an α2/Mcm1 binding site approximately 200 bp upstream of the start site of the 35S rRNA::T7 minigene (Fig. 1). At this location, the α2/Mcm1 operator is

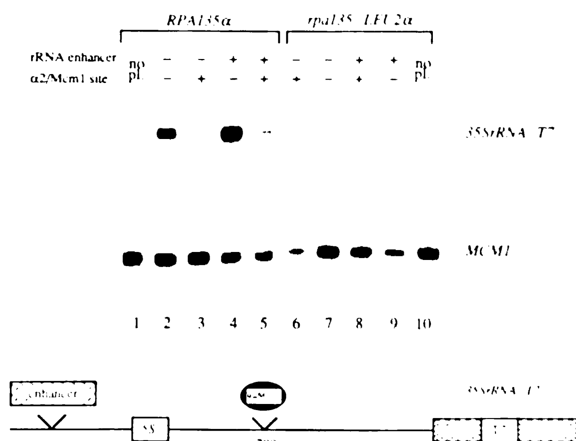


FIG. 2. Evidence that transcription of the 35S rRNA::T7 reporter gene is accomplished by RNA polymerase I. Test promoter constructs were transformed into yeast strains NOY396 (lanes 1 to 5) and NOY446 (lanes 6 to 10). These strains are isogenic except at the *RPA135* locus. RNA was isolated from strains transformed with no plasmid (no pl.; lanes 1 and 10), pBH74 (lanes 2 and 9), pBH104 (lanes 3 and 8), pBH75 (lanes 4 and 7), or pBH101 (lanes 5 and 6). The relevant features of these plasmids are indicated above the lanes. Samples were electrophoresed and blotted. The upper panel shows hybridization of the filter with a ^{32}P-labeled riboprobe that hybridizes to the T7 DNA marking the 35S rRNA::T7 test promoter constructs; the lower panel shows the same blot stripped and rehybridized with an oligonucleotide probe to the *MCM1* gene.

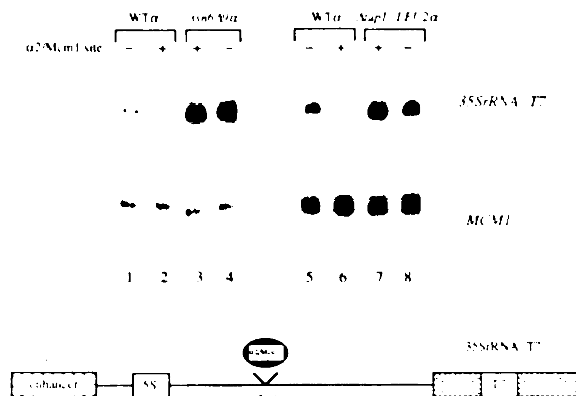


FIG. 3. Evidence that α2 repression of RNA polymerase I transcription requires *SSN6* and *TUP1*. *MAT*α yeast strains isogenic except at the loci indicated were transformed with test promoter constructs. RNA was isolated from strains transformed with no plasmid (no pl.; lane 1), pBH75 (lanes 2, 5, 6, and 9), or pBH101 (lanes 3, 4, 7, and 8). The relevant features of these plasmids are indicated above the lanes. Samples were electrophoresed and blotted. The upper panel shows hybridization of the filter with a ^{32}P-labeled riboprobe that hybridizes to the T7 DNA marking the 35S rRNA::T7 test promoter constructs; the lower panel shows the same blot stripped and rehybridized with an oligonucleotide probe to the *MCM1* gene. WT, wild type.

at least 50 bp upstream of any essential class I promoter elements.

RNA isolated from strains transformed with these constructs was analyzed by Northern blotting. Transcripts from the test 35S rRNA::T7 constructs were detected by hybridization with a riboprobe complementary to the T7 DNA. Accuracy of sample loading was monitored by rehybridizing the same blot with an oligonucleotide probe that detects the *MCM1* transcript, which should be present at the same level in all cells examined.

The experiment whose results are shown in Fig. 1 shows that α2 represses transcription of test 35S rRNA::T7 constructs that contain an α2/Mcm1 binding site (lanes 4 and 7). The presence of an α2/Mcm1 site has no effect in **a** cells, which lack α2 (lanes 2 and 3; lanes 8 and 9). Apparently, Mcm1 on its own does not stimulate RNA polymerase I transcription. This finding is consistent with previous observations that class II activators do not affect RNA polymerase I transcription (89). Moreover, binding of Mcm1 alone to the α2/Mcm1 site (in **a** cells) does not appear to interfere with transcription of the 35S rRNA::T7 fusion gene. Presumably, this means that operator occupancy per se is insufficient for repression of class I transcription, as it is for repression of class II transcription.

Although it does not bear on the interpretation of our results, we note that the presence of the RNA polymerase I enhancer had little or no effect in the strains used in this study (Fig. 1; compare lanes 2 to 5 with lanes 6 to 9).

We ruled out the possibility that our fusion constructs might fortuitously be transcribed by RNA polymerase II by transforming the same reporter constructs into a strain disrupted for *RPA135*, the gene encoding the second-largest subunit of RNA polymerase I (78, 79). These strains survive (on galactose) because they carry a high-copy-number plasmid with the wild-type 35S rRNA gene under the control of the *GAL7* promoter. With transcription of the 35S rRNA gene being directed by RNA polymerase II, RNA polymerase I is dispensable. Figure 2 shows that our test 35S rRNA::T7 constructs are not transcribed in strains lacking functional RNA polymerase I (lanes 6 to 10). The same constructs are transcribed (lanes 1 to 5), and repressed if they contain an α2/Mcm1 binding site (lanes 2 and 5), in otherwise isogenic strains whose *RPA135* gene is still intact. Thus, the observed transcription of our 35S rRNA::T7 reporter gene is carried out by RNA polymerase I. We conclude that α2 represses transcription by RNA polymerase I.

Repression of RNA polymerase I transcription requires Ssn6 and Tup1. α2 repression of class II genes requires the Ssn6/Tup1 repressor complex. In the absence of Ssn6, α2 and Mcm1 occupy the operator, but RNA polymerase II transcription is not repressed (49). We determined whether α2 repression of RNA polymerase I transcription showed the same requirement for Ssn6 and Tup1.

The experiment whose results are shown in Fig. 3 shows that Ssn6 and Tup1 are required for repression of RNA polymerase I transcription. Strains that have intact *SSN6* and *TUP1* genes repress the reporter constructs (lanes 2 and 6). In contrast, otherwise isogenic strains bearing the *ssn6*Δ*9* allele, which eliminates Ssn6 function (91), do not repress the 35S rRNA::T7 reporter gene (lane 3). Strains carrying a Δ*tup1*::*LEU2* disruption allele also do not repress the 35S rRNA::T7 reporter (lane 7). By these criteria, α2 appears to repress both RNA polymerase I and RNA polymerase II transcription through the same pathway.

α2 does not repress RNA polymerase III transcription of the *SNR6* promoter. We next examined whether α2 could repress RNA polymerase III transcription. We examined transcription of the *SNR6* gene, which encodes the yeast U6 snRNA. To distinguish transcription of our test constructs from endogenous U6 transcripts, we used strains carrying the *SNR6*5′Sp allele. In this allele, the 5′ stem-loop of the *S.*

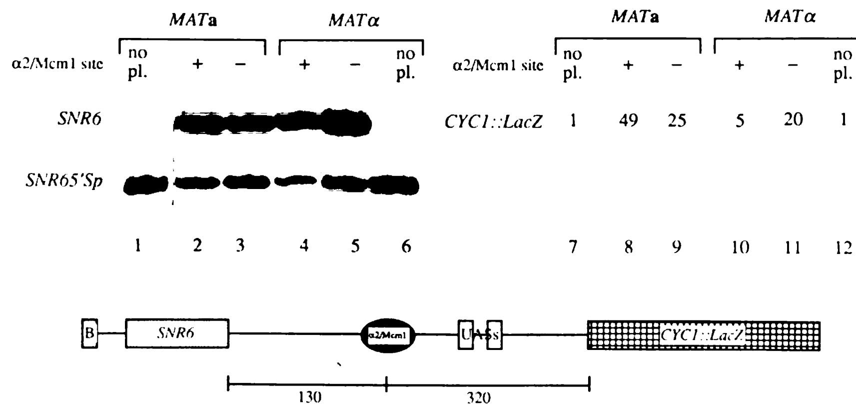


FIG. 4. Evidence that α2 does not repress RNA polymerase III transcription of the *SNR6* gene. Test promoter constructs were transformed into yeast strain BHY32 or BHY33. RNA was isolated from strains transformed with no plasmid (no pl.; lanes 1 and 6), pBH109 (lanes 2 and 4), or pBH97 (lanes 3 and 5). The relevant features of these plasmids are indicated above the lanes. Expression of the *SNR6* gene was assayed by primer extension analysis. A truncated *SNR65'*Sp transcript serves as an internal control for RNA levels in the individual samples. Expression of the *CYC1::lacZ* fusion gene was detected by β-galactosidase activity levels in liquid culture (lanes 7 to 12).

cerevisiae U6 has been substituted with that from *S. pombe*. The resulting transcript is shorter by 13 nucleotides but is fully functional (7a, 62). The endogenous U6 transcript in these strains is thus detectably shorter than transcripts from our test promoter constructs, which contain the wild-type *SNR6* gene.

We transformed yeast strains of both mating types with test constructs containing an α2/Mcm1 operator 130 bp upstream of the *SNR6* transcription start site (Fig. 4). As a control, the RNA polymerase II-transcribed *CYC1::lacZ* fusion gene (37) was positioned on the other side of the α2/Mcm1 operator (Fig. 4). The *SNR6* and *CYC1::lacZ* genes on these constructs are transcribed divergently. The *CYC1::lacZ* fusion is sensitive to α2 repression (43, 47) and thus provides a control ensuring that a functional repression complex can assemble at the operator. Expression of the *SNR6* gene is detected by primer extension. *CYC1::lacZ* expression is detected by β-galactosidase assay in liquid culture.

Figure 4 shows that transcription of the *SNR6* gene is not affected by an α2/Mcm1 operator at −130 (lanes 2 and 4). The *CYC1::lacZ* gene, on the other hand, is repressed approximately fivefold in *MAT*α cells (compare lane 10 with lanes 9 and 11), as expected from previous observations (47). This result shows that an intact repression complex forms at the α2/Mcm1 site but fails to repress RNA polymerase III transcription. Constructs lacking the *CYC1::lacZ* gene, or those in which the α2/Mcm1 operator is located downstream of the *SNR6* transcription unit but upstream of the B box, give identical results (data not shown). In each case, we find that α2 does not repress RNA polymerase III transcription of *SNR6*.

