REGULATION AND VARIATION OF SUBUNITS OF RNA POLYMERASE II IN *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

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RNA polymerase (RNAP) uses ribonucleoside triphosphates as a substrate to form RNA chains. The RNA is a faithful copy of one strand of a double-stranded DNA template along which RNAP moves while making the RNA. This process (called transcription) is highly regulated such that DNA-encoded genes are transcribed at a large variety of rates and at different times in response to cues within and outside the cell. The goal of my thesis work was to investigate the mechanisms that regulate the synthesis of RNAP in yeast cells. I focused on the form of RNAP (RNAPII) of the yeast, *Saccharomyces cerevisiae*, that is responsible primarily for transcribing genes that encode proteins. I have shown that a 10-fold reduction in the level of the largest subunit of RNAPII, and likely the level of RNAPII itself, causes slow growth, temperature-sensitivity, and the inability to grow on medium lacking inositol. Hence, the level of RNAPII must be carefully maintained for normal cell growth. I next examined elements that control the synthesis of RNAPII. I have demonstrated that the DNA sequences which are upstream of the genes encoding the two largest subunits of RNAPII, namely *RPO21* and *RPO22*, contain binding sites for two abundant transcription factors called Abf1p and Reb1p, and thymidine-rich sequences downstream of these binding sites. Both the binding sites and the T-rich regions are important for the expression of these genes. An examination of the upstream sequences of other RNAPII subunit genes revealed binding sites for Abf1p and Reb1p as well as nearby thymidine-rich sequences. This may indicate that there is a mechanism for the coordinate synthesis of RNAPII subunit genes. I sought evidence for a feedback regulatory mechanism that may control the synthesis of RNAPII. Either the underproduction of Rpo21p, or the depletion of Fcp1p, an RNAPII phosphatase that has a critical role in transcription, leads to a 5-fold increase in the expression of a reporter gene that is controlled by *RPO21* regulatory sequences. The increase is not observed with other subunits. I discuss the implications of these results and future directions.

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2µm	2 micron-circle plasmid origin of replication
ARS	autonomously replicating sequence
bp	base pair
CEN	DNA sequences conferring centromeric properties
DNA	deoxyribonucleic acid
kb	kilobase
kDa	kilodalton
µg	microgram
mRNA	messenger RNA
ng	nanogram
nm	nanometer
ORF	open reading frame
O.D.	optical density
RNA	ribonucleic acid
RNAP	RNA polymerase
rDNA	ribosomal DNA
r-protein	ribosomal protein
rRNA	ribosomal RNA
tRNA	transfer RNA
ts	temperature sensitive

This thesis is dedicated to the loving memory of my uncle, Lou Haaksma. Lou died, at the age of 65, on January 20, 1999. This happened to be the day that I defended this thesis. I know you are having a hearty laugh Oom Lou: your tax dollars at work!!

CHAPTER 1

The Biosynthesis of RNA Polymerases

Gene expression is essential for cellular proliferation, differentiation and homeostasis. An entire complement of molecules must be synthesized by the cell simply to replace its components during the cell cycle. Furthermore, the continual degradation of proteins and RNAs creates a constant demand for replacements. Protein and RNA synthesis begin with transcription, the process whereby RNA polymerase (RNAP) moves along a DNA molecule, catalyzing the synthesis of a nascent RNA chain that has a sequence that is complementary to one strand of the DNA template. Although functional links between RNAP and transcript processing, translation and DNA repair have been proposed or described, the only known catalytic activity of the enzyme involves the synthesis (or, under some conditions, the 3'-end cleavage) of RNA molecules. The process of transcription is highly regulated. Many proteins are involved in enhancing or repressing transcription, either at specific genes (*e.g.*, Gal4p, WT1) or at many genes (*e.g.*, the SWI/SNF complex, histone proteins).

Despite their importance in the transcriptional process, surprisingly little is known about the biosynthetic regulation of the RNAPs in the cells of eukaryotes. In eukaryotic cells, there are three forms of RNAP (RNAPI, II and III). RNAPII is the enzyme that synthesizes messenger RNA (mRNA) as well as some small nuclear RNAs (snRNAs). In my thesis, I address the question of what factors are involved in the biosynthetic regulation of RNAPII and what effects a reduction in RNAPII has on cell growth in the budding yeast, *Saccharomyces cerevisiae*.

I. Scope

DNA-dependent RNA polymerase synthesizes RNA molecules. In a purified system, the core enzyme (see below) is capable, on its own, of RNA-chain extension using double-stranded or single-stranded DNA as a template and four ribonucleotides as substrates (Chamberlin 1976). This core enzyme is insufficient, however, to carry out the normal *in vivo* functions of promoter-dependent transcription initiation or

termination of transcription. For these functions, a variety of protein factors are required, which are not necessary for RNA chain polymerization *per se* (Orphanides, *et al.* 1996). The core enzyme is also assisted by elongation factors that increase the rate of RNA chain synthesis (Reines, *et al.* 1996). The studies described in my thesis focus on the genes encoding the two largest subunits of RNAPII of *Saccharomyces cerevisiae*. Since these subunits are part of the core enzyme, I shall concentrate on information that pertains mostly to core subunits.

I shall begin by briefly describing the function and composition of RNAP in prokaryotes and eukaryotes. The next section reviews studies that concern the biosynthesis of RNAP, beginning with prokaryotes, followed by information obtained from multicellular organisms. Next I describe investigations concerning the biosynthesis and maintenance of subunits of RNAPs in *S. cerevisiae*. This literature review concludes with a summary of information concerning a family of transcription factors, two of which I have shown are important for the expression of subunits of RNAPII in yeast. The last section of this chapter describes the rationale for the investigations reported here and outlines the chapters that follow.

II. Structure and function of RNA polymerase

A. Composition of RNAP

Prokaryotic cells, such as *Escherichia coli*, have a single form of DNA-dependent RNAP that is capable of synthesizing all types of RNA (rRNA, tRNA and mRNA) (Yura and Ishihama 1979). The core of this enzyme, composed of four protein molecules, includes two large subunits called β' and β, and a smaller subunit, α, which is present as a dimer (Ishihama 1990; Yura and Ishihama 1979). An additional subunit, called σ, is required for RNAP to recognize and initiate at promoters (Burgess,

et al. 1969). Core RNAP in combination with one of several types of σ factor forms a complex called the holoenzyme (Helmann and Chamberlin 1988).

The structure of RNAP in eukaryotic cells is more complex. There are three forms of RNAP. RNAPII of *S. cerevisiae*, the subject of this thesis, is composed of 13 proteins that are encoded by 12 different genes. The enzyme has some similarity to RNAP of *E. coli* in that it contains two large subunits, Rpo21p[1] and Rpo22p, that share significant amino-acid sequence identity with β' and β, respectively (Allison, *et al.* 1985; Jokerst, *et al.* 1989; Sweetser, *et al.* 1987). In contrast to the prokaryotic enzyme, RNAPII does not contain a dimer of α-like subunits, but rather a heterodimer composed of Rpo23p, the third-largest subunit, and Rpo211p (Svetlov, *et al.* 1998; Ulmasov, *et al.* 1996). Both Rpo23p and Rpo211p share sequence homology with the α subunit (Kolodziej and Young 1989; Woychik, *et al.* 1993). Table 1.1 is a summary of the genes that encode subunits of yeast RNAPII.

Six of the RNAPII subunits (the products of five genes) are also part of RNAPI and RNAPIII. These subunits are Rpo25p (two per polymerase molecule) (Woychik, *et al.* 1990), Rpo26p (Archambault, *et al.* 1990; Woychik, *et al.* 1990), Rpo28p (Woychik,

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[1] Yeast RNAP subunits have been described in the literature by several systems of nomenclature (see Table 1.1). In this thesis, I shall use the following system: All RNAP core subunit genes begin with the letters *RPO* (RNA polymerase). The next number indicates the kind of nuclear RNAP, either 1, 2 or 3. The final single-, or double-digit number indicates the specific subunit, designated by its size, largest to smallest. In the case of RNAPII, the 12 subunits are numbered from 1 to 12. Therefore, the gene encoding the fifth-largest subunit of RNAPII is called *RPO25* while the gene encoding the 11th-largest subunit of RNAPIII is called *RPO311*. In keeping with the standard nomenclature for *S. cerevisiae*, a wild-type gene is written in uppercase letters and italics, while the protein encoded by that gene has only the first letter capitalized and a "p" at the end (e.g. *GAL4*, Gal4p).

Table 1.1 Genes encoding subunits of RNAPII of *S. cerevisiae*

RNAPII subunit gene	**Other names**	**Homologue in *E. coli*: gene (subunit)**	**Essential for viability in *S. cerevisiae*?**	**Common to RNAPI, II and III, or unique to RNAPII?**
RPO21	*RPB1, RPB220, SIT1, SUA8*	*rpoC* (β')	YES	unique
RPO22	*RPB2, RPB150, SIT2, SOH2*	*rpoB* (β)	YES	unique
RPO23	*RPB3*	*rpoA* (α)	YES	unique
RPO24	*RPB4*		NO	unique
RPO25	*RPB5*		YES	common
RPO26	*RPB6*		YES	common
RPO27	*RPB7*		YES	unique
RPO28	*RPB8*		YES	common
RPO29	*RPB9, SHI1, SSU73*		NO	unique
RPO210	*RPB10*		YES	common
RPO211	*RPB11*	*rpoA* (α)	YES	unique
RPO212	*RPB12, RPC10*		YES	common

et al. 1990), Rpo210p (Woychik and Young 1990) and Rpo212p (Carles, *et al.* 1991; Treich, *et al.* 1992a).

The remaining three subunits are found only in RNAPII. Rpo24p and Rpo27p are subunits that can interact directly with one another (Edwards, *et al.* 1991). Rpo24p is not essential for growth of yeast (and hence for normal RNA chain synthesis) at optimal growth conditions (Woychik and Young 1989). In contrast, Rpo27p is essential (McKune, *et al.* 1993). Rpo29p is the only other non-essential subunit (Woychik, *et al.* 1991). It has a role in start-site selection *in vivo* (Furter-Graves, *et al.* 1994; Hull, *et al.* 1995) and transcriptional elongation *in vitro* (Awrey, *et al.* 1997).

B. Function of RNAP

Prior to RNA chain synthesis, the *E. coli* RNAP core enzyme binds one of a family of proteins called σ (Helmann and Chamberlin 1988). The particular σ factor that is bound determines the sites (promoters) on the chromosome with which the RNAP molecule can interact with high enough specificity to begin RNA chain polymerization. The σ factor that is most often used by cells growing at optimal conditions is called σ^{70}, which is encoded by *rpoD* (Burgess, *et al.* 1987).

Transcription initiates through a number of biochemically well-defined steps that start with the formation of the closed complex. At this stage, RNAP holoenzyme is bound tightly and specifically at the promoter with the DNA in a double helical conformation (Chamberlin 1974). The promoter contains two specific six-nucleotide-long DNA sequences centred 10 and 33 bp upstream of the start site of transcription (Siebenlist, *et al.* 1980). The σ component of the holoenzyme is necessary for RNAP to interact specifically with these sequences (Burgess, *et al.* 1969; Gardella, *et al.* 1989; Siegele, *et al.* 1989). Binding is followed by open complex formation, characterized by limited melting of the DNA template, which extends from 10 bp upstream of the transcriptional start-site to about 2 bp past it (Siebenlist 1979).

Ribonucleotides that can form basepairs with the template strand of DNA enter the active site of the enzyme. The enzyme catalyzes the formation of a phosphodiester bond, which involves the nucleophilic attack of the 3' OH group from the first nucleotide on the α-phosphate on the 5' end of the incoming ribonucleoside triphosphate. The reaction results in the release of pyrophosphate and the formation of a chemical bond between the first two initiating nucleotides (reviewed in Richardson and Greenblatt 1996). Chain extension continues with the subsequent addition, to the 3'-end of the nascent RNA chain, of nucleotides that can base pair with the next base in the DNA template. The RNAP molecule produces a number of short RNA chains (abortive transcripts) because of repeated attempts at the beginning of the transcription cycle to clear the promoter region (McClure and Cech 1978). When the polymerase molecule is finally able to leave the promoter region (promoter clearance) it releases σ factor (Travers and Burgess 1969). An elongation factor, called NusA, binds the elongating RNAP complex, increasing the length of pauses at certain locations on the template. This is thought to be important for coupling transcription and translation (reviewed in Richardson and Greenblatt 1996). Chain synthesis continues in a highly stable manner. When RNAP is stalled at particular sites on the template, elongation factors GreA and GreB can facilitate a limited cleavage of the 3'-end of the RNA, realigning the active site with the end of the transcript, and thus allowing the polymerase to resume synthesis (Borukhov, *et al.* 1993). RNA chain synthesis is completed when the polymerase encounters template-encoded signals (terminators) that cause RNAP to arrest and then to disengage from the template, freeing the RNA chain for its designated cellular role. Depending on the type of terminator, termination may be dependent on a protein factor called ρ (Roberts 1969), or it may require only a template-encoded signal (reviewed in Richardson and Greenblatt 1996).

The transcription cycle in eukaryotic cells is highly analogous to that of *E. coli.* The core enzyme, although capable of synthesizing RNA, is not able to recognize promoter sequences with high specificity, initiate transcription or leave the promoter region. In order to carry out these functions, RNAPII is assisted by several ancillary proteins called the general transcription factors. The transcription factors that are critical for basal-level transcription *in vitro* are TFIIB, TFIID, TFIIE, TFIIF and TFIIH (for a recent review, see Orphanides, *et al.* 1996). Two models have been proposed for the mechanism of transcriptional initiation by RNAPII. The first model proposed a step-wise assembly of the initiation complex, beginning with the recognition of the TATA-box on the promoter by a small subunit of TFIID, called TBP. TFIIB binds to the TFIID-DNA complex, forming a binding site for RNAPII that is already bound to TFIIF. TFIIE is the next factor to join the assembly, providing a binding site for TFIIH (Buratowski, *et al.* 1989; Buratowski, *et al.* 1991).

More recently, evidence has accumulated for the existence of a "holoenzyme", a large (pre-formed) complex that, in addition to core RNAPII, contains some or all of the general transcription factors as well as other proteins that are necessary for the regulation of RNAPII activity via repressing and activating proteins (recently reviewed in Greenblatt 1997; Orphanides, *et al.* 1996). In light of this finding, an alternative model for the step-wise assembly of the initiation complex suggests that, in one step, the (pre-formed) holoenzyme is able to recognize the promoter and begin the initiation process. Open-complex formation and promoter clearance are dependent on holoenzyme proteins. A helicase activity of TFIIH is required to generate the open complex, while a kinase activity of the same factor phosphorylates the carboxy-terminal domain (CTD) of the largest subunit of RNAPII (see below). This is thought to be necessary for promoter clearance and processive elongation (Orphanides, *et al.* 1996). Chain elongation continues with the assistance of many elongation factors (*e.g.*, TFIIS, Elongin, TFIIF, P-TEFb, DSIF) (Marshall, *et al.* 1996; Reines, *et al.* 1996;

Wada, *et al.* 1998) followed by the termination of transcription. Elongation and termination are not as well-defined as the initiation step of transcription but, there are some similarities to analogous events in prokaryotic cells.

C. β'/Rpo21p and β/Rpo22p in the transcription reaction.

The two largest subunits of the single prokaryotic and three eukaryotic nuclear RNAPs serve several functions in the transcription cycle. Eight regions, labelled A to H, are especially well-conserved among the largest subunits of prokaryotic and eukaryotic RNAPs (Jokerst, *et al.* 1989). The largest subunit of eukaryotic RNAPIIs has an additional well-conserved domain at the C-terminus of the protein. This domain, called the CTD, is an imperfect repeat of a heptapeptide sequence: Tyr Ser Pro Thr Ser Pro Ser (Allison, *et al.* 1985). There are 26 repeats in *S. cerevisiae* and 52 in mice and humans. The second-largest subunit has nine regions (A to I) that are well-conserved in enzymes from prokaryotic and eukaryotic cells (Sweetser, *et al.* 1987).

On its own, the β' subunit is able to bind to DNA (Zillig, *et al.* 1976). Interactions between the largest subunits and DNA have also been investigated in the context of the entire RNAP molecule. RNAPIII of *S. cerevisiae* was assembled, along with general transcription factors, into an initiation complex on a *SUP4* $tRNA^{tyr}$ gene whereupon the template was cross-linked to proteins at specific sites. The two largest subunits of RNAPIII were cross-linked to the template at positions ranging from 17 bp upstream to 16 bp downstream of the transcriptional start site (Bartholomew, *et al.* 1993). Similar results were obtained using human RNAPII and a template (Adenovirus major late promoter) with photo-activated affinity probes positioned along the DNA up to 19 bp upstream of the start site (Coulombe, *et al.* 1994; Robert, *et al.* 1996). Specific contacts have been mapped between DNA that lies 6 nucleotides downstream of the active site of the enzyme and an N-terminal domain (region A) of β' that coordinates zinc ions (Nudler, *et al.* 1996). This same site in the template is also

in contact with region B at the N-terminus of β. A second region of β (conserved region I) is in contact with the template at the site of ribonucleotide incorporation (Nudler, *et al.* 1996). These results fit well with genetic data that indicate an interaction between the zinc-binding domain (region A) of the largest subunit of RNAPI of *S. cerevisiae* and the zinc-binding domain (near region I) that is at the C-terminus of the second-largest subunit (Yano and Nomura 1991). DNA-binding has also been demonstrated for region C of the largest subunit of RNAPII of Drosophila (Kontermann, *et al.* 1993).

The largest subunits of RNAP make extensive contacts with the RNA product. When immobilized on nitrocellulose filters, the largest subunits of RNAPII of Drosophila are both able to bind to RNA (Gundelfinger 1983). Cross-linking studies with nucleotide derivatives incorporated into the nascent RNA show that it is in contact with conserved regions of β' and β (Nudler, *et al.* 1998). The β' subunit contacts the growing end of the RNA chain at region D during elongation and region G when RNAP is arrested (Markovtsov, *et al.* 1996). In the case of β the contacts are with regions D, H and I (Grachev, *et al.* 1989; Markovtsov, *et al.* 1996; Mustaev, *et al.* 1991; Severinov, *et al.* 1995b). Interactions of the initiating nucleotide with domains H and I were also observed in the second-largest subunit of RNAPII in *S. cerevisiae* (Riva, *et al.* 1990; Treich, *et al.* 1992b).

The two largest subunits of RNAP have also been implicated as the site of RNA catalysis. Early evidence for this came from the observation that the binding site for rifampicin, an antibiotic that inhibits the transition from transcriptional initiation to elongation, was located in the β subunit (McClure and Cech 1978). Streptolydigin, an antibiotic that also inhibits transcriptional elongation, binds β and mutations that confer resistance to the drug are found between regions C and D of β and in region F of β' (Severinov, *et al.* 1995a, and references therein). The fungal toxin, α-amanitin, inhibits the activity of RNAPII at the translocation step that occurs after the formation of the first phosphodiester bond of the RNA chain (de Mercoyrol, *et al.* 1989). This drug

binds the largest subunit, likely near region F, since mutations that confer resistance to α-amanitin map exclusively to that region. The cross-linking of the initiating nucleotide to specific regions of the two largest subunits implicates them as the site for catalysis (see above). Further evidence for localization of the catalytic center to regions in the two largest subunits can be inferred by the following results. A Mg^{2+} ion at the catalytic center is essential for RNA synthesis. When Mg^{2+} was replaced by Fe^{2+}, an ion that induces peptide cleavage, the β and β' subunits were cleaved in regions shown to contact the initiating nucleotide (see above) as well as in conserved region F of β' (Mustaev, *et al.* 1997).

The catalytic activity of RNAP is linked to its ability to promote an efficient termination of transcription. Mutations affecting the termination process map to conserved regions in β and β' (Landick 1997; Weilbaecher, *et al.* 1994). A mutational analysis of the largest subunit of RNAPIII in *S. cerevisiae* showed that conserved regions D and F are linked to the catalytic activity of that enzyme, particularly at the stage after initiation and during conversion to an actively elongating enzyme (Dieci, *et al.* 1995; Thuillier, *et al.* 1996). RNAPIII with amino-acid substitutions in region F of the largest subunit showed an increase in the intrinsic ribonucleolytic activity of the enzyme, providing more evidence for catalytic activity residing, at least in part, in the largest subunit (Thuillier, *et al.* 1996). Finally, mutations in various locations of the second-largest subunit of RNAPIII have an effect on the elongation and termination activities of that enzyme (James and Hall 1990; James, *et al.* 1991; Shaaban, *et al.* 1995).

Genetic experiments have defined regions of Rpo21p that are involved in the selection of transcriptional start-sites. Mutations in conserved region D result in a shift in the normal start-site pattern such that downstream sites are used more efficiently (Berroteran, *et al.* 1994). Mutations in conserved region G have the opposite effect, causing a slight shift towards the use of start-sites upstream of the major initiation site

of *HIS4* (Hekmatpanah and Young 1991). These results fit well with the data showing that these same regions of β' cross-link to the initiating nucleotide (Markovtsov, *et al.* 1996). The second-largest subunit of RNAPII also has a role in start-site selection since some mutations in *RPO22* cause an increase in downstream start-site use on *HIS4* (Arndt, *et al.* 1989). Genetic studies have also revealed that Rpo21p contains part or all the binding site for elongation factor TFIIS (Archambault, *et al.* 1992). The genetic interaction of TFIIS with the G-H region of Rpo21p was supported by *in vitro* data showing that mutations in this region disrupt the interaction of these two proteins (Wu, *et al.* 1996).

A major difference between β' and Rpo21p is the seven-amino-acids repeat (CTD) that is found at the C-terminus of the latter. This domain is involved in the initiation process, probably through its interaction with other components of the holoenzyme. A factor known as mediator is a protein sub-fraction of the holoenzyme that interacts directly with the CTD (Bjorklund and Kim 1996). Kinases such as Kin28p and Srb10p directly phosphorylate the CTD (Feaver, *et al.* 1994; Hengartner, *et al.* 1998). This phosphorylation is thought to be important in the process of promoter clearance (Greenblatt 1997) and in the regulation of holoenzyme formation (Hengartner, *et al.* 1998). The CTD is also important for efficient splicing and 3'-processing of mRNA since expression of RNAPII lacking the CTD in mammalian cells results in a marked reduction in both processes (McCracken, *et al.* 1997). Furthermore, pre-mRNA splicing factors can bind to the CTD and *in vitro* splicing in a mammalian extract can be inhibited by the addition of antibodies that recognize the CTD (reviewed in Steinmetz 1997).

Structural studies of RNAP have begun to reveal the physical basis for some of the biochemically and genetically defined functions of the two largest subunits. Electron crystallography of two-dimensional crystals of RNAP from *E. coli*, and RNAPI and RNAPII from *S. cerevisiae*, revealed two prominent projections in the molecule

that form a cleft or channel that could accommodate a double-stranded DNA template (Darst, *et al.* 1989; Darst, *et al.* 1991; Schultz, *et al.* 1993). The second-largest subunit was mapped to one of these projections through the detection of electron densities that were caused by a monoclonal antibody directed against a particular epitope of that subunit (Klinger, *et al.* 1996). Preliminary data concerning the X-ray crystal structure of yeast RNAPII that lacks the fourth- and seventh-largest subunits have been described recently. The diffraction data reveal a symmetry that suggests that the largest subunits of RNAPII may have a similar fold. Although this is not apparent from direct sequence alignments, there is a striking correlation between the predicted secondary structures of the two proteins (Fu, *et al.* 1998).

III. Biosynthesis of RNAP

In this section I review investigations of the regulation of the biosynthesis of RNAP in a variety of organisms. RNAP has a central role in cellular growth, it may be that evolutionary constraints have conserved mechanisms that control the amount and rate of synthesis of RNAP. There are large differences between, for example, yeast cells and bacterial cells, making conservation at the level of molecules unlikely. General themes governing the regulation of synthesis, however, may have been maintained. I shall first describe the synthesis of RNAP in *E. coli*, then discuss data concerning multi-cellular eukaryotes. I conclude this section with a summary of the subject for yeast cells.

A. RNAP synthesis in *E. coli*

Three genes encode components of the RNAP core in *E. coli*. The genes for β and β' are located in the same operon (*rplKAJLrpoBC*) and are called *rpoB* and *rpoC*. The gene for the α subunit, *rpoA*, is in a separate operon. When *E. coli* cells grow in

log phase, the synthesis rates of α, β and β' are maintained at a level that results in a subunit ratio of 2.8 : 1 : 1 (Ishihama and Fukuda 1980; Pedersen, *et al.* 1978). This matches closely the 2 : 1 : 1 ratio of these subunits found in core RNAP. A number of mechanisms, which are not understood in detail, regulate the expression of the RNAP subunits. I shall begin by describing the structure of the genes and mRNA transcripts encoding the RNAP. Next I summarize data concerning the regulation of the synthesis of these subunits.

i) RNAP core subunits: operon structure and transcripts

The operon of *E. coli*, from which β and β' are expressed, includes six genes. The promoter-proximal genes encode proteins from the large subunit of the ribosome. In order of their location on the chromosome they are: L11 (*rplK*), L1 (*rplA*), L10 (*rplJ*) and L12 (*rplL*). Downstream of these genes are *rpoB* and *rpoC*. pL11, the first of two major promoters in this operon, can produce bicistronic transcripts that terminate downstream of *rplA*, tetracistronic transcripts (Friesen, *et al.* 1983) that terminate at an attenuator (see below) which is located downstream of *rplL* and immediately upstream of *rpoB*, or transcripts containing all six coding regions, terminating downstream of *rpoC* (Downing and Dennis 1987). The second major promoter (pL10) is located between *rplA* and *rplJ*. This promoter can produce transcripts that terminate at the attenuator, or continue to the end of *rpoC* (Downing and Dennis 1987). Since no major promoters have been mapped either *in vitro* or *in vivo* in the region after *rplL* and upstream of *rpoB* or between *rpoB* and *rpoC*, the expression of β and β' is likely to be dependent on the L11 and L10 promoters.

One of the interesting features of this operon is the *in vivo* ratio of the various transcripts. Transcripts encoding only the ribosomal proteins are five times more abundant than those that also encode β and β' (Dennis 1977a; Downing and Dennis 1987). As alluded to above, this is the result of a DNA sequence, called an attenuator,

located downstream of *rplL* and immediately upstream of *rpoB*. The attenuator acts through a RNA stem-loop structure that forms after RNAP has transcribed the DNA sequence that encodes the attenuator (Barry, *et al.* 1980). This RNA structure then normally causes the termination of 80% of the transcripts coming from the upstream promoters (Barry, *et al.* 1979; Dennis 1984; Downing and Dennis 1987). Approximately half of the transcripts that continue past the attenuator are processed by an endoribonuclease called RNase III, creating a new 5'-end in approximately one half of the transcripts containing *rpoB* and *rpoC* (Barry, *et al.* 1980). It is not clear if this processing has physiological significance for the regulation of RNAP synthesis since expression of β and β' is not affected in a strain that lacks RNase III processing activity (Dennis 1984).

The *rpoA* gene (encoding the α subunit) is the fourth cistron in an operon with four r-protein genes, *rpsM* (S13), *rpsK* (S11), *rpsD* (S4) and *rplQ* (L17). The operon produces one transcript (see Thomas, *et al.* 1987, and references therein).

ii) Regulation of β, β' and α synthesis

Although the amounts of β, β' and α in the cell roughly reflect their stoichiometries in RNAP, α is in excess. The rate of synthesis of the transcripts encoding α is five times higher than that of transcripts encoding β and β' (Dennis 1977b). The steady-state level of α was measured for cells growing at four different rates and was always higher than twice the molar amount of β (Pedersen, *et al.* 1978). This result led to the suggestion that RNAP synthesis is limited by the production of β and β' (reviewed in Bremer and Dennis 1996). One of the first indications of a regulatory mechanism controlling synthesis of RNAP came from experiments with strains carrying the transducing phage λd*rif*^{d}18. This defective phage carries, in part, *rpoB* with a dominant mutation conferring resistance to the antibiotic (and RNAP inhibitor) rifampicin. Cells carrying this phage as a lysogen have two copies of *rpoB*,

but express the same level of β subunit as a cell without λd*rif*^{d}18 (Kirschbaum 1973). Moreover, induction of the lysogen increases the level of β only two-fold, even though *rpoB* was replicated to a high copy-number along with other phage sequences (Kirschbaum 1973). Therefore, during balanced growth, production of β is modified to maintain a level that is near to that of a cell with a single copy of *rpoB*. This modification may be at one or more stages of synthesis, including increased degradation of excess subunit.

Evidence for transcriptional regulation

Studies with strains carrying mutations in *rpoC* support the existence of a feedback mechanism for the control of β and β' synthesis. The ts4 mutation in *rpoC* confers slow cell death (*i.e.*, arrest occurs after a few cell divisions) at high temperature and affects the stability of RNAP *in vivo*. Although the rate of degradation of both β and β' increases, the rate of synthesis of these subunits also increases by up to five-fold (Kirschbaum, *et al.* 1975). Growth at high temperature (42°) of a strain (XH56) with a different mutation in *rpoC* results in a defect in transcriptional initiation. At a semi-permissive temperature this strain exhibited an increase in the synthesis of β and β' that was due partly to an increase in the synthesis of mRNA for *rpoBC* (Dennis 1977a). This result points to the *activity* of RNAP as a determinant for control of synthesis rather than simply the *physical amount* of the enzyme. The result is not a general effect since, at a semi-permissive temperature for growth, the transcription of most genes decreases (Downing and Dennis 1991). Transcription from the promoters for L11 and L10, however, increases two-fold. In addition, the level of transcripts containing *rpoB* and *rpoC* increases five-fold. The increase in the transcripts was not a result of increased mRNA stability, since the stability of *rpoB* and *rpoC* transcripts at these conditions actually decreases by two-fold. The main cause of the increase in *rpoB* and

rpoC message was a relaxation of the termination occurring at the attenuator (Downing and Dennis 1991).

This experiment and similar ones in which rifampicin was used to reduce the activity of RNAP *in vivo* have been criticized because the tool used to probe regulation may affect the activity of RNAP itself. Hence the relaxation of attenuation may simply be a consequence of the mutant RNAP activity or of rifampicin interaction with elongating RNAP. To avoid these problems, Steward and Linn (1992) devised an experiment that tests the attenuator activity in a strain with normal RNAP activity. A series of plasmids was constructed in which promoters of varying strengths were used to replace sequences immediately upstream of the start-site of transcription for *rplJLrpoBC*. Next, a *lacZ* reporter gene was inserted either just upstream or just downstream of the attenuator of each fusion gene. The resulting gene fusions were tested for their levels of expression. Promoters driving the expression of genes lacking the attenuator varied in strength over a 27-fold range. The same promoters driving the expression of genes that included the attenuator varied only over a nine-fold range. As a control, when terminators from other genes replaced the attenuator, the range of promoter strengths was proportional to the same genes lacking the terminators. The authors concluded that the efficiency of the attenuator changed in response to the strength of the promoter, terminating the transcripts which were initiated at strong promoters and allowing readthrough from the weak promoters. When levels of RNAP are low, loading of the *rplKAJLrpoBC* operon is reduced. It was suggested that under these conditions the attenuator allows a greater percentage of transcriptional readthrough, leading to higher levels of β and β' to compensate for the reduced level of RNAP (Steward and Linn 1992).

The attenuator may be regulated by an anti-termination mechanism similar to that described for the expression of genes of the phage λ. In the case of λ, when RNAP encounters a DNA sequence called a *nut* site, a transcript is produced that is

able to bind factors that can modify RNAP activity, allowing it to read through terminator elements located further downstream (reviewed in Greenblatt, *et al.* 1993). An element like a *nut* site may exist upstream of the attenuator. The rate of transcription of the *rplKAJLrpoBC* operon or perhaps the level of RNAP activity in the cell may stimulate the RNA signal produced from the site (and perhaps protein factors) to modify RNAP to read through the attenuator. There is some support for this hypothesis since a strain with a mutation in *nusA*, a gene important for N-mediated λ anti-termination, shows a decreased readthrough at the attenuator (Ralling and Linn 1987). Also, NusA and NusG (another factor required for λ anti-termination) stimulate readthrough at the attenuator in a dose dependent manner *in vitro* (Linn and Greenblatt 1992). A recent study (Steward, *et al.* 1997) shows that the mechanism regulating the activity of the attenuator differs from N-mediated anti-termination in that RNAP that reads through the attenuator is not modified to be able to read efficiently through a subsequent downstream terminator.

An additional form of transcriptional regulation of the *rplKAJLrpoBC* operon has been described (Dykxhoorn, *et al.* 1996). Strains bearing plasmids that over-produce all or combinations of the subunits of RNAP were constructed. The steady-state level of β, β', α and σ^{70} were measured to demonstrate that subunit levels were indeed elevated *in vivo*. Reporter genes in single-copy (carried on λ integrated in the chromosome) were introduced into the subunit over-producing strains. The reporters were fusions of *lacZ* (encoding β-galactosidase) to various transcriptional and translational control elements in *rplKAJLrpoBC* . When all the core or holoenzyme subunits of RNAP were over-produced, a 3.3-fold decrease in β-galactosidase activity was observed from a gene fusion driven by the two major promoters of the operon. This gene fusion included the attenuator, which was shown to relax termination of transcripts when RNAP activity decreased (see above). Gene fusions that did not include the attenuator still showed a 2.5-fold decrease in expression, suggesting that

repression was mostly a result of decreased promoter activity and only partially caused by an increase in termination at the attenuator (Dykxhoorn, *et al.* 1996). The repression at the L11 and L10 promoters was not a general consequence of an increase in the level of RNAP since expression of an unrelated (*tet-lacZ*) gene was not repressed. A similar approach in an earlier study also indicates that overproduction of RNAP *in vivo* reduces the synthesis rates of the β and β' subunits (Bedwell and Nomura 1986).

Therefore, two forms of transcriptional regulation modulate the expression of *rpoB* and *rpoC*. First, an attenuator upstream of these genes can relax to allow increased readthrough when RNAP activity is low; second, the strength of the promoters required for *rpoBC* expression (upstream of *rplK* and *rplJ*) is reduced when there is an excess of RNAP in the cell.

Evidence for translational regulation

In addition to transcriptional regulation, there is evidence that RNAP synthesis in *E. coli* is regulated at the level of translation. Dennis and Fiil (1979) constructed *E. coli* strains that carried plasmids with portions of the *rplKAJLrpoBC* operon. The plasmid frequency was 7 to 12 copies per cell. Although the amount of *rpoBC* mRNA increased by six-fold, the amount of β and β' subunits increased only two-fold (Dennis and Fiil 1979). Since the level of *rpoBC* mRNA is increased without a concomitant increase in the steady-state level of β and β', there exists a mechanism that controls the expression of β and β' at the post-transcriptional level. Although these experiments identified a post-transcriptional regulation of RNAP synthesis, they did not address the mechanism of this regulation. In these experiments, like some of the studies of transcriptional regulation, RNAP was altered such that the cell was resistant to rifampicin. Although it is possible that the mutation altered the transcriptional

regulation of RNAP synthesis, rifampicin resistance is less likely to affect post-transcriptional regulation.

The transcriptional regulation described in the preceding section was not seen in these experiments. Under normal conditions, the attenuator operates at near maximum capacity, terminating 80% of transcripts (Steward and Linn 1992). If β and β' levels are increased by, for example, increasing the copy number of *rpoBC*, then the attenuator is not able to compensate by *increasing* the level of termination (Dennis 1984). That form of regulation is observed primarily when RNAP activity is decreased, leading to a relaxation of termination at the attenuator, and an increase in *rpoBC* mRNA.

A mutational analysis of the intergenic region of *rplL* and *rpoB* provides support for translational regulation of β synthesis (Dennis 1984). Plasmids were constructed bearing the genes *rpJLrpoB* under the control of the promoter upstream of *rplJ*. The intergenic region contained either wild-type sequences or a deletion of the attenuator that is upstream of the RNase III processing site. Compared to a strain without a plasmid, the introduction of *rplJLrpoB* increases the level of β mRNA, but β subunit increases at a lower rate. Removal of the attenuator increases the level of β mRNA a further 2.5-fold, but the efficiency of the translation of β mRNA is reduced further. This is not a consequence of the removal of sequences required for binding the ribosome since 5'-processing of the β mRNA by RNase III was not disturbed by removal of the attenuator sequence. Hence over-expression of β has a negative effect on the translation of β mRNA (Dennis 1984).