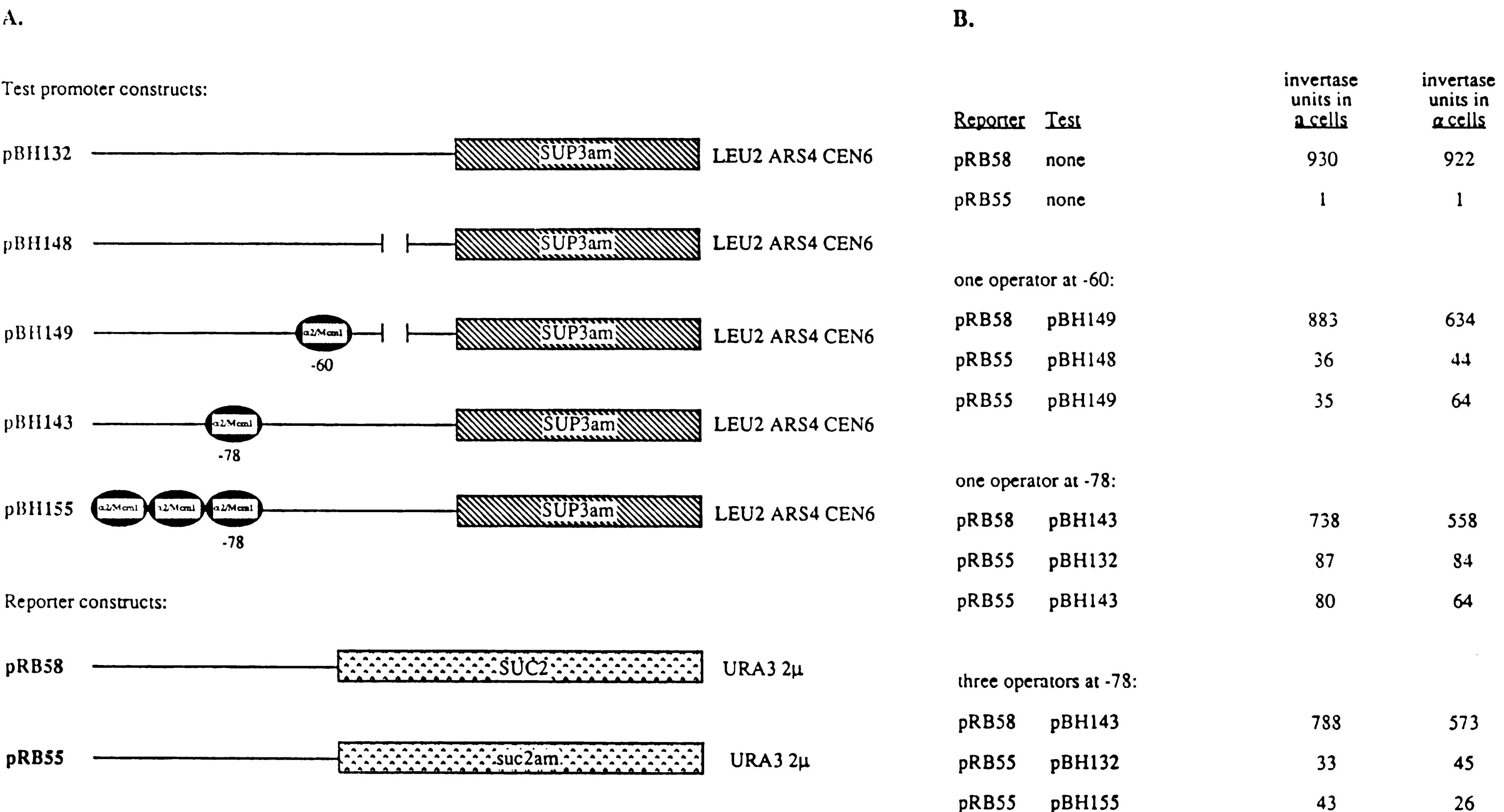
α2 does not repress RNA polymerase III transcription of the *SUP3*(am) promoter. It remained possible that the *SNR6* promoter is uniquely resistant to repression by α2. To generalize our results to other class III transcription units, we examined the effect of upstream α2/Mcm1 sites on the activity of the *SUP3*(am) amber suppressor tRNA gene. Figure 5A shows the constructs used in assays of *SUP3*(am) activity. One or three α2/Mcm1 operators were cloned 78 bp upstream of the *SUP3*(am) transcription start site. Deletion of 18 bp of polylinker DNA from the single-operator construct created a third test promoter construct, with the α2/Mcm1 site at −60 relative to the transcription start. Unfortunately, cross-reactivity with other yeast tRNAs prevents direct analysis of *SUP3*(am) expression (38, 88). We were able to determine the level of expression of the *SUP3*(am) gene, however, by assaying invertase activity in strains carrying an amber mutation in the *SUC2* invertase gene. In such strains, invertase activity reflects the level of *SUP3*(am) tRNA available to suppress the *suc2* amber mutation (88). Each test promoter construct was cotransformed with a reporter plasmid bearing either the wild-type *SUC2* gene or the *suc2-215*(am) amber mutant allele (15) into wild-type **a** and α cells. Invertase activity was determined in liquid cultures.

Figure 5B shows that invertase levels are unaffected by the presence of α2/Mcm1 operators upstream of the *SUP3*(am) gene. Operators at −53 or −84 also had no effect on *SUP3*(am) expression (data not shown). Apparently, binding of Mcm1 alone in **a** cells or α2/Mcm1 in α cells has no significant effect on the level of transcription of the *SUP3*(am) tRNA gene. Combining this finding with the data for the *SNR6* gene, we conclude that α2 does not repress transcription of at least two RNA polymerase III-transcribed genes.

DISCUSSION

We have shown that the yeast α2 repressor, which is usually responsible for repression of genes transcribed by RNA polymerase II, can also repress transcription by RNA polymerase I but not by RNA polymerase III. Moreover, the data suggest that α2 represses transcription by RNA polymerases I and II by the same pathway, indicating that the target of α2 repression is common to RNA polymerases I and II. This allows us to rule out several specific models of α2 repression.

For example, our results argue against involvement of the carboxy-terminal domain (CTD) of RNA polymerase II in α2 repression. The CTD of the largest subunit of yeast RNA polymerase II consists of 26 or 27 copies of a heptapeptide repeat. Phosphorylation of this tail is believed to control the transition from transcription initiation to elongation (17, 56, 60, 61, 81). In principle, α2 could repress RNA polymerase II transcription by preventing phosphorylation of the CTD,



Reporter	Test	invertase units in a cells	invertase units in α cells
pRB58	none	930	922
pRB55	none	1	1
one operator at -60:			
pRB58	pBH149	883	634
pRB55	pBH148	36	44
pRB55	pBH149	35	64
one operator at -78:			
pRB58	pBH143	738	558
pRB55	pBH132	87	84
pRB55	pBH143	80	64
three operators at -78:			
pRB58	pBH143	788	573
pRB55	pBH132	33	45
pRB55	pBH155	43	26

FIG. 5. Evidence that α2 does not repress RNA polymerase III transcription of the *SUP3*(am) gene. (A) Test promoter constructs and reporter plasmids used in analyses of *SUP3*(am) transcription. (B) Assay in which test promoter constructs were cotransformed with reporter constructs into isogenic wild-type strains. Expression of the *SUP3*(am) gene was detected by its ability to suppress an amber mutation in the *SUC2* invertase gene.

thus interfering with promoter escape. However, our results show that α2 can repress transcription by RNA polymerase I. Since RNA polymerase I does not have an analogous CTD, we conclude that the RNA polymerase II CTD is not required to mediate α2 repression.

We can similarly eliminate most of the class II basal transcription factors (e.g., TFIIA and TFIIF) as targets of α2 repression, since they are involved only in RNA polymerase II transcription. Also, polymerase subunits specific to RNA polymerase II are not required in order to mediate α2 repression.

Our data also argue against models of repression by interference with upstream activators. The results show that α2 can repress transcription by RNA polymerases whose activation systems cannot be interchanged. RNA polymerases I and II do not respond to the same transcriptional activators (12, 89), yet both are repressed by α2. This fact argues against the simplest models of activator interference.

We can also rule out the possibility that α2 repression involves packaging of DNA into inaccessible chromatin. Chromatin structure is postulated to be involved in transcriptional position effects, wherein gene expression is affected by chromosomal location. Such position effects are observed in yeast cells at the silent mating-type loci and at telomeres (3, 35, 36, 52, 77). Both of these regions have complex chromatin structure, and both can silence genes that are transcriptionally active when located elsewhere in the genome (7, 36, 63, 76, 100, 111). The products of the *SIR* genes mediate transcriptional repression both at telomeres and at the silent mating-type loci (35, 39, 51, 84). As all three RNA polymerases rely on recognition of specific DNA elements for assembly of functional transcription complexes, we might expect DNA packaging to repress transcription of all three gene classes. Consistent with this idea, Schnell and Rine (88) have shown that *SIR* repression effectively inhibits RNA polymerase III transcription of the *SUP3(am)* gene when this gene is integrated at the silent mating-type locus *HMR***a**. On the other hand, we have shown in this report that the same gene is not repressed when α2/Mcm1 operators are positioned upstream. Morse et al. (73) have similarly found that an α2/Mcm1 site 90 bp upstream of the start site of the RNA polymerase III-transcribed *sup4-o* $tRNA^{Tyr}$ gene does not interfere with its transcription. For another class III gene, *SNR6*, we were able to show that a functional repression complex assembles at the upstream α2/Mcm1 operator, repressing a divergently transcribed class II gene, but has no effect on transcription by RNA polymerase III. Thus, α2 repression and *SIR* repression are distinguished by their effects on RNA polymerase III transcription.

Roth et al. (85, 86) have postulated that α2 might repress transcription not by organizing regions of DNA into complex chromatin structures but by positioning a single nucleosome over important promoter elements. Analyses of chromatin containing an α2/Mcm1 site show that a nucleosome is positioned adjacent to the α2/Mcm1 operator in α cells but not in **a** cells (85, 86, 96). Morse et al. (73) have further demonstrated a correlation between nucleosome positioning and transcriptional repression by showing that the RNA polymerase III-transcribed *sup4-o* $tRNA^{Tyr}$ gene, which is resistant to α2 repression, is also not incorporated into a nucleosome. However, other observations indicate that destabilization of positioned nucleosomes by mutation (86) or depletion (39a) of histone H4 has only a modest effect on α2 repression. Thus, while nucleosome positioning may contribute to α2 repression, as suggested by Roth et al. (85, 86), it is unlikely to be the sole mechanism.

The simplest interpretation of our results says that a factor common to RNA polymerases I and II mediates α2 repression. Recent work on components of the basal transcription machinery offers intriguing possibilities. For example, the TATA-binding protein (TBP or TFIID), originally identified as a component of the class II basal machinery, is required for transcription by all three polymerases (22, 23, 58, 66, 92, 107). TBP associates with different accessory factors, termed TBP accessory factors (TAFs), to create complexes specific to each RNA polymerase (22, 28, 82, 103). Given that α2 can repress transcription by RNA polymerases I and II but not III, we would postulate that α2 repression targets either a TAF specific to class I and class II TBP/TAF complexes or a surface of TBP that is inaccessible in class III TBP/TAF complexes.

Class I, II, and III basal transcription complexes may have other common features. In particular, homologs of the class II transcription factor TFIIB may be involved in transcription by the other polymerases. A TFIIB homolog required for RNA polymerase III transcription has been isolated as a suppressor of TBP temperature-sensitive alleles and also as a suppressor of an A-box mutation (10, 21, 59). It will be interesting to determine whether a third member of the TFIIB family is involved in class I transcription. Specific regions of homology between class I and class II TFIIBs, distinct from the class III TFIIB, might suggest interaction domains for α2 repression machinery.

Given these observations, we propose that α2 repression involves interaction with components of the basal transcription machinery that are common to RNA polymerases I and II. According to this idea, α2 directs transcriptional repression by recruiting the Ssn6/Tup1 general repressor complex to promoters containing an α2/Mcm1 operator. Interaction of Ssn6/Tup1 with basal transcription factors, perhaps TBP or TFIIB, interferes with further assembly of a functional transcription complex and thus represses transcription.

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Appendix A

Transcriptional interference by α2 bound within the *SUP3am* promoter region

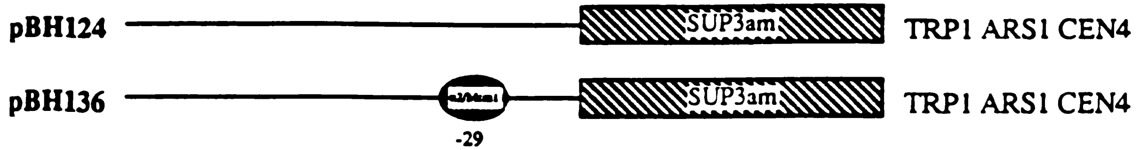
As mentioned in the introduction to Chapter 3, DNA up to -45 from the transcription start site of class III genes must be accessible to the RNA polymerase III transcriptional machinery. Insertion of recognition sites for DNA-binding proteins within this region can interfere with expression of the downstream gene, presumably due to competition between the DNA binding protein and the RNA polymerase III transcriptional machinery for access to the DNA (Syroid *et al.* 1982; Shaw and Olson 1984; Marschalek and Dingermann 1988; Dingermann *et al.* 1992).

The experiment of Figure A-1 shows that insertion of an α2/Mcm1 recognition site 29 bp upstream of the transcription start site of the *SUP3am* gene interferes with the expression of that gene. The test promoter constructs used in these experiments are diagrammed in Figure A-1A. We determined the level of expression of the *SUP3am* gene on these constructs by assaying invertase activity in strains carrying an *amber* mutation in the *SUC2* invertase gene. Each of the diagrammed test promoter constructs was cotransformed with a reporter plasmid bearing with the wild type *SUC2* gene or the *suc2-215am amber* mutant allele (Carlson and Botstein 1982). Invertase activity was determined in liquid culture. In α cells, invertase activity is reduced approximately 5 fold when the test promoter construct contains the α2/Mcm1 operator. In **a** cells, there may also be a small effect in the presence of the α2/Mcm1 operator, presumably due to Mcm1 binding to the operator in the absence of α2. Perhaps because Mcm1 alone does not compete as effectively with the RNA polymerase III transcription machinery for access to the DNA as does the α2/Mcm1 complex, the transcriptional interference observed in **a** cells is less dramatic and more variable than the effect seen in α cells.

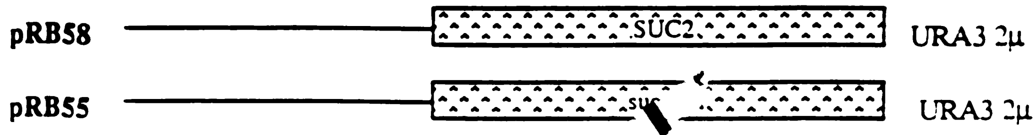
Figure A-1

A.

Test promoter constructs:



Reporter constructs:



B.

Reporter	Test	invertase units in a cells	fold interference	invertase units in α cells	fold interference
pRB58	pBH136	801		850	
pRB55	pBH124	90		100	
pRB55	pBH136	47	**1.9**	19	**5.3**

Figure A-1. Transcriptional interference by α2 bound within the promoter region of the *SUP3am* gene.

A. Constructs used to assay transcriptional interference by α2 bound within the promoter region of the *SUP3am* gene. pBH136 was made by dropping the 36 bp α2/Mcm1 operator into the unique *Sal* I site in pBH124 (described in Chapter 3 Materials and Methods).

B. Test promoter constructs and reporter constructs were cotransformed into yeast strain EG123 (Tatchell *et al* 1981; Silicano and Tatchell 1984). Invertase assays in liquid culture were performed as described in Chapter 3 Materials and Methods.

Unlike true $\alpha 2$ repression, transcriptional interference does not require the N-terminal region of $\alpha 2$ (Figure A-2). Mutational studies (Hall and Johnson 1987; K. Komachi and A.D.J., unpublished) have revealed that the N-terminal domain of $\alpha 2$ is required, in addition to the homeodomain responsible for DNA binding, to mediate $\alpha 2$ repression from an $\alpha 2$/Mcm1 operator. In the experiment of Figure A-2, mutant versions of $\alpha 2$ that are disrupted in the N-terminal domain and are incapable of directing repression of RNA polymerase II-transcribed genes (Hall and Johnson 1987; K.Komachi and A.D.J., unpublished) were transformed on a high copy vector into yeast strains that themselves make no $\alpha 2$. The *SUP3am* test promoter constructs and reporter constructs used in the experiment of Figure A-1 were also transformed into these strains and invertase activities were determined in liquid culture. Point mutations ($\alpha 2^{thr4}$, $\alpha 2^{lys71}$), and even a small deletion ($\alpha 2^{\Delta 3\text{-}6}$::LacZ), within the N-terminal region of $\alpha 2$ did not affect the ability of $\alpha 2$ to interfere with RNA polymerase III transctipion of the *SUP3am* gene from a binding site within the promoter region (Figure A-2). It therefore appears that only the DNA-binding activity of $\alpha 2$ is required for transcriptional interference.

The observation of transcriptional interference reveals that $\alpha 2$, or more likely the $\alpha 2$/Mcm1 complex, is able to compete effectively with the RNA polymerase III machinery for access to DNA sequences. Presumably, the more stable the $\alpha 2$/Mcm1 complex is, the more effectively it will interfere with RNA polymerase III transcription.

The results presented in Figure A-3 suggest that Tup1 and/or Ssn6 may stabilize the $\alpha 2$/Mcm1 complex on the DNA. When the test promoter constructs and reporter constructs diagrammed in Figure A-1A were cotransformed into yeast strains disrupted either for *TUP1* or *SSN6*, strong transcriptional interference was not observed. This result suggests that the $\alpha 2$/Mcm1 complex is less stable on the DNA (less able to compete effectively with the RNA polymerase III transcription machinery) in the absence of Tup1 or Ssn6. This observation could be explained by proposing that Tup1 and/or Ssn6

Reporter	Test	CV13	fold int.	$\alpha 2^{\Delta 3-6}$:: LacZ	fold int.	$\alpha 2^{thr4}$	fold int.	$\alpha 2^{lys71}$	fold int.
pRB58	pBH136	1420		1307		1369		1038	
pRB55	pBH124	113		91		139		106	
pRB55	pBH136	65	**1.7**	11	**8.3**	8	**17.4**	30	**3.5**

Figure A-2. Transcriptional interference by N-terminal mutants of α2

High copy expression vectors of α2 mutants in CV13 (YEp13) were provided by K. Komachi. Expression vectors, test promoter constructs, and reporter constructs were co-transformed into yeast strain KTX23αK8 (*matΔ trp1 leu2 ura3 his4 suc2 gal2*, created by deletion of MATα from EG123 (Tatchell *et al* 1981; Silicano and Tatchell 1984). Invertase assays were performed in liquid culture as described in Chapter 3 Materials and Methods.

Reporter	Test	invertase units in **a** cells	fold interference	invertase units in α cells	fold interference
tup1Δ::LEU2					
pRB58	pBH136	2279		1829	
pRB55	pBH124	521		565	
pRB55	pBH136	596	**0.9**	847	**0.7**
ssn6Δ9					
pRB58	pBH136	3245		4051	
pRB55	pBH124	296		497	
pRB55	pBH136	421	**0.7**	225	**2.2**

Figure A-3. Lack of transcriptional interference in the absence of Tup1 or Ssn6

Test promoter constructs and reporter constructs diagrammed in Figure A-1A were co-transformed into yeast strains made by C. Keleher (Keleher *et al.* 1992). These strains are disrupted for *SSN6* or *TUP1* but otherwise isogenic to EG123 (Tatchell *et al.* 1981; Silicano and Tatchell 1984). Invertase assays were performed in liquid culture as described in Chapter 3 Materials and Methods.

directly contact the α2/Mcm1 complex, and the energy of that interaction contributes to the overall stability of the complex.

Alternatively, it is possible that the size of the protein complex assembled at the α2/Mcm1 operator contributes to the efficiency of transcriptional interference. Loss of Tup1 or Ssn6 would then reduce the extent of transcriptional interference because a smaller complex assembles at the α2/Mcm1 operator. This possibility seems unlikely since the experiment of Figure A-2 shows that the $\alpha2^{\Delta3\text{-}6}$::LacZ allele, wherein an N-terminal deletion mutant of α2 is fused to the large ß-galactosidase moiety, is no more effective at directing transcriptional interference than are simple α2 point mutants.

The N-terminal α2 point mutants are thought to be defective in their ability to interact with Tup1 (K.Komachi, personal communication). Why, then, are they able to direct transcirptional interference when wild type α2 does not direct transcriptional interference in the absence of Tup1? The simplest explanation is that the high copy vectors used in the experiment of Figure A-2 to express the N-terminal α2 mutants produced enough protein that either the DNA occupancy was so high that contributions from Tup1 became negligible or the defect in interaction with Tup1 was overcome by the high levels of mutant α2 protein.

The simplest interpretation of the data presented here is that occupancy of the DNA is sufficient for transcriptional interference and that Tup1 and/or Ssn6 contributes to the stability of the α2/Mcm1 complex on the DNA, perhaps by making direct physical contact with α2 and/or Mcm1.

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Chapter 4

Transcriptional repression directed by the yeast α2 protein *in vitro*

The α2 protein, a homeodomain protein involved in specifying cell type in the budding yeast *Saccharomyces cerevisiae*, is a transcriptional repressor. α2 functions *in vivo* when its operator is placed in a variety of positions upstream of the transcription start.[1] In this report, we show transcriptional repression *in vitro* directed by the α2 protein. A point mutant of α2 that is defective for repression *in vivo*, but is competent for DNA binding, also fails to repress transcription *in vitro*. Repression by α2 *in vivo* depends on several other proteins (including Mcm1, Ssn6, and Tup1), and at least some of these components are also required for α2 repression *in vitro*. Given that our system includes neither transcriptional activators nor a chromatin assembly step, we argue that α2 represses transcription by interfering with the general transcription machinery.

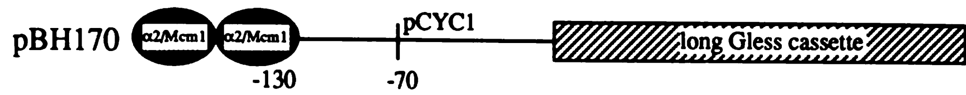
The α2 protein is involved in cell-type determination in the yeast *Saccharomyces cerevisiae*. In the α and **a**/α cell types, α2 binds to DNA cooperatively with the SRF-like protein Mcm1,[2-5] and recruits the Ssn6/Tup1 repressor complex[6-8] to shut off expression of the **a**-specific genes, which are required only in the **a** cell type. We report the faithful reproduction of α2 repression *in vitro.*

Figure 1A diagrams the DNA templates used to detect α2 repression *in vitro.* A reporter template with two **a**-specific gene operators (which consist of binding sites for α2 and Mcm1[1,9]) upstream of the *CYC1* promoter[10] and a control template lacking upstream operators were transcribed in a whole cell extract prepared from yeast cells that themselves produce no α2, but that carry plasmids overexpressing *SSN6* and *TUP1*. As shown in Figure 1B, addition of α2 protein purified from *Escherichia coli* (*E. coli*) to the transcription reaction resulted in an approximately two-fold decrease in transcription from the reporter template but had no effect on expression of the control template in the same reaction.

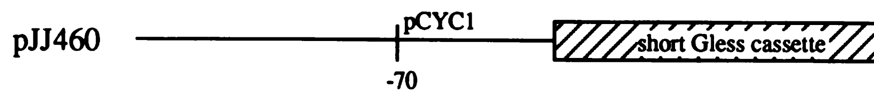
The experiment of Figure 2 argues that our *in vitro* system accurately mimics the α2 repression observed *in vivo*. A point mutant of α2, $\alpha2^{ser10}$, that is defective for α2

A.

reporter:



control:



B.