An examination of *rpoB* amber mutants in strains carrying the weak *supE* suppressor shows that, 90% of the time, translation of *rpoB* mRNA gives a truncated (non-functional) protein. The truncated and full-length forms of β produced in this strain were quantitated. It was shown that the rate of translation of the *rpoB* mRNA increases by 3-fold (Dennis, *et al.* 1985) compared to a strain without the amber

mutation in *rpoB*. The over-all increase in translation compensates for the reduction in functional β subunit, thereby generating sufficient amounts of full-length β. This result is particularly intriguing since it suggests that a reduction in the level of β subunit can be compensated by an increase in the rate of translation of *rpoB* mRNA. Hence, both transcriptional and translational mechanisms are employed to respond to a decrease in the level of a RNAP, or of a particular subunit.

An extensive *in vivo* analysis of β and β' synthesis indicates that the artificial over-production of either or both of β and β' results in a 2- to 8-fold repression of a gene with *lacZ* fused in frame to the first 134 codons of *rpoB* (Dykxhoorn, *et al.* 1996). This repression represents the sum of potential transcriptional and translational regulation. The *translational* contribution (a repression of 2- to 4-fold) was calculated by subtracting the amount of repression observed using a similar gene that did not include the leader or coding sequences of *rpoB* (and hence could not be regulated by translation) (Dykxhoorn, *et al.* 1996). A two-fold translational repression was also seen with a *lacZ*-fusion gene containing the leader and 245 codons of *rpoC*. It is noteworthy that over-production of all the components of the holoenzyme did not have any effect on translation, but repressed only at the level of transcription (see above) (Dykxhoorn, *et al.* 1996). This may indicate that translational repression mediated by β or β' is possible only if the subunit is not already in the holoenzyme. Perhaps β or β' interferes with the translation of *rpoB* and *rpoC* mRNA by binding to the message. Formation of the holoenzyme may block the ability of β or β' to bind to *rpoB* or *rpoC* mRNA (Dykxhoorn, *et al.* 1996).

A series of 62 linker-scanning mutations that spanned the 400 bp flanking the *rpoB* translation initiation start site was tested for its effect on the translational repression imposed by over-expression of β and β' (Dykxhoorn, *et al.* 1997). Surprisingly, no significant diminution of repression was observed. The authors suggested that redundant repression sites in the *rpoB* transcript affected singly by β or

β' are present and hence the effect of mutation of one subunit-binding site is compensated for by the other. Consistent with this hypothesis, mutation of nucleotides (+13 to +25) downstream of the start codon reduced the translational repression resulting from the over-expression of only the β' subunit (Dykxhoorn, *et al.* 1997).

The mechanism of the regulation of RNAP synthesis has also been examined *in vitro*. Experiments using coupled transcription and translation of *rpoB* show that addition of excess holoenzyme or an assembly precursor, $\alpha_2\beta$, inhibits the synthesis of β and β' without affecting the synthesis of other proteins encoded by the template (Fukuda, *et al.* 1978). This inhibition is at the post-transcriptional level since the relative ratio of *rpoBC* mRNA to that of genes with unaffected expression was not altered (Kajitani, *et al.* 1980). Peacock *et al.* (1983) extended these results using purified mRNA encoding the ribosomal protein L10 and the β subunit of RNAP. Purified ribosomes, elongation factors and charged tRNAs necessary to make the dipeptides that begin the peptide chains of L10 and β were added to the RNA template. Addition of RNAP holoenzyme inhibits the formation of the dipeptide of the β subunit but does not affect the synthesis of the dipeptide of L10 (Peacock, *et al.* 1983). These data suggest that RNAP can act directly as a repressor of the translation of β mRNA.

Studies of the synthesis of the α subunit show that increased gene dosage of the operon that includes *rpoA* leads to an increase in *rpoA* mRNA and in the level of α subunit (Thomas, *et al.* 1987). These data suggest that α subunit synthesis is not regulated tightly. The operon that includes *rpoA* has four r-protein genes (see above). The expression of these genes is regulated at the level of translation by r-protein S4, one of the gene products of this operon. Interestingly, α subunit synthesis is not affected by this regulation, suggesting that α translation is independent of the translation of the other genes on the operon (Thomas, *et al.* 1987).

iii) Growth-rate control and the stringent response

The growth rate of *E. coli* depends on the availability of nutrients. When nutrients become plentiful, the growth rate increases. This increase is possible because the concentration of ribosomes and RNAP increases as well, helping manufacture cellular components required to achieve a higher rate of macromolecular synthesis and a shorter time for cell division (reviewed in Bremer and Dennis 1996). Growth-rate control refers to the mechanism coupling the rate of cellular growth to ribosome and perhaps RNAP synthesis.

When the growth rate of bacterial cells increases, the concentration of ribosomes increases at a faster rate than the concentration of the bulk of protein (Schaechter, *et al.* 1958). This increase is controlled by the rate of synthesis of ribosomal RNA (rRNA) (reviewed in Gourse, *et al.* 1996). When the growth rate is low, rRNA production is reduced, leading to free ribosomal protein (r-protein). A subset of r-proteins that bind to rRNA in the ribosome binds to the leader sequences of different mRNAs, each of which encode several r-proteins. Translation of the messages is inhibited by this binding, resulting in a feedback regulation. If the growth rate increases, the concentration of rRNA also increases, allowing the rRNA to sequester free r-proteins into functional ribosomes. The feedback inhibition on r-protein mRNAs is lifted allowing production of more r-protein (reviewed in Nomura, *et al.* 1984).

The key to growth-rate control is the production of rRNA. Recently, an elegant model has emerged to explain this coupling (Gaal, *et al.* 1997; Roberts 1997). In order that RNAP transcribe rDNA, the enzyme must bind to the promoter and form an open complex. The open complex at rDNA promoters is unusually sensitive to the level of GTP or ATP in the cell, since these nucleotides are the initiating residues for rRNA chain synthesis. Also, a promoter sequence exists that makes open complex formation highly unstable when GTP or ATP (depending on the specific promoter) concentrations are low (Gaal, *et al.* 1997). When nutrients are plentiful, respiration

leads to an increase in the production of high-energy molecules such as GTP and ATP. These allow for stable, productive open complexes at rDNA promoters, leading to an increase in synthesis of rRNA. An increase in rRNA results in an increase in the concentration of ribosomes.

A secondary level of macromolecular regulation is called the stringent response (reviewed in Gallant 1979). When *E. coli* cells experience a sudden shift to growth conditions with a lower level of nutrients, the production of rRNA declines sharply. This mode of regulation is thought to be controlled by guanosine tetraphosphate and guanosine pentaphosphate (ppGpp and pppGpp) (Gallant 1979). These molecules are produced by an enzyme (RelA) whose activity is stimulated when ribosomes encounter tRNA molecules that are not charged with amino acids. The ppGpp molecules are thought to reduce rRNA synthesis by binding directly to RNAP and inhibiting its ability to initiate transcription of rDNA (reviewed in Condon, *et al.* 1995). There is also evidence that ppGpp slows the elongation rate of RNAP that is transcribing mRNA (Vogel, *et al.* 1992), whereas the elongation rate for rRNA is not reduced. This led to a model whereby the amount of RNAP in the cell is reduced because RNAP molecules are sequestered in slow elongation complexes. This, in turn, leads to a reduction in rRNA levels since less RNAP is available to initiate at rDNA promoters (Jensen and Pedersen 1990). The transcription of genes encoding r-proteins is under stringent control (Dennis and Nomura 1975). ppGpp may also affect the ability of RNAP to initiate at r-protein gene promoters (Lindahl, *et al.* 1976).

How is the level of RNAP in *E. coli* cells affected by the growth rate and the stringent response? Unfortunately, there is no clear consensus among researchers. Early attempts at measuring the levels of the subunits of RNAP in cells grown at different rates indicate that the levels of $\alpha : \beta : \beta'$ are maintained at the ratio of 2-2.2 : 1 : 1. Furthermore, as the growth rate increases, the level of these subunits increases in proportion to the growth rate, accumulating faster than the rate of bulk protein

(Iwakura, *et al.* 1974; Matzura, *et al.* 1973; Shepherd, *et al.* 1980). A more recent study, however, concludes that the rate of synthesis of the β and β' subunits does not increase as growth rate increases (Ralling, *et al.* 1985). As the growth rate increases, the mass of the cell increases, a consequence of higher rates of gene expression. As an alternative to an increase in the amount of RNAP, the increased demand for more transcription may be accommodated by a shift in the pool of inactive versus active RNAP (Shepherd, *et al.* 1980). Indeed, there is strong evidence for an excess of RNAP in the cell, suggesting that RNA synthesis is not limited by the concentration of RNAP (Matzura, *et al.* 1973).

With respect to the stringent response, the rate of synthesis of the RNAP subunits decreased when cells were shifted to a poor growth medium (Iwakura and Ishihama 1975). This decrease does not, however, occur at the level of transcription. Maher and Dennis (1977) demonstrated that the rate of synthesis of the mRNA encoding the r-proteins in the *rplKAJLrpoBC* operon is reduced by a shift to poor nutrient conditions. Similar to rRNA, these mRNAs show a stringent response. The mRNA for *rpoBC* did not decrease under the same conditions, suggesting that these genes were not under stringent control (Maher and Dennis 1977). The apparent paradox of the differential synthesis of transcripts of genes that are part of the same mRNA can be resolved if the attenuator is sensitive to growth conditions, becoming relaxed during a nutrient down-shift. Similar to the β and β' subunits, α subunit synthesis does not show a stringent response, suggesting the existence of a mechanism that allows *rpoA* expression to escape the control that is exerted on the r-protein genes in the α operon (Thomas, *et al.* 1987).

How might the multiple mechanisms for the regulation of RNAP production in *E. coli* benefit the cell? Energy wasted on producing excess enzyme could be conserved and channeled into the production of other cellular components. The concentration of

RNAP in the cell may affect also the expression of genes, affecting the rate of initiation at promoters for which binding of the holoenzyme is rate-limiting for transcription. An excess of RNAP may lead, therefore, to an inappropriate increase in the expression of certain genes. Similarly, the competition by the various σ factors for binding with the core polymerase may be affected by the relative level of RNAP. An alteration in the distribution of these holoenzymes may alter normal gene expression patterns. Further characterization of the molecular basis for measuring the RNAP activity level (in the case of transcriptional regulation) and the physical level of the β and β' subunits (leading to translational regulation) may lead to the discovery of similar mechanisms in eukaryotic cells, helping to define another level of homeostatic control of gene expression.

B. Synthesis of RNAP in animal cells

In contrast to the extensive studies of the regulation of the synthesis of RNAP in *E. coli*, there is relatively little information available on the production of RNAPI, II and III in eukaryotic cells. Studies that focus on changes in the *activity* level of the three polymerases have been reviewed elsewhere (Sentenac 1985). It is unclear whether the experimental data from these studies reflect changes in the synthesis of RNAPs or rather a change in the catalytic properties of pre-existing RNAP. Since this thesis is primarily concerned with the regulation of the synthesis of RNAPII, I shall concentrate on reviewing studies that give information about the regulation of the biosynthesis of this RNAP. Below, I describe the available evidence concerning multi-cellular eukaryotes and the following section concentrates on RNAP synthesis in yeast.

i) Regulation of RNAPII synthesis in mammalian cells

The fungal toxin, α-amanitin, inhibits the activity of RNAPII from mammalian cells. A mutant cell line (Ama102) from rat myoblast cells was selected for its

resistance to α-amanitin. Partially-purified RNAPII from these cells was tested for sensitivity to the inhibitor. Titration of the RNAPII activity with α-amanitin indicated that both sensitive (wild type) and resistant forms of RNAPII were present in the extract. At a concentration of 0.1 μg/mL α-amanitin, there was 30% of the RNAPII activity compared to that detected without the inhibitor (Somers, *et al.* 1975b). This was an unexpected result since Ama102 cells grow at the same rate either in the absence or in the presence of up to 3 μg/mL of α-amanitin. The level of α-amanitin-resistant RNAPII activity from Ama102 cells grown in the presence of α-amanitin for 4 days is three-fold higher than that of Ama102 cells grown without the inhibitor (Somers, *et al.* 1975b). This effect on RNAPII activity is completely reversible and RNAPI levels are unaffected by treatment of the cells with α-amanitin. It was suggested that a mechanism exists in the cell to compensate for the loss of RNAPII activity that results from inactivation by α-amanitin of at least half of the RNAPII molecules (*i.e.*, those carrying the largest subunit expressed from a gene that did not have the mutation that conferred α-amanitin resistance) (Somers, *et al.* 1975b).

This phenomenon was investigated further with resistant cells from a Chinese hamster ovary (CHO) cell line (Guialis, *et al.* 1977). The cell line Ama1 contains RNAPII that is highly resistant to α-amanitin. This cell line was fused to wild-type cells, generating hybrid cell lines that are Ama^r/Ama^s or $Ama^r/Ama^s/Ama^s$. In agreement with the results from the rat myoblast cell lines (Somers, *et al.* 1975b), hybrid cells that grow in the presence of α-amanitin show an increase in the amount of RNAPII activity resistant to the drug but the total amount of RNAPII activity does not change. It was proposed that the amount of the form of RNAPII that was sensitive to α-amanitin was reduced while the amount of the resistant form increased during growth in the presence of α-amanitin. This hypothesis was tested by quantifying the amount of RNAPII present in the cell. Antibodies that bind both the α-amanitin-resistant and sensitive forms of RNAPII were used to immunoprecipitate the enzyme from the bulk of

cellular protein. The total amount of RNAPII (either resistant or sensitive to α-amanitin) remained constant during growth in the presence of the inhibitor (Guialis, *et al.* 1977). This suggested two possible explanations, (i) the increase in activity of α-amanitin-resistant RNAPII is the result of a physical increase in the level of this form of the enzyme matched by a corresponding decrease in the mass of α-amanitin-sensitive RNAPII or (ii) the resistant form of RNAPII is modified such that its specific activity increased. The first model was supported by the observation that the amount of the sensitive form of RNAPII, capable of binding radioactively-labelled γ-amanitin (a toxin related to α-amanitin), decreases with time as hybrid cells are incubated in the presence of the drug (Guialis, *et al.* 1977). This decrease follows the same kinetics as the decrease in α-amanitin-sensitive polymerase activity seen under the same conditions.

A similar set of experiments (Crerar and Pearson 1977) with rat myoblast cells containing α-amanitin-resistant and wild-type forms of RNAPII also supports the following model: in the presence of α-amanitin, wild-type RNAPII is degraded rapidly, whereas the amount of RNAPII that is resistant to α-amanitin increases, either by becoming more stable or through an increase in the rate of synthesis. These results point to an *in vivo* mechanism for regulating levels of RNAPII such that a constant amount of the enzyme is maintained.

The parental cell lines that produced the α-amanitin-resistant rat myoblast cells have either two or four copies of the gene that encodes the largest subunit of RNAPII. Careful measurements with radioactively-labelled γ-amanitin showed that the amounts of RNAPII in these parental cell lines were equal, despite the difference in gene-copy number. This observation supports further the existence of a compensatory mechanism that maintains the amount of RNAPII at a constant level (Crerar, *et al.* 1977).

A more recent study (Nguyen, *et al.* 1996) shows that mammalian cells treated with α-amanitin exhibit a large decrease in the amount of the largest subunit of RNAPII. In contrast, the fifth- and eighth-largest subunits, which are common to all polymerases, are only slightly reduced. The degradation is not dependent on new protein synthesis since the effect is seen in the presence of cycloheximide, an inhibitor of protein synthesis. Furthermore, the degradation is specific to a form of RNAPII capable of binding the inhibitor since RNAPII containing a largest subunit encoded by a gene with a mutation that conferred resistance to α-amanitin is not degraded in the presence of the inhibitor (Nguyen, *et al.* 1996). The signal for degradation does not seem to be a general block of transcription since α-amanitin-promoted degradation is partially suppressed by the addition of actinomycin D, a DNA intercalator that inhibits all transcription. Although binding of α-amanitin directs the degradation of the largest subunit *in vivo*, binding is not sufficient for the effect since, when isolated nuclei were treated with the inhibitor, inhibition of RNAPII activity was seen, but degradation was not (Nguyen, *et al.* 1996). Degradation therefore, is not the mechanism of RNAPII inhibition, but rather is a secondary effect of the inhibition *in vivo*.

Guialis *et al.*, (1979) radioactively labelled the protein of CHO cells containing wild-type and α-amanitin resistant forms of RNAPII. Immunoprecipitation of the enzyme allowed the detection of the two largest subunits of RNAPII as well as five smaller subunits (Guialis, *et al.* 1979). When the cells were incubated in α-amanitin, the amount of radioactively-labelled largest and second-largest subunits decreased, indicating the existence of a mechanism for degrading the drug-sensitive form of the enzyme (Guialis, *et al.* 1979). This fits well with the findings of Nguyen *et al.* (1996) that were mentioned above. In addition, a 20 minute pulse-labelling experiment showed that the rate of synthesis of the largest subunit increases by up to 2.3-fold in $Ama^r/Ama^s/Ama^s$ cells that have been incubated in α-amanitin for 24 hours. An increase is not detected for the second-largest subunit, but is seen for 25 kDa and 20.5

kDa subunits (probable homologues of the yeast subunits, Rpo25p and Rpo27p, respectively). Three other subunits, which are most likely Rpo23p, Rpo26p and Rpo28p homologues, do not show an increase in the rate of synthesis after growth in α-amanitin (Guialis, *et al.* 1979). These papers present compelling evidence for the existence of a feedback mechanism that controls the level of RNAPII in mammalian cells. The mechanism is important for maintaining a constant level of RNAPII and acts at the level of enhanced subunit degradation as well as through an increase in the *de novo* synthesis of some subunits. It is not known if the regulation of this synthesis is at the transcriptional or post-transcriptional level.

Another indication of the regulation of RNAPII synthesis in mammalian cells comes from recent studies of human cells that are resistant to the chemotherapeutic drug, doxorubicin. The gene encoding the human homologue of Rpo211p was identified in a screen for genes whose expression is down-regulated in doxorubicin-resistant human carcinoma cell lines (Fanciulli, *et al.* 1996). The same group showed that the human homologue of Rpo23p also is down-regulated by doxorubicin (Fanciulli, *et al.* 1998). As mentioned previously, these subunits form a heterodimer and behave as the functional homologue of the α dimer of bacterial RNAP. The subunits have identical patterns of expression in various human tissues, showing the greatest expression in heart, muscle and pancreas (Fanciulli, *et al.* 1998). This coordinate regulation is maintained at the transcriptional level, however, it is not known which stage of transcript synthesis or degradation is affected. Unfortunately, these studies do not indicate the effect of doxorubicin on, or the tissue specificity of, the expression of the largest or second-largest subunits of RNAPII. It is unclear, therefore, if the third and eleventh subunits share a specific form of co-regulation, or if all of the RNAPII subunits are expressed in the same manner. Indeed, an independent study indicates that transcripts for human *RPB4*, *RPB5*, *RPB6* and *RPB7* also are expressed

at a higher level in heart and muscle compared to other tissues such as lung, liver and kidney (Khazak, *et al.* 1998, and reference therein).

Although RNAPII subunit genes are expressed at different levels in different tissues, there is little evidence regarding changes in RNAPII amounts within a particular cell type. I have mentioned studies concerning hamster or rat cells grown in the presence of α-amanitin. In those experiments RNAPII that is sensitive to the toxin is degraded while a resistant form of RNAPII is over-produced (see above). The net result is a constant amount of RNAPII in the cell. A recent study suggests that, under certain conditions, the amount of RNAPII in heart cells can increase. Cardiac cells of living mice can be induced to enter a state called hypertrophy which is characterized by an increase in the cell volume and mass that is the result of an increase in cellular protein content. To induce cardiac hypertrophy, the aortas of mice were narrowed by a surgical procedure, resulting in pressure on the heart. The amount of the largest of subunit of RNAPII (both the hypo- and hyper-phosphorylated forms) increased by at least 3-fold relative to loading controls (Abdellatif, *et al.* 1998). The kinase (cdk7) of the general transcription factor TFIIH was also upregulated.

ii) Regulation of RNAPII in *Caenorhabditis elegans*

Strains of *C. elegans* with one, two, or three copies of the gene (*ama-1*) encoding the largest subunit of RNAPII were constructed to test for regulation of the expression of this subunit (Dalley, *et al.* 1993). The activity of RNAPII and the steady-state level of the largest subunit is constant in all three strains. In contrast, the steady-state level of the mRNA of ama-1varies in direct proportion with the gene-copy number (Dalley, *et al.* 1993). These results indicate the existence of a post-transcriptional mechanism that regulates the expression of the largest subunit of RNAPII in *C. elegans*. The authors suggest that the mechanism may act at the level of polypeptide synthesis (rather than protein degradation) since previous studies with rat myoblast

cells (Guialis, *et al.* 1979; see above) indicate that those eukaryotic cells compensate for a reduction in RNAPII activity by increased synthesis of the largest subunit of RNAPII.

iii) Regulation of RNAP synthesis: effects of cellular differentiation and hormones

As mentioned above, the physical amount of RNAPII in cells can be quantified by measuring the amount of radioactively labelled γ-amanitin that is bound to the enzyme. This technique was used to demonstrate that the number of RNAPII molecules decreases by 200-fold during spermatogenesis in salmon sperm cells (Gillam, *et al.* 1979). RNAPII was purified from the brine shrimp, *Artemia salina*, at different stages during the development of larvae. The number of RNAPII molecules per cell decreases by almost 6-fold during a 48 hour period (Bagshaw, *et al.* 1978).

The γ-amanitin- binding technique was also used to demonstrate that the number of RNAPII molecules in cells from rat uteri increased by 40% after the cells were treated with the hormone estradiol for 24 hours (Courvalin, *et al.* 1976). There is a two-fold increase in the number of RNAPII molecules in the liver cells of roosters that are treated with estrogen for 24 hours (Kastern, *et al.* 1981). Soybean seedlings that are treated for 48 hours with auxin, a plant hormone, undergo an increase in cell number (as indicated by an increase in DNA content) but the amount of RNAPI per genome increases 3 to 6-fold. RNAPII levels increase 2-fold during the same treatment. In this experiment, the amount of RNAPs could be measured directly through a purification protocol (Guilfoyle 1980). These experiments demonstrate clearly that the amount of RNAPs in eukaryotic cells is regulated in response to varied conditions such as cellular differentiation or the presence of hormones.

C. Synthesis of RNAP in yeast cells

The nuclear RNAPs of *S. cerevisiae* are the most intensively studied among eukaryotic organisms. For each enzyme, the subunits and their corresponding genes have been defined. However, it is possible that subunits unable to withstand the process of purification have been overlooked. Less is known about the synthesis of these enzymes. As mentioned above, the three forms of RNAP have five common subunits. Although the function of these subunits is not yet understood, it has been suggested that they may serve to coordinate the synthesis of the three enzymes (Woychik, *et al.* 1990). Although coordinate synthesis of the three RNAPs may occur under certain conditions, the levels of RNAPI and II do not seem to be coordinated for yeast growing at different rates. The specific activity of RNAPI increases with increasing growth rate while that of RNAPII is relatively constant (Carter and Dawes 1975; Sebastian, *et al.* 1973). Furthermore, the level of RNAPI is constant throughout the cell cycle while the level of RNAPII peaks at the G2 stage (Carter and Dawes 1975; Sebastian, *et al.* 1974). In these studies, the RNAPs were separated chromatographically using a DEAE Sephadex column. Since only two of the three RNAPs were resolved, and the relative purity of the RNAP in the column fractions is not indicated, the specific activities may be affected by co-purifying proteins. This problem was circumvented by J. M. Buhler, who showed, by immunoprecipitation of individual RNAPs, that the number of RNAPI molecules increases from 14,000 to 27,000 in cells growing with doubling times of 2.5 hours and 1.5 hours, respectively. In contrast, the number of RNAPII molecules remains relatively constant (11,000 and 14,000, respectively; reported in Sentenac and Hall 1982). These data suggest that mechanisms exist to regulate the level of RNAPs in the cell. The nature of the mechanisms is not understood.

One approach to understanding regulatory mechanisms is to begin by identifying DNA elements that are important for the expression of genes that encode

RNAP subunits. The following section describes what is known about DNA elements important for the expression of genes encoding subunits of RNAPs in yeast.

i) Promoter studies of yeast RNAP subunit genes

Studies of yeast genes reveal three main categories of DNA elements that are important for expression. These are the TATA box, which binds the general transcription factor TBP (see above), the initiator element, which includes the template nucleotide coding for the start of the mRNA chain, and upstream elements that may activate (UASs; upstream activation sequences) or repress (operators) transcription (reviewed in Guarente 1987; Guarente 1992; Struhl 1989).

Studies of functional upstream activating sequences of RNAP subunit genes have focused only on three genes: *RPO31*, *RPC40* and *RPO26*. *RPO31* (*RPC160*) encodes the largest subunit of RNAPIII, which is a homologue of the RNAPII subunit, Rpo21p (Allison, *et al.* 1985). *RPC40* encodes a subunit found in both RNAPI and RNAPIII. This subunit, along with Rpc19p, provides the function of the bacterial α RNAP subunit (Thuriaux and Sentenac 1992). *RPO26* encodes a subunit common to all three nuclear RNAPs in yeast (see above).

The DNA sequence of the upstream region of *RPO31* was used as a probe to identify proteins from a yeast extract that form specific DNA-protein complexes (Della Seta, *et al.* 1990b). Electrophoretic mobility-shift assays (EMSA) indicate the formation of three complexes, two of which are due to Abf1p binding (described below in greater detail). The third complex reflects binding by an unidentified protein in the partially purified protein fraction that was used in the experiment. The sites of binding were mapped by DNase I footprinting to two distinct DNA sequences that match the consensus-binding sequence for Abf1p (Della Seta, *et al.* 1990b). The two complexes can be competed by DNA sequences from the upstream region of *RPC40*, indicating the presence of an Abf1p-binding site there as well. Indeed, DNase I footprinting

reveals a single binding site for Abf1p in this sequence. The biological of significance of the complex formed on the *RPC40* promoter was tested by measuring the expression of an *RPC40-lacZ* reporter gene that did or did not contain the binding site. A plasmid that contains 5'-sequences of *RPC40* ending just upstream of the Abf1p-binding site (144 bp upstream of the transcriptional start-site) has 25% of the activity of a similar plasmid that contains 338 bp of upstream sequences. This result suggests that the sequences between -338 and -144 contain DNA elements that are necessary for maximum expression. Deletion of the Abf1p-binding site (to -103) reduces β-galactosidase activity to below a detectable level. Although it is not clear what the limit of detection was for this experiment, the results suggest that the Abf1p site contributes to the normal expression of *RPC40*. A 34 bp oligonucleotide that contains the *RPC40* Abf1p-binding site was inserted upstream of a *CYC1-lacZ* fusion gene that has had the wild-type *CYC1* UAS removed. The Abf1p-binding site causes a 10-fold increase in the expression of this heterologous promoter. Mutation of the sequence that abrogates binding of Abf1p to the site drastically reduces the ability of the DNA sequence to activate the expression of *CYC1-lacZ*. These data suggest that Abf1p can form specific complexes on the upstream regions of two RNAPIII subunit genes and is important for the expression of at least *RPC40* (Della Seta, *et al.* 1990b).

A screen for mutations in *RPO26* that are synthetically-lethal with the *rpo21-4* mutation revealed mutations in the upstream region of *RPO26* (Nouraini, *et al.* 1996b). As the mutations disrupted the sequence of a putative Abf1p-binding site, purified Abf1p and DNA with and without the mutation was used in an EMSA. The mutated sequence is defective in binding Abf1p *in vitro*. Furthermore, the binding site is important for the expression of *RPO26* since the mRNA level of a reporter gene that has the mutated site is only 60% of the wild-type level. Consistent with this result, a strain with the sole copy of *RPO26* mutated at the Abf1p-binding site has a cold-sensitive growth phenotype (Nouraini, *et al.* 1996b). The mutation of the Abf1p site

also causes a shift in the use of transcriptional start sites of *RPO26*, suggesting that Abf1p has a role in start-site selection (Nouraini, *et al.* 1996b).

The experiments described above demonstrate the functional significance of DNA elements in the upstream regions of RNAP subunit genes. In another study, DNA sequences of various RNAP subunit genes were screened for putative activating elements. Putative Abf1p-binding sites are found upstream of *RPO12* (*RPA135*), *RPO32* (*RET1*), *RPC82*, and *RPC19* (Dequard-Chablat, *et al.* 1991). Since *RPC40* and *RPO26* were shown to have Abf1p-binding sites that are important for the expression of these genes, the sites found upstream of these other RNAPI and III subunit genes suggest that Abf1p may have a general role in coordinating the expression of the genes encoding RNAP I and III. Other putative activating elements include sites that may bind the transcription factor Rap1p (Shore 1994). These are found upstream of *RPO11*, *RPA49*, *RPO32* , *RPC53* and *RPC40* (Dequard-Chablat, *et al.* 1991). Finally, a putative activating element with the consensus site TG(C/A)GATGAG was found upstream of *RPO11*, *RPO12*, *RPA49*, *RPO32*, *RPC82*, *RPC53* (two sites), *RPC34*, *RPC31*, *RPC40*, and *RPC19* (Dequard-Chablat, *et al.* 1991). This element is called the PAC box (RNA Polymerase A and C) since, so far, it is found exclusively upstream of genes encoding subunits of RNAP I and III. None of the genes encoding subunits shared by all three RNAPs has an upstream PAC box (Dequard-Chablat, *et al.* 1991). There is no evidence to indicate that the PAC box affects the expression of the genes that have it, nor has a protein that binds the site been identified. For this reason, it is unclear if it has any biological significance. Nevertheless, the fact that it is found exclusively upstream of RNAP I and III subunit genes suggests it may play an important role in coordinating the synthesis of two forms of RNAP, namely, those that are involved in the production of RNA molecules required for the synthesis of protein. In conclusion, Abf1p binding sites upstream of two RNAPIII

subunit genes are important for normal expression of these genes and may play a role in coordinating the expression of RNAP subunits.

ii) Regulation of Rpo24p and Rpo27p

The fourth- (Rpo24p) and seventh- (Rpo27p) largest subunits of RNAPII form a heterodimeric complex *in vitro* and can be dissociated from purified RNAPII by treatment with urea (Edwards, *et al.* 1991). RNAPII that lacks these subunits cannot initiate transcription at promoters but is capable of non-specific polynucleotide-template dependent RNA chain extension (Edwards, *et al.* 1991). Choder (1993) showed that increasing the expression of Rpo24p *in vivo* does not affect the growth of cells in log phase. Expression of *RPO24* on a high-copy plasmid does, however, increase the growth rate of cells that have reached the "slow-growth" phase of yeast that comes after log phase and before stationary phase. These cells have undergone a "diauxic shift", switching from glucose as carbon source (which has been depleted) to ethanol, a poorer carbon source that is the byproduct of fermentative growth. When cells reach the slow-growth phase, the level of *RPO24* mRNA is reduced two-fold. In stationary phase the level is reduced by another factor of two (Choder 1993). Cells which over-express Rpo24p in log phase (up to 25-fold higher than the wild-type level) show a 6-fold decrease in Rpo24p after the diauxic shift. By the time cells reach stationary phase, the level of Rpo24p in this strain is comparable to the level of a strain that has only one copy of *RPO24* (Choder 1993). These data indicate that two mechanisms may control the synthesis of Rpo24p in cells leaving log phase: the level of mRNA is regulated and, even more strikingly, the level of protein is tightly controlled, leading to a Rpo24p level that is 6-fold lower than would be expected from the *RPO24* mRNA available. The regulation is specific since Rpo22p can be over-expressed in stationary phase cells (Choder 1993). Despite the strong regulation of the level of Rpo24p after the diauxic shift, it is important to note that there is virtually no regulation

during log-phase growth. Introduction of the over-expression plasmid results in 24 copies of *RPO24* per cell. The level of *RPO24* mRNA increases by an equal amount, as does the level of protein (Choder 1993). Therefore, potential mechanisms for regulating the level of RNAPII subunits during log phase growth might not regulate all of the subunits.

A screen for genes which, when expressed in high copy, could induce psuedohyphal growth in yeast, revealed the gene encoding Rpo27p (Khazak, *et al.* 1995). Although regulation of the synthesis of Rpo27p has not yet been demonstrated, the finding suggests that the level of Rpo27p must be carefully controlled in order to maintain the growth morphology that is suitable for the conditions in which yeast are growing.

iii) Stability/degradation of RNAP subunits

The level of RNAP in cells is the result, not only of the synthesis of new subunits, but also of the turnover or degradation of pre-existing subunits. In the case of mammalian cells grown in the presence of α-amanitin (see above), RNAPII that is sensitive to the drug is rapidly degraded, suggesting that degradation is a mechanism to regulate the level of RNAPII in cells. There is no direct evidence that degradation is an active regulatory mechanism in the biosynthesis of yeast RNAPs. However, factors that influence the stability of these enzymes have been identified and may be involved in regulatory pathways.

Rsp5p is a ubiquitin-protein ligase that adds ubiquitin peptides to Rpo21p (Huibregtse, *et al.* 1997). Ubiquitination targets proteins for degradation by the 26S proteasome. Rsp5p binds either directly or indirectly to the CTD of Rpo21p and this binding is not affected by the phosphorylation state of this domain. When the level of Rsp5p is reduced by 30-fold *in vivo* (by expressing the gene from the glucose-repressed control sequences of *GAL1*) the level of Rpo21p increases 5-fold

(Huibregtse, *et al.* 1997). Rsp5p interacts with Rpo21p whether present as a free subunit, or a part of RNAPII. The recovery of Rsp5p-containing protein complexes indicates that 10% of Rpo21p in the cell is bound to Rsp5p. Rpo22p is not ubiquinated by Rsp5p (Huibregtse, *et al.* 1997). These data suggest that the level of Rpo21p is affected significantly by the degradation machinery of the cell.

PUP3 encodes a subunit of the yeast proteasome. A mutant allele of *PUP3* was identified as a suppressor of the temperature-sensitive growth phenotype conferred by *rpo26-31*, a mutation in *RPO26* (Nouraini, *et al.* 1997). Suppression by alteration of a proteasome subunit supports a role for the proteasome in setting the level of RNAPs in the cell and fits well with the evidence concerning Rpo21p and Rsp5p. Since Rpo26p is a subunit of all three nuclear RNAPs, proteasome-mediated degradation may also be important in controlling the levels of RNAPI and III.

The *rpo26-31* mutation results in a reduction in the steady-state level of Rpo26p (Nouraini, *et al.* 1996a). The steady-state levels of Rpo11p and Rpo21p are also reduced in this mutant, suggesting that Rpo26p has a role in maintaining the level of subunits of at least RNAPI and II. The altered Rpo26p subunit causes a defect in the assembly of RNAPII suggesting that non-functional sub-assemblies of RNAPII may accumulate in this mutant. These may be targeted for degradation by the proteasome degradation pathway (Nouraini, *et al.* 1996a). Since alteration of Pup3p can partially suppress the growth defect of a *rpo26-31* mutant, it may do so by slowing the degradation of RNAPII sub-assemblies, allowing more time for functional enzyme to assemble (Nouraini, *et al.* 1996a).

The twelfth-largest subunit of RNAPI, encoded by *RPO112* (*RPA12*, *RRN4*) is a homologue of the Rpo29p subunit of RNAPII (Nogi, *et al.* 1993). Like the RNAPII subunit, it is not essential for cell viability. Deletion of the gene does, however, cause a reduction in the steady-state level of Rpo11p (Nogi, *et al.* 1993). Hence, like Rpo26p, Rpo112p has a role in setting the level of RNAPI in the cell.

In conclusion, the degradation of RNAPs in the cell is affected by other proteins, some of them subunits of the RNAPs, whereas others are components of the degradation machinery. Any of these could be part of a mechanism used by the cell to regulate the level of RNAPs under various growth conditions.

IV. Gene-specific DNA-binding transcription factors

In this thesis I describe experiments that suggest two DNA-binding proteins are important for the expression of Rpo21p and Rpo22p. These factors, Abf1p and Reb1p, are members of a family of abundant multi-functional proteins that also includes Rap1p (Shore 1994). In this section I shall give a description of these proteins.

A. Abf1p

Abf1p has been identified numerous times due to its interaction with many DNA elements in the chromosomes of yeast. The name of the gene, *ABF1*, signifies one class of these interactions, namely, ARS binding factor (Diffley and Stillman 1988). ARS (autonomously replicating sequence) elements are the sites on the chromosome where DNA replication is initiated (Toyn, *et al.* 1995). Binding of Abf1p is important for the function of some ARSs (Toyn, *et al.* 1995). Abf1p also binds one of three elements that are important for the function of a transcriptional silencer (*HMR*). Silencers suppress the transcription of genes found at the silent mating loci on chromosome III (Shore, *et al.* 1987).

Abf1p was purified on the basis of its ability to bind to *ARS1* (Diffley and Stillman 1988). The gene encoding Abf1p was identified and shown to be essential for growth in yeast (Diffley and Stillman 1989; Rhode, *et al.* 1989). The predicted molecular weight of Abf1p (82 kDa) is considerably less than 135 kDa, the size estimated from its migration during SDS-PAGE (Diffley and Stillman 1988). The N-

terminal region contains a zinc-finger motif that is often found in the DNA-binding domain of transcription factors (Johnson and McKnight 1989). Removal of zinc from purified Abf1p makes the protein unable to bind DNA *in vitro* (Diffley and Stillman 1989). The consensus of DNA elements that bind Abf1p is 5' RTCRYNNNNNACG 3' (Della Seta, *et al.* 1990b; Halfter, *et al.* 1989). An Abf1p-binding site, upstream of *SPT15*, has a variation in the third residue of the consensus (C3 to A), yet binds Abf1p as well as sites that match the consensus. 5' sequences that were not detected as part of the consensus likely increase the binding of Abf1p to this variant binding site (Schroeder and Weil 1998a).

Rap1p (Shore 1994) has significant sequence identity with Abf1p, although it has a different kind of DNA-binding domain (not a zinc-finger) and binds to an entirely different kind of DNA element. The C-terminal regions of Abf1p and Rap1p may be interchanged and still support growth of yeast cells, although replacement of the C-terminus of Rap1p with that of Abf1p results in very poor growth (Gonçalves, *et al.* 1996). Purified Abf1p induces a 120° bend in DNA upon binding to its recognition site (McBroom and Sadowski 1994).

DNA elements that bind Abf1p *in vitro* also activate the transcription of genes. Although relatively poor activators on their own, Abf1p-binding sites can act in concert with adjacent T-rich regions to achieve a high level of gene activation (Buchman and Kornberg 1990; Gonçalves, *et al.* 1995). Abf1p-binding sites are important for the expression of the meiosis-specific genes, *HOP1* and *SMK1*. Similar sites are likely involved in the expression of other meiosis-specific genes (Gailus-Durner, *et al.* 1996; Pierce, *et al.* 1998). The expression of *COX6* is repressed by glucose and activated by non-fermentable carbon sources; an Abf1p-binding site is important for this regulation (Trawick, *et al.* 1992). The phosphorylation of Abf1p is affected by the carbon source of the growth medium, suggesting that post-translational modifications influence the role of Abf1p in transcriptional activation (Silve, *et al.* 1992). There are 137 genes that

encode subunits of the ribosome. Most of these genes contain one or more binding sites for Rap1p, however, 12 that do not, contain Abf1p-binding sites (Planta and Mager 1998). These sites are likely to be significant since the Abf1p-binding sites upstream of r-protein genes *RPS28A* (Gonçalves, *et al.* 1995), *RPL3* (Hamil, *et al.* 1988) and *RPL4A* (Della Seta, *et al.* 1990a) are important for the expression of these genes. Abf1p and Rap1p may, therefore, help coordinate the expression of r-protein genes.

Abf1p also has a role in the expression of genes encoding subunits of RNAPs (see above; this thesis). This, and recent evidence for the importance of Abf1p for the expression of *SPT15*, encoding the TATA-box binding protein (Schroeder and Weil 1998a), point to a mechanism for coordinating the expression of genes that are important for the synthesis of RNA and protein. Abf1p may also be important for the expression of genes transcribed by RNAPI. Mutation of a weak Abf1p-binding site in the rRNA gene enhancer causes at least a 5-fold decrease in activation (Kang, *et al.* 1995). Others, using a different system, showed that the site may have a role in rRNA transcription, but it is redundant with other elements in the enhancer (Morrow, *et al.* 1993a). Mutation of the Abf1p-binding site in the upstream region of *QCR8* alters the sensitivity of this DNA to nuclease digestion, suggesting that Abf1p promotes a disruption of the nucleosome pattern (De Winde, *et al.* 1993). Therefore, transcriptional activation by Abf1p may involve counteracting the repressing effect of nucleosomes.

Genetic experiments suggest that Abf1p, and not an unidentified protein with similar DNA-binding properties, is the protein involved in the function of Abf1p-binding sites. Mutations in *ABF1* result in defects in DNA synthesis (Rhode, *et al.* 1992), in transcriptional activation (Rhode, *et al.* 1992; Schroeder and Weil 1998b), and in silencing of *HMR* (Loo, *et al.* 1995). Fusion of the C-terminal domain of Abf1p to a

heterologous DNA-binding domain allows activation of *ARS1* activity if the binding site for the fusion protein is included in the *ARS1* (Li, *et al.* 1998).

B. Reb1p

Reb1p (rRNA enhancer binding protein) was identified as a protein that binds to the enhancers that are located between tandem repeats of rDNA genes (Morrow, *et al.* 1989). A second protein, called Reb2p, also binds the enhancer and is identical to Abf1p (see above). Reb1p was also identified as Y, a protein that binds to the UAS of the divergent genes, *GAL1* and *GAL10* (Fedor, *et al.* 1988). Reb1p is encoded by a gene (*REB1*) that is essential for yeast growth and has a predicted molecular weight of 92 kDa (Ju, *et al.* 1990). Like Abf1p, the protein runs on SDS-PAGE as a considerably larger protein (125 kDa) (Ju, *et al.* 1990). The polypeptide sequence does not show significant similarity to that of other proteins except for the C-terminal DNA-binding domain, which has some resemblance to the DNA-binding domains of the oncogene Myb and the yeast transcription factor Bas1p. This class of DNA-binding domain has a characteristic repeat of tryptophan residues (Morrow, *et al.* 1993b).

Liaw *et al.* (1994) screened double-stranded oligonucleotides containing 18 random nucleotides for those that could be retained on an affinity column bearing a GST-Reb1p fusion protein. The core consensus obtained from 35 of the retained oligos was 5' YYACCCG 3' (Liaw and Brandl 1994). This sequence matches closely the consensus-binding sequence (5' YNNYYACCCG 3') that had been derived from Reb1p binding sites in *CEN4* and upstream of *GAL1* and *PYK* (Chasman, *et al.* 1990). Binding sites for Reb1p are also found upstream of *TRP1*, *RAP1*, and *ACT1*, as well as in sequences that are adjacent to telomeres (Chasman, *et al.* 1990).

A Reb1p-binding site in the rDNA enhancer and one immediately upstream of the 35S rRNA promoter are separated by a 2 kb spacer that includes the RNAPIII-transcribed 5S RNA gene. These Reb1p-binding sites act in concert to stimulate rDNA

transcription (Kulkens, *et al.* 1992). It has been proposed that the separate Reb1p binding sites are brought together and attached to the nucleolar matrix, making separate loops for DNA containing the RNAPI and III transcribed regions. This protein-DNA structure may facilitate the recycling of RNAPI, after termination, directly onto the promoter of the adjacent rDNA gene (Kulkens, *et al.* 1992). The Reb1p binding site in the rDNA enhancer is important for the termination of upstream rRNA transcripts. This binding site, in concert with other elements, causes RNAPI to pause and terminate transcription at a site 17 nucleotides upstream of the Reb1p-binding site (Lang and Reeder 1993).

Reb1p-binding sites are also important for the expression of genes transcribed by RNAPII. Similar to Abf1p-binding sites, Reb1p-binding sites are able to activate weakly the expression of a reporter gene that has no other enhancer elements. In combination with a T-rich region, however, a Reb1p-binding site strongly enhances promoter activity (Chasman, *et al.* 1990). Reb1p-binding sites are important for the expression of *ACT1* (McLean, *et al.* 1995) and the derepression of *ILV1* (Remacle and Holmberg 1992). In concert with binding sites for Rap1p and Gcr1p, a Reb1p-binding site activates the high level of expression of the glycolytic gene, *TPI1* (Scott and Baker 1993). When interposed between the UAS and promoter of *CYC1*, a Reb1p-binding site reduces gene expression (Wang, *et al.* 1990). Deletion of *SIN3*, a gene that has positive and negative effects on the expression of various genes, results in approximately a 5-fold reduction in Reb1p-binding activity if cells are grown in galactose medium (Wang, *et al.* 1990). The reduction in binding activity does not, however, effect the stimulatory or repressing activity of Reb1p-binding sites (Wang, *et al.* 1990). Reb1p is autoregulated. Three binding sites for Reb1p in the *REB1* gene act as positive and negative elements for *REB1* expression (Wang and Warner 1998).

The role of Reb1p in transcriptional activation or repression of RNAPII-transcribed genes is not clear. The protein may influence the positioning of

nucleosomes. The Reb1p-binding site of the *GAL1* UAS, introduced into yeast on a minichromosome, creates a nucleosome-free region of 160 bp. This region is flanked by ordered nucleosomes (Fedor, *et al.* 1988). Reb1p may, therefore, have a role in counteracting the repressive activity of nucleosomes by i) freeing portions of an upstream region from nucleosomes, allowing other DNA-binding factors to have access to the region, and/or ii) determining the location of flanking nucleosomes, such that other important DNA elements are made accessible to transcription factors.

V. Thesis rationale and outline

In this introduction I have described investigations of the regulation of RNAP synthesis in organisms ranging from *E. coli* to humans. The evidence for the existence of specific regulatory pathways is strong. There is, however, little information concerning the mechanisms through which these pathways operate.

Understanding the regulatory mechanisms for RNAP synthesis is important for a number of reasons. RNAPII (the focus of this thesis) is essential for the first step of eukaryotic gene expression, *i.e.,* transcription. A multitude of signaling pathways converge upon this enzyme, determining the timing and rate of gene expression. It stands to reason that the level of RNAPII activity is important for the proper expression of genes. One way to regulate this activity is by controlling the physical amount of RNAPII in the cell. Hence, if we wish to understand the mechanisms of gene expression, we ought to know about the biosynthesis of its key component, RNAPII. Three other key cellular processes are DNA synthesis, pre-mRNA processing (*e.g.,* splicing) and protein synthesis. Growth of cells is altered dramatically during the development of organisms or simply by changes in the cellular environment. Understanding how the cells respond to these changes includes a knowledge of how key components of the biosynthetic machinery work in unison. It is likely that

mechanisms exist to coordinate these activities, and biosynthesis of the machinery itself is an obvious place to look for such coordination. Hence an investigation of the biosynthesis of RNAPII may provide insight into global cellular regulatory schemes. Finally, RNAPII is a multi-subunit enzyme. The assembly of functional RNAPII requires the availability of the various subunits in appropriate concentrations. Over- or under-production of a subset of subunits would lead to the wasteful production of incomplete enzyme. Understanding the coordination of the synthesis of RNAPII subunits may give us insight into the coordination of the synthesis of other multi-component enzymes, and indeed, the coordination of different enzymatic processes.

For these reasons I have chosen to investigate the biosynthesis of RNAPII. This thesis begins with a study of the significance of maintaining a normal level of RNAPII in the cell by the characterization of the effect of underproducing Rpo21p, the largest subunit (CHAPTER 2). This study was a collaborative effort between myself and Jacques Archambault. In CHAPTER 3, I describe my characterization of the regulatory elements of the genes encoding the two largest subunits of RNAPII. CHAPTER 4 describes my investigation of potential feedback mechanisms acting on the expression of RNAPII subunit genes (and other genes). This was done by testing the expression of various reporter plasmids in a strain that underproduces Rpo21p or in a strain in which Fcp1p, a protein that is important for the normal activity of the RNAPII holoenzyme, can be depleted. This thesis concludes with a discussion of my results and suggestions of how this work may be continued (CHAPTER 5).

CHAPTER 2

Underproduction of the Largest Subunit of RNA Polymerase II causes Temperature Sensitivity, Slow Growth, and Inositol Auxotrophy in *Saccharomyces cerevisiae*.

Attribution of contributions:

This chapter is a modified version of an article, with the same title, published in 1996 in *Genetics* **142**: 737-747. Jacques Archambault and I are co-first authors of that paper. James Friesen is the other author. Jacques Archambault wrote the first draft of the article. I contributed the construction of *lacZ*-fusion genes diagrammed in Figure 2.1. Dr. Archambault constructed the other plasmids in that figure. I constructed the yeast strains, YF1971 and YF1733. I planned and carried out the experiments shown in Figure 2.2, Figure 2.3, Figure 2.4 and Figure 2.6. I prepared the cultures for the experiment shown in Figure 2.5. Dr. Archambault prepared yeast extracts and carried out the protein blot experiment shown in Figure 2.5.

ABSTRACT

In the yeast *Saccharomyces cerevisiae* mutations in genes encoding subunits of RNA polymerase II (RNAPII) often give rise to a set of pleiotropic phenotypes that includes temperature-sensitivity, slow growth and inositol auxotrophy. In this study, we show that these phenotypes can be brought about by a reduction in the intracellular concentration of RNAPII. Underproduction of RNAPII was achieved by expressing the gene (*RPO21*), encoding the largest subunit of the enzyme, from the *LEU2* promoter or a weaker derivative of it. These promoters can be repressed by the addition of leucine to the growth medium. We found that cells that underproduced Rpo21p were unable to derepress fully the expression of a reporter gene under the control of the *INO1* UAS. Our results indicate that the phenotypes of temperature-sensitivity, slow growth and inositol auxotrophy can all be caused by reducing the steady-state amount of RNAPII. These results also lead to the prediction that some of the previously-identified RNAPII mutations which confer this same set of phenotypes affect the assembly and/or stability of the enzyme. We propose a model to explain the hypersensitivity of *INO1* transcription to mutations that affect components of the RNAPII transcriptional machinery.

INTRODUCTION

RNA polymerase II (RNAPII), the enzyme responsible for the transcription of all protein-encoding genes in eukaryotes, is a multisubunit enzyme whose structure has been conserved during evolution (reviewed in Sawadogo and Sentenac 1990; Young 1991; Archambault and Friesen 1993). In the yeast *Saccharomyces cerevisiae*, RNAPII is made up of two large subunits that are similar to the two large subunits, β' and β, of *E.*

coli RNAP, as well as 10 smaller subunits, five of which also are components of RNAPI and RNAPIII.

Mutational studies aimed at identifying functional regions of RNAPII have yielded two broad categories of mutations thus far (reviewed in Archambault and Friesen 1993). One category comprises mutations that affect the assembly of the enzyme and/or its stability (Himmelfarb *et al.* 1987; Archambault *et al.* 1990; Kolodziej and Young, 1991). These mutations could affect either the stability of individual subunits or their ability to engage in critical protein-protein interactions required for the proper assembly of the enzyme or for the maintenance of its structure once assembled. The other category comprises mutations that affect specific aspects of the transcription cycle. Mutations have been isolated that affect the positioning of the transcription start-site (Hekmatpanah and Young 1991; Berroteran *et al.* 1994; Furter-Graves *et al.* 1994; Hull *et al.* 1995), response to transactivator proteins (Arndt *et al.* 1989; Allison and Ingles 1989; Scafe *et al.* 1990a) or the ability to interact with regulatory factors such as elongation factor TFIIS (Archambault *et al.* 1992b).

A major limitation of the mutational approach is that often it is difficult to determine whether the phenotypes imposed by a given mutation are a consequence of an assembly/stability defect, a transcription defect *per se*, or a combination of both. This is exemplified by the observation that inositol auxotrophy is often associated with mutations affecting RNAPII, regardless of the type of selection scheme that is used to isolate them. Mutations located throughout the genes encoding the two largest subunits (Arndt *et al.* 1989; Nonet and Young 1989; Scafe *et al.* 1990a; 1990b; 1990c; Archambault *et al.* 1992a; Berroteran *et al.* 1994), the sixth-largest subunit (S. Nouraini, J. Archambault and J. D. Friesen; unpublished data), or deletion of the gene encoding the fourth-largest subunit of RNAPII (Woychik and Young 1989) give rise to inositol auxotrophy. It was first recognized by Arndt *et al.* (1989) that the inositol auxotrophy can be explained by the finding that the gene *INO1*, encoding inositol-1-

phosphate synthase, is not derepressed in mutant-polymerase cells starved for inositol. This is in contrast to many other genes, which are transcribed at normal levels in these mutant cells.

Why do so many mutations that occur in many different regions of the enzyme affect transcription of *INO1* and what is the mechanism underlying this phenotype? Archambault *et al.* (1992) argued previously that a reduction, by any means, of the amount of assembled RNAPII would result in an Ino$^-$ phenotype (Archambault *et al.* 1992a). This suggestion was based largely on the observation that the Ino$^-$ and temperature-sensitive (ts) phenotypes imposed by a mutation, *rpo21-4*, in the gene encoding the largest subunit of RNAPII, could be suppressed by overproducing the sixth-largest subunit of the enzyme (Archambault *et al.* 1990; 1992a). This result and others (Archambault *et al.* 1990; 1992a) are consistent with the hypothesis that more RNAPII can be assembled in the suppressed strain than in the mutant strain and therefore that the Ino$^-$ and ts phenotypes are due to a decrease in the level of assembled RNAPII in the mutant strain. However, in these studies, as in other mutational studies, it is difficult to determine whether the mutation that affects the largest subunit has an effect only on the assembly/stability of the enzyme and not on transcription *per se*.

In the present study we sought to gain more direct evidence in support of the suggestion that a decrease in the level of assembled RNAPII is sufficient to confer inositol auxotrophy and temperature-sensitivity. We constructed yeast strains in which the assembly of RNAPII was reduced by limiting the availability of one of its subunits, in this case the largest one. We found that cells that underproduced the largest subunit of RNAPII were inositol auxotrophs because they failed fully to induce transcription of *INO1* when starved for inositol. In addition, we found that growth of these cells was slow at temperatures ranging from 23° to 30° and was inhibited at high temperature. Our results indicate that a reduction in the steady-state amount of RNAPII

is sufficient to cause temperature-sensitivity, slow growth and inositol auxotrophy. This suggests that some of the previously-identified mutations affecting RNAPII, which conferred this set of phenotypes, affect the assembly/stability of the enzyme. We propose a model to explain why transcription of *INO1* is particularly sensitive to mutations affecting the RNAPII transcriptional machinery.

MATERIALS AND METHODS

Strains and growth media: *Saccharomyces cerevisiae* strains used in this study are listed in Table 2.1. Growth media and yeast manipulation were as described previously (Sherman *et al.* 1986). Yeast transformation was performed essentially as described (Ito *et al.* 1983). Repression of the *LEU2* promoter was accomplished by growing cells in medium containing leucine, isoleucine and threonine at the indicated concentrations.

Plasmids: All DNA manipulations were performed essentially as described (Maniatis *et al.* 1982). Plasmid DJ20 contains a 7.0 kb *Hin*dIII DNA fragment encompassing *RPO21* cloned into the *Hin*dIII site of pFL39 (*TRP1 CEN6 ARS*; Bonneaud *et al.* 1991). Plasmid JA469 that carries the *pLEU2-RPO21* allele contains a 400 bp fragment encompassing the *LEU2* promoter and its 5'-untranslated region (5'-UT) cloned upstream of the *RPO21* ORF. For construction of this plasmid, the *LEU2* promoter and 5'-UT were amplified by the polymerase chain reaction (PCR) using plasmid JJ250 (Jones and Prakash 1990) as a template and two oligonucleotides. The downstream oligonucleotide was mutagenic in such a way as to create an *Nco*1 restriction site (5'-CC**ATG**G-3') at the translation-initiation ATG of *LEU2*. The amplified fragment was digested with *Eco*R1 and *Nco*1, which cut the PCR fragment immediately upstream of the *LEU2* promoter (position -400) and at the initiator ATG, respectively; this fragment

Table 2.1. *Saccharomyces cerevisiae* strains used in this study.

Strain[a]	Relevant genotype
W303-1a	*MAT*a *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*
W303-1b	*MAT*α *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*
AJ1	W303-1a with [YEp13; *LEU2*]
YF1733	W303-1b with *rpo21::ADE2* [pJAY47; *pGAL-RPO21 URA3*]
YF1960	YF1733 with *LEU2*
AJ2	YF1733 with [YEp13; *LEU2*]
YF1971	W303-1b with *pLEU2-RPO21 LEU2*
YF2122	W303-1b with *LEU2* [pFL39; *TRP1*]

[a] Strain W303-1a and W303-1b were obtained from R. Rothstein.

was cloned between the *Eco*R1 and *Nco*1 sites of plasmid JAY432 so as to replace the *GAL10* promoter fragment of this plasmid with the *LEU2* promoter. In order to construct plasmid JAY432, a 5.4 kb *Nco*1-*Hin*dIII *RPO21* fragment (from nucleotide +1 to 5415; in this numbering system the "A" residue of the translation-initiation ATG is at position +1) containing the entire *RPO21* ORF, which had been mutated previously so as to contain an *Nco*1 site at the initiator ATG (Archambault *et al.* 1992a), was fused downstream of the *GAL10* promoter (carried on a *Eco*R1-*Nco*1 fragment; Archambault *et al.* 1992a). The resulting *GAL10-RPO21* fragment (*Eco*R1-*Hin*dIII) was inserted between the *Eco*R1 and *Hin*dIII sites of pFL39. Plasmid JA443, which carries the *pLEU2Δ-RPO21* allele, was constructed by digestion of plasmid JA469 (*pLEU2-RPO21*) with *Ssp*1 and *Nco*1, blunting of the ends with the Klenow fragment of DNA polymerase I, followed by religation. In this process the DNA sequences located between the *Ssp*1 site in the promoter and the *Nco*1 site in the ATG of *pLEU2-RPO21*

were deleted. Versions of the plasmids carrying *pLEU2-RPO21* and *pLEU2Δ-RPO21*, respectively, were constructed that lack the translation-initiation codon of *RPO21* by digestion of these plasmids with *Nco*1 (which cleaves at the ATG) and treatment with S1 nuclease followed by religation. Plasmid JAY47 carries the same *pGAL-RPO21* fragment as plasmid JAY59 in addition to a 1.6 kb *Hin*dIII-*Eco*R1 fragment downstream of *RPO21* cloned into the *Eco*R1 site of plasmid INT2 (*URA3 CEN1 ARS1*; Percival-Smith and Segall 1986). Assays for promoter strength used plasmids based on pFL39, which were constructed so as to fuse either the promoters of *LEU2*, *LEU2Δ* or *RPO21* along with the first 662 nucleotides of the *RPO21* ORF in frame to the *lacZ* gene of *E. coli*. These fusion genes were constructed by replacing in pDJ20 (*RPO21*), pJA469 (*pLEU2-RPO21*) and pJA443 (*pLEU2Δ-RPO21*) the *Spe*1-*Hin*dIII fragment that encodes the C-terminal part of the *RPO21* ORF and 3'-UT by a *Xba*1-*Stu*1 fragment (a *Hin*dIII linker was attached to the *Stu*1 end) encoding *lacZ* from YEp356R (Myers *et al.* 1986).

Transfer of the *pLEU2-RPO21* allele to the endogenous *RPO21* chromosomal location was accomplished by creating plasmid DJ58 that carries a *RPO21* fragment (from -1583 to +5415) in which sequences between - 722 to +1 were replaced by the *LEU2* promoter and 5'-UT. pDJ58 was constructed by inserting a *Hin*dIII-*Bst*E2 *RPO21* fragment (from -1583 to -722) upstream of the *LEU2* promoter in plasmid JA469 (*pLEU2-RPO21*).

Transfer of the *pLEU2-RPO21* allele to the chromosomal *RPO21* locus: A *Hin*dIII-*Bgl*II fragment from pDJ58 containing the *RPO21* upstream sequences (-1583 to -722), the *LEU2* promoter and the beginning of the *RPO21* ORF (+1 to +3408) was introduced into yeast strain YF1960 (*MATα rpo21::ADE2 LEU2* [pJAY47; *pGAL-RPO21*]). YF1960 was constructed in three steps. First, strain W303-1b was transformed with a single-copy episomal plasmid (pJAY47; *pGAL-RPO21 URA3*) in

which *RPO21* is expressed from the *GAL10* promoter. Second, the chromosomal copy of *RPO21* was disrupted by the insertion of *ADE2* between the *Bst*E2 (-722) and *Spe*1 (+662) sites of *RPO21* to create strain YF1733. Growth of this strain is maintained only on medium containing galactose since the maintenance plasmid expresses *RPO21* from the *GAL10* promoter. Third, strain YF1733 was made prototrophic for leucine by transformation with a *LEU2* fragment and selection for Leu^+ transformants in order to create strain YF1960. Following introduction of the *pLEU2-RPO21* fragment into strain YF1960, Ade^- transformants (pink colored) that were able to grow on glucose medium lacking leucine were selected in order to select for transformants in which the chromosomal *RPO21* allele was replaced by the *pLEU2-RPO21* allele. Replacement of *RPO21* by *pLEU2-RPO21* was confirmed by PCR. One transformant carrying the *pLEU2-RPO21* allele in the chromosome was then streaked on medium that lacked leucine and contained uracil in order to screen for cells that had lost the maintenance plasmid (pJAY47). One such Ura^- clone (YF1971, *MATα pLEU2-RPO21 LEU2*) was used in this study.

β-galactosidase assays: β-galactosidase activity was measured as described by Miller (1972). Cells were grown in selective medium with the indicated concentration of leucine, isoleucine and threonine. For each measurement, β-galactosidase activity was determined on three independent cultures.

Immunoblot (western blot) analysis: Whole-cell extracts were prepared from cultures grown to an optical density (600 nm) of 0.5 essentially as described previously (Tyers *et al.* 1992). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to immunoblot analysis essentially as described (Harlow and Lane 1989). The presence of the largest subunit of RNAPII (Rpo21p) was detected using the monoclonal antibody (MAb) 8WG16

directed against the Rpo21 polypeptide (Thompson *et al.* 1989). As a loading control, the same blot was exposed to a rabbit polyclonal antiserum directed against the yeast BiP protein (kindly provided by Dr. D. Williams, University of Toronto). The secondary antibody was peroxidase-conjugated goat anti-mouse when using mAb 8WG16, or anti-rabbit when using anti-BiP antiserum. Detection was by the enhanced chemiluminescence method as described by the manufacturer (Amersham). Efforts to quantify the amount of Rpo21 polypeptide present in *pLEU2-RPO21* cells grown in the presence of leucine relative to the level detected in wild-type cells were made by immunoblotting serial dilutions of whole-cell extracts and comparing multiple exposures.

RESULTS

Expression of the largest subunit of RNAPII from the *LEU2* promoter: In order to test the hypothesis that a reduction in the level of RNAPII could confer temperature-sensitivity, slow growth and inositol auxotrophy, we set out to construct yeast strains in which the level of expression of the largest subunit of RNAPII (the *RPO21* gene product) would be limiting for enzyme assembly. This could be accomplished by fusing the ORF and downstream sequences of *RPO21* to a heterologous promoter of lower strength than that of *RPO21*. We first tried the promoter of *LEU2*, a gene involved in leucine biosynthesis (Andreadis *et al.* 1984), because it has been characterized extensively and its activity can be repressed by the addition of leucine to the growth medium (Andreadis *et al.* 1984; Tu and Casadaban 1990). Maximal repression of the *LEU2* promoter can be achieved by supplementing the growth medium with isoleucine and threonine in addition to leucine (Tu and Casadaban 1990). This regulation of *LEU2* transcription is mediated through a well-defined upstream activating sequence (UAS) that binds the *LEU3* gene product

(Friden and Schimmel 1988; Tu and Casadaban, 1990). A series of gene fusions was constructed (Figure 2.1). We first fused the promoter and 5'-untranslated sequence of *LEU2* upstream of the *RPO21* ORF (see Materials and Methods); we named this allele *pLEU2-RPO21*. A second allele, similar to *pLEU2-RPO21* but lacking sequences located between the *LEU2* UAS and translation-initiation codon, was also created (see Materials and Methods). This allele, which we named *pLEU2Δ-RPO21*, lacks a DNA element required for maximal expression of *LEU2* but not for its regulation by *LEU3* (Tu and Casadaban, 1990). It was shown previously that a *LEU2* promoter that lacks this element is weaker than the wild-type *LEU2* promoter but is still regulated by leucine (Tu and Casadaban 1990).

Strength of the pLEU2 and pLEU2Δ promoters: The strength of the pLEU2 and pLEU2Δ promoters relative to that of *RPO21* were compared by fusing these three promoters, along with their 5'-untranslated regions and the first 662 nucleotides of the *RPO21* ORF, in frame to the *lacZ* gene of *E. coli*. These fusion genes were introduced into yeast strain AJ1 (W303-1a [YEp13, *LEU2*]) and the amount of β-galactosidase activity was measured (Figure 2.2). In medium lacking leucine, the condition under which activity of the *LEU2* promoter is maximal, the *LEU2* promoter directed expression of 13.4 units of β-galactosidase, a level of activity similar to that measured in strains that express *lacZ* from the *RPO21* promoter. However, in contrast to the expression of *RPO21-lacZ*, that of *pLEU2-lacZ* was repressed about 40-fold by the addition of leucine, isoleucine and threonine to the growth medium. The magnitude of this regulation is similar to that reported previously for *LEU2* (Tu and Casadaban 1990). As expected, pLEU2Δ directed the expression of lower levels of β-galactosidase activity in medium lacking leucine but still retained the ability to be repressed by leucine. From these results, we anticipated that the transcription of

Figure 2.1. Structure of the fusion genes used in this study. The top line represents the structure of the *LEU2* gene, which comprises the promoter and 5'-untranslated region (5'-UT) from nucleotide -400 to +1 (+1 being the position of the first nucleotide of the initiator ATG) fused to the *LEU2* open-reading-frame (ORF). The two arrows indicate the position of the transcription start-sites mapped previously (Andreadis *et al.* 1984). The two black boxes indicate the location of the UAS and a TATA element that are required for maximal expression of *LEU2* (Tu and Casadaban 1990). The next four lines represent the structure of the fusion genes in which the *RPO21* ORF was fused either to the intact *LEU2* promoter and its 5'-UT (plasmid JA469) or to a deletion-derivative that lacks the "TATA" element because of a deletion of nucleotides -120 to -1 (plasmid JA443). "ΔATG" indicates alleles in which the initiator ATG was deleted. The sixth line represent the structure of the *RPO21* gene which comprises the promoter and 5'-UT from position -1583 to +1 (hatched box) fused to the *RPO21* ORF (plasmid DJ20). The seventh, eighth and ninth lines represent the structure of the *lacZ* fusions that were derived from *pLEU2-RPO21*, *pLEU2Δ-RPO21* and *RPO21*, respectively.