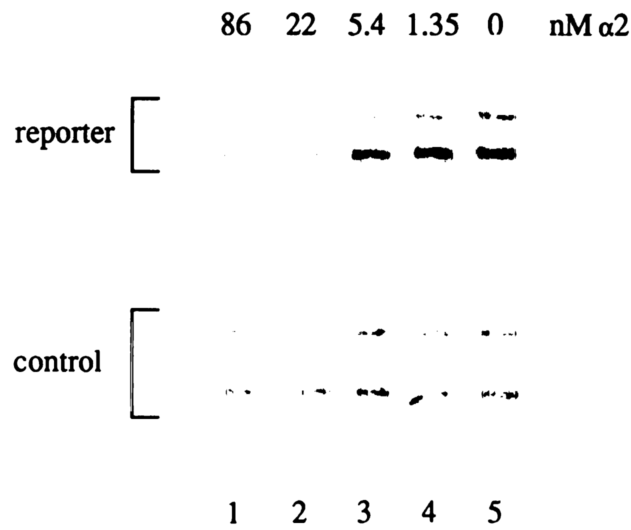


Figure 4-I

Figure 4-1. Transcriptional repression *in vitro* by the yeast α2 protein.

A. Constructs used as templates in *in vitro* transcription reactions. pBH170 was made by insertion of 2 copies of a 32 base pair (bp) oligonucleotide containing the α2/Mcm1 operator into the unique Pst I site in pJJ469.[22] pJJ460 has been described elsewhere.[23]

B. α2 repression *in vitro*. The constructs diagrammed in A were used in run-on transcription reactions in whole cell extracts from yeast strain KTX23αX8 (*matΔ trp1 leu2 ura3 his4 suc2Δ gal2*, created by deletion of MATα from EG123[24,25] carrying plasmids pLN113-3[26] and pKK391 (see Figure 3 legend). Purified α2 protein, prepared by A. Mak as previously described,[27] was added at the concentrations indicated.

EXTRACT PREPARATION: Whole cell extracts from yeast were prepared as described[23,28] except that cells were lysed by bead beating for seven twenty second intervals interrupted by 1 minute rests, and extract proteins were resuspended in and dialysed against 150 mM potassium glutamate, 20mM HEPES pH 7.5, 20% v/v glycerol, 10 mM magnesium sulfate, 10mM EGTA, 5 mM DTT, 1 mM PMSF, 2 mM benzamidine hydrochloride. All solutions were DEPC treated to inactivate RNase.

TRANSCRIPTION REACTIONS: Transcription reactions were performed in 50 mM HEPES pH 7.5, 90 mM potassium glutamate, 10% v/v glycerol, 10 mM magnesium acetate, 5 mM EGTA, 2.5 mM DTT. Reactions contained 0.5 U RNasin (Promega), 30 U RNase T1, 0.47 U creatine kinase, 30mM creatine phosphate, 50 ng of each template, and 1100 ng of pGEM3 (Promega) competitor DNA. Purified α2 protein was added to the reactions at the indicated concentrations. Approximately 160 μg of extract was added and the mixture was incubated for 30 minutes at 23°C to allow the α2/Mcm1 complex to assemble on the DNA. Transcription was initiatied by the addition of nucleotides to final concentrations of: 0.5 mM CTP, 0.5 mM ATP, 0.1 mM UTP, 200 μCi/ml α^{32}P-UTP 3000 Ci/mmol. After 10 minutes, reactions were stopped by addition of 4.3 V of 0.6% SDS. 40 μg of proteinase K was added and reactions were incubated at 37°C for 30 minutes. Reactions were precipitated with 0.5 V 5 M ammonium acetate, 3 V of ethanol, and 40

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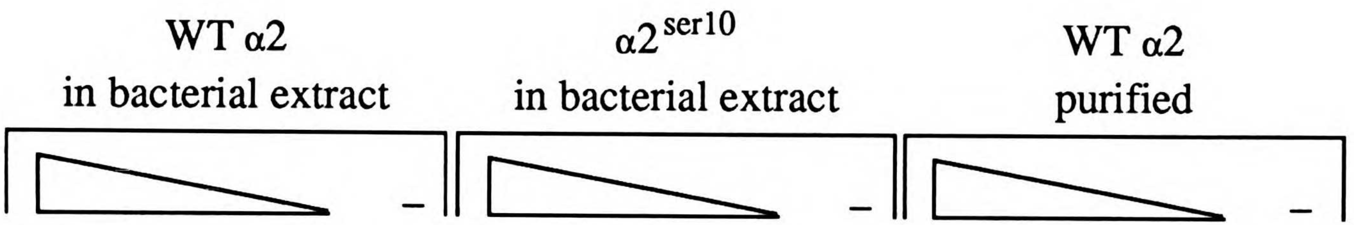

Figure 4-2

Figure 4-2. A point mutant of $\alpha2$, $\alpha2^{ser10}$, does not repress transcription *in vitro*. Extracts made from *matΔ* strains overexpressing *SSN6* and *TUP1* were used in run-on transcription reactions. Wild type $\alpha2$ in a bacterial extract, $\alpha2^{ser10}$ in a bacterial extract, or wild type $\alpha2$ purified from *E. coli* were added in increasing concentrations (in four fold steps) as indicated. The concentrations of purified $\alpha2$ range from 5.4 nM (lane 14) to 346 nM (lane 11). The isolation and preparation of $\alpha2^{ser10}$ will be described elsewhere (K. Komachi and A.D.J., personal communication).

repression *in vivo* (K. Komachi and A.D.J., personal communication), fails to mediate repression *in vitro*. The $\alpha 2^{ser10}$ point mutant is indistinguishable from wild type α2 in its ability to bind to DNA either alone or with Mcm1 (data not shown). Thus, as is the case *in vivo*,[8] operator occupancy alone is insufficient for α2 repression *in vitro*. Rather, a functional repression complex, presumably including Ssn6 and Tup1, must assemble to mediate transcriptional repression.

Consistent with this idea, we observed α2 repression *in vitro* only when the transcription reactions contained extracts prepared from strains overexpressing *SSN6* and *TUP1* (Figure 3). When transcription reactions were performed in extracts from non-overproducing strains, expression of the reporter template was unaffected by the addition of α2 protein to levels (22-86 nM) at which DNA binding studies (not shown) show full occupancy of the α2/Mcm1 operators in our *in vitro* transcription reactions. Apparently, extracts from non-overproducing strains contain insufficient levels of Ssn6 and/or Tup1 proteins to give detectable repression *in vitro*. The combined observations that α2 repression *in vitro* requires a wild type α2 protein and that repression is not observed in extracts from strains that do not overexpress *SSN6* and *TUP1* suggest that *in vitro* repression is not artifactual, but rather accurately reflects the phenomenon observed *in vivo*.

Although we routinely observed only 2 to 4 fold repression of the reporter template *in vitro*, we believe it to be significant, especially since the $\alpha 2^{ser10}$ mutant does not show this effect. Approximately 1% of the available templates are transcribed in our *in vitro* system (data not shown). Two- to four- fold repression is consistent with 50-75% of those templates being fully repressed. We believe that this effect represents a significant number of functional repression complexes forming in our *in vitro* system.

The observation of α2 repression in this *in vitro* system suggests a model for the mechanism of α2 repression (Figure 4). Our *in vitro* transcription system includes no added activator protein, nor does the DNA template contain a binding site for any known

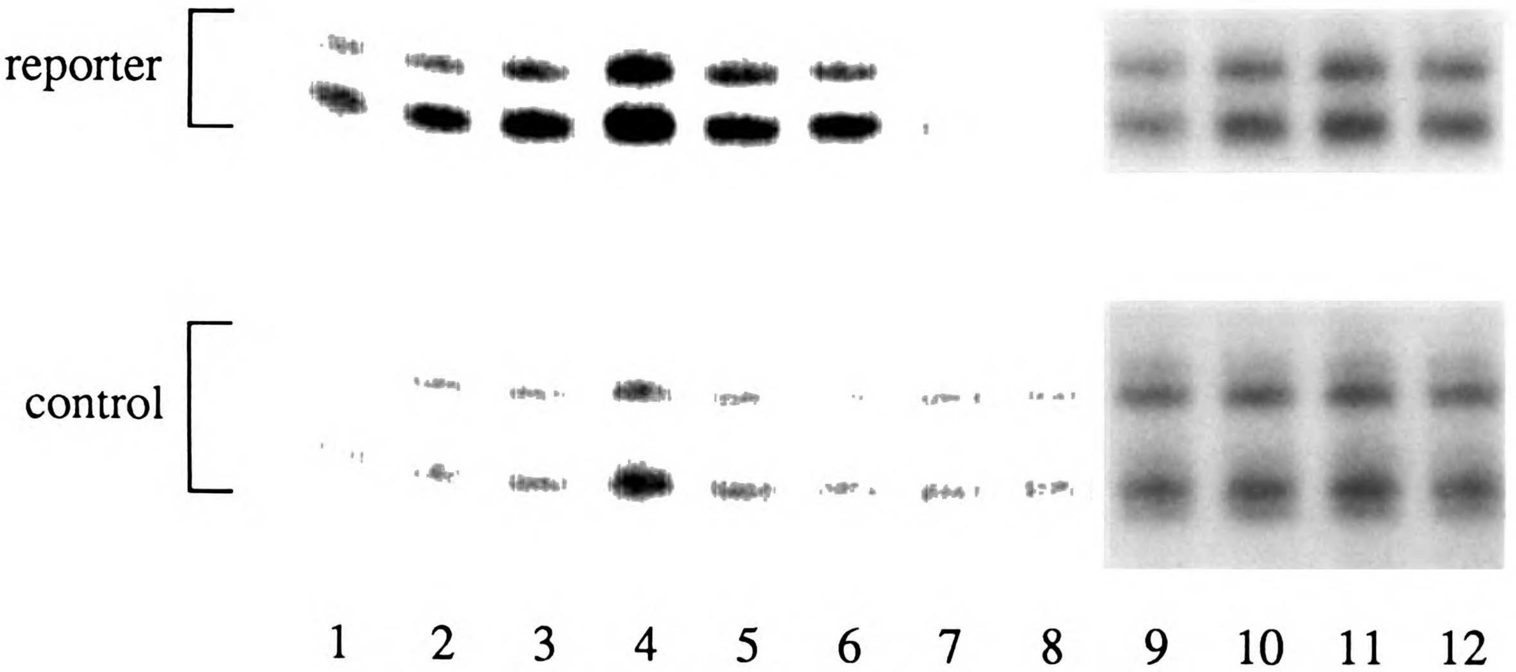

Figure 4-3

Figure 4-3. α2 repression only in extracts of strains overexpressing *SSN6* and *TUP1*. Extracts prepared from *matΔ* strains carrying no plasmid (lanes 1-6) or *matΔ* strains carrying plasmids overexpressing *SSN6* (pLN113-3) and *TUP1* (pKK391; lanes 7-12) were used in run-on transcription reactions to which the indicated amounts of purified α2 protein were added. *SSN6* expression is controlled by its own promoter on pLN113-3. *TUP1* expression is driven by the Gal10 promoter on pKK391. pKK391 was made by cloning a PCR fragment containing the entire *TUP1* coding sequence into the BamHI site of pΔSJ, a SalI/XhoI deletion of pSJ101.[29] Extracts from non-overproducing strains were prepared from cells grown in YEPD. Extracts from strains carrying *SSN6* and *TUP1* overproducing plasmids were prepared from cells grown to OD_{600} 1.0-1.5 in selective media supplemented with 2% glucose. The pGal promoter driving *TUP1* expression was then induced by four to six hours of growth in selective media supplemented with 2% glucose-free galactose.

transcriptional activator. Although it is formally possible that some yeast activator protein fortuitously binds to our templates and stimulates transcription, we find this possibility to be unlikely. The same fragment of the *CYC1* promoter is inactive for transcription *in vivo*,[1,10] which suggests that an activator capable of stimulating transcription from this DNA is not present, at least not at significant levels, in yeast. We therefore believe that α2 directs repression in our *in vitro* system in the absence of transcriptional activation. Thus, we conclude that, unlike at least some other transcriptional repressors,[11-14] α2 does not repress transcription by interfering with activator binding or function. Rather, we believe that α2 repression involves interference with the general transcription machinery. This idea is consistent with the observation that α2 can repress transcription by both RNA polymerases I and II[15] even though the activation systems for these RNA polymerases cannot be interchanged.[16,17] Although it is possible that interference with transcriptional activators also contributes to α2 repression *in vivo*, our results indicate that it cannot be the sole mechanism.