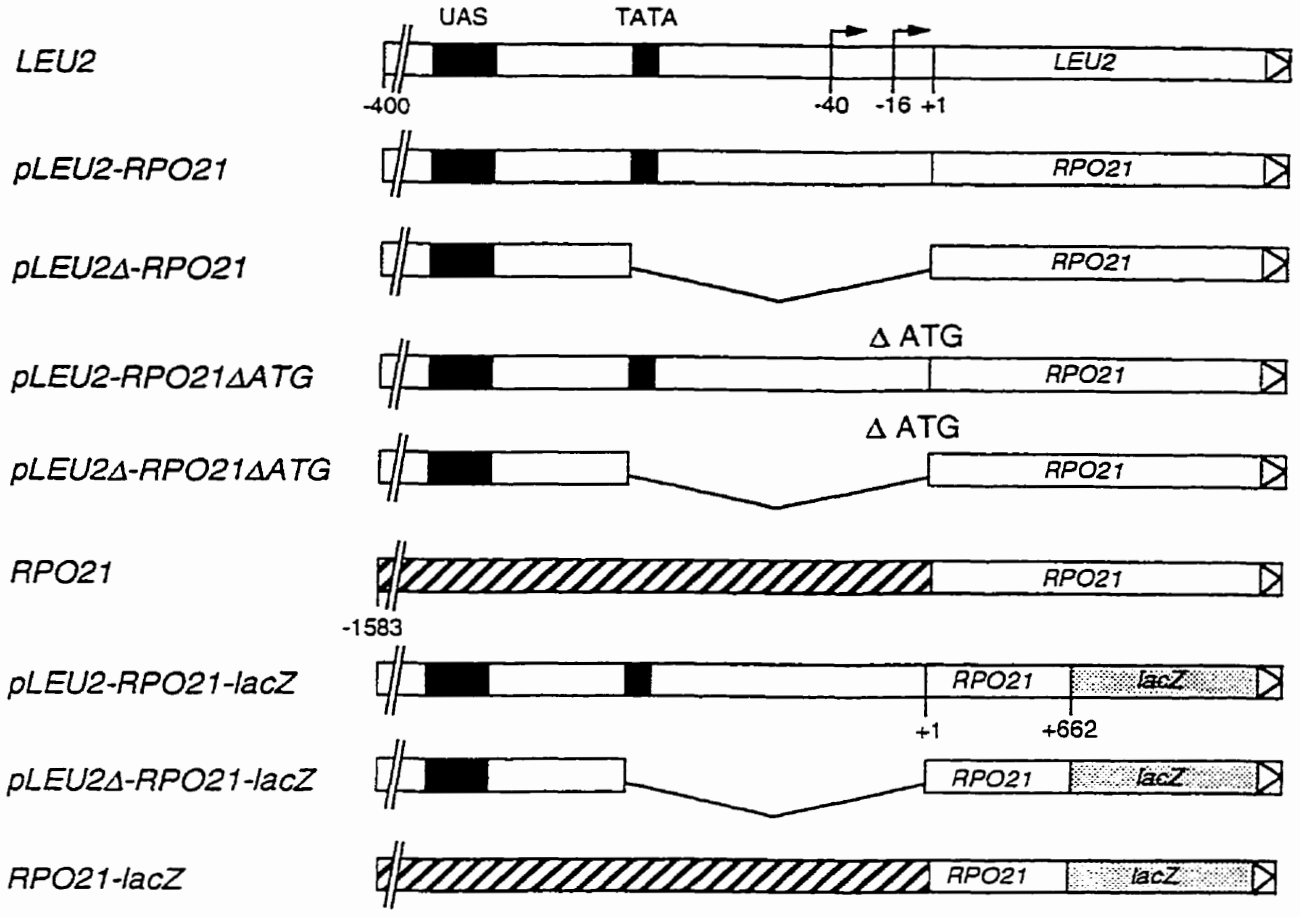
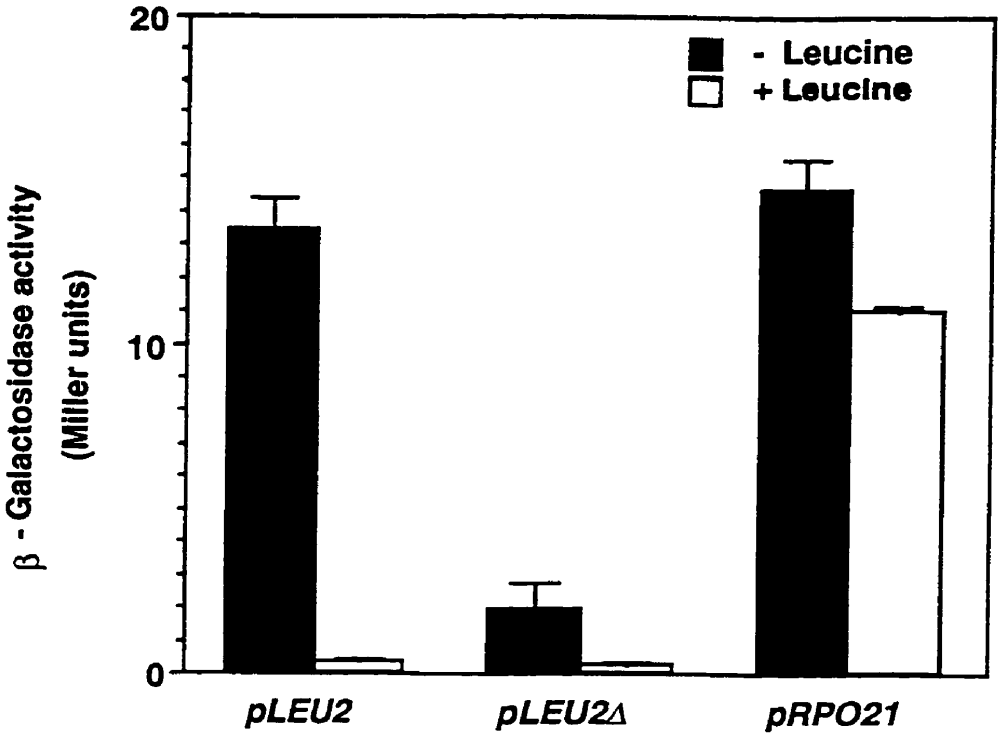

UAS
TATA
LEU2
LEU2
-400
-40
-16
+1
pLEU2-RPO21
RPO21
pLEU2Δ-RPO21
RPO21
Δ ATG
pLEU2-RPO21ΔATG
RPO21
Δ ATG
pLEU2Δ-RPO21ΔATG
RPO21
RPO21
RPO21
-1583
pLEU2-RPO21-lacZ
RPO21
lacZ
+1
+662
pLEU2Δ-RPO21-lacZ
RPO21
lacZ
RPO21-lacZ
RPO21
lacZ

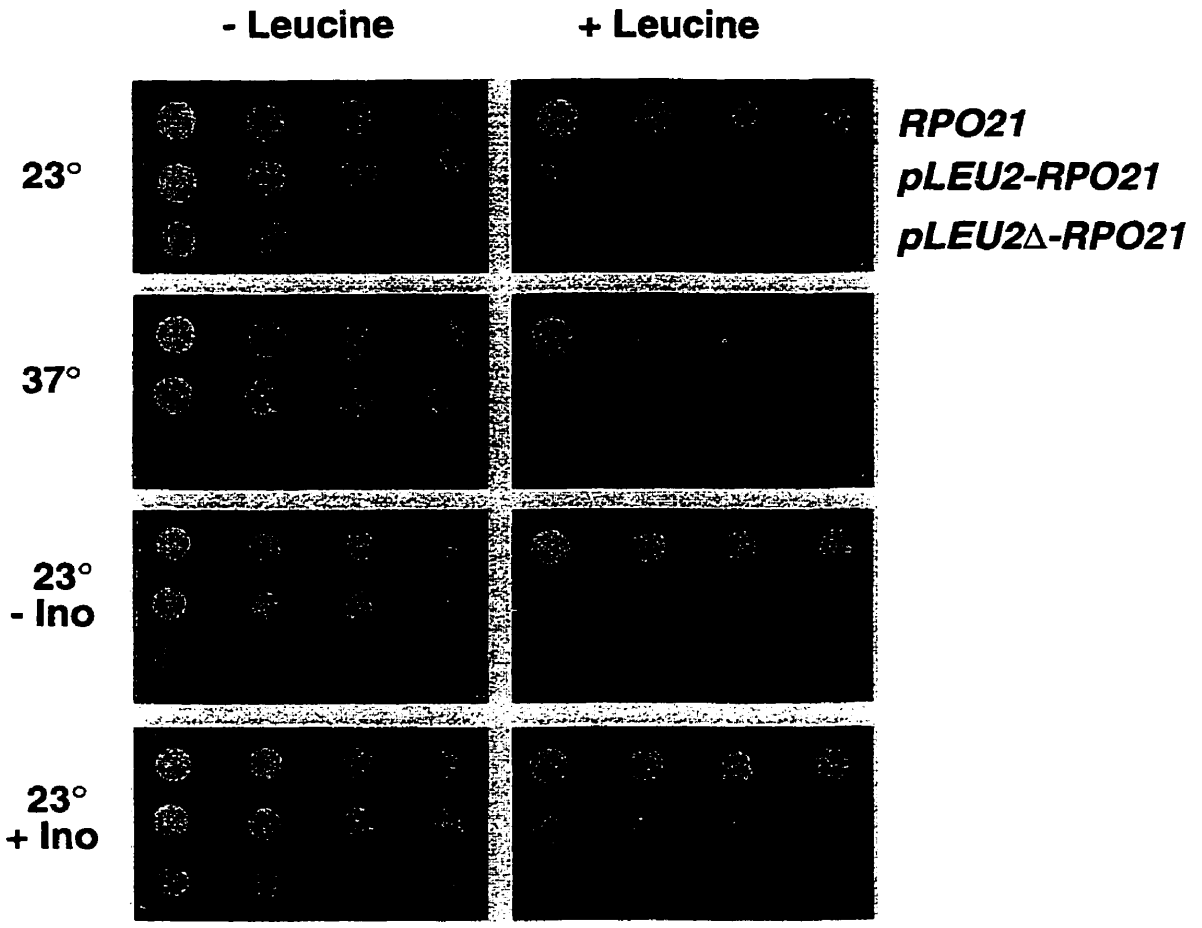
Figure 2.2. Strength of the pLEU2, pLEU2Δ and pRPO21 promoters. Levels of β-galactosidase activity expressed in Miller units were determined in strain AJ1 expressing a *RPO21-lacZ* fusion protein from either the pLEU2, pLEU2Δ or RPO21 promoter on a single-copy replicating vector. Cells were grown either in the absence (filled bars) or presence (hatched bars) of 2.0 mM leucine, isoleucine and threonine.


20
10
0
β - Galactosidase activity
(Miller units)
- Leucine
+ Leucine
pLEU2
pLEU2Δ
pRPO21

RPO21 from the *LEU2* promoter would be near wild-type levels (*i.e.*, the levels obtained with the *RPO21* promoter) in medium lacking leucine. A significant decrease in the level of transcription of *RPO21* can be achieved by addition of leucine to the growth medium of cells expressing *RPO21* from the *LEU2* promoter or by expressing *RPO21* from the *LEU2Δ* promoter.

Ability of *pLEU2-RPO21* and *pLEU2Δ-RPO21* to support growth of yeast cells: We next tested the ability of the *pLEU2-RPO21* and *pLEU2Δ-RPO21* alleles to support cell growth by complementing a *RPO21* null allele (*rpo21::ADE2*). As a control, a similar plasmid containing the entire *RPO21* gene expressed from its endogenous promoter was also used in these experiments. These alleles, carried on a single-copy replicating plasmid (pFL39; *TRP1 CEN6 ARS*), were introduced into yeast strain AJ2 (*rpo21::ADE2* [pJAY47; *URA3 pGAL-RPO21*][YEp13; *LEU2*]) by selection for tryptophan prototrophy on medium containing galactose (to allow for expression of *pGAL-RPO21* from the maintenance plasmid) and lacking leucine (to derepress the pLEU2 and pLEU2Δ promoters). We obtained strains that lacked the maintenance plasmid by growing these transformants for several generations in glucose-medium containing uracil and lacking leucine and subsequently by screening for uracil-auxotrophy on glucose medium at 23°. As can be seen in Figure 2.3, Ura$^-$ cells carrying the *pLEU2-RPO21* allele were able to grow as well as cells carrying a wild-type *RPO21* gene on solid medium lacking leucine. This finding was consistent with our previous observation (see above) that the strength of the *LEU2* promoter is similar to that of pRPO21 when leucine is absent from the growth medium. However, when leucine was added to the medium or when *RPO21* was transcribed from the weaker pLEU2Δ promoter, cell growth was impaired. We also showed that deleting the translation-initiation codon in *pLEU2-RPO21* and *pLEU2Δ-RPO21* (see Materials and Methods) abolished their ability to support growth, indicating that

Figure 2.3. Phenotypes of cells expressing *RPO21* from either the pLEU2, pLEU2Δ or pRPO21 promoters. Plasmids carrying the *pLEU2-RPO21, pLEU2Δ-RPO21,* or *RPO21* allele were introduced into yeast strain AJ2 (*MATα rpo21::ADE2* [pJAY47; *pGAL-RPO21*][YEp13; *LEU2*]. Transformed strains were screened for loss of the maintenance plasmid (pJAY47) and were tested for their ability to support growth on solid medium containing glucose at 23° and 37° and on glucose medium that either lacked (-Ino) or contained 10 μM inositol (+Ino) at 23°. Repression of the *LEU2* promoter was accomplished by adding 2.0 mM leucine, isoleucine and threonine to the growth medium. Serial dilutions of the cell culture were spotted on the plates; these contained 10 000, 2000, 400 and 80 cells respectively.

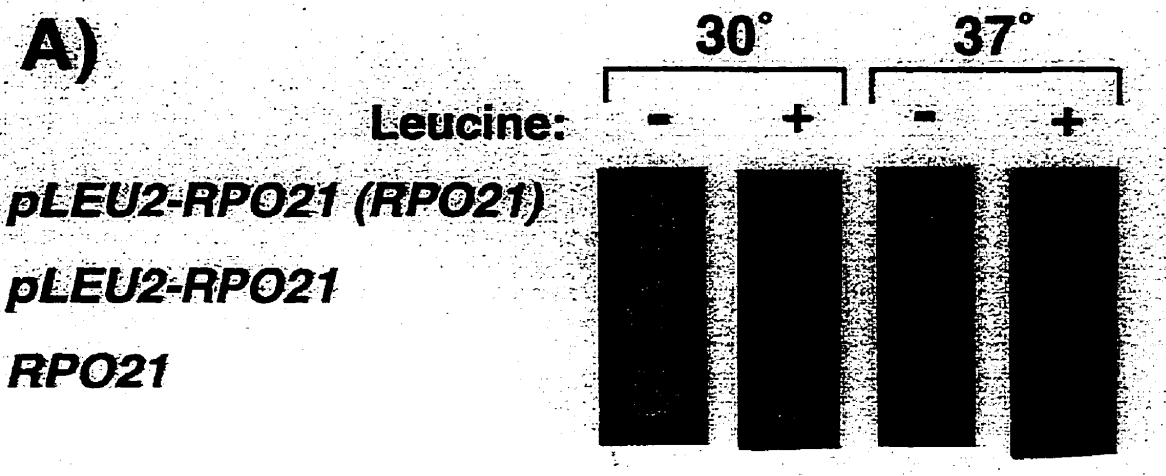

- Leucine
+ Leucine
23°
37°
23°
- Ino
23°
+ Ino
RPO21
pLEU2-RPO21
pLEU2Δ-RPO21

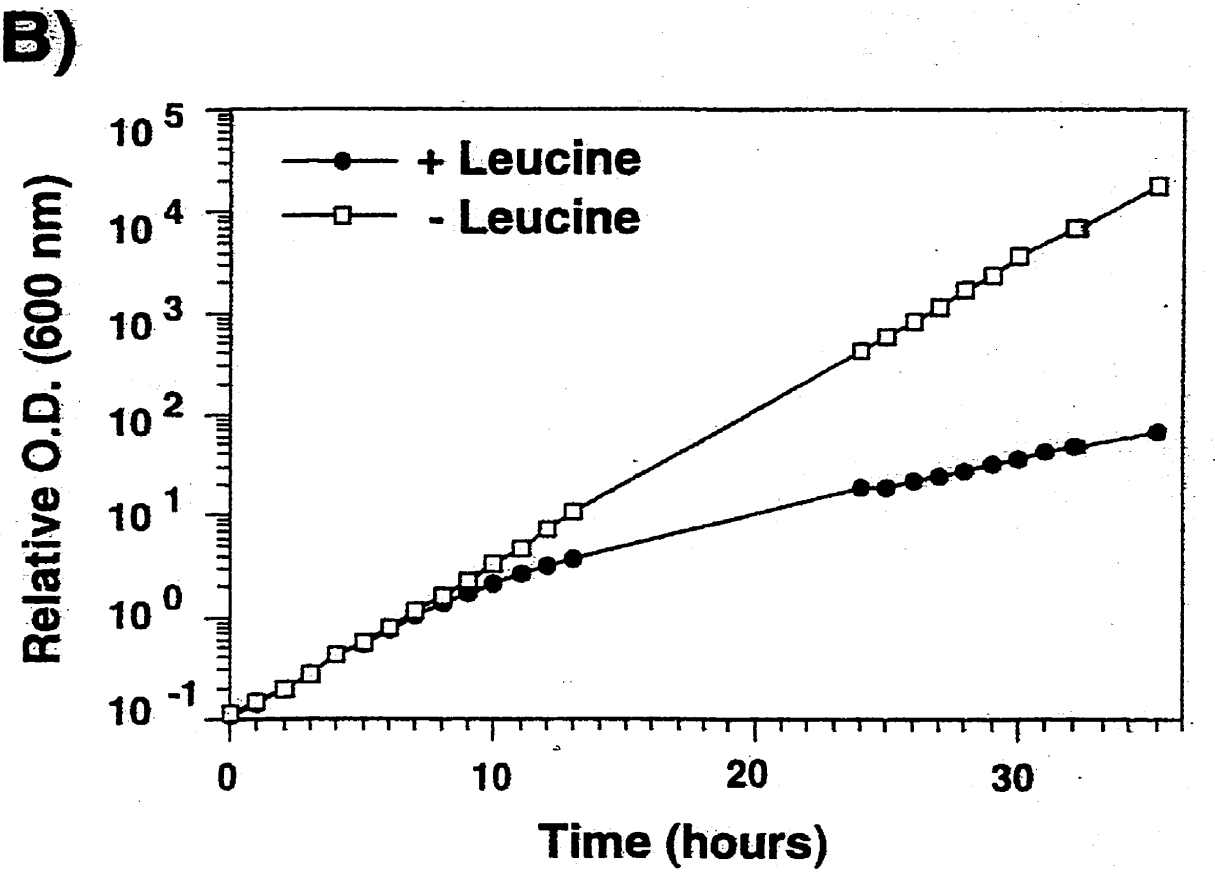
complementation of *rpo21::ADE2* requires that the translation of the fusion-gene mRNAs be initiated at the correct AUG codon (data not shown). These results indicated that a substantial decrease in the level of transcription of *RPO21* cannot be tolerated without reducing the growth rate.

Cells that underproduce the Rpo21 polypeptide are temperature-sensitive for growth and auxotrophic for inositol: We next tested cells that express *RPO21* from the pLEU2 or pLEU2Δ promoters for their ability to grow at high temperature (37°) or on medium lacking inositol (at 23°). We found that cells expressing *RPO21* from the *LEU2* promoter grew as well as wild-type cells at high temperature and on medium lacking inositol under conditions where the pLEU2 promoter was derepressed (*i.e.*, when leucine was absent from the growth medium) (Figure 2.3). This was not surprising since our analysis of the strength of the *RPO21* and *LEU2* promoters suggested that, under these growth conditions, *RPO21* is expressed at similar levels in both strains. However, when leucine, isoleucine and threonine were added to the growth medium, *pLEU2-RPO21* cells grew extremely poorly at high temperature or at 23° on medium lacking inositol (Figure 2.3). Addition of 10 μM inositol to the growth medium relieved the Ino$^-$ phenotype (Figure 2.3). Similar results were observed for cells expressing *RPO21* from the pLEU2Δ promoter. These results indicated that underproduction of the largest subunit of RNAPII is sufficient to confer thermosensitivity and inositol auxotrophy.

Transfer of the *pLEU2-RPO21* allele to the *RPO21* chromosomal location: We constructed a yeast strain in which the endogenous *RPO21* gene was replaced by the *pLEU2-RPO21* allele (see Materials and Methods). This strain grew as well as a wild-type strain at 30° and 37° when grown in the absence of leucine, isoleucine and threonine (Figure 2.4A). In contrast, this strain grew slowly at 30° and was

Figure 2.4. Effect of leucine on the growth of yeast cells in which the chromosomal *RPO21* promoter was replaced by the *LEU2* promoter. (A) A yeast strain was constructed in which the *RPO21* locus was replaced by the *pLEU2-RPO21* allele (see Materials and Methods). This strain was then transformed with either a single-copy plasmid (pDJ20) carrying wild-type *RPO21* or with a control plasmid (pFL39). In the figure, these transformed cells are designated *pLEU2-RPO21 (RPO21)* and *pLEU2-RPO21*, respectively. These transformed strains, along with a wild-type strain (in which the chromosomal copy of *RPO21* is wild type; strain YF2122) were tested for their ability to grow on solid medium containing glucose at 30° and 37° in the presence (+Leucine) or absence (-Leucine) of 2 mM leucine, isoleucine and threonine. (B) Portions of a culture of pLEU2-RPO21 cells grown in liquid medium at 30° were inoculated at time zero in medium lacking (- Leucine) or containing (+ Leucine) 0.5 mM leucine, isoleucine and threonine. The cells were diluted into fresh medium, when appropriate, to ensure continued logarithmic growth. Growth was determined by absorbance at 600 nm.


A)
30°
37°
Leucine:
-
+
-
+
pLEU2-RPO21 (RPO21)
pLEU2-RPO21
RPO21

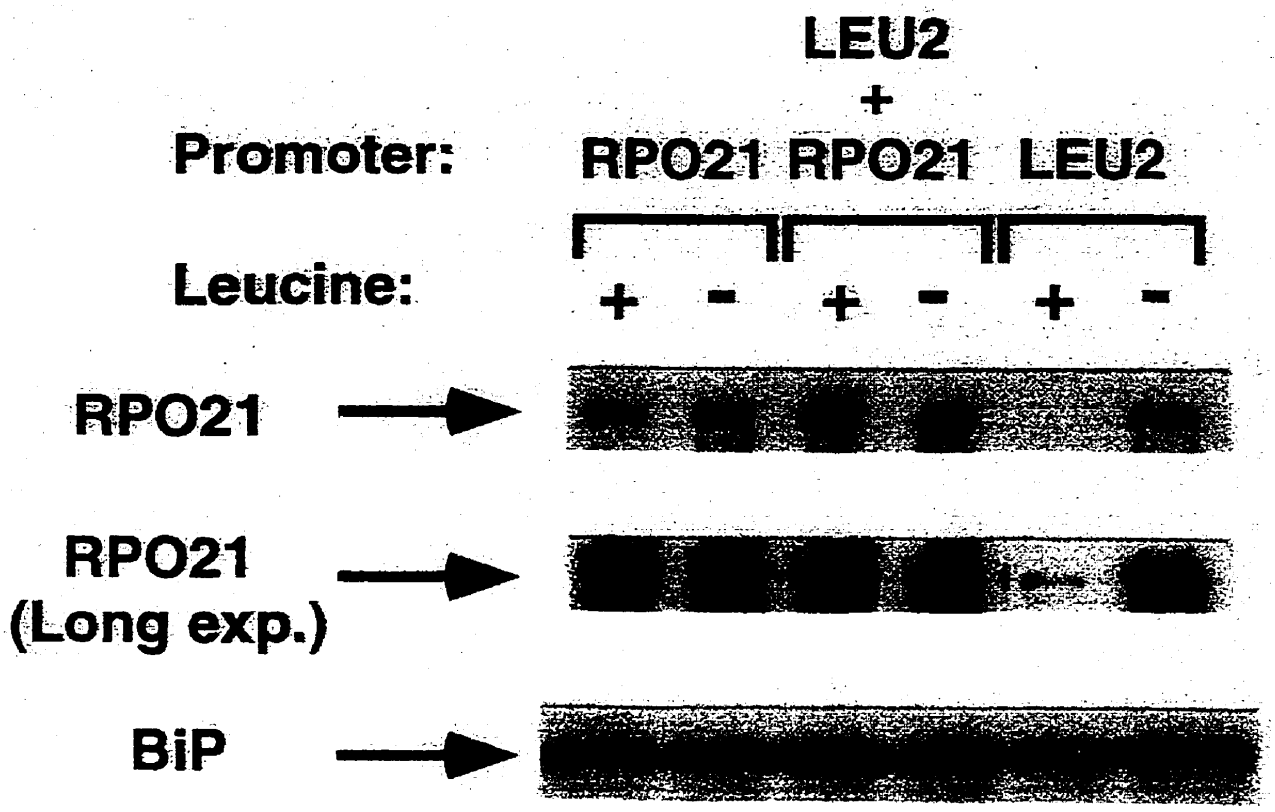

B)
+ Leucine
- Leucine
Relative O.D. (600 nm)
10 5
10 4
10 3
10 2
10 1
10 0
10 -1
0
10
20
30
Time (hours)

temperature-sensitive at 37° when leucine, isoleucine and threonine were present in the growth medium (Figure 2.4A). Both, the slow-growth and temperature-sensitive phenotypes could be complemented by introducing a wild-type copy of *RPO21* into this *pLEU2-RPO21* strain. These results are consistent with those obtained in Figure 2.3.

The *pLEU2-RPO21* strain was then used to measure the difference in growth rate between cells grown in the absence or presence of leucine at 30°. As can be seen in Figure 2.4B, *pLEU2-RPO21* cells grown in the absence of leucine had a doubling time of 2 hr, similar to that of wild-type cells (data not shown). Addition of leucine, isoleucine and threonine to the medium increased the doubling time to 5.5 hr. This doubling time was reached approximately 10 to 15 hr after the addition of leucine, isoleucine and threonine to the medium and presumably represents the time required to reduce the cellular content of RNAPII synthesized before the addition of these amino acids (time zero).

We verified by immunoblot analysis that the addition of leucine, isoleucine and threonine to the growth medium reduced the amount of *RPO21* gene product in these cells (Figure 2.5). When grown in the absence of leucine, *pLEU2-RPO21* cells had a similar amount of *RPO21* polypeptide as wild-type cells. In contrast, *pLEU2-RPO21* cells grown in the presence of leucine, isoleucine and threonine had significantly less *RPO21* protein than wild-type cells. We obtained a gross estimate of this difference by comparing the level of *RPO21* to the amount of BiP protein (Nicholson *et al.* 1990) present in these extracts (see Materials and Methods). We found that *pLEU2-RPO21* cells had approximately 10-fold less Rpo21 polypeptide than wild-type cells. However, this could be an underestimate of the difference, since a suboptimal amount of Rpo21p could indirectly reduce expression of BiP. As a control, we also showed that *pLEU2-RPO21* cells which had been transformed with a single-copy plasmid expressing wild-type *RPO21* did not show a reduction in the steady-state level of

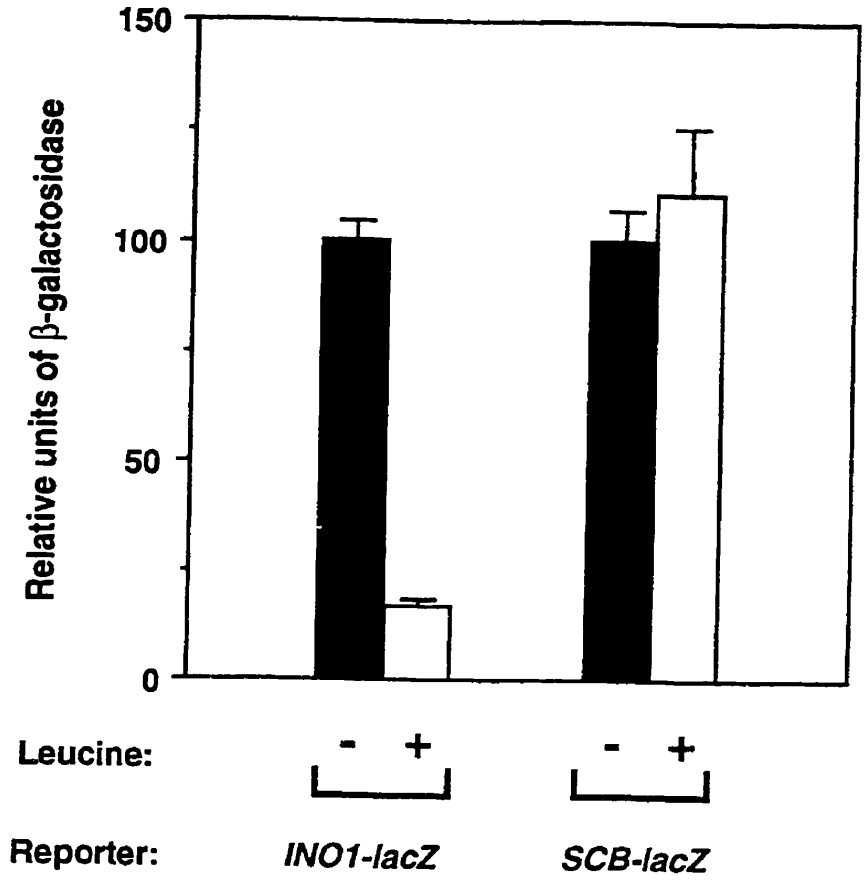
Figure 2.5. Amount of Rpo21 protein in strains carrying the *pLEU2-RPO21* allele. The Rpo21 protein was detected by immunoblot analysis using the mAb 8WG16 antibody in total cellular extracts prepared from cultures of *pLEU2-RPO21* cells (pLEU2), or from the same strain carrying a wild-type*RPO21* gene on a single copy plasmid (pDJ20) (pLEU2 + pRPO21), or from a wild-type strain (pRPO21). Cells were grown either in the presence (+) or absence (-) of leucine, isoleucine and threonine. The amount of Rpo21 protein present in *pLEU2-RPO21* cells grown in the presence of leucine was detected only after a longer exposure of the autoradiogram (RPO21 long exp.). As a control, the amount of the BiP protein was also detected in these extracts using a rabbit polyclonal antiserum directed against yeast BiP.


LEU2
+
Promoter:
RPO21 RPO21 LEU2
Leucine:
+ - + - + -
RPO21
RPO21
(Long exp.)
BiP

RPO21 polypeptide when grown under repressing conditions (Figure 2.5, pLEU2 + pRPO21).

***INO1* expression is reduced in cells that underproduce Rpo21p:** We determined the ability of *pLEU2-RPO21* cells to induce expression of *INO1* when starved for inositol, by measuring β-galactosidase activity in cells carrying an *INO1-lacZ* fusion gene. This fusion gene carries the *INO1* UAS placed upstream of the *CYC1* TATA-box and transcriptional-initiation sites (pJH359; Lopes *et al.* 1991). A similar reporter gene (pBA259; Andrews and Herskowitz 1989) carrying four SCB binding sites (which bind the transcription factor SWI4/SWI6) instead of the *INO1* UAS was used as a control in these experiments. As can be seen in Figure 2.6, *pLEU2-RPO21* cells showed a six-fold reduction in β-galactosidase activity produced from the *INO1-lacZ* reporter gene when grown in the presence of leucine, isoleucine and threonine. In contrast, expression of the *SCB-lacZ* gene, which is also driven by the *CYC1* promoter but under the control of a different UAS, was unaffected by the addition of these amino acids to the growth medium. These results indicated that the inositol auxotrophy of *pLEU2-RPO21* cells, which are grown in the presence of leucine, isoleucine and threonine, is caused by a reduction in the level of *INO1* expression. The inositol auxotrophy and reduction in *INO1* expression were not detected in *pLEU2-RPO21* cells that had been transformed with a plasmid bearing a wild-type copy of *RPO21* (data not shown), indicating that underproduction of Rpo21p is sufficient to bring about these phenotypes. Furthermore, since the *INO1-lacZ* fusion used in this study contains only the *INO1* UAS and not its promoter, the failure to induce expression of *INO1-lacZ* must be due to an inability to activate its transcription in response to the signals originating from the *INO1* UAS. A previous study also indicated that the failure of some RNAPII mutants to transcribe *INO1* is the result of an inability to respond to the *INO1* UAS (Scafe *et al.* 1990a).

Figure 2.6. Expression of *INO1-lacZ* and *SCB-lacZ* fusion genes in *pLEU2-RPO21* cells. Relative β-galactosidase activity were determined for *pLEU2-RPO21* cells expressing either an *INO1-lacZ* or a *SCB-lacZ* fusion gene. Cells were grown in the absence of inositol and in the presence (+) or absence (-) of 2.0 mM leucine, isoleucine and threonine. For each reporter gene, the level of β-galactosidase activity measured for cells grown in the absence of leucine, isoleucine and threonine was assigned a value of 100.


150
100
50
0
Relative units of β-galactosidase
Leucine:
- +
- +
Reporter:
INO1-lacZ
SCB-lacZ

The magnitude (six-fold) of the reduction in the expression of *INO1-lacZ* detected in cells that underproduce Rpo21p is similar to that brought about by other mutations that affect components of the transcriptional machinery, such as those that truncate the carboxy-terminal domain (CTD) of RNAPII, the *rpb1-1* mutation, or a deletion of the *SRB2* gene. These last three mutations reduce expression of *INO1-lacZ* approximately four-fold (Koleske *et al.* 1992).

DISCUSSION

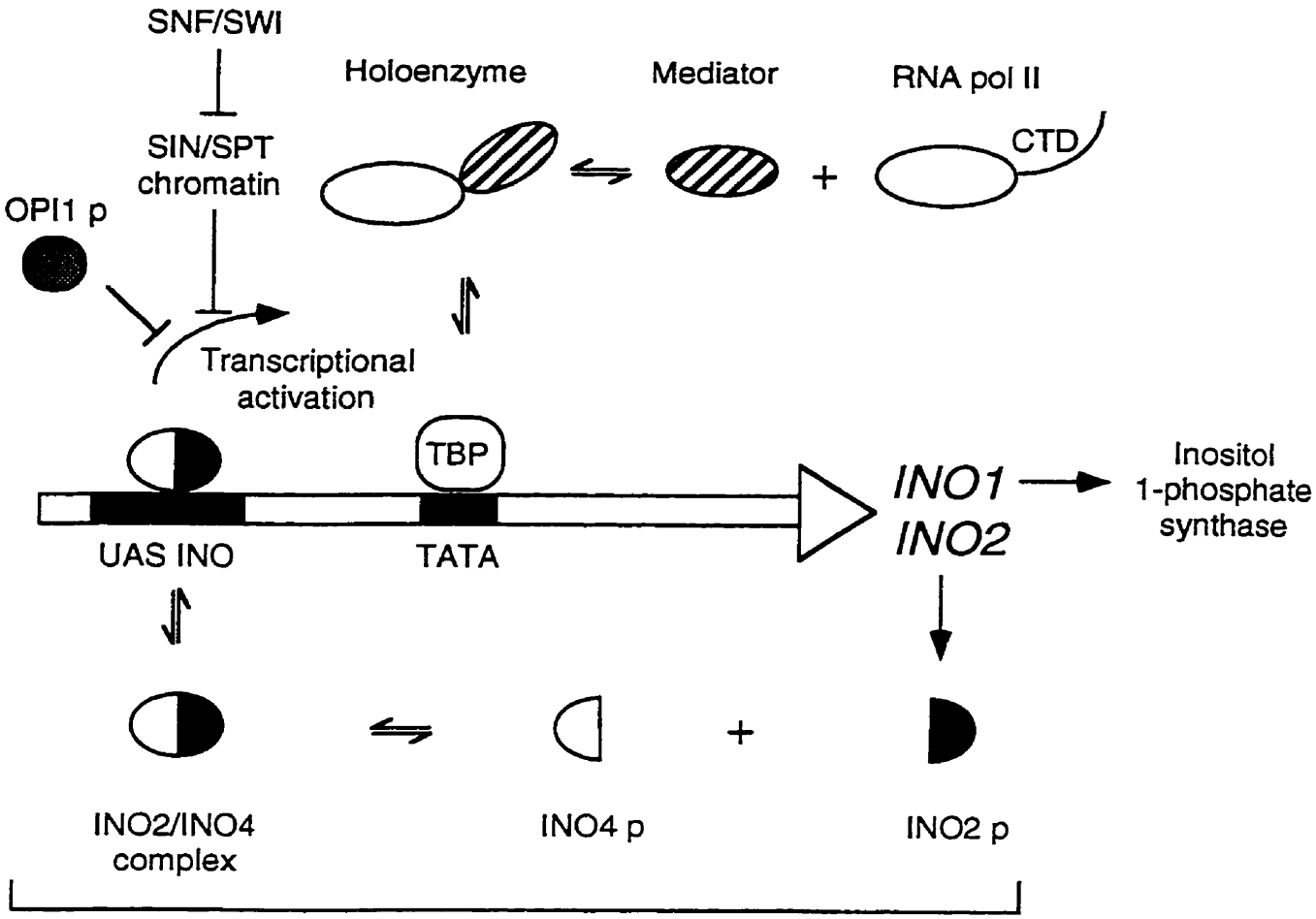
The results described in this study indicate that an approximately 10-fold reduction in the steady-state amount of Rpo21p is not a lethal event but rather is sufficient to confer phenotypes often associated with mutations affecting RNAPII, such as temperature-sensitivity, slow growth and inositol auxotrophy. Since the only known function of Rpo21p is as a component of RNAPII, we infer that the phenotypes caused by underproduction of Rpo21p are a consequence of a reduction in the intracellular amount of RNAPII. Because we did not measure directly the extent by which RNAPII is reduced in cells that underproduce Rpo21p, we cannot rule out the possibility that a 10-fold reduction in the level of Rpo21p does not lead to a concomitant 10-fold reduction in the level of RNAPII. However, it is unlikely that a substantial reduction in the amount of Rpo21p would cause only a marginal reduction in the level of RNAPII since Rpo21p is not produced in excess in wild-type yeast cells (Kolodziej and Young 1991). The finding that *pLEU2-RPO21* cells grown under repressing conditions produce approximately 10-fold less Rpo21p than wild type cells (Figure 2.5), even though the pLEU2 promoter is about 40 times weaker that the *RPO21* promoter (Figure 2.2), may indicate that a compensatory mechanism exists which maintains the required amount of Rpo21p protein when transcription of the gene is reduced. Such a compensatory mechanism has been described previously in mammalian cell lines

(Somers *et al.* 1975; Guialis *et al.* 1977; 1979) and in *Caenorhabditis elegans* (Dalley *et al.* 1993). See also CHAPTER 4.

Our finding that reducing the steady-state level of RNAPII is sufficient to confer temperature-sensitivity, slow growth and inositol auxotrophy leads to the prediction that some of the previously-isolated RNAPII mutations which confer this same set of phenotypes may have affected the assembly/stability of the enzyme. Our results also provide an explanation as to why many mutations in different subunits and in different locations of RNAPII impose a similar set of phenotypes. Our results do not exclude the possibility that these previously-identified RNAPII mutations may also have affected other aspects of RNAPII function in addition to affecting its assembly/stability. Also, temperature-sensitivity, slow growth and inositol auxotrophy can be caused by RNAPII mutations that do not affect the assembly of the enzyme. One such example is mutations that truncate the CTD of the largest subunit of RNAPII (Allison *et al.* 1988; Nonet *et al.* 1987). The similarities between CTD-truncation mutations and mutations that reduce the level of RNAPII, such as those described in this study, are discussed below.

Regulation of *INO1* transcription: Our results indicate that a decrease in the intracellular level of RNAPII affects transcription from the *CYC1* promoter when it is placed under the control of the *INO1* UAS but not when controlled by SCB elements (which bind the transcription factor Swi4p/Swi6p; Andrews and Herskowitz 1989). How is transcription regulated by the *INO1* UAS and why is this regulation altered in cells that underproduce RNAPII? It was shown previously that transcription of *INO1* is controlled by three regulatory genes, *INO2*, *INO4* and *OPI1* (Figure 2.7; reviewed in Carman and Henry 1989). The *INO2* and *INO4* gene products are positive activators of *INO1* expression and form a complex that binds a DNA element which is present in two copies in the *INO1* UAS (Hirsh and Henry 1986; Lopes *et al.* 1991; Ambroziak and

Figure 2.7. Model for the transcription of *INO1* and *INO2*. This model emphasizes the roles of certain proteins (discussed in the text) involved in regulating transcription-initiation by RNAPII at the *INO1* and *INO2* promoters. Transcription from these two promoters requires two DNA elements, the UAS INO and the TATA box, which are bound by the Ino2p/Ino4p complex and by the general initiation factor TBP, respectively. Transcription from the *INO1* and *INO2* promoters requires formation of the RNAPII holoenzyme, which is depicted as an association between the mediator complex and the CTD of RNAPII. Initiation of transcription by RNAPII requires that the holoenzyme be recruited to the promoter, at least in part by an association with TBP, in response to the transcriptional activation signal of the Ino2p/Ino4p complex. The activity of this complex is regulated negatively by the *OPI1* protein. Transcriptional activation by the Ino2p/Ino4p complex is also subject to the negative regulation of chromatin, which may prevent it, or TBP, from binding to DNA. This negative effect of chromatin is alleviated by the SNF/SWI complex. Wild-type-level expression of *INO1* and *INO2* also requires that the *INO2* protein be synthesized in sufficient amount and that it associates with the *INO4* protein. Since the levels of the *INO2* protein are limiting for *INO1* and *INO2* transcription, small variations in the intracellular concentration of *INO2* protein are amplified by the *INO2* autoregulatory loop. In this model, the hypersensitivity of *INO1* transcription to mutations affecting components of the transcriptional machinery is explained by the amplification of small transcriptional defects by the *INO2* autoregulatory loop.



INO2 autoregulatory loop

(amplification of small decrease in transcription of *INO1* and *INO2*)

Henry 1994). These same DNA sequences are also required for the negative regulation of *INO1* expression by the *OPI1* gene (Ashburner and Lopes 1995a). It has been suggested that the *OPI1* protein represses transcription by antagonizing the ability of the Ino2p/Ino4p complex to activate transcription in a manner similar to that of Gal80p on the Gal4p transactivator protein (Ashburner and Lopes 1995a). Interestingly, the promoter of *INO2* also contains a binding site for the Ino2p/Ino4p complex and is also subject to the positive and negative regulation of *INO2*, *INO4* and *OPI1* (Ashburner and Lopes 1995a).

Transcription of *INO1* is also subject to the regulatory effect of chromatin and proteins involved in remodeling chromatin. Mutations in *SWI1(ADR6)*, *SWI2 (SNF2)*, *SWI3*, *SNF5* and *SNF6*, which encode proteins that are part of a large complex (Peterson *et al.* 1994; Coté *et al.* 1994) required to overcome the negative regulatory effect of chromatin at certain promoters (Hirschhorn *et al.* 1992; reviewed in Peterson and Tamkun 1995), affect the expression of many unrelated genes, including *INO1* (Peterson and Herskowitz 1992; reviewed in Winston and Carlson 1992; Figure 2.7). Mutations that were isolated previously as suppressors of mutations affecting the SWI/SNF complex, such as mutations in *SIN2* encoding histone H3, *SIN1(SPT2)* encoding an HMG1-like protein (Kruger and Herskowitz 1991) or *SIN3* (Hudak *et al.* 1994), also affect expression of *INO1*. In contrast to the *SWI/SNF* genes, *SIN1*, *SIN2* and *SIN3* are required for repression of *INO1* when inositol is present in the growth medium.

Why is expression of *INO1* particularly sensitive to mutations affecting the transcriptional machinery?: The fact that transcription of *INO1* is determined by the level of transcription of the positive regulator *INO2* (Ashburner and Lopes 1995b), and that *INO2* is under the control of an autoregulatory loop (Ashburner and Lopes 1995a), could explain why transcription of *INO1* is particularly sensitive to a

decrease in the levels of RNAPII. In this case, a small reduction in the ability of the transcription machinery to respond to the regulatory signal from the *INO2* UAS would result in decreased expression of *INO2* which in turn would lead to a much larger reduction in the transcription of *INO1* and *INO2* itself. Amplification of slight transcriptional defects would certainly be accentuated by the fact that the levels of *INO2* transcription (and presumably of *INO2* protein) appear to be rate-limiting for transcription of *INO1* and that the *INO2* promoter is the weakest yeast promoter characterized thus far (Ashburner and Lopes 1995a). In this model, therefore, the sensitivity of *INO1* expression to a decrease in the level of RNAPII, or to any other type of mutation affecting components of the transcriptional machinery, is explained by the fact that a slight defect in transcription of *INO1* and *INO2* is amplified by the *INO2* autoregulatory loop (Figure 2.7). In general, it may be that the transcription of genes whose regulatory proteins are limiting for their expression and are under the control of an autoregulatory loop, are more sensitive to mutations that affect components of the transcriptional machinery.

Underproduction of RNAPII compared to truncation of the CTD:

Transcription of *INO1* also requires a set of proteins encoded by the *SRB* genes (*SRB*: Suppressor of RNAPII [B]) that were identified in a search for suppressors of mutations that shorten the CTD of the largest subunit of RNAPII (Nonet and Young 1989; Thompson *et al.* 1993; Figure 2.7). Several of the *SRB* polypeptides (Srb2p, 4p, 5p, 6p, and 7p) are components of a multisubunit complex termed the mediator, which also contains other global regulators of transcription such as Sug1p, Gal11p and the general initiation factor TFIIF (Kim *et al.* 1994). The mediator can also be purified as part of a much larger complex containing RNAPII (Kim *et al.* 1994) and, under certain purification conditions, TFIIB, TFIIH and Srb8p, 9p, 10p and 11p (Thompson *et al.* 1993; Koleske and Young 1994). This large complex, termed the "RNAPII

holoenzyme" (reviewed in Koleske and Young 1995) can respond to transcriptional-activator (Kim *et al.* 1994) and repressor (Kuchin *et al.* 1995; Wahi and Johnson 1995) proteins. The RNAPII holoenzyme is thought to be the form of RNAPII that is recruited at the promoter of most yeast genes (Thompson and Young 1995), at least in part through interactions with the TATA-box binding protein (TBP) (Koleske *et al.* 1992; Thompson *et al.* 1993) and with activator proteins (Hengartner *et al.* 1995).

In this context, it is worth considering why yeast cells carrying mutations that shorten the CTD have similar phenotypes as cells underproducing RNAPII, namely temperature-sensitivity, slow growth and inositol auxotrophy. Mutations that truncate the CTD are unlikely to affect the steady-state level of the enzyme; this suggestion is supported by the finding that mutations that truncate the CTD do not reduce the stability of the largest subunit (Nonet *et al.* 1987; Allison *et al.* 1988) but rather affect the ability of RNAPII to respond to the regulatory signals of certain UASs (including the *INO1* UAS) (Allison and Ingles 1989; Scafe *et al.* 1990a). However, because the CTD participates in the assembly of the RNAPII holoenzyme, it is possible that mutations that shorten the CTD affect the ability of RNAPII to interact with the mediator complex. In support of this hypothesis is the fact that the mediator can be dissociated from RNAPII using a monoclonal antibody directed against the CTD (Kim *et al.* 1994). Therefore, the phenotypes imposed by mutations that shorten the CTD may result from reduced RNAPII holoenzyme assembly in these mutant cells. Similarly, underproduction of the largest subunit of RNAPII, as was done in this study, would also lead to a reduction in the amount of RNAPII holoenzyme, in this case not by affecting an interaction with the mediator complex, but by reducing the amount of RNAPII available to assemble into the holoenzyme. Although speculative, these similarities provide an explanation as to why truncation of the CTD and underproduction of RNAPII impose similar phenotypes. Finally, since the holoenzyme is recruited to promoters, at least in part through an association with TBP (Koleske *et al.* 1992;

Thompson *et al.* 1993), it is not surprising that mutations in TBP also cause an Ino⁻ phenotype (Arndt *et al.* 1995).

With these ideas in mind, we tested whether a dominant mutation in *SRB2* (*SRB2-1*), that was isolated on the basis of its ability to suppress the phenotypes of CTD-truncation mutations, would suppress the phenotypes of cells that underproduce Rpo21p. We found that *SRB2-1* was unable to suppress the slow-growth phenotype of cells that underproduce RNAPII (data not shown). This result was, perhaps, not surprising since *SRB2-1* suppresses specifically CTD-truncation mutations and not any other mutations in *RPO21*, some of which might affect the assembly/stability of the enzyme since they confer inositol auxotrophy. Similarly, we found that the phenotypes of cells that underproduce RNAPII were not suppressed by lengthening the CTD or by overproducing the sixth-largest subunit of RNAPII (Rpo26p) (data not shown). Lengthening the CTD was shown previously to suppress weakly the effects of mutations that reduced the activity of the transcriptional activator protein Gal4p (Allison and Ingles 1989). Over-expression of Rpo26p was shown to suppress, in an allele-specific manner, the inositol auxotrophy, temperature-sensitivity and slow-growth phenotypes imposed by the *rpo21-4* mutation, a mutation that is thought to affect the assembly/stability of RNAPII (Archambault *et al.* 1990; Archambault *et al.* 1992a). These results suggested that suppression by *SRB2-1*, or by lengthening of the CTD or by overexpression of *RPO26* in those previous studies was not brought about by bypassing the requirement for a threshold level of RNAPII.

Underproduction of RNAPIII: A finding of this work and of previous studies of RNAPII mutants is that the transcription of specific genes such as *INO1* is affected to a greater extent than that of other genes such as *SCB-lacZ* by alterations in the structure or levels of RNAPII. This differential effect on gene-expression is not unique to RNAPII. In the case of RNAPIII, it was found that mutations affecting the largest subunit

(Gudenus *et al.* 1988), or some of the smaller subunits, Rpc82p (Chiannilkulchai *et al.* 1992), Rpc53p (Mann *et al.* 1992), Rpc34p (Stettler *et al.* 1992) and Rpc31p (Mosrin *et al.* 1990), lead to a reduction in the amount of tRNAs, while that of 5S RNA was relatively unchanged. It was suggested that RNAPIII either has a lower affinity for the initiation complexes assembled at tRNA genes than for those assembled at the 5S RNA gene or that the enzyme is more stable in the nucleolus (where 5S RNA is synthesized) than in the nucleoplasm (where tRNAs are synthesized).

CHAPTER 3

Similar Upstream Regulatory Elements of Genes that Encode the Two Largest Subunits of RNA Polymerase II in *Saccharomyces cerevisiae.*

Attribution of contributions:

This chapter is a modified version of an article, with the same title, published in 1996 in *Nucleic Acids Research*, **24**: 4543-4551. The authors of that paper are me, Jacques Archambault, Omid Mostachfi and James Friesen. Jacques Archambault initiated this study by constructing the original *RPO21*- and *RPO22-lacZ* fusion genes. He was a mentor and instructor. Omid Mostachfi worked under my supervision during the summer of 1995. He assisted in the construction of the plasmids used in Figure 3.3. With these exceptions, I am responsible for the experiments and constructions (plasmids and yeast strains) of this study.

ABSTRACT

I have determined the location of *cis*-acting elements that are important for the expression of *RPO21* and *RPO22*, genes that encode the two largest subunits of RNA polymerase II (RNAPII) in *Saccharomyces cerevisiae*. A series of 5'-end deletions and nucleotide substitutions in the upstream regions of *RPO21* and *RPO22* were tested for their effect on the expression of *lacZ* fusions of these genes. Deletion of sequences from -723 to -693 in *RPO21*, which disrupted two Reb1p-binding sites and an Abf1p-binding site, resulted in a 10-fold decrease in expression. A T-rich region downstream of these sites was also important for expression. Deletion of sequences from -437 to -392 in the *RPO22*-upstream, which resulted in a 30-fold decrease in expression, indicated that the Reb1p- and Abf1p-binding sites in this region were important for *RPO22* expression, as was a T-rich sequence immediately downstream of these sites. The *RPO21* and *RPO22* upstream regions were capable of interacting *in vitro* (gel-mobility-shift assays) with Reb1p and Abf1p. The similarities in the type and organization of elements in the upstream regions of *RPO21* and *RPO22* suggest that expression of these genes may be regulated coordinately.

INTRODUCTION

RNA polymerase II (RNAPII) is responsible for the synthesis of mRNAs and some small nuclear RNAs in eukaryotic cells. In the yeast *Saccharomyces cerevisiae*, the enzyme is composed of subunits encoded by 12 different genes that are scattered throughout the yeast genome (Archambault and Friesen 1993; Young 1991). Five of the subunits are also found in RNAPI, which synthesizes rRNA, and in RNAPIII, which synthesizes tRNA, 5S RNA and U6 snRNA.

Little is known about the biosynthesis of RNAPII subunits in yeast, despite the fact that this enzyme plays a central role in the expression of thousands of genes whose transcript levels are likely to be influenced by the cellular amount of active RNAPII (CHAPTER 2; Archambault, *et al.* 1996). For this reason it is important to understand the mechanisms involved in maintaining the appropriate amount of enzyme. A consideration of the biosynthesis of multi-subunit enzymes such as RNAPII leads to the question of whether subunit synthesis is coordinated so as to produce the required molar ratio of subunits. In *Escherichia coli*, coordinate synthesis of the three proteins that make up the core RNAP results in subunit levels that reflect closely the stoichiometry of each subunit in the enzyme (Yura and Ishihama 1979). Although the mechanisms involved in this regulation are not understood fully, it is clear that coordinate synthesis of the two largest subunits of bacterial RNAP, β and β', is due in part to the fact that the genes encoding these subunits lie in the same operon and thus are transcribed from the same promoters (Yura and Ishihama 1979).

One mechanism for generating similar amounts of expression from genes that act in a common biochemical pathway is the use of similar transcriptional control elements. For example, in *S. cerevisiae* the Gal4p transcription factor acts through a defined upstream regulatory sequence (UAS) to control the expression of a number of genes required for galactose metabolism (Johnston 1987). Transcription of ribosomal-protein genes is controlled in a similar manner. In this case, the expression of many of the yeast genes that encode protein subunits of the ribosome is controlled by similar *cis*-acting elements, which are located upstream of the start site of transcription. These elements often contain one or two binding sites for Rap1p (Shore 1994) or Abf1p (Buchman, *et al.* 1988), transcription factors that have important roles in coordinating the expression of ribosomal-protein genes (Planta and Raue 1988).

A similar mechanism may affect the expression of genes encoding subunits of RNAPIII. An analysis of the upstream regions of *RPC160* and *RPC40*, two of the genes

encoding subunits of RNAPIII in *S. cerevisiae*, has shown that binding sites for the transcription factor Abf1p are present (Della Seta, *et al.* 1990b). A deletion analysis of the upstream region of *RPC40* suggested that the binding site for Abf1p plays a significant role in the expression of this gene (Della Seta, *et al.* 1990b). It is possible that Abf1p has a role in ensuring a similar level of expression from genes encoding subunits of RNAPIII.

In CHAPTER 2, I showed that cells producing 10-fold less Rpo21p than normal are viable, but are slow growing, temperature sensitive, and auxotrophic for inositol. In this chapter, I extend these results through an investigation of the biosynthesis of RNAPII in *S. cerevisiae* . My approach has been to determine which elements in the upstream regions of the genes encoding the two largest subunits of RNAPII, namely *RPO21* (also called *RPB1*) and *RPO22* (also called *RPB2*), are important for their expression. I describe a similarity in the type, organization and function of *cis*-acting elements in the two promoters.

MATERIALS AND METHODS

Strains and Media

The *Saccharomyces cerevisiae* strain used in this study was W303-1a (*MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*). Growth media were prepared and yeast transformations were performed as described (Hill, *et al.* 1991; Sherman, *et al.* 1986). All plasmids were propagated in *Escherichia coli* strain XL-Blue (Bullock, *et al.* 1987).

Plasmids

DNA manipulations were performed essentially as described (Maniatis, *et al.* 1982). pYF1495 is a yeast shuttle-vector (*LEU2*; 2μm origin of replication) carrying an in-

frame fusion of *RPO21* and *lacZ*. It was constructed in several steps: (i) a 3.2 kb *Bam*HI-*Nco*I fragment containing a fusion of *lacI* and *lacZ* sequences from pHK413 (obtained from D. Harbrecht) was cloned into the *Bam*HI and *Pvu*II sites of YEp13 (*LEU2*, 2μm), creating pAV200 (constructed by A. Vassarotti); (ii) pJAY36, bearing a 7 kb *Hin*dIII fragment containing *RPO21*, was modified by adding a *Bam*HI linker at the *Avr*II site at +171 (the A of the *RPO21* initiation codon is +1), forming pJAY61 (the linker regenerated the *Avr*II site); (iii) a.1.8 kb *Hin*dIII-*Bam*HI fragment containing the *RPO21* sequences from -1585 to +171 from pJAY61 was cloned into the *Hin*dIII and *Bam*HI sites of pAV200, creating pJAY63; (iv) a 600 bp *Sal*I fragment containing the f1 origin of replication was inserted at the *Xho*I site of pJAY63 to create pYF863. The orientation of the f1 origin allows for the generation of single-stranded DNA of the non-coding strand of *RPO21*; (v) a 1.2 kb region between the *LEU2* ORF and 2μm sequences, which contains undesired restriction sites, was removed from pYF863 as follows: the plasmid was digested with *Sal*I and *Pst*I, the ends were blunted with the Klenow fragment of DNA polymerase I and the plasmid was religated to create pYF1495.

A series of 5'-end deletions was generated in the *RPO21*-upstream region by digestion of pJAY36 with *Hin*dIII and *Bal*31 exonuclease. The ends were filled with Klenow fragment of DNA polymerase I and *Hin*dIII linkers were ligated to the ends. *Hin*dIII-*Avr*II fragments from these derivatives of pJAY36 were cloned into the same sites of pYF1495, generating the series of 5'-end deletions shown in Figure 3.1. The *rpo21-ΔUAS* allele was constructed by: (i) inserting a *Hin*dIII linker at an end-filled *Bst*EII site after -724 in the *RPO21*-upstream region; (ii) cutting the resulting plasmid with *Hin*dIII to isolate a 860 bp *Hin*dIII-*Hin*dIII fragment containing *RPO21* sequences from -1585 to -724; (iii) cloning this fragment in the *Hin*dIII site of a derivative of pYF1495 that has a 5' deletion in the upstream region of *RPO21* up to -692. The resulting internal deletion/insertion is shown in Figure 3.2A (BOTTOM).

pYF1476 is a yeast shuttle-vector (*LEU2*; 2μm origin of replication) carrying an in-frame fusion of *RPO22* and *lacZ*. It was constructed in several steps: (i) a 1.3 kb *Hin*dIII-*Pst*I fragment carrying the upstream region and first 81 nucleotides of the *RPO22* ORF was cloned into the same sites of pUC19. This plasmid was cleaved with *Sal*I, the ends were filled and the plasmid was re-ligated, creating pJAY66 (ii) a 1.3 kb *Hin*dIII-*Bam*HI fragment from pJAY66 (containing sequences from approximately -1200 to +81 of *RPO22*) was cloned into the same sites of pAV300 (identical to pAV200 except that *lacI* and *lacZ* sequences were derived from pHK414), creating pYF1476.

5'-end deletions of the *RPO22*-upstream region were generated by cleaving pJAY66 at unique sites in the upstream region of *RPO22*, filling the ends, adding *Hin*dIII linkers, digesting with *Hin*dIII, and religating the DNA. *Hin*dIII-*Bam*HI fragments from the resulting plasmids were cloned into the same sites of pYF1476, generating a series of 5'-end deletions in the upstream region of *RPO22*.

Plasmids with mutations in the *RPO21* UAS (Figure 3.2B) were generated by using oligonucleotides with UAS element mutations and a second downstream oligonucleotide to amplify the *RPO21* UAS sequence by PCR using a wild-type or, in some cases, previously mutated template. The PCR product was subcloned into pYF1495. The amplified region was sequenced to confirm that the mutated plasmids contained only the desired mutations. The mutations introduced in the putative Abf1p-binding site changed TC to GT at the second and third positions (Figure 3.2A; TOP). This change resulted in a new *Sna*BI-restriction-enzyme site, which was used to construct the 5'-end deletion shown in line 5 of Figure 3.1.

Mutations in the upstream elements of *RPO22* were generated using a PCR-based site-directed mutagenesis technique that was described previously (Barettino, *et al.* 1994). The amplified region was sequenced to confirm that the mutated plasmids contained only the desired mutations. One of the site-directed mutations

introduced three nucleotide substitutions in the putative Abf1p-binding site in the *RPO22*-upstream region. At position -383, a G is changed to A and at positions -393 and -392, CG is replaced with AA (see Figure 3.3B, TOP). The latter two substitutions created a new *Eco*RI site at -394 to -389 in the *RPO22*-upstream region. The new *Eco*RI site was used to create a 5'-deletion (to -391) in the upstream region of *RPO22* shown in the fourth line of Figure 3.3A. The asterisk in Figure 3.3A refers to the additional G to A substitution at -383 in the upstream region, which, as mentioned above, introduced another alteration in the Abf1p-binding site. The *rpo22-ΔUAS* allele was created by: (i) subcloning a *Hin*dIII-*Hin*cII fragment containing sequences of the *RPO22*-upstream region from -1200 to -438 into the *Hin*dIII-*Eco*RV sites of the vector pBSKS+; (ii) employing the novel *Eco*RI site generated in the *RPO22*-upstream region (see above), a 500 bp *Eco*RI-*Bam*HI fragment containing *RPO22* sequences from -391 to +82 was subcloned into the same sites of the pBSKS+ plasmid bearing the *RPO22* sequences from -1200 to -438; (iii) a 1.3 kb *Hin*dIII-*Bam*HI fragment from the resulting plasmid was subcloned into pYF1476, creating a *RPO22-lacZ* fusion gene with the deletion/insertion mutation shown in Figure 3.3B (BOTTOM). Note that this allele also contains the G to A substitution at -383.

Plasmids were generated with the UAS of *RPO21* cloned upstream of a UAS-less *CYC1-lacZ* gene. The vector, pDJ22, was derived from pLG670-Z (Guarente and Ptashne 1981), a yeast shuttle-vector (*URA3*, 2μm) containing a fusion of *CYC1*-upstream sequences to the *lacZ* gene. pDJ22 was created by deleting sequences in pLG670-Z from the *Sma*I site at the 3' end of *URA3* to the *Sph*I site 12 bp upstream of the 5'-most TATA box of *CYC1* and replacing them with a *Bgl*II linker. DNA fragments from wild-type and mutated *RPO21*-upstream sequences were amplified by PCR using two oligonucleotides, DA23 (5'-GGGGATCCGACTATCATACGGTAACC-3') and DA24 (5'-GGGGATCCACCGACAATCGTCTTTAG-3'). Amplified products contained sequences from -740 to -674 that encompass the UAS of *RPO21*. The PCR products

were digested with *Bam*HI and were cloned into the *Bgl*I site of pDJ22. Resulting plasmids were sequenced to confirm the number of inserts, insert orientation and the absence of unwanted mutations.

β-galactosidase assays

β-galactosidase activity was measured as described by Miller (1972). Cells were grown in selective medium at 30°C. For each measurement, β-galactosidase activity was determined on at least three independent cultures.

Electrophoretic-mobility-shift assays (EMSA)

Assays were performed as described (Buchman and Kornberg 1990) except that DNA-protein mixtures were separated on 5% acrylamide gels. Probes and non-labeled-competitor DNA from *RPO21* were generated by PCR using the primers DA23 and DA24, described above. The non-labeled-competitor DNA from the *RPO22* -upstream region was a *Hin*dIII-*Rsa*I fragment containing -437 to -209 of *RPO22*-upstream sequence. The probe with the Reb1p site from the *GAL1-GAL10* intergenic region (provided by C. Brandl) was a 104 bp *Bam*HI-*Eco*RI fragment from plasmid *his3*-GG227 (Liaw and Brandl 1994). The probe containing the Abf1p site from *MAT*a (provided by L. McBroom) was the 136 bp *Bam*HI fragment of pB2ABF1 (McBroom and Sadowski 1994). Probes were end-labeled using Klenow fragment of DNA polymerase I and [α-32P]dATP.

Proteins used for gel-shift assays were either: (i) purified yeast Abf1p, provided by A. Buchman (Buchman and Kornberg 1990) or (ii) an extract from an *E. coli* strain expressing *REB1* (Morrow, *et al.* 1993b)(strain provided by J. Warner) or (iii) yeast whole-cell extract from a wild-type yeast strain (provided by S. Nouraini) that was prepared as described (Adman, *et al.* 1972). Estimations of the relative intensities of complexes were obtained by PhosphorImager analysis, using ImageQuant software

(Figure 3.4A), or by scanning autoradiographs and quantifying band intensities using NIH image software (Figure 3.5).

Nucleotide-Sequence Analysis

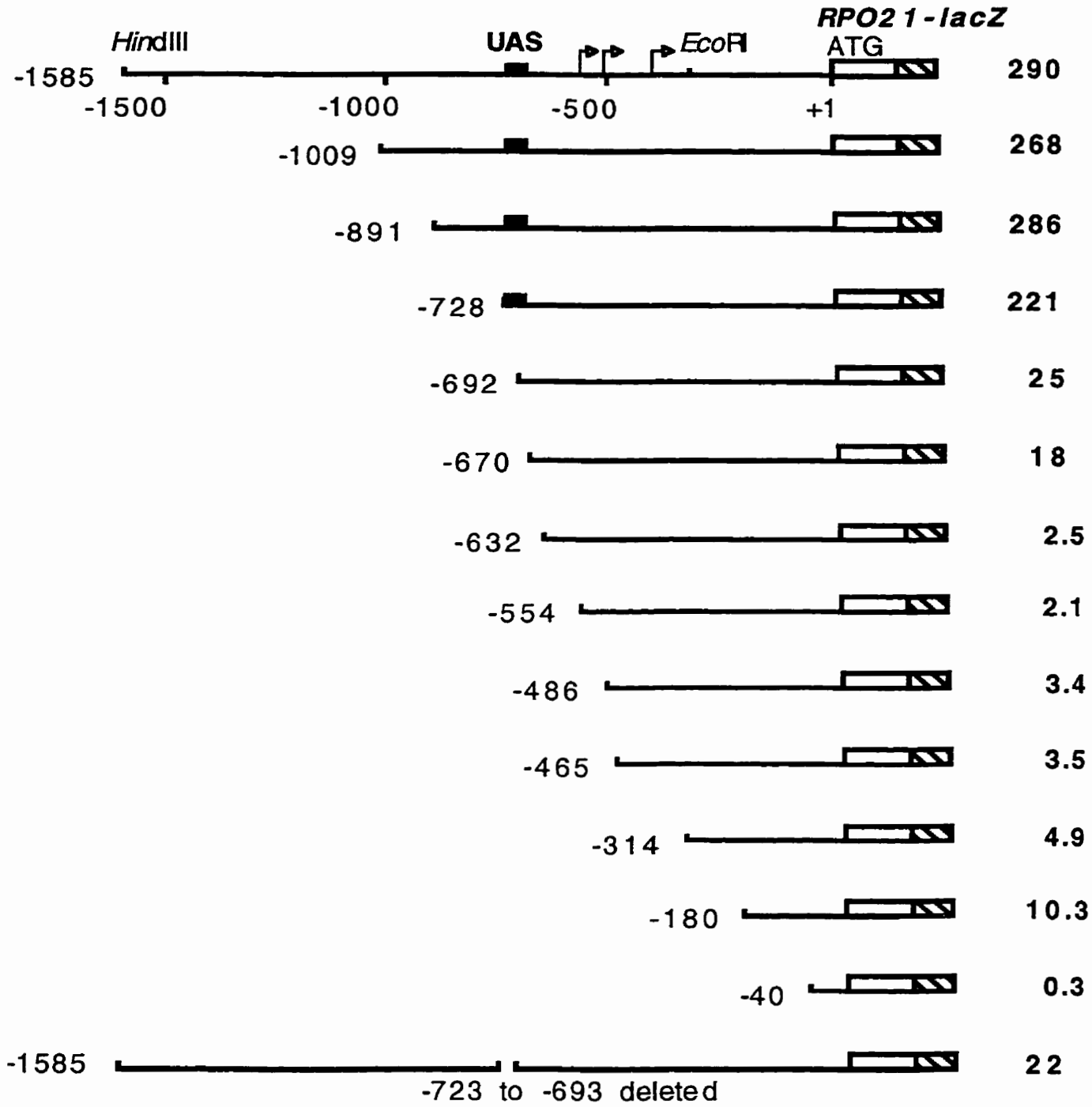
The *RPO21*-upstream region and various constructs were sequenced by the chain-termination method(Sanger, *et al.* 1977), using a combination of promoter deletions and primers that had been synthesized on the basis of the *RPO21* or *RPO22* sequences. Single- or double-stranded DNA templates were used.

RESULTS

Deletion and sequence analyses of the *RPO21*-upstream region

The transcripts expressed from *RPO21* have unusually long leader sequences with transcriptional start sites at -565, -510, and -400 (the A of the initiation codon is defined as +1) (Nonet, *et al.* 1987a). In order to locate DNA sequence elements in the upstream region of *RPO21* that are important for *RPO21* expression, a plasmid (pYF1495) was constructed that contains an in-frame fusion of *RPO21* to the *lacZ* gene of *E. coli*. In this plasmid approximately 1600 bp of *RPO21*-upstream sequences as well as the first 57 codons of the open-reading frame (ORF) are fused to *lacZ* (Figure 3.1). The upstream sequences used in constructing this plasmid probably contain all sequences that are important for the expression of *RPO21*, since they include the transcriptional start site furthest from the initiation codon as well as an additional 1000 bp of upstream sequence. A series of 5'-end deletions was made in the *RPO21*-upstream region in pYF1495 and the resulting plasmids were introduced into the wild-type yeast strain, W303-1a. β–galactosidase assays performed on the transformants indicated that deletion of sequences upstream of position -728 did not have a significant effect on the expression of *RPO21-lacZ* (Figure 3.1).

Figure 3.1. Deletion analysis of the promoter of *RPO21*. A 1585 bp fragment of *RPO21*-upstream sequences as well as the first 57 codons of the *RPO21* ORF was fused in-frame to *lacZ*. A black box indicates the position of the *RPO21* UAS (see text). Right-angled arrows show the locations of transcriptional start sites as determined by S1-nuclease analysis (Laurent and Carlson 1992). A series of 5'-end deletions was created in the upstream region of the fusion gene and tested for β-galactosidase activity in a wild-type yeast strain (W303-1a). Numbers on the left side of each of the diagrammed constructs indicate the coordinate (the A of the initiation codon is +1) of the 5'-end of each deletion. The bottom line shows a construct with an internal deletion of the indicated base pairs. Numbers to the right of each construct indicate the amount of β-galactosidase activity (in Miller units) measured in strains bearing the construct. Values are the mean of determinations from at least three independent cultures. The standard deviation for each value was less than 20%.


RPO21-lacZ
HindIII
UAS
EcoRI
ATG
-1585
-1500
-1000
-500
+1
290
-1009
268
-891
286
-728
221
-692
25
-670
18
-632
2.5
-554
2.1
-486
3.4
-465
3.5
-314
4.9
-180
10.3
-40
0.3
-1585
22
-723 to -693 deleted

The sequences downstream of -315, including the *RPO21* ORF, have been determined previously by Allison, *et al.* (Allison, *et al.* 1985). The DNA sequence of the region from -1585 to -315 was determined (data not shown) and indicated an ORF extending from -1557 to -817. The presence of the ORF is consistent with my observations that these sequences do not have a role in the expression of *RPO21*.

In contrast, a further deletion to -692 resulted in a 10-fold decrease in expression (Figure 3.1). Sequences from -728 to -692 contain all or part of three putative binding sites for yeast transcription factors (Figure 3.2A TOP). The two 5'-most elements match closely the consensus sequence for binding sites of Reb1p (Grf2p) (Chasman, *et al.* 1990). The third, and 3'-most element, matches closely the consensus sequence for a binding site for Abf1p (Dorsman, *et al.* 1990). I shall refer to the sequences that encompass these three putative transcription-factor-binding sites as the *RPO21* UAS.

Deleting sequences up to -632 resulted in an additional 10-fold decrease in gene expression. Removal of 38 bp from the 5'-end of *RPO21-lacZ* that has 670 bp of upstream sequences resulted in a seven-fold decrease in expression (Figure 3.1, compare lines 6 and 7). The removed sequences are part of a region (-674 to -622), downstream of the UAS, which is rich in thymidine residues (30 of 52). The sequences spanning the region of the UAS and transcriptional start sites were scanned for potential binding sites for the TATA-binding component of the general transcription factor, TFIID. No matches to the consensus binding site were found, although the sequence is rich in adenine and thymidine residues. I conclude that sequences downstream of -728 are sufficient for the full expression of *RPO21*, and that two regions are important for *RPO21* expression: (i) the *RPO21* UAS, composed of two putative Reb1p-binding sites and one putative Abf1p-binding site; (ii) a T-rich sequence downstream of this UAS.

In order to test whether sequences upstream of position -728 act redundantly with the sequences between -728 and -692, a plasmid was constructed that carries the entire 1585 bp *RPO21*-upstream sequence fused to *lacZ*, except that sequences from -723 to -693 inclusive were deleted and replaced by a *Hin*dIII linker (Figure 3.1, *rpo21-ΔUAS* in Figure 3.2A). A strain bearing this plasmid showed a 10-fold decrease in *RPO21-lacZ* expression compared to one carrying the wild-type promoter; the reduction in expression was comparable to that seen with a deletion up to -692. Therefore, removal of sequences from -723 to -693 is sufficient to cause a 10-fold decrease in *RPO21* expression and these sequences are necessary for full expression of *RPO21*.

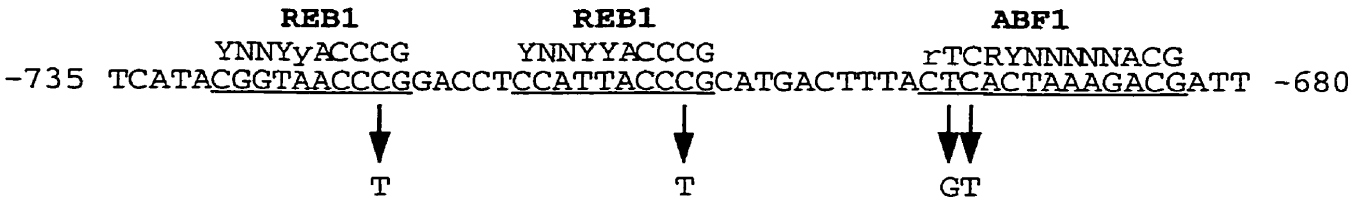
Mutational analysis of the *RPO21* UAS

Site-directed mutagenesis was used to introduce substitutions in the *RPO21* UAS (Figure 3.2). Plasmids were constructed that contain 1585 bp of *RPO21*-upstream region with mutations in the three elements of the UAS (Figure 3.2B, line h), mutations in any single site (Figure 3.2B, lines b-d) or mutations in any two of the sites (Figure 3.2B, lines e-g). The chosen mutations (Figure 3.2A, TOP) have been shown in other Reb1p- and Abf1p-binding sites (Chasman, *et al.* 1990; Della Seta, *et al.* 1990b; Halfter, *et al.* 1989) to reduce protein binding *in vitro* or transcriptional activation activity *in vivo*.

When mutations were introduced into all three sites (Figure 3.2B, line h), a 10-fold decrease in expression was observed, equal to the decrease observed with a deletion that removed all or part of the three elements that make up the UAS (Figure 3.2B, line i). When any one of the three elements was wild-type, and the other two were mutated, 50% to 70% of wild-type activity was observed (Figure 3.2B, lines e, f and g). Finally, activation of transcription by UAS sequences with mutations in any single element (the other two being wild-type) was at levels that ranged from 70% to

Figure 3.2. Mutational analysis of the *RPO21* UAS. (A) The consensus binding sites of Reb1p and Abf1p are indicated above the sequence of the *RPO21* UAS. Lower case letters indicate variations from the consenses. Nucleotide substitutions introduced into the *RPO21* UAS are shown below the sequence (Figure 3.2A, TOP). The sequences that were removed, as well as 12 bp added in an internal deletion in the *RPO21* UAS (*rpo21-ΔUAS*), are indicated (Figure 3.2A, BOTTOM). (B) Line (a) is a representation of the wild-type *RPO21* UAS. In subsequent lines (b-h) the absence of a black box indicates the introduction of nucleotide substitutions at the respective site(s). Line (i) shows the internal deletion of the *RPO21* UAS. Line (j) represents a UAS-less *CYC1-lacZ* fusion gene with no insert upstream of the TATA box. The first column of numbers ("*RPO21*") shows the levels of β-galactosidase activity due to plasmids bearing 1585 bp of *RPO21*-upstream sequences with the indicated mutations in the *RPO21* UAS. The second column of numbers ("forward") shows the levels of β-galactosidase activity due to plasmids bearing a UAS-less *CYC1-lacZ* fusion gene with 79 bp inserts bearing the *RPO21* UAS cloned 12 bp upstream of the 5'-most TATA box of *CYC1*. The last column ("reverse") indicates the activity of a *CYC1-lacZ* fusion with the *RPO21* UAS inserted upstream in the opposite orientation as in the upstream region of *RPO21*.