Similarly, since our transcription reactions did not include a chromatin assembly step, it is unlikely that chromatin assembly is essential to α2 repression. Chromatin structure may contribute to α2 repression *in vivo*,[18-20] but does not easily account for the repression observed *in vitro*.

The *Drosophila even-skipped* (*eve*) protein has recently been shown to interfere with the extent of pre-initiation complex formation by the general transcription machinery in *Drosophila* extracts.[21] We expect that interference with the general transcription machinery will prove to be a mechanism used by many transcriptional repressors to block gene expression. Most eukaryotic genes respond to several different transcriptional activators. Repression of such genes by interference with transcriptional activation could in principle require a dedicated repressor for each individual activator. Interference with the general transcription machinery, in contrast, would provide an efficient way to prevent expression of genes controlled by multiple activators.

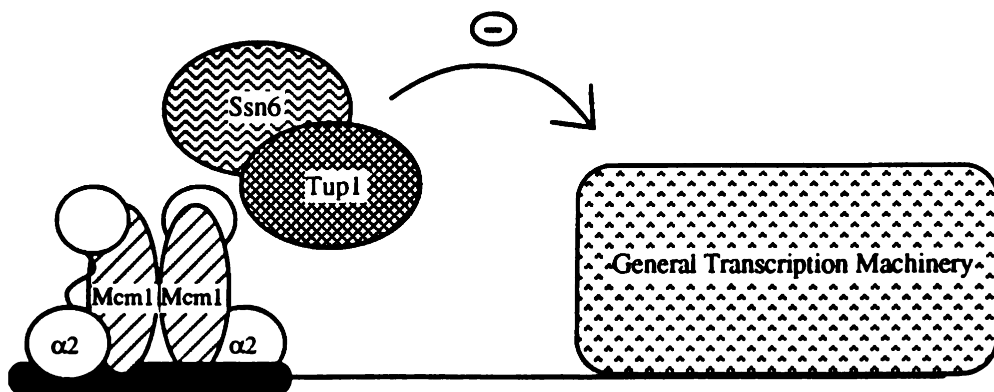

Figure 4-4: Model for transcriptional repression by the yeast α2 protein

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Appendix B

DNA binding by $\alpha2$ and Mcm1 under *in vitro* transcription conditions

In order to determine at what concentrations of added $\alpha2$ the $\alpha2$/Mcm1 operators in our *in vitro* transcription reactions became fully occupied, we performed DNase I protection studies under the conditions used in our *in vitro* transcription reactions.

As a first step, we performed DNase I protection experiments with purified $\alpha2$ and Mcm1 proteins and no extract (Figure B-1A). All other conditions (salt concentrations, template and competitor DNA concentrations, presence of RNasin and RNase T1, etc.) were the same as those used in the *in vitro* transcription reactions (see Legend to Figure 4-1). An end-labelled 86 bp fragment containing a single $\alpha2$/Mcm1 operator was incubated with purified $\alpha2$ and Mcm1 proteins under *in vitro* transcription conditions. DNAse I was added at a concentration empirically determined (data not shown) to give a clean ladder of fragments after ten minutes of digestion. In the absence of Mcm1 (lanes 7-13), $\alpha2$ fully protected its DNA binding sites from DNase I digestion at approximately 346 nM protein. In the presence of Mcm1 (lanes 1-6), full operator occupancy occurred between 22 and 86 nM $\alpha2$.

We then repeated the experiment in the presence of whole cell extract from yeast (Figure C-1B). In the absence of added $\alpha2$ protein (lane 8), the center region of the probe was already protected, presumably by Mcm1 in the extracts[2]. When purified $\alpha2$ protein was added to the reactions, one cluster of bands became protected. Full protection was achieved at approximately 22 nM $\alpha2$ (lane 3). Although these experiments were performed with extracts prepared from yeast cells of α mating type, there did not seem to be any $\alpha2$ DNA binding activity in the absence of added protein (lane 8), consistent with previous observations that $\alpha2$ DNA binding activity is not detectable in whole cell extracts from yeast[2].

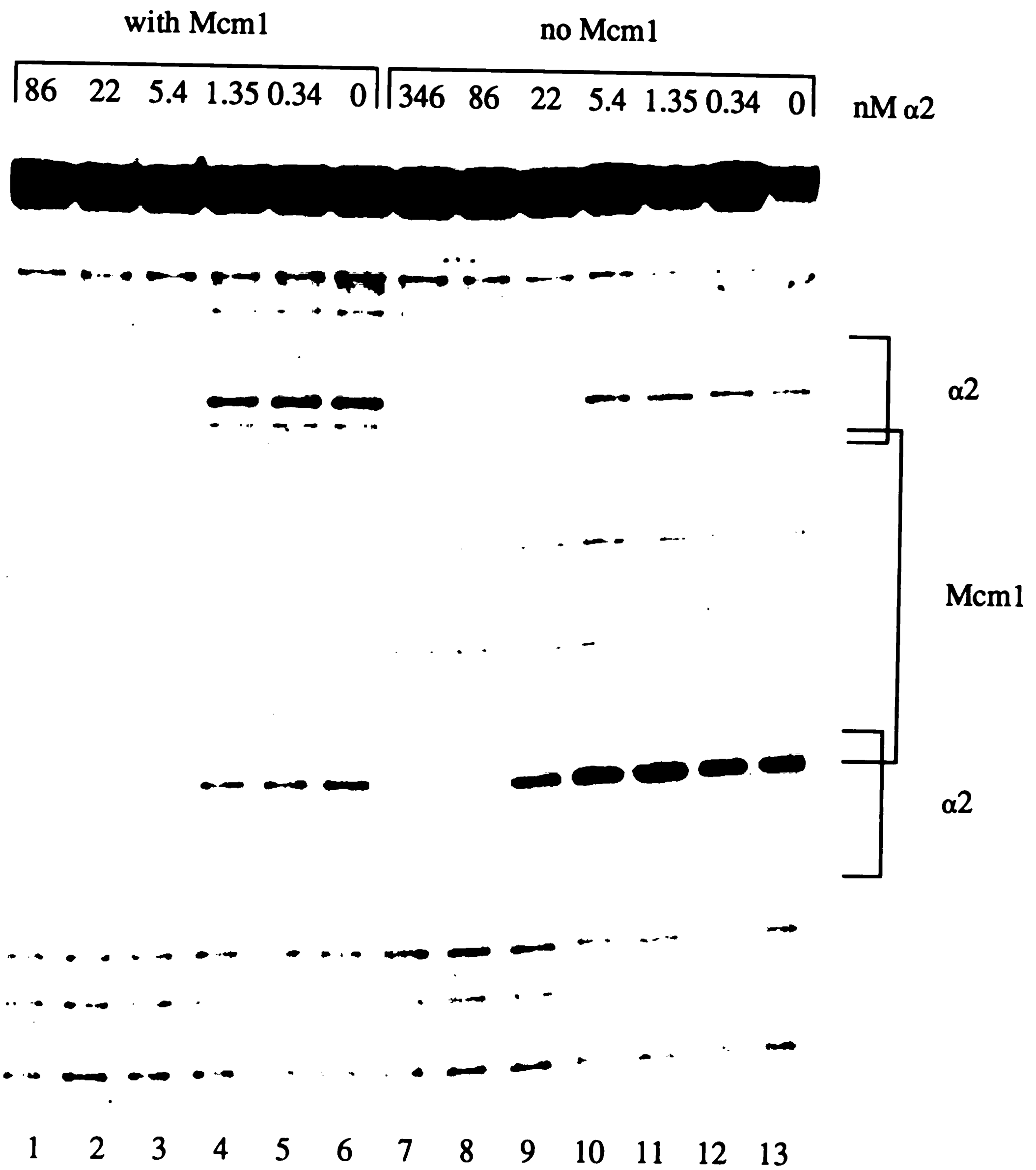


Figure B-IA

B.