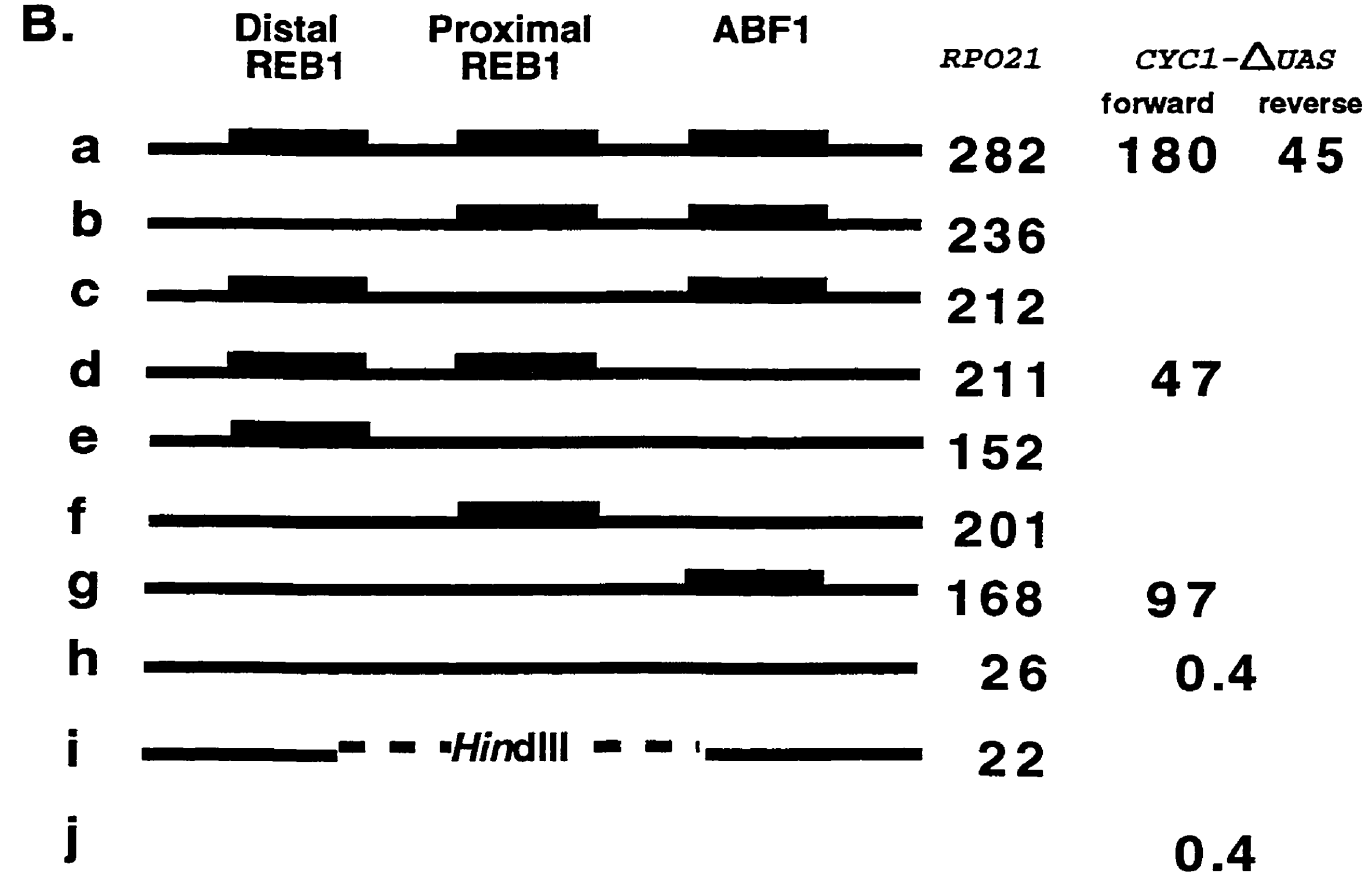
A.



rpo21- Δ*UAS*

-735 TCATACGGTAAC.....gcaagcttgcgt.....ACTAAAGACGATT -680
*Hin*dIII

B.



85% of wild-type. These data suggest that the *RPO21* UAS is composed of three distinct functional elements, all of which are necessary for full activity and all of which are partially redundant.

Activity of the *RPO21* UAS in a heterologous promoter

A DNA fragment carrying the *RPO21* UAS was inserted in a position upstream of the *CYC1* basal promoter and start sites of a *CYC1-lacZ* fusion gene devoid of other upstream-activating sequences. The DNA fragment used in these experiments spans nucleotides -740 to -674 and contains the two putative Reb1p-sites and the putative Abf1p-binding site.

Insertion of the wild-type *RPO21* UAS stimulated expression by greater than 400-fold over background (Figure 3.2B; compare line [a] under "forward" to line [j]). When the UAS was inserted in the reverse orientation, expression was enhanced by greater than 100-fold compared to basal level (Figure 3.2B; compare line [a] under "reverse" to line [j]). DNA sequences that are able to increase the expression of another promoter in an orientation-independent manner meet the criteria of upstream-activating sequences (Struhl 1989); therefore, sequences from -740 to -674 in the *RPO21*-upstream region behave as a *bona fide* UAS.

I tested whether the contribution of the putative Reb1p- and Abf1p-binding sites was the same in the context of the *CYC1* promoter as in the *RPO21* promoter. The expression was determined of *RPO21* UAS-*CYC1-lacZ* fusion genes with mutations in either the single putative Abf1p-binding site (Figure 3.2B, row d) or in the two putative Reb1p-binding sites (Figure 3.2B, row g). A UAS with mutations in the putative Abf1p-binding site activated the reporter gene to 26% of the wild-type level, whereas a UAS with mutations in the two putative Reb1p-binding sites had 54% of wild-type activity. Mutations in all three sites reduced the activity of the UAS to background levels (Figure 3.2B, row h). Hence, in the context of the *CYC1* promoter, the combined

transcriptional activity due to individually acting components of the *RPO21* UAS was less than the activity of the UAS as a whole. Therefore, in contrast to their activity in the *RPO21* promoter, the elements do not act in a partially-redundant manner in the context of the *CYC1* promoter. A possible explanation for this behavior is discussed below.

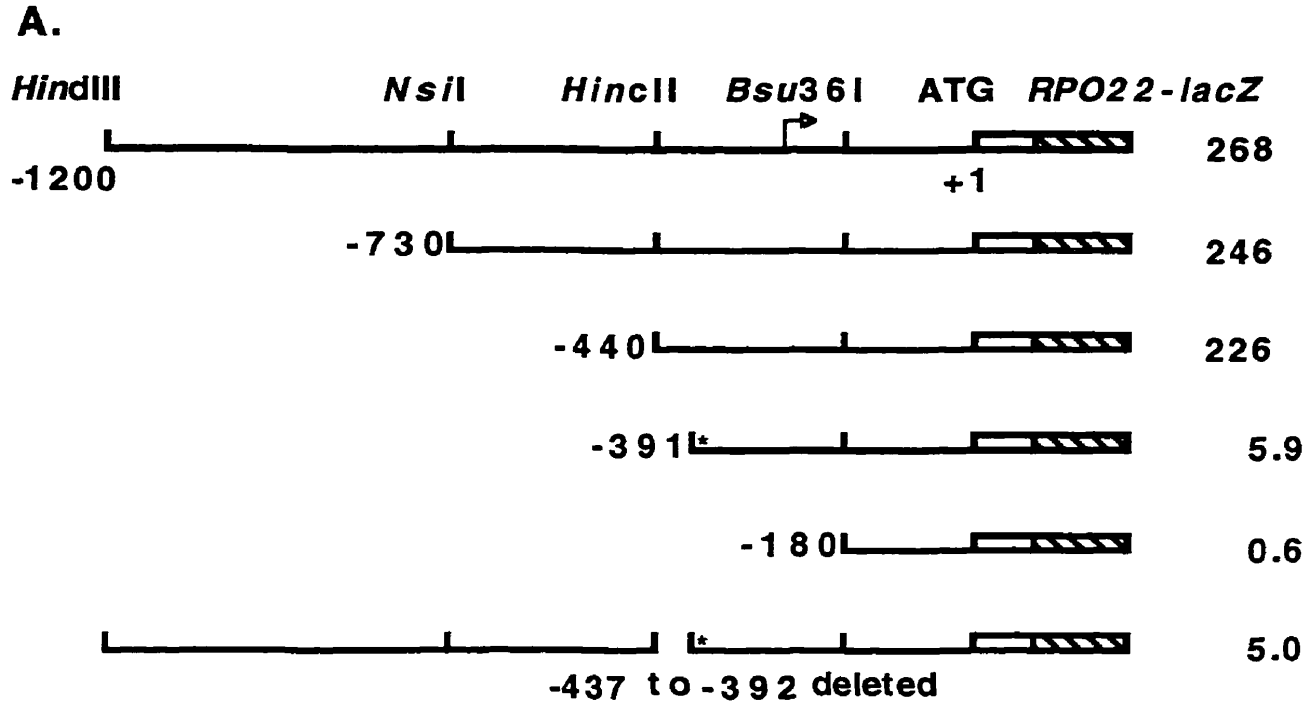
Deletion analysis of the *RPO22* promoter

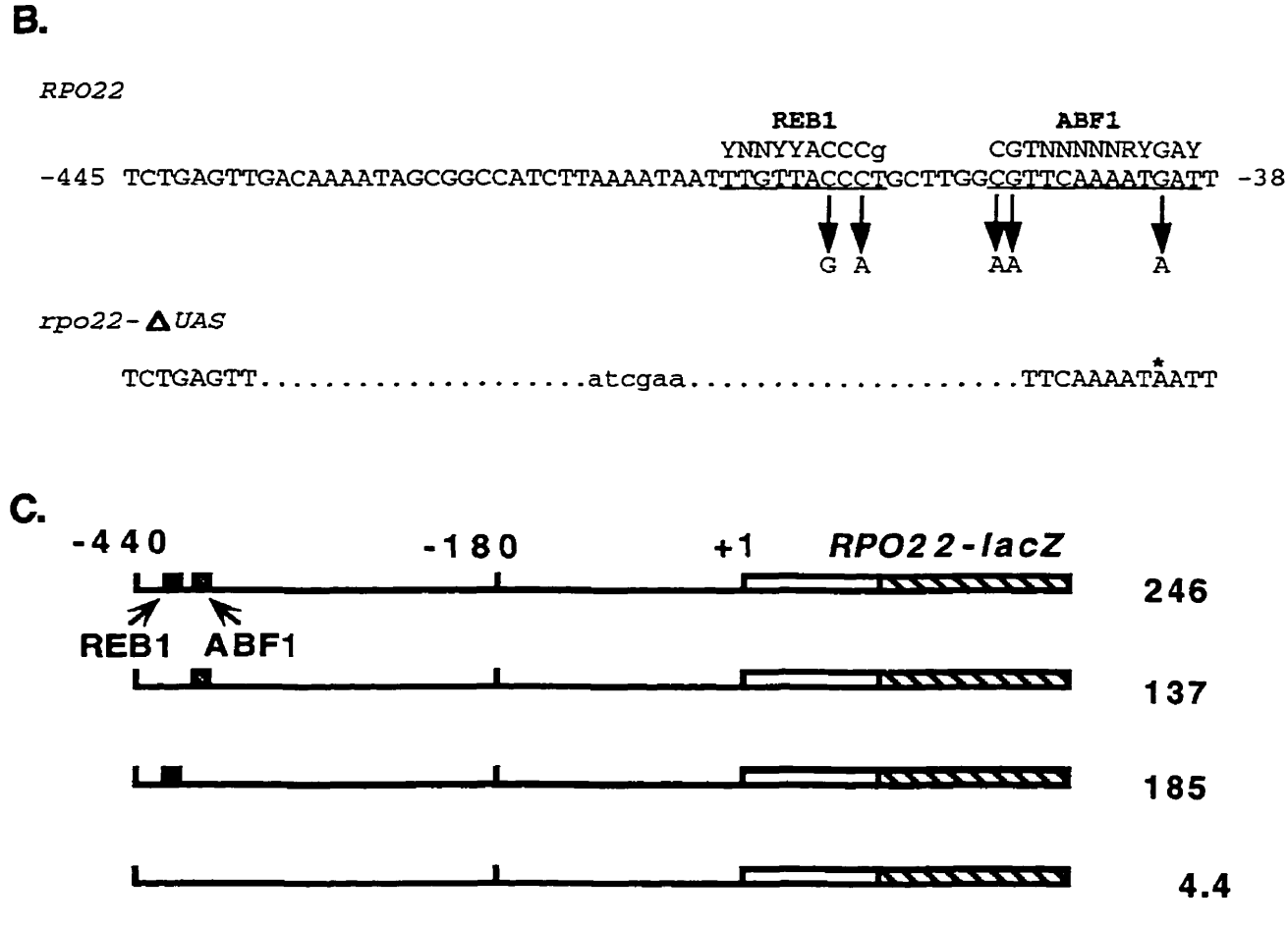
A plasmid (pYF1476) was constructed with 1200 bp of *RPO22*-upstream sequences as well as 27 codons of the ORF fused in-frame to *lacZ*. It has been shown by S1-nuclease analysis of *RPO22* transcripts that the start site of transcription is at -260 +/- 25 bp (the A of the initiation codon of *RPO22* is defined as +1) (Sweetser, *et al.* 1987).

β–galactosidase assays of a series of 5'-end deletions revealed that sequences upstream of -440 were not required for full expression (Figure 3.3A). The removal of 46 bp to position -391 resulted in more than a 30-fold decrease in expression. The DNA sequences of the *RPO22*-upstream region (Sweetser, *et al.* 1987) that lie between -410 and -380 resemble the consensus-binding sites for Reb1p and Abf1p (Figure 3.3B); sites with the same non-conformity with the Reb1p consensus have been shown previously to bind Reb1p (Carmen and Holland 1994; Chasman, *et al.* 1990). The sequences that match the Abf1p-consensus-binding site are in the opposite orientation to those in the UAS of *RPO21*. Since a deletion that removed all or part of the putative Reb1p- and Abf1p-binding sites resulted in decreased expression of *RPO22-lacZ*, this region is important for the expression of *RPO22*. I refer to this region as the *RPO22* UAS.

The T-rich region that lies downstream of the UAS (-380 to -345; 23 of 36) may be important for the expression of *RPO22*, since a deletion of 211 bp encompassing this region resulted in a 10-fold decrease in expression (Figure 3.3A). My results, however, do not exclude the possibility that removal of sequences immediately

Figure 3.3. Deletion and mutational analyses of the promoter of *RPO22*. (A) A 1200 bp fragment of upstream sequences and 27 codons of the ORF of *RPO22* were fused in-frame with *lacZ*. The location of unique restriction-enzyme sites used to construct 5'-end deletions in the *RPO22*-upstream region are shown. The right-angled arrow indicates the location (+/- 25 bp) of the start sites of transcription (Sweetser, *et al.* 1987) as determined by S1-nuclease analysis. The numbers on the right are β-galactosidase levels (Miller units) obtained from wild-type yeast strains (W303-1a) carrying each plasmid. (B) (TOP) The consensus binding sites of Reb1p and Abf1p are indicated above the sequence of the *RPO22* UAS. The lower case letter indicates a deviation from the consensus. Mutations introduced into the Reb1p and Abf1p sites are indicated below the sequence. (BOTTOM) Dotted lines indicate sequences deleted and lower case letters indicate sequences added to form *rpo22-ΔUAS*. The asterisk (also shown in the fourth and sixth lines of Figure 3.3A) indicates an additional nucleotide substitution (G to A at -383) that was introduced in the construction of these alleles. The substitution alters the binding site for Abf1p. (C) The top line is a diagram of an *RPO22-lacZ* fusion gene with 440 bp of upstream sequences that contains the wild-type UAS with the Reb1p- and Abf1p-binding sites indicated by filled boxes. The next three lines are diagrams of the same region except that nucleotide substitutions in the Reb1p- or Abf1p-binding sites are indicated by the absence of one or both boxes. The β-galactosidase activity obtained with strains containing these constructs is shown on the right.


A.
HindIII
NsiI
HincII
Bsu36I
ATG
RPO22-lacZ
-1200
+1
268
-730
246
-440
226
-391
5.9
-180
0.6
5.0
-437 to -392 deleted


B.
RPO22
REB1
YNNYYACCCg
ABF1
CGTNNNNNRYGAY
-445 TCTGAGTTGACAAAATAGCGGCCATCTTAAAATAATTTGTTACCCTGCTTGGCGTTCAAAATGATT -380
G A
AA
A
rpo22-ΔUAS
TCTGAGTT.....atcgaa.....TTCAAAATAATT
C.
-440
-180
+1
RPO22-lacZ
246
REB1
ABF1
137
185
4.4

downstream of the T-rich tract (-344 to -180) also contributes to the observed decrease in expression. Deletion of sequences from -437 to -392 (*rpo22-ΔUAS*, Figure 3.3B) resulted in a level of expression similar to that of a deletion of all sequences up to -391. Thus, these sequences (-437 to -392) are important for the expression of *RPO22*. A scan of sequences containing the UAS and start-site region of *RPO22* revealed no match to the consensus binding site of TFIID.

Nucleotide substitutions were made in either or both of the putative Reb1p- and Abf1p-binding sites (Figure 3.3B, TOP). The substitutions in the putative Reb1p-binding site alter highly-conserved residues in the consensus binding site (Liaw and Brandl 1994). The changes introduced into the putative Abf1-binding site have been shown to reduce binding of Abf1p *in vitro* (Goel and Pearlman 1988; Halfter, *et al.* 1989) and activation of transcription *in vivo* (Goel and Pearlman 1988).

Mutation of the putative Abf1p- and Reb1p-binding sites resulted in a 56-fold decrease in expression, similar to that observed with deletion of the *RPO22* UAS (Figure 3.3C). Mutation of only the putative Abf1p-binding site resulted in retention of 75% of wild-type activity; mutation of only the putative Reb1p-binding site yielded a UAS with 55% of wild-type activity. Together, these results show that: (i) both putative binding sites are necessary for full activity of the UAS; (ii) these elements are partially redundant in their effects on the expression of *RPO22*; (iii) the T-rich sequence may be important for the expression of *RPO22*.

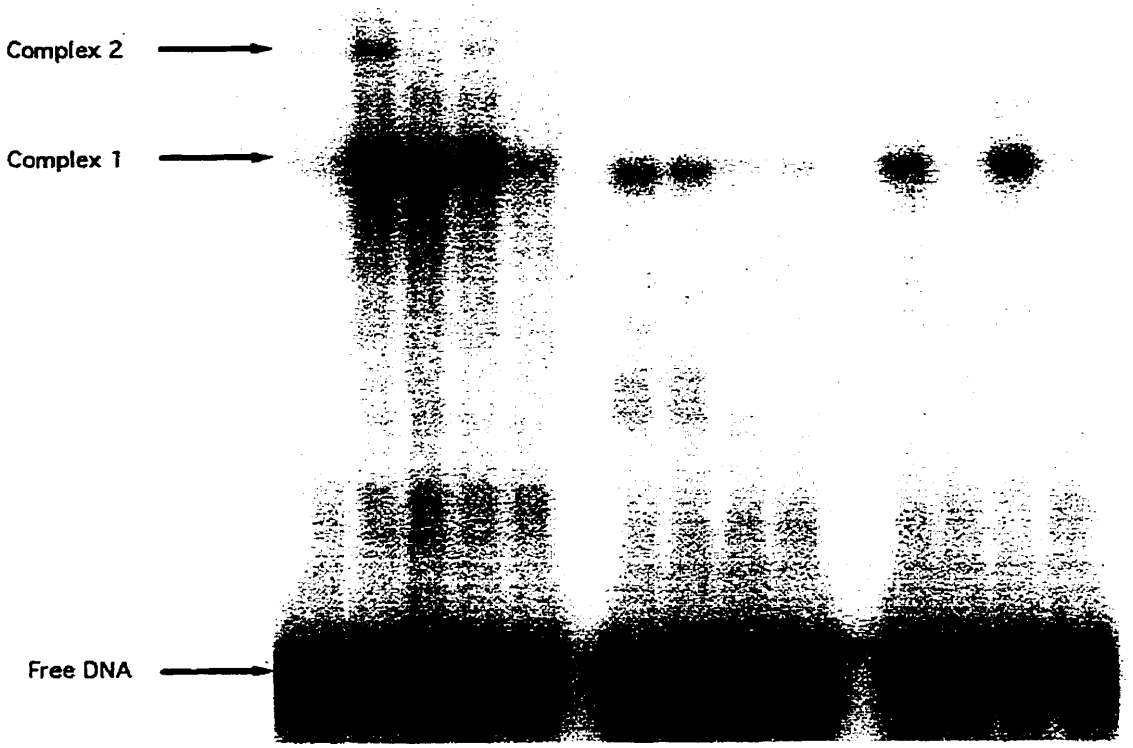
Electrophoretic-mobility-shift-assay (EMSA) analysis of the *RPO21* UAS

DNA fragments derived from the promoters of *RPO21* and *RPO22* were used as probes or competing DNA in electrophoretic-mobility-shift assays (EMSA) (Fried and Crothers 1981). Incubation of a DNA fragment containing the wild-type *RPO21* UAS with a crude yeast whole-cell extract (WCE), revealed two DNA-protein complexes (complex 1 and 2, Figure 3.4A, lane 2). Mutation of the Abf1p-binding site (lane 3) or

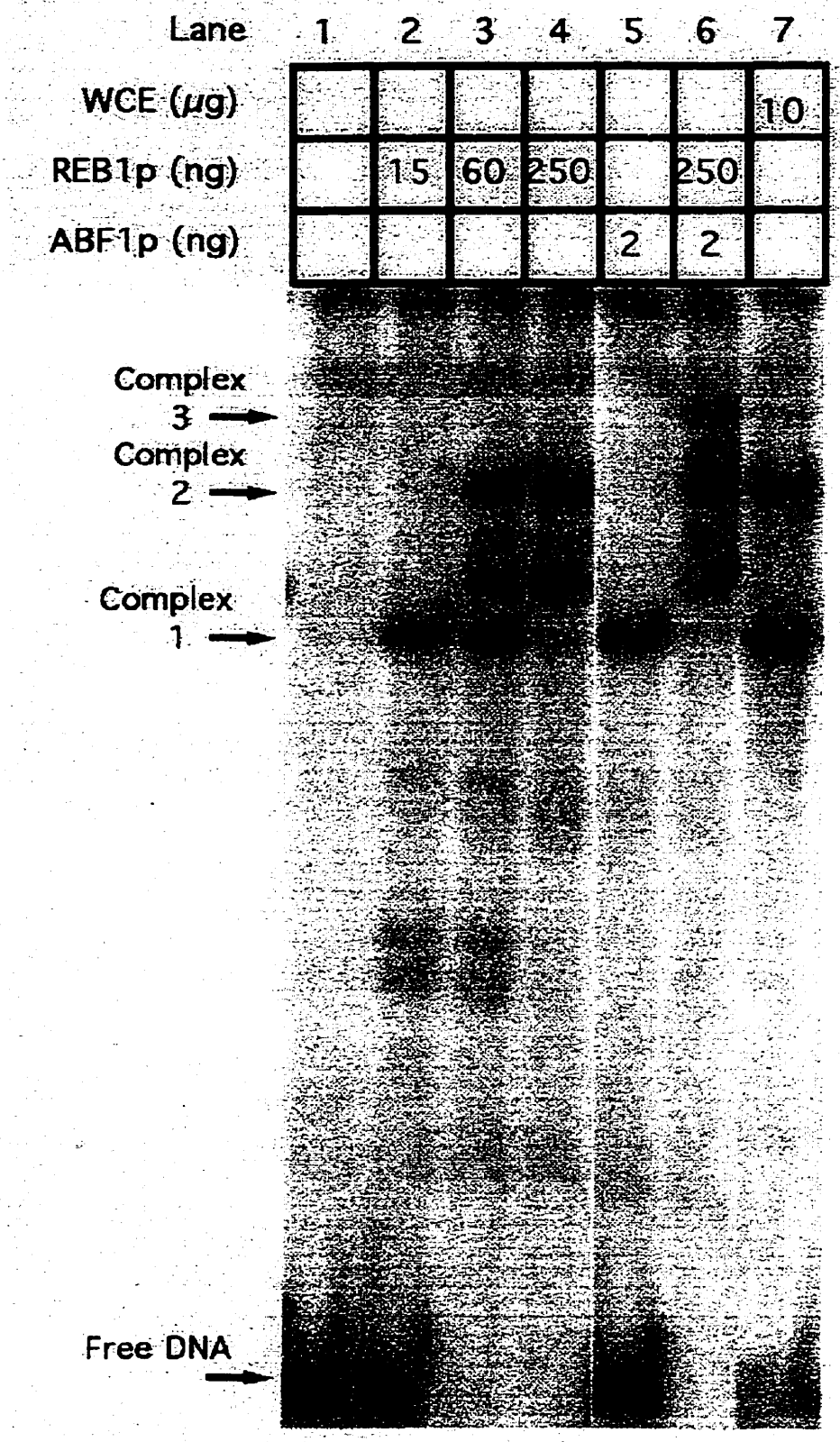
Figure 3.4. Electrophoretic-mobility-shift assays with the *RPO21* UAS. A) A 79 bp fragment of DNA containing the UAS of *RPO21* or altered versions of it containing either mutations in the two Reb1p-binding sites, mutations in the Abf1p-binding site or mutations in all three elements (Figure 3.2A) was labeled radioactively. The probes had similar specific activities (less than a 15% variation). The top three rows of the panel indicate whether the *RPO21* UAS probe for that lane (1.5 ng) contains the wild-type sequence (+) or nucleotide substitutions (-) in the indicated elements. The next three rows indicate the amount and source of proteins combined with the probe. WCE indicates yeast whole-cell extract. REB1p indicates an extract from *E. coli* over-expressing *REB1*. ABF1p indicates highly-purified Abf1p from yeast cells. Free DNA and two complexes are indicated by arrows. B) Multiple protein-DNA complexes form on the UAS of *RPO21*. A 79 bp DNA fragment (1 ng per lane) containing the wild-type *RPO21* UAS was labeled radioactively and combined with the proteins indicated at the top of the figure. The locations of three major complexes formed and the free DNA are indicated by arrows. Two complexes that migrated between complexes 1 and 2 are likely the result of the two Reb1p-binding sites being occupied by proteolytic fragments of Reb1p, or by one intact Reb1p molecule and a proteolytic fragment at the other site. These complexes are likely not formed only by intact Reb1p molecules since the complexes were not observed when a yeast whole-cell extract was used (lane 7).

A.

	Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Probe:	Distal REB1	+	+	+	-	-		+	+	-	-		+	+	-	-
	Proximal REB1	+	+	+	-	-		+	+	-	-		+	+	-	-
	ABF1	+	+	-	+	-		+	-	+	-		+	-	+	-
Protein:	WCE (μg)		5	5	5	5										
	REB1p (ng)							25	25	25	25					
	ABF1p (ng)												1	1	1	1



B.


Lane 1 2 3 4 5 6 7
WCE (μg) 10
REB1p (ng) 15 60 250 250
ABF1p (ng) 2 2
Complex 3
Complex 2
Complex 1
Free DNA

of the two Reb1p-binding sites (lane 4) caused a modest (less than two-fold) reduction in the amount of complex 1. In contrast, mutations in all three elements abolished complex 2 and reduced complex 1 by more than 10-fold (compare lanes 2 and 5). I conclude that the Reb1p and Abf1p elements of the UAS of *RPO21* are able to bind to proteins in a sequence-specific manner. Furthermore, mutations that reduce the activity of the UAS *in vivo* (Figure 3.2B) also reduce the strength of DNA-protein interactions *in vitro*.

An extract from an *E. coli* strain over-expressing *REB1* (provided by J. Warner) (Morrow, *et al.* 1993b) was used to test whether the *RPO21* UAS can bind Reb1p. With the wild-type *RPO21* UAS, a complex migrated at the same position as complex 1 (Figure 3.4A, compare lanes 2 and 7). Two additional faster-migrating complexes were also observed; these probably result from interactions between the probe and proteolytic fragments of Reb1p. Formation of complex 1 with a probe that carried mutations in both potential Reb1p-binding sites was reduced by 10-fold (lane 9). Mutations in the putative Abf1p-binding site had no significant effect on the interaction of Reb1p with the probe (lane 8) and a probe with mutations in all three elements showed a pattern similar to that with mutations in the two Reb1p-binding sites (lane 10). These data suggest that Reb1p can bind specifically *in vitro* to the UAS of *RPO21*. Although the mutations in the Reb1p sites did not completely abolish complex formation *in vitro*, the reduction in binding is sufficient to impair their function in the *RPO21* promoter *in vivo* (see Figure 3.2B). Residual binding of Reb1p to the mutated sites *in vivo* may account for the unexpectedly large activation of the *CYC1* promoter by the *RPO21* UAS carrying mutations in both Reb1p sites (leaving a single Abf1p site, Figure 3.2B; row g), perhaps through a synergistic interaction between Abf1p and Reb1p (see Discussion).

When wild-type *RPO21* UAS DNA was combined with purified Abf1p (Buchman and Kornberg 1990) a single major complex was observed that migrated in a position

corresponding to complex 1 (Figure 3.4A, compare lanes 2 and 12) and this was reduced 15-fold by mutations in the putative Abf1p-binding site (lane 13). Mutations in the two Reb1p-binding sites showed no effect (lane 14), while mutations in all three sites showed a similar pattern to that seen with mutations in only the Abf1p site (lane 15). These data suggest that Abf1p interacts specifically with the UAS of *RPO21*. Mutations that disrupt the function of the element *in vivo* (Figure 3.2B), also disrupt the interaction of Abf1p with the UAS *in vitro*.

The suggestion that the UAS of *RPO21* contains two Reb1p-binding sites was tested by adding to a wild-type *RPO21* UAS probe increasing amounts of extract from *E. coli* expressing *REB1*. At low levels of Reb1p, a single major complex (complex 1) was seen, which was chased into the slower-migrating complex 2 with the addition of increasing Reb1p concentration (Figure 3.4B, lanes 2-4). This complex migrated to the same position as complex 2 (Figure 3.4A, lane 2 and Figure 3.4B, lane 7). I conclude that the *RPO21* UAS has two binding sites for Reb1p.

The observation that elements of the *RPO21* UAS are partially redundant *in vivo* (Figure 3.2B) may reflect the fact that only a subset of the three sites is occupied at one time. Radioactively-labeled wild-type *RPO21* UAS was incubated with purified Abf1p and an extract from *E. coli* expressing *REB1* in order to test this possibility. The presence of a third complex (complex 3, Figure 3.4B, lane 6) at a higher gel position suggested that all three sites in the UAS can be occupied simultaneously *in vitro*. Complex 3 was also detected with a yeast whole-cell extract (lane 7).

These results may indicate that complex 1, which is formed with a yeast whole-cell extract, is a mixture of two complexes, each of which includes one molecule of Abf1p or one molecule of Reb1p. This hypothesis is supported by the observation that complex 1 is reduced by the introduction of mutations in all three binding sites (Figure 3.4A, lane 5). Similarly, complex 2 probably contains a mixture of probe bound either to two molecules of Reb1p, or to one molecule of Abf1p and one of Reb1p. This

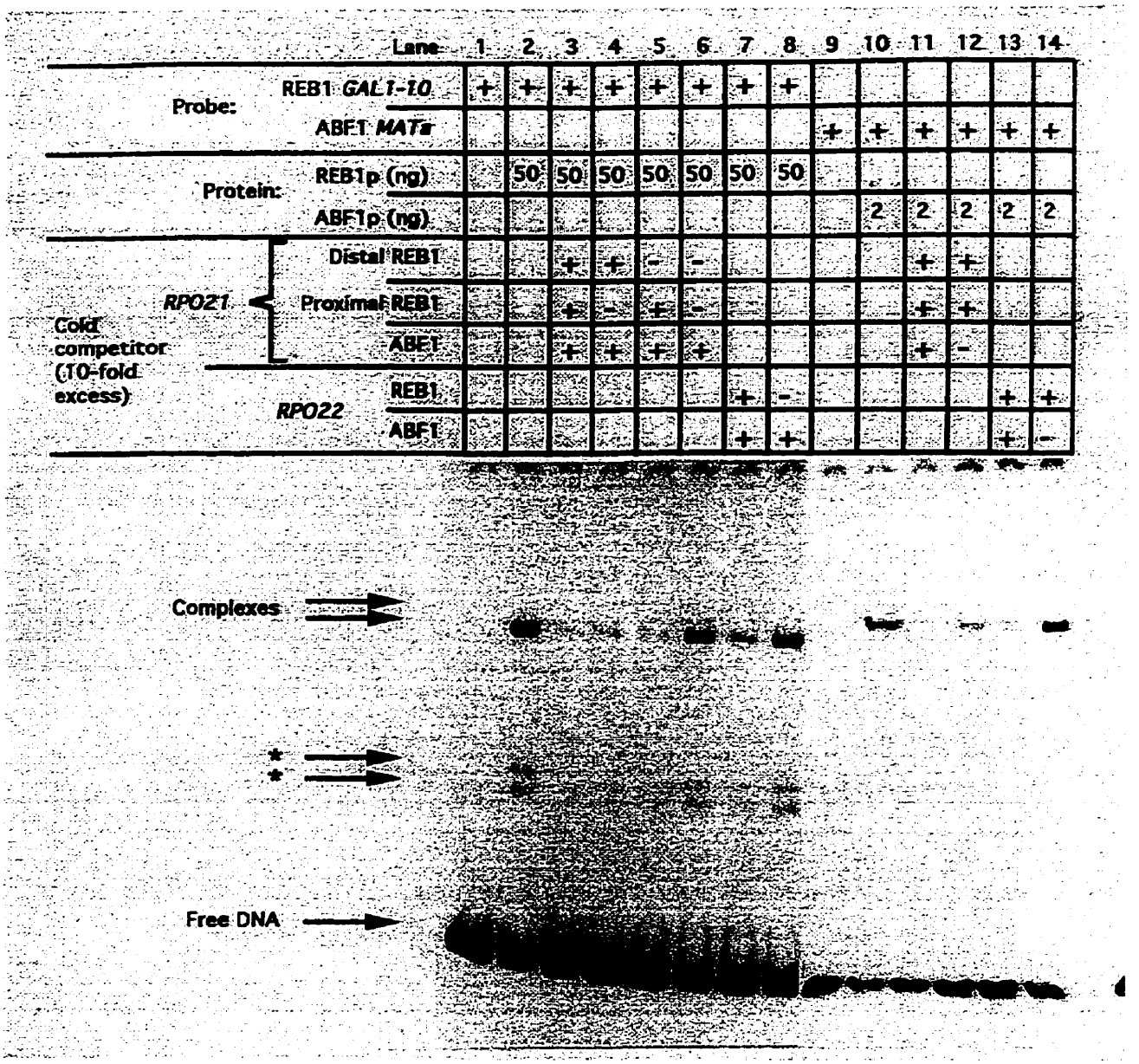
hypothesis is supported by the observation of a reduction in complex 2 when either the Abf1p-binding site (Figure 3.4A, lane 3) or both of the Reb1p-binding sites (Figure 3.4A, lane 4) are mutated. Finally, complex 3 (Figure 3.4B, lane 7) may represent the *RPO21* UAS probe bound to two molecules of Reb1p and one molecule of Abf1p. Regardless of the exact composition of each complex, the formation of three complexes is clear and these complexes are abolished by mutations that have been shown to reduce the binding of Abf1p and Reb1p. Thus, I conclude that the *RPO21* UAS contains two sites that bind Reb1p and one site that binds Abf1p.

Competition of Reb1p- and Abf1p-DNA complexes with the *RPO21* UAS and *RPO22* UAS

Reb1p was combined with a radioactively-labeled DNA fragment containing a known Reb1p-binding site from the *GAL1-GAL10* intergenic region (Liaw and Brandl 1994); DNA fragments containing the UASs of *RPO21* and *RPO22* were used as non-labeled competitors. As expected, a single major complex was formed, which was reduced 15-fold by the addition of a 10-fold molar excess of non-labeled wild-type *RPO21* UAS (Figure 3.5, compare lanes 2 and 3). Non-labeled competitor carrying mutations in both Reb1p-binding sites reduced the amount of complex by less than two-fold (lane 6). Competitors with a mutation in only one Reb1p-binding site (either one) were almost as effective as the wild-type UAS, suggesting that both sites are able to bind Reb1p (lanes 4 and 5). A 10-fold molar excess of a DNA fragment with the wild-type UAS of *RPO22* was able to compete for binding of Reb1p to the Reb1p-binding site of *GAL1-GAL10* (Figure 3.5, lane 7); however, DNA with mutations in the putative Reb1p-binding site of *RPO22* failed to compete (lane 8).

These data suggest that the UAS of *RPO22* can interact with Reb1p *in vitro* in a sequence-specific manner, since mutations that impair the activity of the Reb1p site *in vivo* (Figure 3.3C) also reduce the ability of the site to interact with Reb1p *in vitro*.

Figure 3.5. Electrophoretic-mobility-shift assays with *RPO21* or *RPO22* UAS DNA as non-labeled competitors. The top two rows of the panel indicate the probe (1 ng) used (+) for that lane. The next two rows indicate the type and amount of protein combined with the probe. The bottom five rows indicate whether the non-labeled competitor for that lane contains the wild-type sequence (+) or nucleotide substitutions (-) in the indicated elements of either the *RPO21* or *RPO22* UAS. No symbol indicates no competitor added in that lane. Free DNA and two complexes are indicated by arrows. An asterisk with an arrow indicates a complex formed likely by an interaction of the probe with a proteolytic fragment of Reb1p (see text).


Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Probe:
REB1 GAL1-10 + + + + + + + +
ABF1 MATa + + + + + + + +
Protein:
REB1p (ng) 50 50 50 50 50 50 50 50
ABF1p (ng) 2 2 2 2 2
Cold competitor (100-fold excess):
RPO21
Distal REB1 + + - - + +
Proximal REB1 + - + - + +
ABF1 + + + + + -
RPO22
REB1 + - + -
ABF1 + + + -
Complexes
*
*
Free DNA

Competition experiments with a complex formed with purified Abf1p and a radioactively-labeled DNA fragment from *MAT*a (McBroom and Sadowski 1994) yielded similar results (Figure 3.5, lanes 10-14), suggesting that the *RPO22* UAS can interact specifically with Abf1p.

DISCUSSION

My results indicate that the expression of two genes, which encode subunits unique to RNAPII in *S. cerevisiae*, is controlled by similar *cis*-acting upstream elements. Mutation of two Reb1p-binding sites and an Abf1p-binding site results in a 10-fold decrease in *RPO21* expression, while mutation of a Reb1p-binding site and an Abf1p-binding site results in more than a 30-fold decrease in the expression of *RPO22*. In both genes, the UAS is immediately upstream of a T-rich sequence which, when removed, decreases expression by an additional 10-fold.

The similar elements in the UASs of *RPO21* and *RPO22* may serve to control coordinate synthesis of stoichiometric amounts of each subunit, which would minimize the wasteful expenditure of cellular energy associated with the production of one subunit in excess of others.

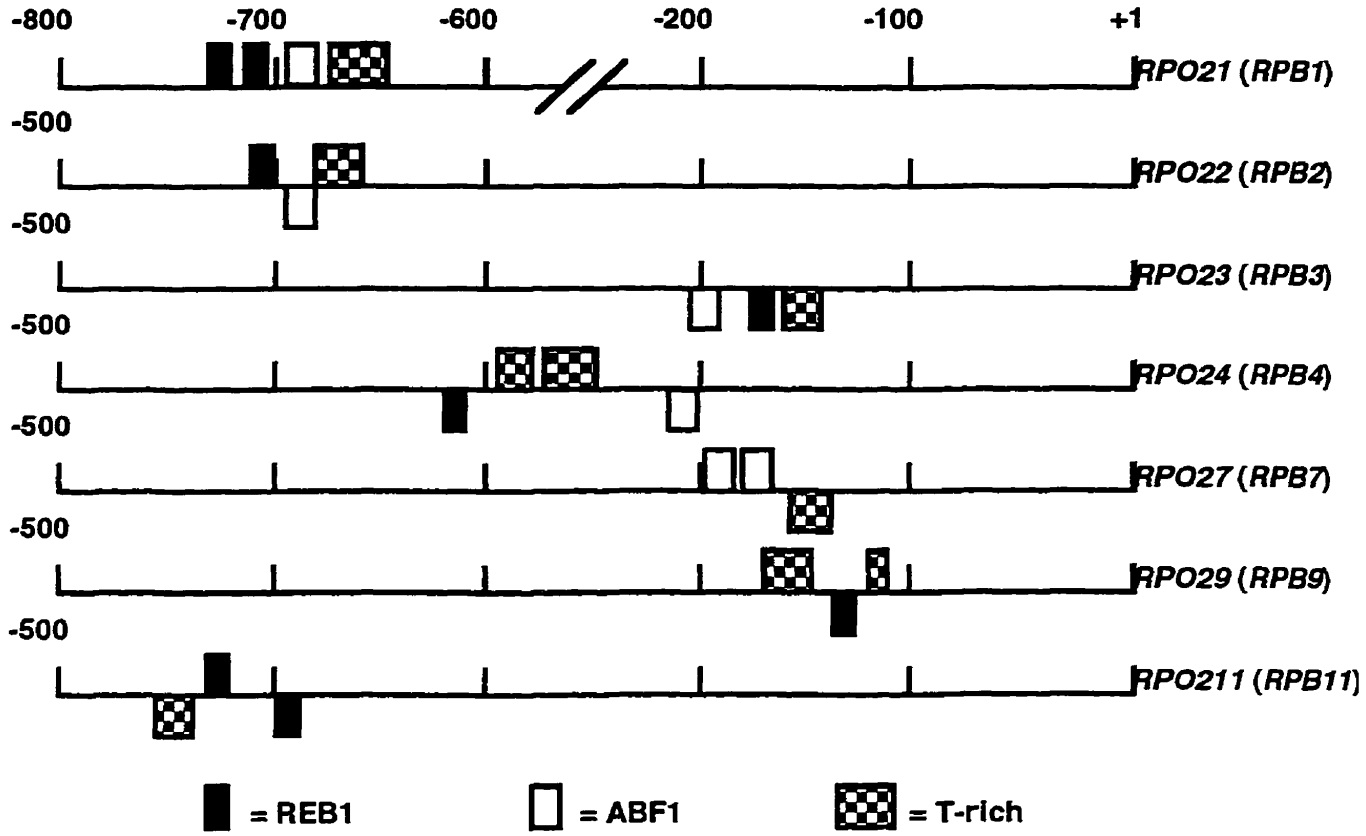
Regulation of *RPO21* and *RPO22* expression is similar in some ways to the regulation of genes that encode components of the ribosome, another multisubunit complex. Ribosomal protein (r-protein) genes are controlled by Abf1p or the related protein, Rap1p (Della Seta, *et al.* 1990a; Hamil, *et al.* 1988; Planta and Raue 1988) and are regulated through the *RAS*/cAMP signal-transduction pathway. The increase in mRNA transcribed from many r-protein genes due to a nutritional upshift depends on two kinds of upstream elements: a T-rich stretch (Gonçalves, *et al.* 1995) and a functional Rap1p- (Gonçalves, *et al.* 1995; Klein and Struhl 1994; Neuman-Silberberg, *et al.* 1995) or Abf1p- (Gonçalves, *et al.* 1995) binding site. The increase in gene expression also requires cAMP-dependent-protein kinases (Klein and Struhl

1994; Neuman-Silberberg, *et al.* 1995) and *de novo* protein synthesis (Neuman-Silberberg, *et al.* 1995), indicating that phosphorylation of Rap1p (Klein and Struhl 1994) may not be sufficient for the response. Abf1p also is phosphorylated when cells are shifted to a rich carbon source (Silve, *et al.* 1992), suggesting that this modification may have a role in the regulation of r-protein gene expression.

Abf1p (TAF, BAF) and Reb1p (Grf2p), members of a family of highly-abundant DNA-binding proteins (which also includes Rap1p; Shore 1994), are involved in many functions. Abf1p binds the promoters of many genes, including those which encode r-proteins (Della Seta, *et al.* 1990a; Hamil, *et al.* 1988) and proteins involved in glycolytic functions (Brindle, *et al.* 1990; Chambers, *et al.* 1990; Holland, *et al.* 1990) and is important for silencing at the *HMR* locus (Buchman, *et al.* 1988; Kimmerly, *et al.* 1988). Abf1p also has a role in DNA replication, since its binding is required for the function of some autonomously-replicating sequences (ARSs), and mutations in ABF1 that confer a temperature-sensitive phenotype on yeast result in mitotically unstable *ARS-CEN* plasmids at the semi-permissive temperature (Rhode, *et al.* 1992). Reb1p (Grf2p)-binding sites are important for the expression and termination of rRNA transcripts (Morrow, *et al.* 1989), have roles in the activation and repression of *ENO1* (Brindle, *et al.* 1990; Carmen and Holland 1994) and were shown to be important for creating a nucleosome-free *GAL1-10* intergenic region (Fedor, *et al.* 1988). Reb1p-binding sites stimulate transcription *in vivo* (Chasman, *et al.* 1990), but reduce the expression of *CYC1* when interposed between the UAS and TATA box (Wang, *et al.* 1990). Reb1p also binds to telomeres and centromeres (Chasman, *et al.* 1990).

How could these multi-purpose proteins function in coordinate expression of genes encoding subunits of RNAPII? As shown in Figure 3.6, subunits unique to RNAPII encode seven genes which (with the exception of *RPO29* (*RPB9*), in which only one potential Reb1p-binding site was found in the upstream sequences) have two

Figure 3.6. Comparison of the upstream regions of the genes encoding subunits unique to RNAPII (*i.e.*, are not present in RNAPI or RNAPIII). Upstream sequences of the indicated genes obtained from the GenBank database or from this study were searched for sequences that matched the consensus-binding sites for Abf1p or Reb1p and for sequences rich in thymidine residues. The bottom of the diagram indicates the type of site represented by the symbol. Symbols positioned above the line indicate that the consensus site is in the same orientation as the top strand while symbols below the line indicate the site is in the opposite orientation.


-800
-700
-600
-200
-100
+1
RPO21 (RPB1)
-500
RPO22 (RPB2)
-500
RPO23 (RPB3)
-500
RPO24 (RPB4)
-500
RPO27 (RPB7)
-500
RPO29 (RPB9)
-500
RPO211 (RPB11)
= REB1
= ABF1
= T-rich

or more potential-binding sites for Reb1p and/or Abf1p and one or two T-rich stretches. In all cases these elements cluster within approximately 100 bp of each other. Although the orientations and relative positions of the sites vary, the similarity of the sequences and relative proximity to each other suggest that they might serve as functional units for the maintenance of similar (coordinate) levels of gene expression under various growth conditions.

My results suggest that a T-rich sequence downstream of the *RPO21* UAS, and perhaps the one downstream of the *RPO22* UAS, are important for the expression of these genes. Homopolymeric dA:dT tracts show weak enhancement of the expression of a minimal *CYC1* promoter but can act synergistically with Abf1p, Reb1p and other (usually upstream) DNA-binding factor sites to enhance gene expression (Buchman and Kornberg 1990; Chasman, *et al.* 1990). The synergistic activity of the elements depends on the distance between them, since activation of gene expression falls rapidly when sequences are interposed between the elements (Chasman, *et al.* 1990). Since they are spaced closely, it is possible that the UASs of *RPO21* and *RPO22* also act in synergy with the downstream T-rich tracts.

My results indicate that an Abf1p-binding site located downstream of two mutated Reb1p-binding sites is able to enhance the expression of a *CYC1*-minimal promoter by 200-fold (Figure 3.2B). This level of enhancement is higher than reported previously for a single Abf1p site. Buchman and Kornberg (Buchman and Kornberg 1990) used a *CYC1-lacZ* reporter plasmid (pCZΔ) similar to the one used in this study, which also contained a cloning site 12 bp upstream of the 5'-most TATA site of the *CYC1* promoter. In a study of the effect of seven different single Abf1p-binding sites, Buchman and Kornberg observed a maximum stimulation of nine-fold (Table 2 in [17]). They did, however, report a synergistic enhancement of up to 56-fold with two adjacent Abf1p sites. It is possible that the large enhancement of transcription which I observe with a single Abf1p site is due to a synergistic interaction between this site and the two

mutated Reb1p sites, which may bind Reb1p at a significant level, albeit reduced compared to normal. In support of this hypothesis, there is residual binding of Reb1p to mutated sites *in vitro* (Figure 3.4A), even though the activity of these sites apparently is destroyed in the context of the promoter of *RPO21*.

I found that the elements of the *RPO21* UAS are partially redundant in their roles as activators of *RPO21* expression. The same elements, however, are not redundant when placed in the context of a minimal promoter. The *RPO21* UAS has a greater effect on the expression of the *CYC1* minimal promoter (400-fold) than it has in its normal context in the promoter of *RPO21* (10-fold). These apparent discrepancies may be due to the presence of other elements in the *RPO21* promoter. It has been suggested that homopolymeric dA:dT tracts may enhance the transcriptional-activating effect of other nearby elements that bind transcription factors by freeing the region of nucleosomes that may interfere with the binding of the factors (Iyer and Struhl 1995). If chromatin interference is a major impediment to the function of DNA-binding factors and a sequence in the promoter is present to counteract chromatin interference, then perhaps the effect of a single DNA-binding factor is not enhanced significantly by the binding of an additional factor. However, if a chromatin-modulating sequence is not present (for example, in a minimal promoter), then the binding of a second or third factor may stimulate the activity of the first factor in counteracting the negative effects of chromatin. This stimulation may be the result of enhanced binding or perhaps through increased interactions with general transcription factors that may also be adversely affected by chromatin (Workman and Buchman 1993).

In CHAPTER 2, I showed that a 10-fold reduction of the level of Rpo21p resulted in cells that, although viable, were slow growing, temperature-sensitive, and auxotrophic for inositol. In this chapter, I have shown that the deletion of the UAS of *RPO21* results in a 10-fold decrease in the expression of a *RPO21-lacZ* reporter gene. A simple inference from these data would be that a deletion of the UAS from the

upstream region of *RPO21* would result in viable cells with slow growth, temperature-sensitivity and inositol auxotrophy. Preliminary data indicate that deletion of the UASs of *RPO21* and *RPO22,* at their respective chromosomal loci, does result in viable yeast cells (data not shown). These strains are not, however, slow growing or auxotrophic for inositol. Both strains have a cold sensitive phenotype (data not shown). The deletion of the *RPO21* UAS does not result in as severe a set of phenotypes as a strain that has 10-fold less Rpo21p. This suggests that the UAS delete strain does not have the 10-fold less Rpo21p that might be predicted from the 10-fold reduction in the expression of *RPO21-lacZ* that is caused by a deletion of the UAS. One possible explanation for this apparent contradiction may be the compensatory effect of a feedback mechanism. Deletion of the UAS of *RPO21* may result in a decrease in the expression of *RPO21* which, in turn, stimulates, through an unknown mechanism, an increase in the expression of *RPO21.* In CHAPTER 4, I show evidence for the existence of such a feedback mechanism.

CHAPTER 4

Regulation of the Expression of the Largest Subunit of RNA Polymerase II in *Saccharomyces cerevisiae*.

Attribution of contributions:

The study presented in this chapter has not yet been published. Michael Kobor constructed the Fcp1p-shut off strain (see Figure 4.5A). Alia Ahmed did the northern blot experiment of Figure 4.4.

ABSTRACT

I investigated the regulation of the expression of *RPO21*, the gene encoding the largest subunit of RNAPII in yeast. I showed that the expression of a reporter gene under the control of *RPO21* upstream sequences was increased by 5-fold when the amount of Rpo21p in the cell was decreased by 10-fold. A similar response was observed when Fcp1p, a component of the CTD phosphatase, was depleted from the cell. The expression of *RPO22-lacZ* was not affected by a reduction in the amount of either Rpo21p or Fcp1p. The expression of an unrelated reporter gene (*PRP8-lacZ*) was not affected by the underproduction of Rpo21p, however, depletion of Fcp1p increased the expression of *PRP8-lacZ* by 6-fold. The expression of *RPO21* is controlled by both positive and negative transcriptional mechanisms and by a post-transcriptional mechanism. The underproduction of Rpo21p causes the UAS and T-rich region of *RPO21* to be less effective in their role in the activation of gene expression. The increase in expression of *RPO21-lacZ* upon depletion of Fcp1p is dependent on DNA sequences upstream of the translation-initiation sequence, indicating that DNA or RNA elements mediate the increase in *RPO21-lacZ* expression. I propose a model for the regulation of *RPO21* expression by the amount of Rpo21p and/or Fcp1p in the cell.

INTRODUCTION

RNA polymerase II (RNAPII) catalyzes the synthesis of messenger RNA (mRNA) and some snRNAs in the nucleus of eukaryotic cells. RNAPII is a multi-subunit enzyme; 12 separate genes encode its subunits in the yeast, *Saccharomyces*

cerevisiae. Five of these genes encode subunits that are also components of RNAPI and RNAPIII (RNAP composition is reviewed in Archambault and Friesen 1993; Thuriaux and Sentenac 1992). The three largest subunits, encoded by *RPO21*, *RPO22* and *RPO23* (also called *RPB1*, *RPB2* and *RPB3*, respectively), have a high degree of similarity with the core subunits (β', β and α) of the single RNAP of prokaryotic cells (Allison, *et al.* 1985; Jokerst, *et al.* 1989; Kolodziej and Young 1989; Sweetser, *et al.* 1987). These subunits have been shown to have roles which, in the case of the two largest subunits, include the binding of DNA (Kontermann, *et al.* 1993; Nudler, *et al.* 1996; Zillig, *et al.* 1976), RNA (Gundelfinger 1983; Markovtsov, *et al.* 1996; Nudler, *et al.* 1998), and the initiating nucleotide substrate (Grachev, *et al.* 1989; Markovtsov, *et al.* 1996; Riva, *et al.* 1990; Severinov, *et al.* 1995b), as well as the formation of the catalytic site for RNA polymerization (Mustaev, *et al.* 1997). The third-largest subunit is essential for the assembly of the enzyme and, in the case of the prokaryotic enzyme, is also a contact site for transcriptional activators (Igarashi and Ishihama 1991; Ishihama 1990). The functions of the remaining subunits in the eukaryotic RNA polymerase are largely unknown, but these may participate in enzyme assembly (Rpo26p; Nouraini, *et al.* 1996a), in transcriptional initiation (Rpo24p and Rpo27p; Edwards, *et al.* 1991) or in modulating the process of elongation (Rpo29p; Awrey, *et al.* 1997).

Despite progress in elucidating the functional aspects of RNAPII, relatively little is known about the synthesis of this enzyme. I showed that the upstream regions of the genes encoding *RPO21* and *RPO22* each contain a UAS with a similar structure. These UASs, and T-rich regions directly downstream of them, are important for the normal expression of these genes (CHAPTER 3). In CHAPTER 2 I showed that underproduction of Rpo21p caused slow growth, temperature sensitivity and an inability to grow in the absence of inositol. In this study I use the strain that underproduces Rpo21p to test for feedback regulation of *RPO21*.

MATERIALS AND METHODS

Strains and Media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 4.1. Growth media were prepared and yeast transformations were performed as described (Hill, *et al.* 1991; Sherman, *et al.* 1986). All plasmids were propagated in *Escherichia coli* strain XL-Blue (Bullock, *et al.* 1987).

Table 4.1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype
YF1971[a]	*MATα pLEU2-RPO21 can1-100 his3-11,15 LEU2 trp1-1 ura3-1 ade2-1 ssd1-d2*
YF1974[a]	*MATα RPO21 can1-100 his3-11,15 LEU2 trp1-1 ura3-1 ade2-1 ssd1-d2*
YF2352[b]	*MATa trp1-1 ura3-52 leu2::PET56 ade2 (pRS314; TRP1 CEN/ARS)*
YF2353[b]	*MATa trp1-1 ura3-52 leu2::PET56 ade2 fcp1::ANB-URLF-FCP1 TRP1*

[a] Strains are congenic with W303-1B. Details of construction are in CHAPTER 2.

[b]Constructed by Michael S. Kobor. YF2352 is ZMY60 (Moqtaderi, *et al.* 1996) that contains pRS314.

Plasmids

DNA manipulations were performed essentially as described (Maniatis, *et al.* 1982). The plasmids used in this study are listed and described in Table 4.2.

Table 4.2 Plasmids used in this study.

Name	Description and construction[a]
pFL39	Yeast/*E. coli* shuttle vector containing *TRP1* and *CEN/ARS* sequences. This plasmid contains the polylinker region of pUC19 (Bonneaud, *et al.* 1991).
pDJ20	7 kb *Hin*dIII DNA fragment encompassing *RPO21* (-1583 to +5415) inserted at the *Hin*dIII site of pFL39. The *Eco*RI site of the polylinker was destroyed by end-filling.
pDJ15	6.6 kb *Eco*RI-*Hin*dIII fragment encompassing *RPO21* (-312 to +5415) inserted at the *Eco*RI and *Hin*dIII sites of pFL39.
YEp356R	Yeast/*E. coli* shuttle vector containing *URA3* and a 2μm origin of replication. This plasmid contains also *lacZ* with a polylinker upstream (Myers, *et al.* 1986).
YEp357R	same as YEp356R except polylinker is in a different register (Myers, *et al.* 1986).
pDJ46	*RPO21-lacZ* on *TRP1 CEN/ARS*. This plasmid contains *RPO21* sequences from -1583 to +660. A *Hin*dIII linker was introduced at the *Stu*I site that is in *URA3* downstream of *lacZ* in YEp356R. A 4 kb *Xba*I-*Hin*dIII DNA fragment that contains *lacZ* and a portion of *URA3* was subcloned from ~~the~~ this plasmid into the *Spe*I (at +660) and *Hin*dIII (at +5415) sites of pDJ20. The resulting plasmid is an in-frame fusion of *RPO21* (-1583 to +660) to *lacZ*.
pDJ48	*RPO21-lacZ* on *TRP1 CEN/ARS*. This plasmid contains *RPO21* sequences from -312 to +660. The same *Xba*I-*Hin*dIII fragment containing *lacZ* that was described for the construction of pDJ46 was subcloned into the *Spe*I and *Hin*dIII sites of pDJ15. The resulting plasmid is an in-frame fusion of *RPO21* (-312 to +660) to *lacZ*.
pDJ80	*RPO21-lacZ* on *URA3* 2μm plasmid. This plasmid contains *RPO21* sequences from -1583 to +171. A 1.8 kb *Hin*dIII-*Bam*HI fragment containing *RPO21* sequences from -1583 to +171 was subcloned into the same sites of YEp356R.

Table 4.2 Plasmids used in this study (continued).

Name	Description and construction[a]
pDJ81	*RPO22-lacZ* on *URA3* 2μm plasimd. This plasmid contains *RPO22* sequences from -1272 to +81. A 1.4 kb *Hin*dIII-*Bam*HI fragment containing *RPO22* sequences from -1272 to +81 was subcloned into the same sites of YEp356R. There are additional sequences (5' GGTCGACTCTAGA 3') between +81 of *RPO22* and the *Bam*HI site of the polylinker of YEp356R. These were from the polylinker of the plasmid from which the *RPO22* DNA fragment was obtained.
pDJ83	*PRP8-lacZ* on *URA3* 2μm plasmid. This plasmid contains *PRP8* sequences from -522 to +783. A 1.3 kb *Hin*dIII-*Bam*HI fragment containing *PRP8* sequences from -522 to +783 was subcloned into the same sites of YEp357R.
pDJ84	*RPO21-lacZ* on *URA3* 2μm plasimd. This plasmid contains *RPO21* sequences from -1583 to +660. A 2.2 kb *Hin*dIII-*Bam*HI fragment of pDJ46 containing *RPO21* sequences from -1583 to +660 was subcloned into the same sites of YEp356R.
pDJ86	*LEU2-RPO21-lacZ* on *URA3* 2μm plasimd. This plasmid is identical to pDJ84 except that: (i) sequences containing *RPO21* sequences from -1583 to -1 are replaced with *LEU2* sequences from -400 to -1. (ii) There are 45 additional bp of polylinker sequence upstream of the *LEU2* sequences that are not present in pDJ84.
pSN318	*RPO26-lacZ* on *URA3* 2μm plasimd. This plasmid contains *RPO26* sequences from -234 to +3. A 0.2 kb *Eco*RI-*Hin*dIII PCR product (restriction sites were from the primer sequences) containing *RPO26* sequences from --234 to +3 was subcloned into the same sites of YEp357 (same as YEp356R, except the polylinker is altered). This plasmid was constructed by Shahrzad Nouraini (unpublished results).
pDJ22	*CYC1-lacZ* on *URA3* 2μm plasmid. This plasmid contains *CYC1* sequences from 12 bp upstream of the TATA-box (-138) to +3 fused to *lacZ*. The construction of this plasmid has already been described in the Materials and Methods section of CHAPTER 3. There is a unique *Bgl*II cloning site located 12 bp upstream of the TATA-box.

Table 4.2 Plasmids used in this study (continued).

Name	Description and construction[a]
pYF1756	*RPO21* UAS and T-rich sequence cloned 12 bp upstream of *CYC1-lacZ* on *URA3* 2μm plasmid. The following sequence, from -738 to -637 of *RPO21*, as well as *Bam*HI restriction sites (underlined), 5' GGATCCGACTATCATACGGTAACCCGGACCTCCATT-ACCCGCATGACTTTACTCACTAAAGACGATTGTCGGTTTTAAACATTTTTTTTTTTTC-ATTTCGACCGTCGGGGATCC 3', was cloned into the *Bgl*II site of pDJ22.
pYF1591	*RPO21* UAS sequence cloned 12 bp upstream of *CYC1-lacZ* on *URA3* 2μm plasmid. The following sequence, from -738 to -672 of *RPO21*, as well as *Bam*HI restriction sites (underlined), 5' GGATCCGACTATCATACGGTAACCCGGACCTCCATTACCCGCATGA-CTTTACTCACTAAAGACGATTGTCGGTGGATCC 3', wss cloned into the *Bgl*II site of pDJ22.

[a] Plasmids were constructed by the author, unless indicated by reference. All numbers referring to sequences are relative to the ATG (+1) of the gene in question.

β-galactosidase assays

β-galactosidase activity was measured as described by Miller (1972). Cells were grown in selective medium at 30°C. For each measurement, β-galactosidase activity was determined on at least three independent cultures.

RNA blot analysis

Total RNA was isolated from yeast, separated on an agarose gel and transferred to a nylon membrane by Alia A. Ahmed as described (Maniatis, *et al.* 1982). I purified a 3.5

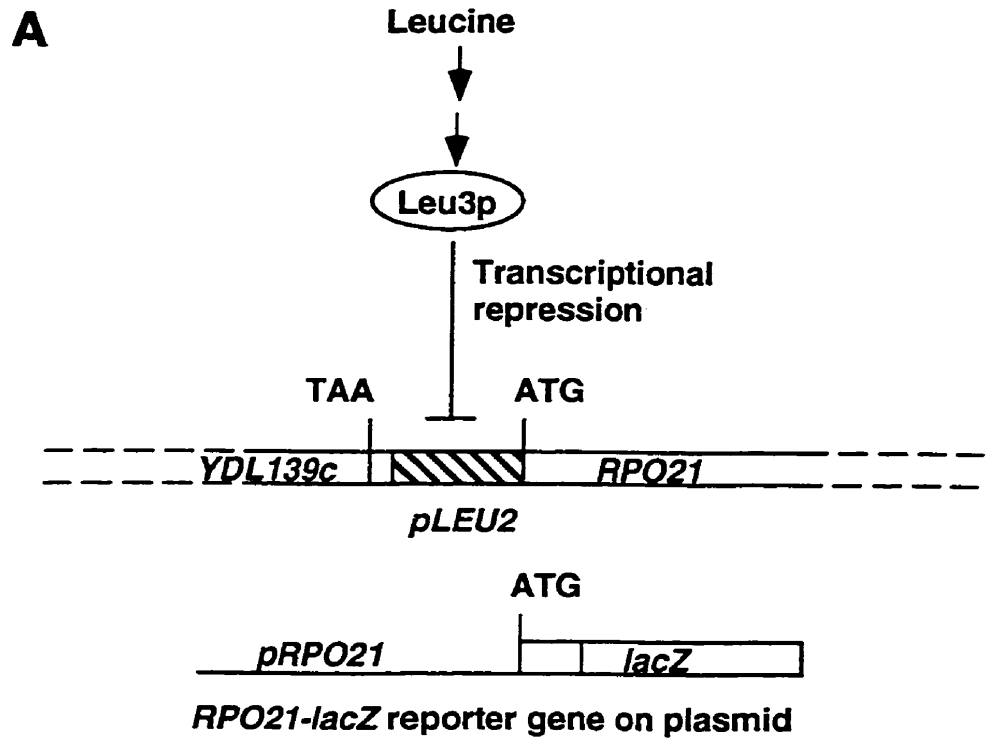
kbp *Eco*RI DNA restriction fragment from plasmid pYF862. The DNA contained the entire open reading frame of the *lacZ* gene from *E. coli*. It also contained some sequences of the *RPO21* gene (-312 to +171). Ms. Ahmed radioactively labelled this DNA, as well as a 144 bp fragment of DNA containing the U6 gene, by the random priming technique (Random primed DNA labeling kit; Boehringer Mannheim). Ms Ahmed used both probes simultaneously in a standard hybridization protocol with the membrane (Maniatis, *et al.* 1982). I quantified the signals that resulted from the northern blot using a phosphoimager and Imagequant software.

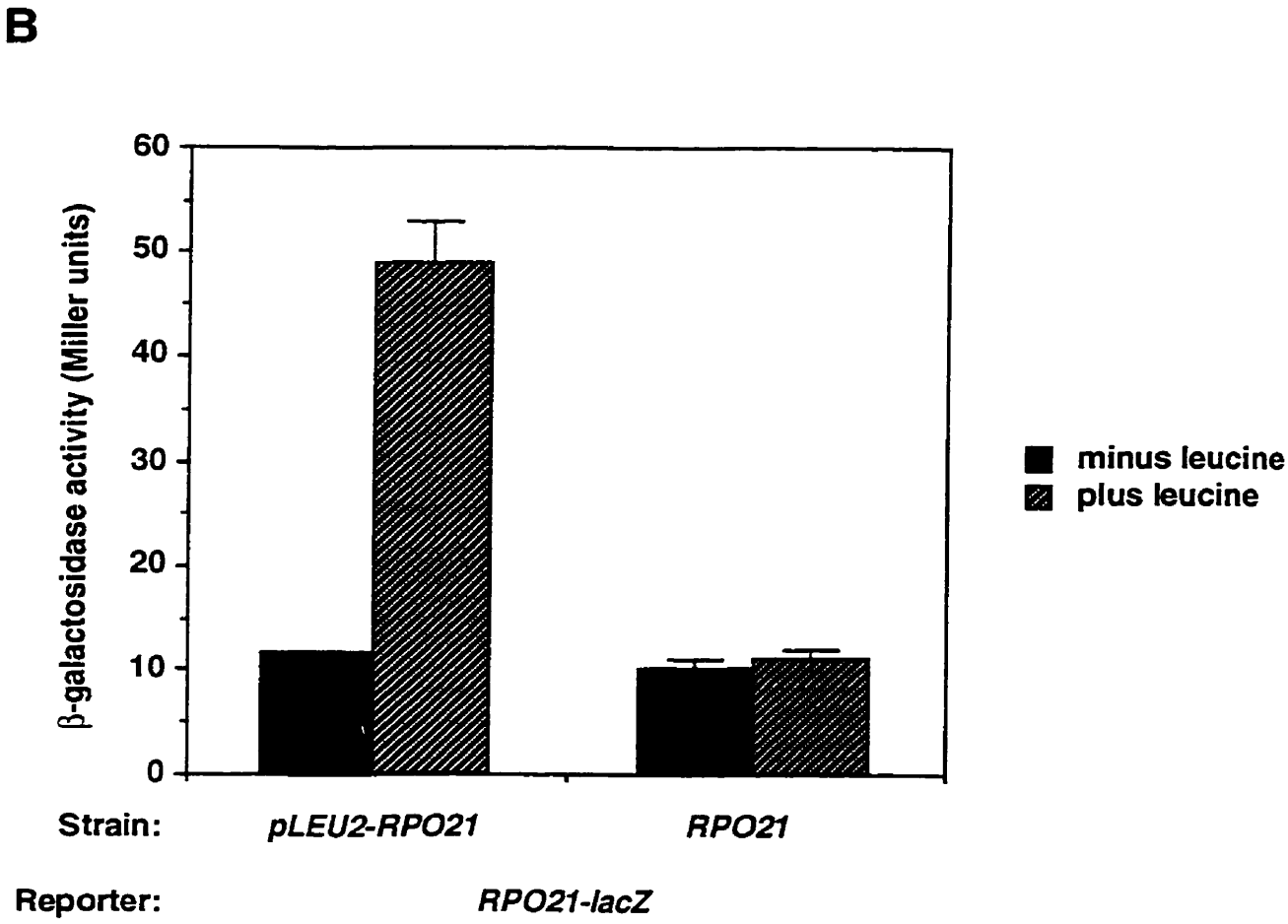
RESULTS

The effect of underproduction of Rpo21p on the expression of *RPO21-lacZ*.

I wished to uncover mechanisms that regulate the expression of *RPO21*. My approach to this problem was to use, as a reporter, a plasmid that contains the regulatory sequences and a small part of the ORF of *RPO21* fused to *lacZ* (CHAPTER 3). This plasmid was introduced into a yeast strain in which the amount of Rpo21p, the largest subunit of RNAPII, could be decreased. As described in CHAPTER 2, when *RPO21* expression is directed by the promoter and 5'-untranslated region of *LEU2*, the level of Rpo21p in cells can be reduced by ten-fold by the addition of leucine, isoleucine and threonine to the growth medium. The strain constructed for that study, YF1971, is a derivative of the wild-type strain, W303-1B, in which approximately 750 bp of DNA sequences upstream of the initiation codon of *RPO21* were replaced with 400 bp of sequences that control the expression of *LEU2* (Figure 4.1A). Growth of YF1971 in the presence of leucine, isoleucine and threonine results in a doubling time of 5.5 hours compared to the normal 2 hours (CHAPTER 2). The change in growth rate correlated with a decrease in the steady-state level of Rpo21p (CHAPTER 2).

Figure 4.1. The effect of underproduction of Rpo21p on the expression of *RPO21-lacZ*. (A) A diagram of the yeast strain (YF1971) used to detect regulation of *RPO21-lacZ*. Chromosomal *RPO21* upstream sequences from -1 to -721 (+1 is the A of the initiation codon) were replaced with upstream sequences of *LEU2* from -1 to -400 (see CHAPTER 2, Materials and Methods). All sequences known to affect the expression of *RPO21* were removed (see CHAPTER 3) up to ~90 bp from the stop codon of the hypothetical ORF (*YDL139c*) that is upstream of *RPO21*. Also indicated is the transcriptional repression (mediated by DNA-binding protein Leu3p) that results from addition of leucine, isoleucine and threonine to the growth medium. Finally, the reporter gene, *RPO21-lacZ*, carried on a high- or low-copy plasmid is also shown. (B) Levels of β-galactosidase activity (expressed in Miller units) were determined in YF1971 (*pLEU2-RPO21*) and YF1974 (*RPO21*). Both strains contained pDJ46, which is *RPO21-lacZ* on a low-copy plasmid. Strains were grown for 12 hours either in the absence or presence of 2 mM leucine, threonine and isoleucine (for the sake of brevity, these three amino acids will be referred to as "leucine" in this and subsequent figures). Each error bar indicates the standard deviation of three independent assays.