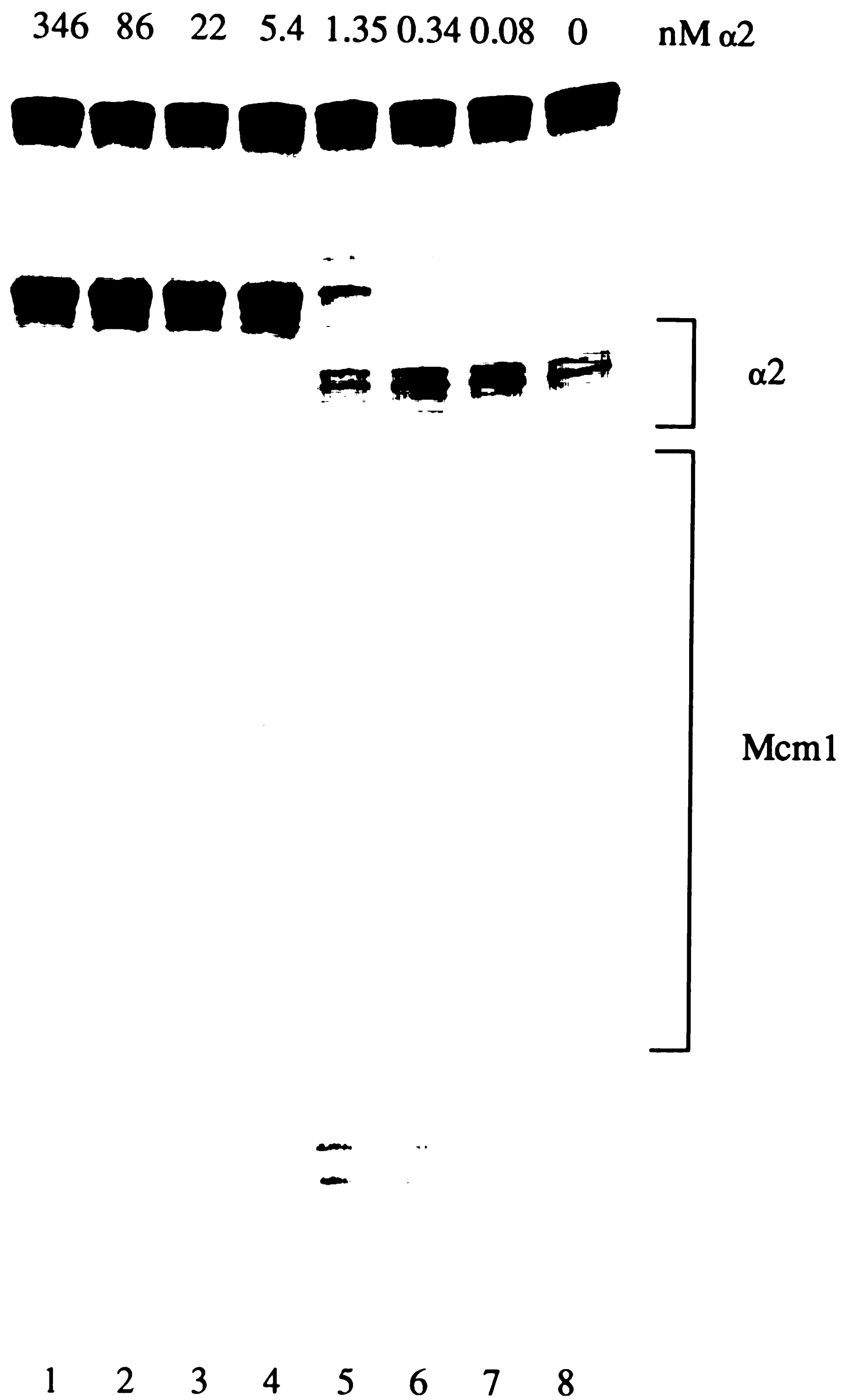


Figure B-IB

Figure B-1. DNA binding by α2 and Mcm1 under *in vitro* transcription conditions

A. DNA binding by purified α2 and Mcm1. α2 protein purified from bacteria by A. Mak as described[27] and Mcm1(1-97) purified from bacteria by A. Vershon were incubated for 30 minutes at approximately 23°C under the reaction conditions used for *in vitro* transcription reactions (see Legend to Figure 4-1) except that no yeast extract was added and an end-labelled 86 bp fragment containing a single α2/Mcm1 operator (prepared as described[27]) was added to a final concentration of approximately 50 pM. DNaseI (Worthington) was added to a final concentration of 4.7 μg/ml and reactions were incubated for 10 minutes at approximately 23°C. Reactions were stopped by addition of 4.3V of 0.6% SDS. 40 μg of proteinase K were added and reactions were incubated at 37°C for 30 minutes. Reactions were precipitated with 0.5V % M ammonium acetate, 3V of ethanol, and 40 μg of carrier tRNA. Products were separated by electrophoresis on an 8% denaturing polyacrylamide gel and examined by autoradiography. The concentrations of α2 protein used are indicated. Mcm1 was used at a 1:375 dilution of the protein stock.

B. DNA binding by purified α2 in a yeast extract. α2 protein purified from bacteria by A. Mak as described[27] was incubated for 30 minutes at approximately 23°C under the reaction conditions used for *in vitro* transcription reactions (see Legend to Figure 4-1) except that the extracts were prepared from yeast strain EG123[24,25] carrying a high copy plasmid that expresses both *SSN6* and *TUP1* from their own promoters (provided by K. Komachi) and the reactions included an end-labelled 86 bp fragment containing a single α2/Mcm1 operator (prepared as described[27]) that was added to a final concentration of approximately 50 pM. DNaseI (Worthington) was added to a final concentration of 47 μg/ml and reactions were incubated for 10 minutes at approximately 23°C. Reactions were stopped by addition of 4.3V of 0.6% SDS. 40 μg of proteinase K were added and

reactions were incubated at 37°C for 30 minutes. Reactions were precipitated with 0.5V % M ammonium acetate, 3V of ethanol, and 40 μg of carrier tRNA. Products were separated by electrophoresis on an 8% denaturing polyacrylamide gel and examined by autoradiography. The concentrations of α2 protein used are indicated.

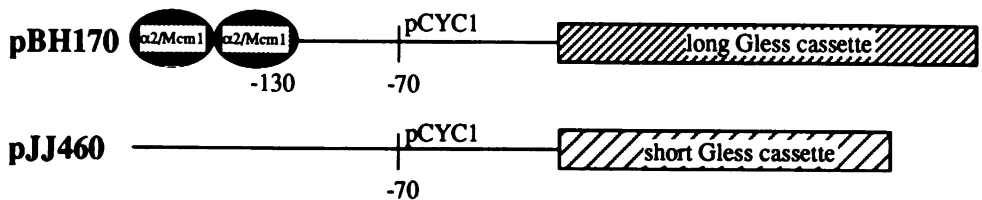
Appendix C

Overexpression of *TUP1* may be sufficient to allow α2 repression *in vitro*

In the experiment of Figure 4-2, we showed that α2 repression *in vitro* requires that *SSN6* and *TUP1* be overexpressed in the strains from which transcription extracts were prepared. We were interested in investigating if overexpression of either *SSN6* or *TUP1* alone were sufficient to allow α2 repression *in vitro*.

Figure C-1 shows the results of *in vitro* transcription reactions carried out in extracts prepared from *matΔ* strains carrying either the *pGal-TUP1* expression vector, the *SSN6* expression vector, or both. As expected, addition of purified α2 protein to the transcription reactions containing the double overexpressing extract results in a decrease of approximately twofold in transcription of the reporter template (pBH170), but has no effect on expression of the control template (pJJ460[23]; lanes 1-4). By contrast, addition of purified α2 protein has no effect on expression of either template in reactions containing extract from yeast cells carrying just the *SSN6* overexpressing plasmid (lanes 9-12). Transcription of the reporter template decreases somewhat (approximately 1.5 fold) as increasing amounts of α2 are added to transcirption reactions containing extract from cells overexpressing *TUP1* alone. It is difficult to determine if α2 repression is significantly reduced in the *TUP1* overexpressing extracts relative to the doubly overexpressing extracts, particularly since the extent of *TUP1* induction during extract preparation could be variable.

A.



B.

Figure C-I

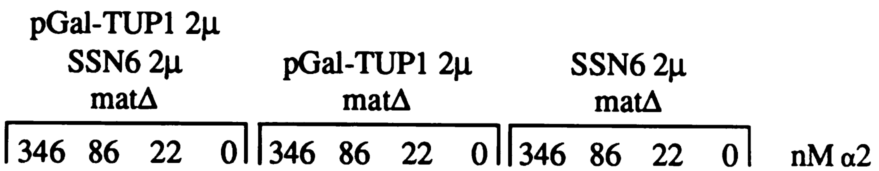


Figure C-1. Overexpression of *TUP1* may be sufficient to allow α2 repression *in vitro.*

A. Constructs used as templates in *in vitro* transcription reactions.

B. *In vitro* transcription reactions. Extracts from *matΔ* strains carrying plasmids overexpressing *SSN6* (pLN113-3[26]; lanes 9-12), *TUP1* (pKK391; lanes 5-8), or both (both plasmids; lanes 1-4) were used in run-on transcription reactions to which the indicated amounts of purified α2 protein were added. *SSN6* expression is controlled by its own promoter on pLN113-3. *TUP1* expression is driven by the Gal10 promoter on pKK391 (see Legend to Figure 4-3). Extracts were prepared from strains grown to OD_{600} 1.0-1.5 in selective media supplemented with 2% glucose. The Gal10 promoter driving *TUP1* expression was induced by four to six hours of growth in selective media supplemented with 2% glucose-free galactose.

Appendix D

In vitro α2 repression on templates linearized just upstream of the α2/Mcm1 operators

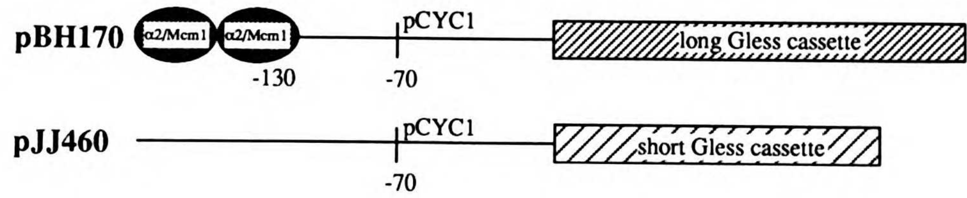
In Chapter 4, we argue that it is unlikely that some yeast activator protein is fortuitously binding to our templates and stimulating transcription in our *in vitro* transcription reactions. To push this argument further, we asked if α2 repression were still effective on templates that had been linearized just upstream (12 bp) of the α2/Mcm1 operators. With just 188 bp, 64 of which are α2/Mcm1 operators, between the start site of transcription and the end of the DNA template, the possibility that some yeast activator is binding to our templates and stimulating transcription becomes highly improbable.

The same templates (pBH170 and pJJ460) used in the experiments presented in Chapter 4 were digested with Hind III. Linear DNAs were purified from an 0.6% agarose gel. Uncut and Hind III-cut templates were used in side-by-side transcription reactions using extracts from strains that themselves produce no α2 protein, but that carry plasmids overexpressing *SSN6* and *TUP1*. The results are presented in Figure D-1. Addition of α2 protein purified from *E. Coli* to the transcription reactions results in a decrease in expression of the reporter template, but not the control template, whether or not the templates had been linearized. The overall expression of both the reporter and the control template decreases when the templates are linearized. It seems unlikely that this decrease reflects differences in the supercoiled state of the templates since whole cell extracts from yeast would be expected to contain many topoisomerases and thus to randomly linearize any added templates. Rather, it is possible that transcription complexes have some difficulty assembling so near the end of a linear template.

The observation of α2 repression *in vitro* on templates linearized just upstream of the α2/Mcm1 operators strengthens the argument that we are observing α2 repression *in*

vitro in the absence of transcriptional activators and thereby strongly suggests that α2 represses transcription not by blocking transcriptional activation but rather by interfering with some component of the general transcription machinery.

A.



B.

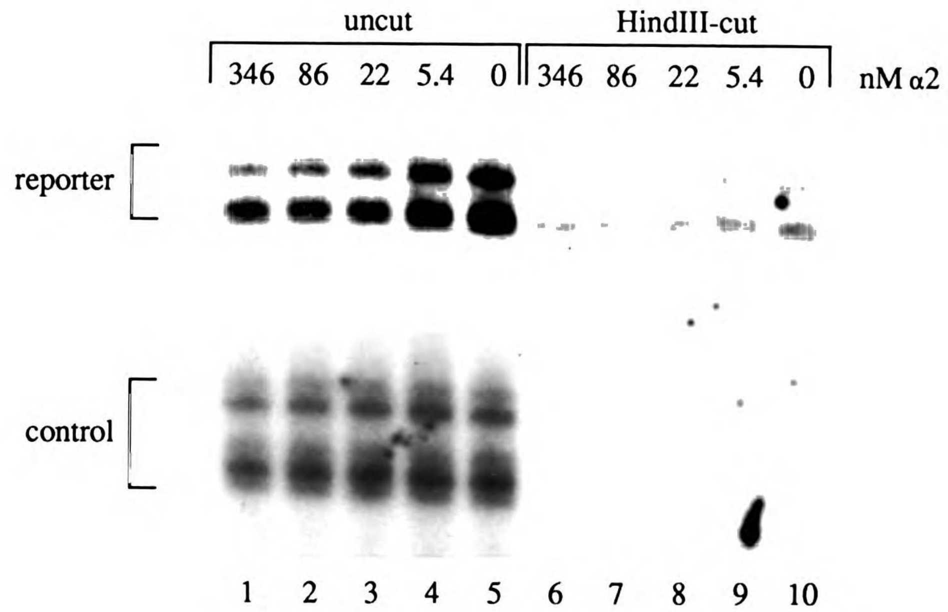


Figure D-I

Figure D-1. *In vitro* α2 repression on templates linearized just upstream of the α2/Mcm1 operators.

A. Constructs used as templates in *in vitro* transcription reactions.

B. *In vitro* transcription reactions. Uncut templates or templates linearized with Hind III were used in *in vitro* transcription reactions in extracts from *matΔ* strains carrying plasmids overexpressing *SSN6* and *TUP1* as described in Chapter 3. α2 protein purified from *E. Coli* was added to the concentrations indicated.

Appendix E

Addition of extra purified basal transcription factors does not overcome α2 repression *in vitro*

Given our model that α2 represses transcription by interfering with the general transcription machinery, we thought it might be possible to overcome α2 repression *in vitro* by adding to the transcription reactions an excess of whichever general component is the target of α2 repression. In the experiment presented in Figure E-1, an aliquot of each of the purified fractions required to reconstitute transcription *in vitro*,[30] was mixed with the yeast extract before transcription reactions were set up. The results show that in no case did addition of a general transcription factor overcome α2 repression *in vitro*. Addition of rYTBP (lanes 7,8) resulted in a dramatic increase in transcription, presumably because TBP is limiting in these reactions,[30] but did not affect the extent of α2 repression. There are many possible explanations for our inability to overcome α2 repression *in vitro* by adding individual general transcription factors to the reactions. For example, perhaps we did not add enough "extra" of the appropriate target factor to overcome repression. Alternatively, it is possible that the interaction between the repression machinery and its target takes place only after the target is assembled into the pre-initiation complex. In this case, adding an extra amount of any one component of the general transcription machinery might not affect the number of assembled "targets" of α2 repression.

Figure E-I

Figure E-1. Addition of extra purified factors does not overcome α2 repression *in vitro*.

A. Constructs used as templates in *in vitro* transcription reactions.

B. *In vitro* transcription reactions. *In vitro* transcription reactions were set up as usual (see Legend to Figure 4-1) using extract from a *matΔ* strain that was carrying plasmids overexpressing *SSN6* and *TUP1*. The extract had been pre-incubated for 30 minutes at approximantely 23°C with the following amount of one of the fractions required to reconstitute transcription *in vitro*,[30] which were generously provided by M. Sayre and L. Henry: no fraction (lanes 1,2,9,10); factor a (14 ng purified to homogeneity from a whole cell yeast extract; lanes 3,4); rYTFIIB (115 ng purified to homogeneity from *E. Coli*; lanes 5,6); rYTBP (171 ng purified to homogeneity from *E. Coli*; lanes 7,8); RNA polymerase II (576 ng purified to homogeneity from a whole cell yeast extract; lanes 11,12); factor g (192 ng purified to homogeneity from a whole cell yeast extract; lanes 13, 14); factor b (1920 ng partially purified from a whole cell yeast extract, lanes 15, 16).

Appendix F

Pre-incubation of a whole cell extract from yeast with Gst-Ssn6 and/or Gst-Tup1 coupled to agarose beads does not deplete transcription activity

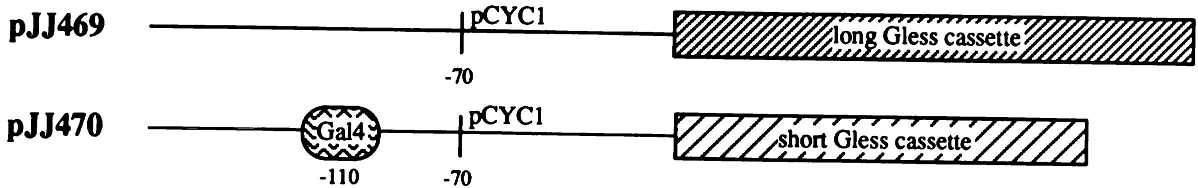
In an effort to gain further evidence that α2 repression involves interference with the general transcription machinery, and eventually to identify which general transcription factor might be contacted by the repression apparatus, we asked whether we could use Gst-Ssn6 and Gst-Tup1 fusion proteins to deplete our extracts of transcription activity. If Ssn6 and/or Tup1 directly contact some component of the RNA polymerase II general transcription machinery, and if that association can be reproduced using Gst-Ssn6 and Gst-Tup1 fusion proteins, it seemed possible that, by incubating our extracts with the Gst-Ssn6 and/or Gst-Tup1 fusion proteins coupled to agarose beads and then removing the beads (and hopefully also any associated proteins) by centrifugation, we could deplete our extracts for whichever general transcription factor interacts with Ssn6 and/or Tup1.

Figure F-1 shows the results of an experiment in which whole cell yeast extracts were pre-incubated with agarose beads coupled to the indicated protein(s) and then the beads were removed by centrifugation before the *in vitro* transcription reactions were set up. For these reactions, we used 30 fold more template DNA than is standard in the hope that small changes in the concentration of one (or a few) of the general transcription factors would be more easily detectable at high template concentration, when many transcription complexes are being assembled. In addition, our reactions contained 14 nM Gal4-VP16 and, as shown in Figure F-1A, the short G-less cassette template that we used in these reactions contained a Gal4 DNA binding site. Thus, we could investigate the ability of Gal4-VP16 to activate transcription in extracts that had been pre-incubated with Gst-Ssn6 and/or Gst-Tup1.

Pre-incubation of extract with beads alone significantly decreases the transcription activity of our extracts (compare lanes 1 and 7). This effect is unlikely to be due to extract proteins sticking nonspecifically to the beads since "blocking" the beads with BSA prior to incubation in the extract does not relieve the effect (data not shown). Pre-incubation of the extract with beads coupled to Gst-Ssn6 (lane 2), beads coupled to Gst-Tup1 (lane 3), beads coupled to a mixture of Gst-Ssn6 and Gst-Tup1 (lane 4), a mixture of beads coupled to Gst-Ssn6 and beads coupled to Gst-Tup1 (lane 5), or beads coupled to Gst (lane 6) does not decrease the transcription activity in the extract any further. Furthermore, activation by Gal4-VP16 was equally effective in all extracts, indicating that no factor essential to Gal4-VP16 activation had been significantly depleted. Thus, we were unable to deplete our extracts of transcription activity, or of the ability to respond to a transcriptional activator, by pre-incubating them with agarose beads coupled to Gst-Ssn6 and/or Gst-Tup1.

Although we were unable to observe an effect of pre-incubating our extracts with Gst-Ssn6 and/or Gst-Tup1, it is still possible that Ssn6 and/or Tup1 do directly contact some component of the general transcription machinery. It is possible, for example, that association of the repression apparatus and its target in the transcription machinery only occurs after a pre-initiation complex has been at least partially assembled. This association might therefore not have occurred during our pre-incubation step since stable pre-initiation complexes do not assemble on the CYC1 promoter in the absence of nucleotides (M. Sayre, personal communication). Alternatively, it is possible that our Gst-Ssn6 and/or Gst-Tup1 fusion proteins are defective for interaction with whatever component of the general transcription machinery might be the target of $\alpha 2$ repression. Thus, these experiments could be repeated when DNA templates that can assemble stable pre-initiation complexes and Gst-Ssn6 and Gst-Tup1 fusions that can complement *SSN6* and *TUP1* deletions, respectively, *in vivo* become available.

A.



B.

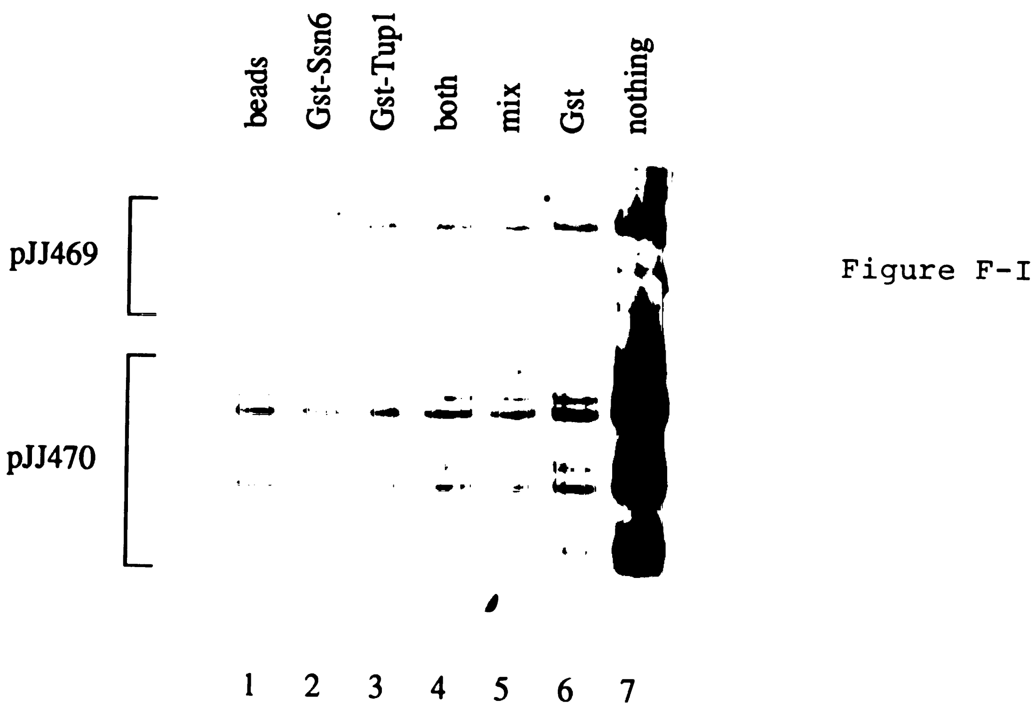


Figure F-I

Figure F-1. Pre-incubation of a whole cell extract from yeast with Gst-Ssn6 and/or Gst-Tup1 coupled to agarose beads does not deplete transcription activity.

A. Constructs used as templates in *in vitro* transcription reactions.

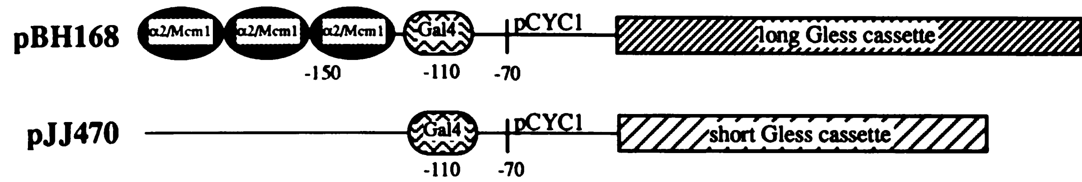
B. *In vitro* transcription reactions. *In vitro* transcription reactions were set up as usual (see Legend to Figure 4-1) except that the amount of template added was increased to 1500 ng each, the amount of pGEM3 competitor DNA was also increased to 1500 ng, Gal4-VP16 purified from *E. Coli* (provided by J. Brickman) was added to 14 nM, the extracts used were prepared from yeast strain EG123[24,25] carrying a high copy plasmid that expresses both *SSN6* and *TUP1* from their own promoters (provided by K. Komachi), and the transcription reactions were incubated for 60 minutes before the addition of SDS to stop the reaction. The extract had been pre-incubated for 30 minutes at approximantely 23°C with the indicated protein(s) coupled to agarose beads. The beads, and hopefully associated proteins, were removed by centrifugation and the "depleted" extracts were stored at -70°C. Gst-Ssn6 and Gst-Tup1 fusion proteins were provided by M. Redd. The Gst-Ssn6 fusion contained Ssn6 amino acids 1-351, which includes eight and a half TPR repeats and therefore might be expected to provide Ssn6 function *in vivo*[31]. The Gst-Tup1 fusion contained full length Tup1.

Appendix G

Addition of purified α2 protein has no effect on *in vitro* transcription activated by Gal4-VP16

We wanted to investigate the effects of added α2 protein on *in vitro* transcription activated by Gal4-VP16. In the experiment presented in Figure G-1, we tested the ability of α2 to repress transcription stimulated by Gal4-VP16 *in vitro*. Figure G-1A diagrams the constructs used in *in vitro* transcription reactions. Both of the templates used contain a Gal4 DNA binding site, and transcription from each is stimulated by the addition of Gal4-VP16 protein purified from *E.Coli* (Figure G-1B, compare lanes 3 and 4 with lanes 1 and 2). The long Gless-cassette template (pBH168) contains three α2/Mcm1 operators upstream of the Gal4 site. When purified α2 protein is added to the reactions, transcription from pBH168, but not from pJJ470, decreases approximately two fold in reactions that do not contain Gal4-VP16 (lanes 1,2). By contrast, addition of purified α2 protein to the reactions that include 14 nM Gal4-VP16 has no effect on transcription from either template (lanes 3,4). It is difficult to interpret the significance of this result since Gal4-VP16 is so potent an activator that it is lethal to yeast cells (J. Brickman, personal communication), so the experiment to make sure that α2 can repress transcription activated by Gal4-VP16 *in vivo* cannot be done. It will be valuable to further investigate the ability of α2 to repress activated transcription *in vitro* with activators that can also be studied *in vivo*.

A.



B.

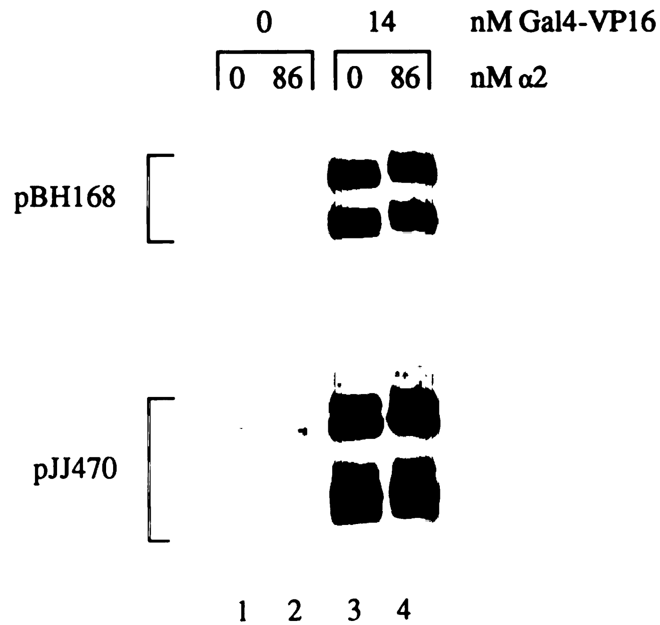


Figure G-I

Figure G-1. Addition of purified α2 protein has no effect on *in vitro* transcription activated by Gal4-VP16

A. Constructs used as templates in *in vitro* transcription reactions.

B. *In vitro* transcription reactions. *In vitro* transcription reactions were set up as usual (see Legend to Figure 4-1) except that the amount of template added was increased to 100 ng each and the amount of pGEM3 competitor DNA was increased to 1500 ng. Gal4-VP16 purified from *E.Coli* (provided by J. Brickman) was added to a final concentration of 14 nM as indicated. All four lanes shown are from the same exposure of the same experiment.

Chapter 5

Discussion

When I began this work, it was known that α2 represses transcription of the **a**-specific genes in yeast by binding to DNA cooperatively with the Mcm1 protein and recruiting the Ssn6/Tup1 complex (Wilson and Herskowitz 1984; Johnson and Herskowitz 1985; Schultz and Carlson 1978; Keleher *et al.* 1988, 1989; Passmore *et al.* 1988, 1989; Ammerer 1990; Mukai *et al.* 1991; Fujita *et al.* 1992; Keleher *et al.* 1992). I undertook experiments designed to give more information about the molecular mechanism by which a repression complex assembled at an α2/Mcm1 operator interferes with transcription from a downstream promoter.

First, I investigated the possibility that chromatin structure might play a role in α2 repression. Utilizing yeast strains whose only copy of the gene encoding histone H4 is under the control of the experimentally regulatable Gal1 promoter (Kayne *et al.* 1988; Kim *et al.* 1988), I asked whether depleting histone H4 from yeast cells interferes with the ability of α2 to repress either a *CYC1::LacZ* reporter gene regulated by an upstream α2 operator, or an endogenous **a**-specific gene. As discussed in Chapter 2, I found that nucleosome depletion has only a modest effect on α2 repression, perhaps decreasing the extent of α2 repression four fold. While the interpretation of these experiments is complicated by the fact that depletion of histone H4 results in a loss of only about half of the chromosomal nucleosomes in yeast cells (Kim *et al.* 1988), the results suggest that nucleosomal structure may not be essential to α2 repression.

In the work presented in Chapter 3, I further investigated the mechanism of α2 repression by asking if could repress transcription by other cellular RNA polymerases. I reasoned that if α2 repression involved some general mechanism like packaging DNA into inaccessible chromatin structures or localizing promoters in some inaccessible region of the nucleus, then it might be effective against any RNA polymerase. On the other hand, if repression involved specific interaction with some component of the RNA polymerase II transcription machinery, α2 might repress only RNA polymerase II transcription. I found that α2 represses transcription by RNA polymerases I and II but

not III. Furthermore, I found that α2 uses the same pathway to repress transcription by RNA polymerases I and II.

The observation that α2 can repress transcription by RNA polymerase I indicates that α2 repression does not require interaction with some component of the general transcription machinery that is unique to RNA polymerase II. Furthermore, since the activation systems for RNA polymerase I and RNA polymerase II are not interchangeable (Schreck *et al.* 1989; Butlin and Quincy 1991), this result argues against models of α2 repression by interference with transcriptional activators.

The data presented in Chapter 3 also indicate that α2 repression probably does not involve packaging of DNA into inaccessible chromatin or localizing promoters in inaccessible regions of the nucleus, as α2 repression is ineffective against RNA polymerase III transcription of at least two different genes. Rather, the results of Chapter 3 suggest that α2 repression involves interaction with some component of the general transcription machinery that is common to RNA polymerases I and II.

In a further attempt to understand the molecular mechanism of α2 repression, I developed an *in vitro* transcription system and asked whether I could reproduce α2 repression *in vitro*. As discussed in Chapter 4, I found that α2 repression could be reproduced *in vitro*, in a whole cell extract from yeast, if *SSN6* and *TUP1* were overexpressed in the yeast cells used to prepare the extract. Moreover, I found that a point mutant of α2 that is defective for α2 repression *in vivo* but is fully competent for DNA binding alone or with Mcm1, fails to repress transcription *in vitro*. These results indicate that I faithfully reproduced α2 repression *in vitro*. Because my *in vitro* system included neither transcriptional activators nor a chromatin assembly step, I conclude that α2 represses transcription by interfering with some component of the general RNA polymerase II transcription machinery.

The development of an *in vitro* α2 repression system provides a tool that will hopefully prove useful in further investigations of the mechanism of α2 repression. It

will be interesting, for example, to examine α2 repression of activated transcription *in vitro*. As discussed in Appendix G, I found that α2 was not able to repress *in vitro* transcription activated by Gal4-VP16. However, Gal4-VP16 is not a physiological yeast activator. In fact, Gal4-VP16 is toxic to yeast. It will be useful to study a more relevant transcriptional activator, perhaps GCN4, whose effects can be examined both *in vivo* and *in vitro*.

An *in vitro* repression system should also help identify the target of α2 repression in the general transcription machinery. Unfortunately, the *CYC1* promoter that I used in my studies is not capable of assembling stable pre-initiation complexes in the absence of nucleotides (M. Sayre, personal communication). A different promoter, one which can assemble stable pre-initiation complexes, could be used to study temporal aspects of α2 repression. That is, one could ask if assembling pre-initiation complexes become resistant to α2 repression after a given amount of time. If so, the point at which pre-initiation complexes become resistant to α2 repression would give information about which step in the assembly of pre-initiation complexes is blocked by α2. Eventually, true order of addition experiments could be done in a purified system to ask which step(s) in the transcription initiation reaction is (are) affected by α2.

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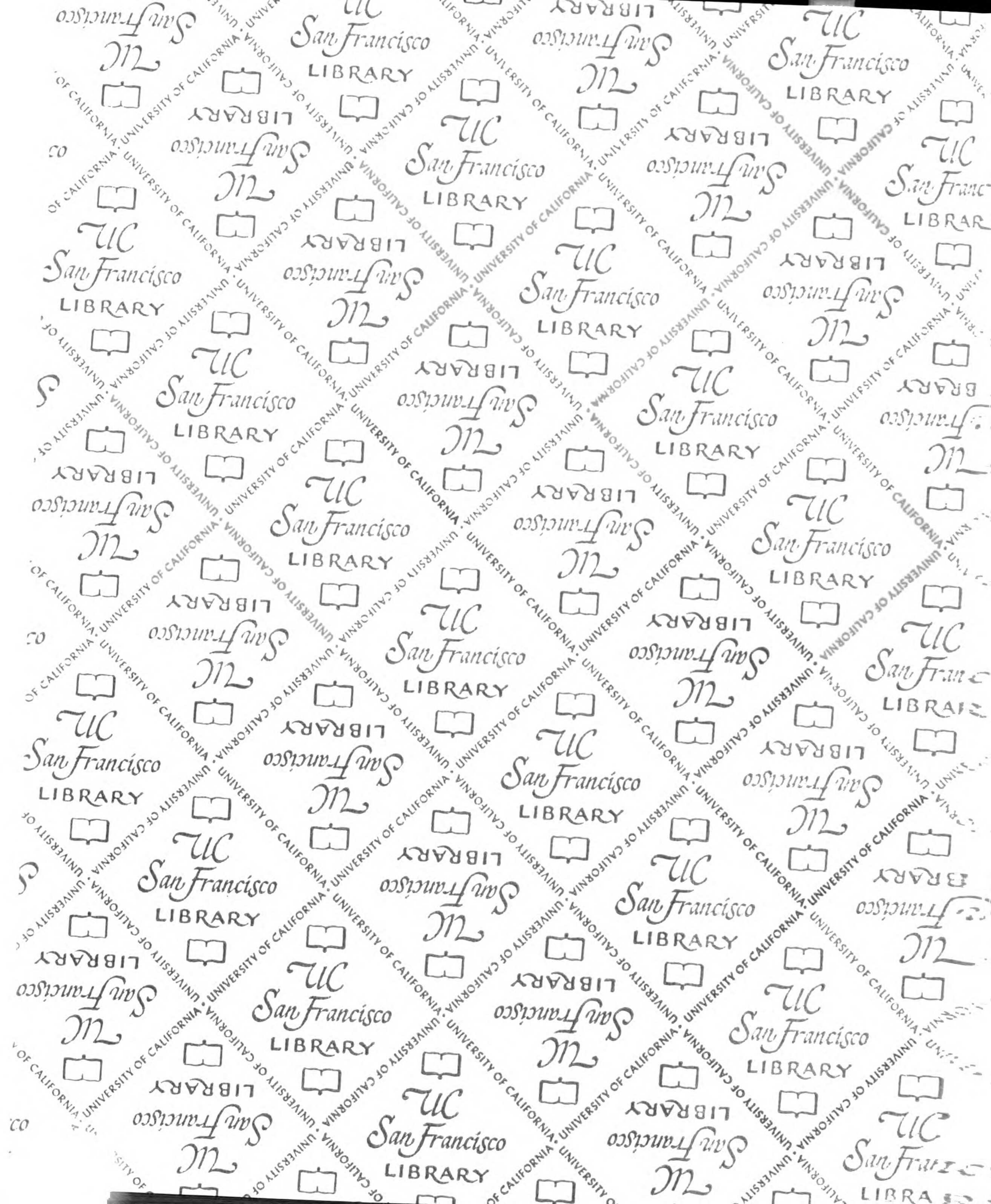
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