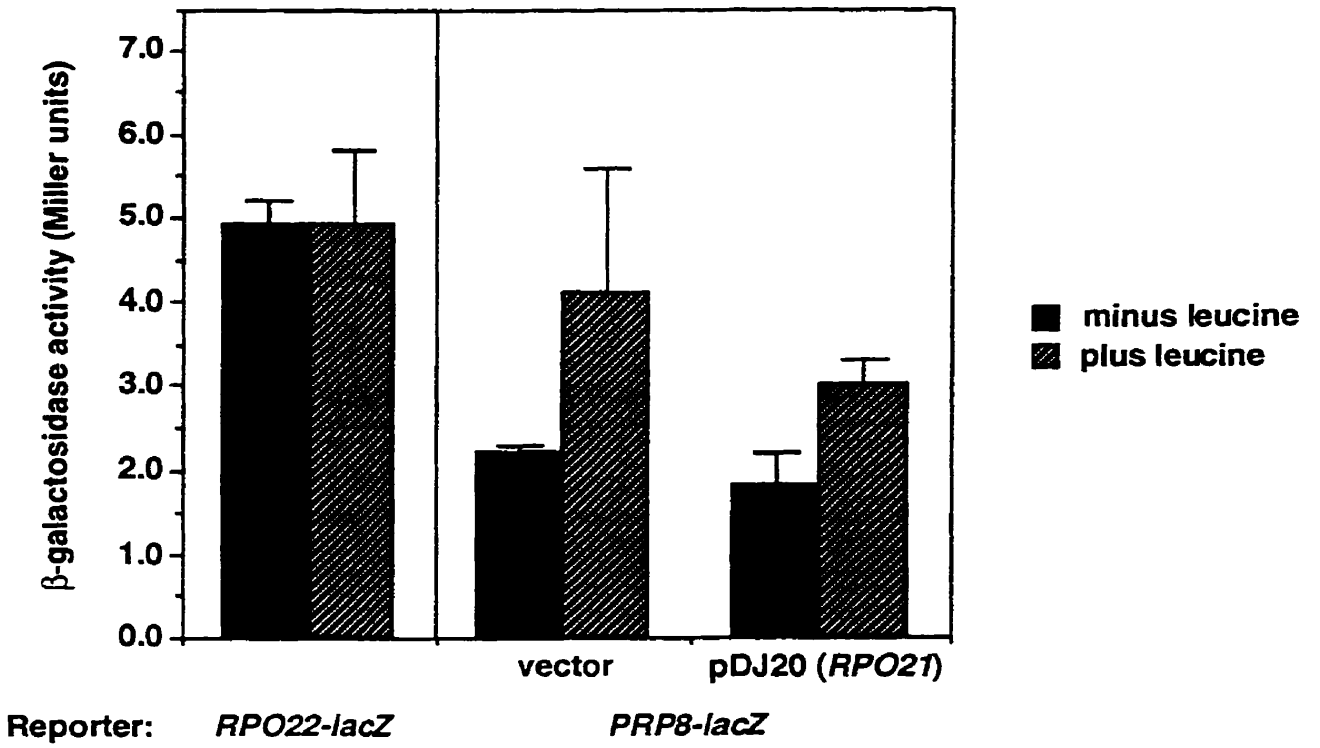

A
Leucine
Leu3p
Transcriptional repression
TAA
ATG
YDL139c
RPO21
pLEU2
ATG
pRPO21
lacZ
RPO21-lacZ reporter gene on plasmid


B
β-galactosidase activity (Miller units)
60
50
40
30
20
10
0
minus leucine
plus leucine
Strain:
pLEU2-RPO21
RPO21
Reporter:
RPO21-lacZ

I tested the effect of a decrease in the level of Rpo21p on the expression of *RPO21-lacZ* by measuring the β-galactosidase activity of strain YF1971 that contains *RPO21-lacZ* on a low-copy plasmid (pDJ46). This strain was grown for 12 hours in the absence or presence of 2 mM leucine, isoleucine and threonine (Figure 4.1B). The expression of the reporter gene, which contains all the sequences required for the full expression of *RPO21* (CHAPTER 3), increased almost five-fold when 2 mM leucine, isoleucine and threonine were added to the growth medium. This increase was not seen in a congenic strain (YF1974) in which *RPO21* expression was controlled by its own upstream sequences (Figure 4.1B). Therefore, a reduction in the level of Rpo21p in the cell caused increased expression of *RPO21-lacZ*. This suggests the existence of a feedback mechanism regulating the expression of *RPO21*.

The effect of underproduction of Rpo21p on the expression of other genes. I showed previously that underproduction of Rpo21p does not affect the level of β-galactosidase activity of YF1971 that contains *CYC1-lacZ* that is activated by 4 SCB elements (CHAPTER 2). In contrast, when the UAS of *INO1* is upstream of *CYC1-lacZ*, expression of the reporter gene was reduced six-fold by underproduction of Rpo21p (CHAPTER 2). I tested the effect of underproduction of Rpo21p on the expression of *RPO22-lacZ*, a reporter gene that is regulated by the upstream sequences required for the normal expression of the second-largest subunit of RNAPII (CHAPTER 3). YF1971, which contained a *RPO22-lacZ* reporter plasmid (pDJ81), was grown for five hours in the absence or presence of 2mM leucine, isoleucine and threonine. The strain was grown for 5 hours because *RPO21-lacZ* expression reached a maximum at that time (data not shown). There was no change in the level of β-galactosidase activity of YF1971 that contained *RPO22-lacZ* whether or not the strain was grown in the presence of 2mM leucine, isoleucine and threonine (Figure 4.2, left part of graph). I also tested, as an unrelated control, the effect of a reduction of the

Figure 4.2. The effect of underproduction of Rpo21p on the expression of *RPO22*- and *PRP8-lacZ* fusion genes. Levels of β-galactosidase activity (expressed in Miller units) were determined in YF1971 (*pLEU2-RPO21*) that contained either *RPO22-lacZ* (pDJ81; 2μm *URA3*) (left part of graph) or *PRP8-lacZ* (pDJ83; 2μm *URA3*) (middle and right part of graph). YF1971 with *PRP8-lacZ* also contained either pFL39 (vector, *TRP1 CEN/ARS* plasmid, middle part of graph) or the pDJ20 (pFL39 containing *RPO21*, right part of graph). Strains were grown for 5 hours either in the absence or presence of 2 mM leucine, threonine and isoleucine. Each error bar indicates the standard deviation of three independent assays.

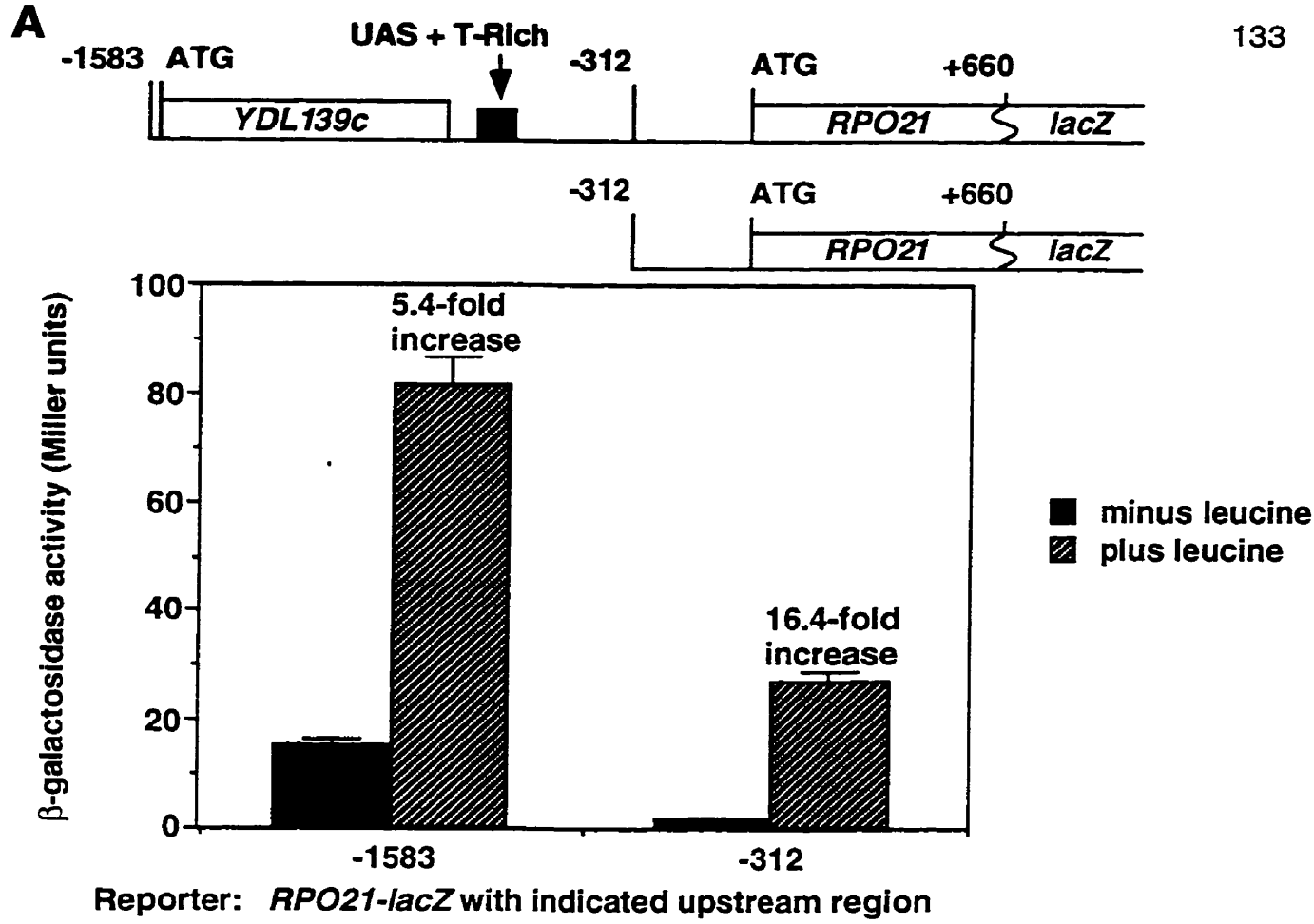

β-galactosidase activity (Miller units)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
minus leucine
plus leucine
vector
pDJ20 (RPO21)
Reporter:
RPO22-lacZ
PRP8-lacZ

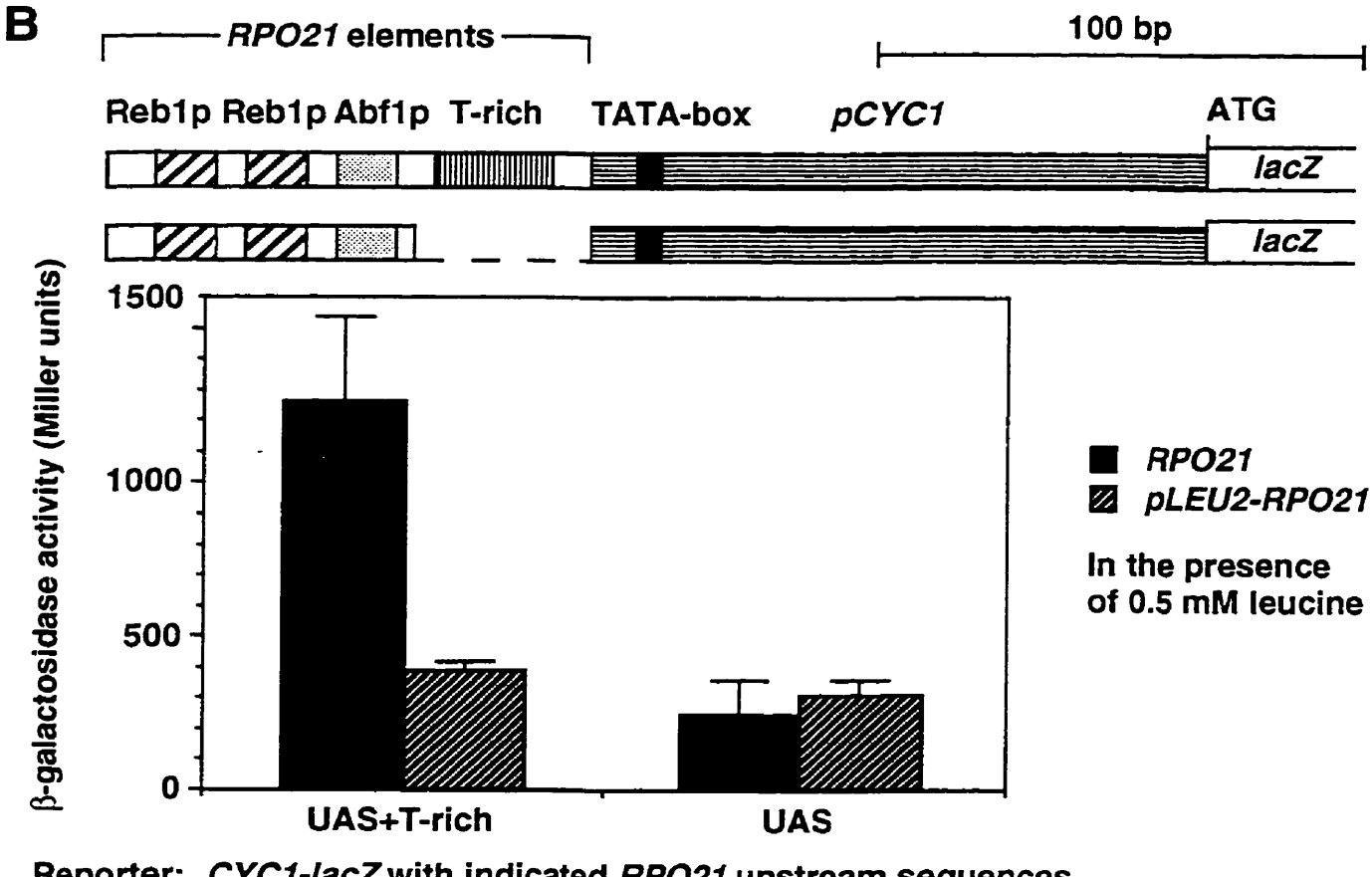
level of Rpo21p on the expression of *PRP8-lacZ*. *PRP8* is a gene that encodes a pre-mRNA splicing factor that is associated with the U5 snRNA (Jackson, *et al.* 1988; Whittaker, *et al.* 1990). Growth in the presence of leucine, isoleucine and threonine of YF1971 that contained a plasmid with *PRP8-lacZ* (pDJ83) resulted in a small (1.9-fold) increase in the level of β-galactosidase activity as compared to the same strain grown in the absence of those amino acids (Figure 4.2, middle of graph). I repeated this experiment with an identical strain that had an additional plasmid (pDJ20) containing *RPO21*. In this case, growth in the presence of leucine, isoleucine and threonine resulted in a similar small increase (1.7-fold) in β-galactosidase activity (Figure 4.2, right part of graph). Since pDJ20 is able to complement fully the reduction in Rpo21p that is caused by growth of YF1971 in the presence of leucine, isoleucine and threonine (CHAPTER 2), it is likely that the increase in β-galactosidase seen in this strain containing *PRP8-lacZ* is not a result of a decrease in the level of Rpo21p. The small increase may be due to the presence of leucine, isoleucine and threonine in the growth medium. Therefore, a reduction in the level of Rpo21p does not alter the expression of either *RPO22-lacZ* or *PRP8-lacZ*.

Identification of sequences in the *RPO21*-upstream region that respond to the underproduction of Rpo21p. I wished to identify the *cis*-acting element(s) that are responsible for the increase in the expression of *RPO21-lacZ* that occurs when Rpo21p is underproduced. As a first step, I tested the expression of *RPO21-lacZ* that has only 312 bp of sequences upstream of the initiator ATG. I showed previously that the removal *RPO21* upstream sequences to -312 resulted in approximately a 50-fold decrease in the expression of *RPO21-lacZ* (CHAPTER 3). The main elements responsible for the positive activity of sequences upstream of -312 are a ~70 bp UAS spanning sequences -738 to -672 and a T-rich region immediately adjacent and downstream of the UAS (-672 to -637). The UAS has two binding sites for Reb1p and

one binding site for Abf1p. Mutation of all three sites destroys the activity of the UAS (CHAPTER 3). These experiments were carried out using a *RPO21-lacZ* reporter gene on a high-copy number (2μm) plasmid with the *LEU2* marker gene. Since YF1971 is a leucine prototroph, I constructed a new set of *RPO21-lacZ* reporter genes, carried on a low-copy *TRP1* plasmid (pFL39). YF1971 transformed with these plasmids (pDJ48, *RPO21-lacZ* with 312 bp of sequence upstream of the initiator ATG) was grown in the presence or absence of 0.5 mM leucine, isoleucine and threonine. The expression of *RPO21-lacZ* (312 bp) increased 16.4-fold (Figure 4.3A) when cells were grown with leucine, isoleucine and threonine relative to levels when cells were grown without supplement. Although a lower (0.5 mM instead of 2 mM) concentration of leucine, isoleucine and threonine was used in this experiment, YF1971 containing *RPO21-lacZ* with 1583 bp of upstream sequence (pDJ46) and grown in 0.5 mM of leucine, isoleucine and threonine, showed a 5.4-fold increase in expression (Figure 4.3A). This is similar to the ~5-fold increase seen when cells were grown in 2 mM leucine, isoleucine and threonine (Figure 4.1B). Two observations were made; i) Removal of sequences to -312 resulted in approximately a 9-fold reduction in the expression of *RPO21-lacZ* when Rpo21p levels were normal (in the absence of leucine, isoleucine and threonine). This reduction is not as great as the approximately 50-fold reduction that was seen previously (CHAPTER 3). This difference may be the caused by the influence of the different vector sequences upstream of *RPO21-lacZ* in that reporter system (based on a YEp13; 2μm *LEU2*) in comparison to those in the vector used in these experiments (based on pFL39; *CEN/ARS TRP1*). ii) When the level of Rpo21p was reduced, the expression of a reporter gene with 312 bp of *RPO21* upstream sequence showed approximately a 3-fold greater increase in expression (16.4-fold vs. 5.4-fold) than a reporter with 1583 bp of *RPO21* upstream sequence. Therefore, while *RPO21* sequences downstream of -312 have a positive (16.4-fold) effect on the expression of *RPO21-lacZ* in response to the underproduction of Rpo21p, sequences

Figure 4.3. The effect of underproduction of Rpo21p on the expression of *lacZ* fusion genes controlled by parts of the upstream region of *RPO21*. (A) Plasmids bearing *RPO21-lacZ* fusion genes that contain upstream sequences extending to -1583 (pDJ46; TOP, first line) or -312 (pDJ48; TOP, second line) were introduced into YF1971 (*pLEU2-RPO21*). Levels of β-galactosidase activity (expressed in Miller units) were determined from strains grown for 12 hours either in the absence or presence of 0.5 mM leucine, threonine and isoleucine. (B) Levels of β-galactosidase activity (expressed in Miller units) were determined from YF1974 (*RPO21*) or YF1971 (*pLEU2-RPO21*) that contained one of two plasmids. Both plasmids included *lacZ* fusion genes driven by the *CYC1* TATA box and start sites. The first plasmid (pYF1756; TOP, first line) has the UAS and T-rich region (-637 to -738) of the *RPO21* upstream region cloned upstream of the basal promoter. The second plasmid (pYF1591; TOP, second line) has only the UAS region (-672 to -738) cloned upstream. Strains were grown for 12 hours in the presence of 0.5 mM leucine, threonine and isoleucine.


A
-1583 ATG
UAS + T-Rich
-312
ATG
+660
YDL139c
RPO21
lacZ
-312
ATG
+660
RPO21
lacZ
β-galactosidase activity (Miller units)
100
80
60
40
20
0
5.4-fold increase
16.4-fold increase
-1583
-312
minus leucine
plus leucine
Reporter: RPO21-lacZ with indicated upstream region


B
RPO21 elements
100 bp
Reb1p Reb1p Abf1p T-rich
TATA-box
pCYC1
ATG
lacZ
lacZ
β-galactosidase activity (Miller units)
1500
1000
500
0
UAS+T-rich
UAS
RPO21
pLEU2-RPO21
In the presence of 0.5 mM leucine
Reporter: CYC1-lacZ with indicated RPO21 upstream sequences

upstream of -312, although important for full expression of the *RPO21* reporter gene, cause a 3-fold *attenuation* of the 16.4-fold increase in the expression of *RPO21-lacZ*.

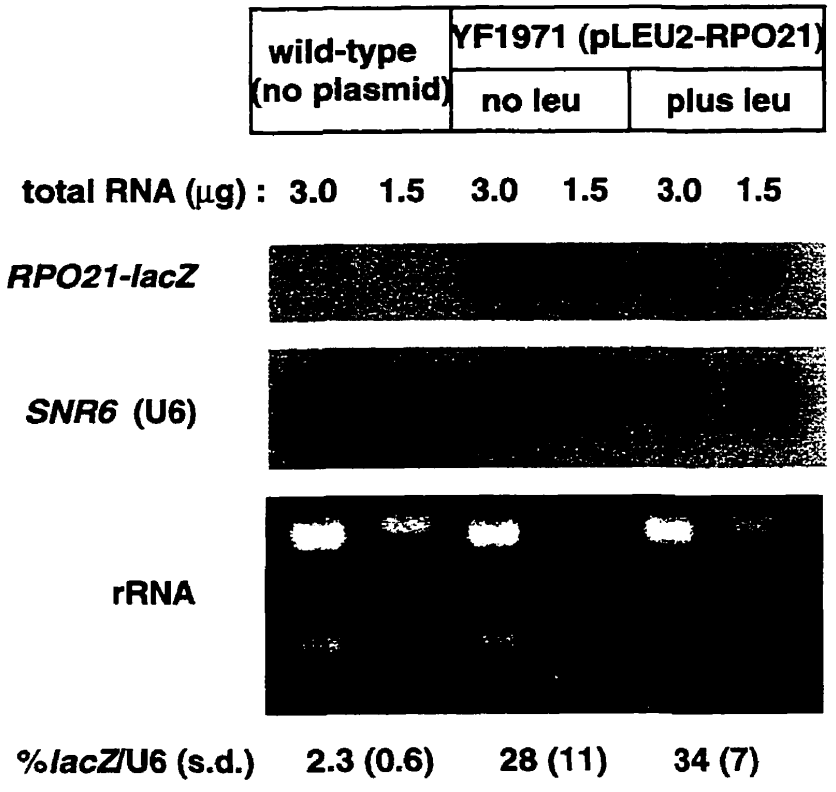
I examined the negative effect of upstream regions of *RPO21* further by testing the response of two specific segments of the *RPO21* UAS region to the underproduction of Rpo21p. Two reporter plasmids were constructed. Both plasmids (*URA3* 2μm) contained *lacZ* fusions to the TATA-box, transcriptional start-sites and 5'-untranslated region of *CYC1*. In place of the *CYC1* UAS was either the *RPO21* UAS and T-rich region (pYF1756), or the *RPO21* UAS alone (pYF1591). YF1974 (*RPO21*) or YF1971 (*pLEU2-RPO21*) that contained pYF1756 was grown for 12 hours in the presence of 0.5 mM leucine, isoleucine and threonine. The same strains, containing instead pYF1591, were grown under the same conditions. I made the following observations concerning the amounts of β-galactosidase activity measured from each strain (Figure 4.3B); i) *CYC1* that is activated by the UAS and T-rich region of *RPO21* has approximately a 5-fold higher level of expression than *CYC1* activated by the *RPO21* UAS alone. This fits well with previous data indicating that both the UAS and T-rich region contribute to the expression of *RPO21* (CHAPTER 3). ii) The level of β-galalctosidase activity (1200 Miller units) generated from the reporter gene containing the *RPO21* UAS and T-rich region is higher than the expression of *RPO21-lacZ* on a high-copy plasmid (300 units; data not shown). This may reflect differences in the efficiency of the *CYC1* basal promoter sequences versus those of *RPO21*, the relative spacing of promoter elements, or the copy number of the plasmids used. iii) A reduction in the amount of Rpo21p reduces the expression of *CYC1* that is activated by the *RPO21* UAS and T-rich region by 3-fold. iv) *CYC1* that is activated by the UAS of *RPO21* alone, is not affected significantly by a reduction in the level of Rpo21p. Together, these results suggest that the level of Rpo21p in the cell has a positive affect on the activity of the UAS and T-rich region of *RPO21*, or possibly on the T-rich region alone. Hence the level of Rpo21p negatively affects elements that are downstream of

-312 and positively affects elements (the UAS and/or the T-rich region) upstream of -312. The net effect is an increase in *RPO21-lacZ* expression when the level of Rpo21p is reduced.

Steady-state level of *RPO21-lacZ* mRNA. Total RNA was isolated from a wild-type strain (S288C) and from YF1971 (*pLEU2-RPO21*) that contains *RPO21-lacZ* (pDJ46). YF1971 with pDJ46 was grown for 10 hours in the absence or presence of 2 mM leucine, isoleucine and threonine. The RNA was separated by gel electrophoresis and transferred to a membrane. The membrane was probed with radioactively-labelled DNA that encoded *lacZ* and with a second fragment of labelled DNA that encoded the RNAPIII-transcribed *SNR6* (U6) (Figure 4.4). The signal from the transcripts of *RPO21-lacZ* were identified as bands that migrated to the same position of the gel in the lanes with RNA from strains that contained pDJ46 but were absent in the lanes from a strain without the plasmid (Figure 4.4; top band). A second set of bands, present in all the lanes, were the signals from the transcripts of *SNR6* (Figure 4.4; lower band). Quantitation of the signals by phosphoimager analysis indicated that the steady-state level of *RPO21-lacZ* mRNA from YF1971 grown in the absence or presence of leucine, isoleucine and threonine, did not increase significantly, relative to the level of U6 RNA (Figure 4.4). Since, under these same conditions, there is a 5-fold increase in the level of β-galactosidase (Figure 4.1B, 3A), the increased activity must be due to a post-transcriptional mechanism.

The effect of depletion of Fcp1p on the expression of *RPO21-lacZ*. Fcp1p was identified as a protein that interacts with Rap74 (Tfg1p), a component of the general transcription factor, TFIIF (Archambault, *et al.* 1997). *FCP1* is essential for yeast growth and the protein is a component of a complex that dephosphorylates the CTD of RNAPII *in vitro* (Archambault, *et al.* 1997). In order to understand the role of

Figure 4.4. The effect of underproduction of Rpo21p on the steady-state level of mRNA from *RPO21-lacZ*. Total RNA was isolated from a wild-type strain with no plasmid (S288C), or from YF1971 (*pLEU2-RPO21*) that contained pDJ46 (*RPO21-lacZ*; *TRP1 CEN/ARS*). The latter strain was grown for 10 hours in the absence or presence of 2mM leucine, isoleucine and threonine. RNA from each strain (3 or 1.5 μg) was separated on an agarose gel and transferred to a membrane. The membrane was probed simultaneously with radioactively-labelled DNA containing the entire 3.5 kb ORF of *lacZ* (as well as ~500 bp of *RPO21* sequence) and with DNA containing the U6 gene (*SNR6*) as a loading control. Amounts of rRNA in each lane are indicated by staining with ethidium bromide. The ratio of *lacZ* mRNA signal to U6 (*SNR6*) signal is expressed as a percentage at the bottom. The standard deviation for each value is indicated in brackets.

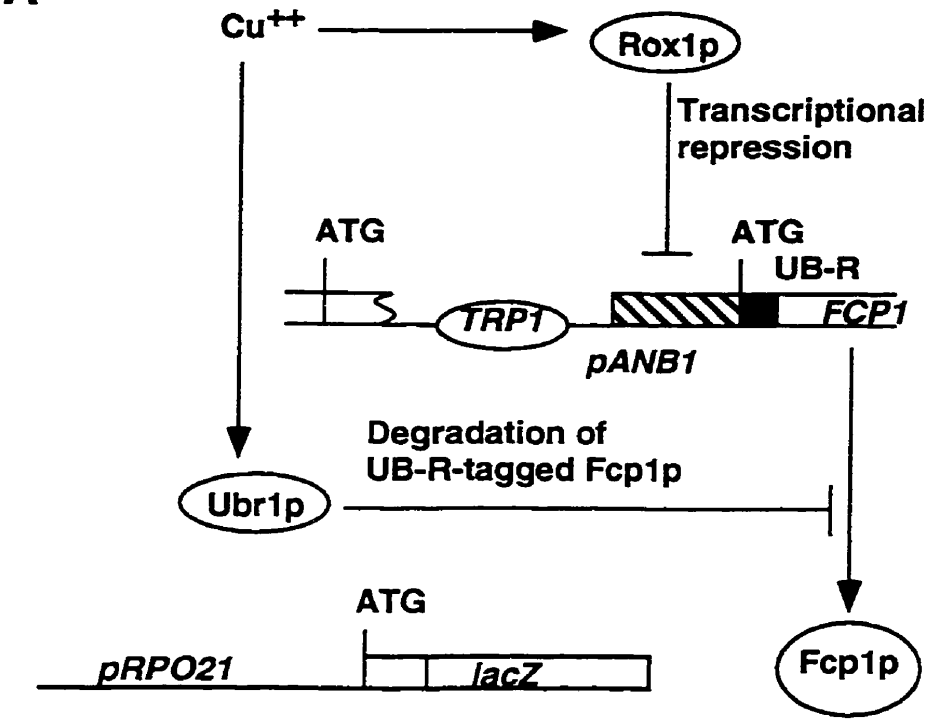

wild-type (no plasmid)
YF1971 (pLEU2-RPO21)
no leu
plus leu
total RNA (μg) : 3.0 1.5 3.0 1.5 3.0 1.5
RPO21-lacZ
SNR6 (U6)
rRNA
%lacZ/U6 (s.d.) 2.3 (0.6) 28 (11) 34 (7)

Fcp1p *in vivo*, Michael S. Kobor (a graduate student in the laboratory of Dr. Jack Greenblatt) constructed a strain that allows for the depletion of Fcp1p from the cell upon the addition of Cu^{++} to the growth medium (Figure 4.5A). I showed that growth of the Fcp1p shut-off strain in the absence of added Cu^{++} results in a growth rate that is similar to a wild-type strain (Figure 4.5B and M. Kobor, personal communication). When I added Cu^{++} to the growth medium, the strain showed normal growth for 3 hours, followed by an increase in the doubling time, and eventually no further cell divisions (Figure 4.5B and M. Kobor, personal communication). Mr. Kobor observed a 5- to 10-fold increase in the steady-state level of Rpo21p when the Fcp1p shut-off strain was grown for up to 8 hours in the presence of Cu^{++} (M. Kobor, personal communication). The steady-state level of another subunit of RNAPII, Rpo24p, was unchanged upon depletion of Fcp1p (M. Kobor, personal communication). Since the increase in the steady-state level of Rpo21p was reminiscent of the increase in *RPO21-lacZ* expression when Rpo21p is underproduced, Mr. Kobor and I entered into a collaboration for the purpose of comparing and contrasting the effects of Rpo21p underproduction and Fcp1p depletion on the expression of *RPO21*.

The growth characteristics of the Fcp1p-depletion strain (YF2353) are different from the *pLEU2-RPO21* strain (YF1971) since the former does not attain a new constant slower rate of growth when Fcp1p is depleted, but rather, eventually ceases growth entirely. For this reason, I measured the level of expression of *RPO21-lacZ* in YF2353 at different times after addition of Cu^{++} to the growth medium (Figure 4.5B). The amount of β-galactosidase activity did not change for the first 2 hours. The level then increased at 5 hours to a maximum that was about 3-fold higher than the level of the same strain that was grown in the absence of added Cu^{++} (Figure 4.5B). After 5 hours, there is a decline in the level of β-galactosidase activity (Figure 4.5B). Therefore, similar to the steady-state level of Rpo21p, the level of expression of *RPO21-lacZ* increases upon the depletion of Fcp1p in YF2353.

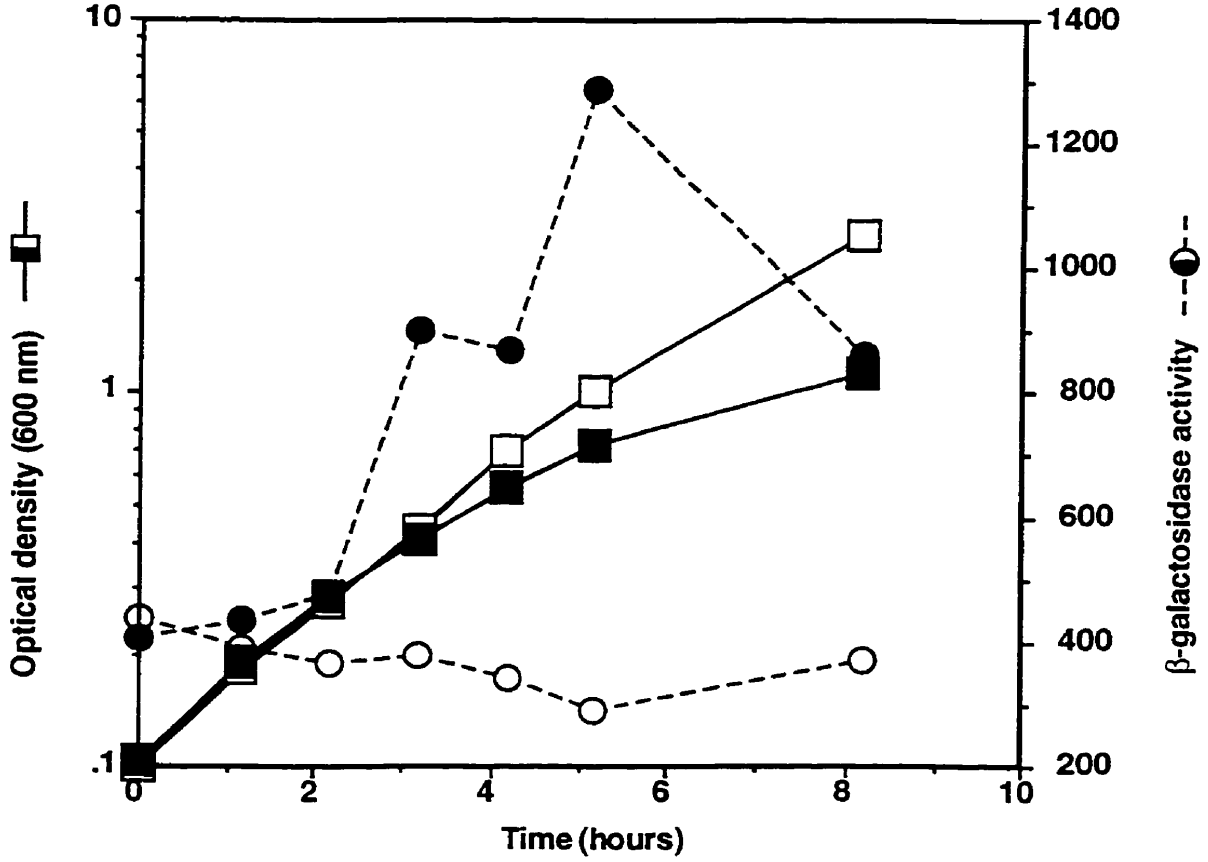
Figure 4.5. The effect of the depletion of Fcp1p on the expression of *RPO21-lacZ*. (A) A diagram of the yeast strain (YF2353) used to deplete Fcp1p. The depletion system originated in Kevin Struhl's laboratory (Moqtaderi, *et al.* 1996) and the Fcp1p-shut off strain was constructed by Michael Kobor (unpublished results). The strain was designed such that addition of Cu^{++} to the growth medium results in transcriptional repression of *FCP1* and in the degradation of pre-existing Fcp1p. Transcriptional repression is achieved by introducing, at the *FCP1* locus, a truncated (non-functional) version of *FCP1* that is under the transcriptional control of the *ANB1* gene (*pANB1*). Homologous recombination results in full-length *FCP1* under the control of *pANB1*, while the original *FCP1* promoter drives the expression of the non-functional fragment of *FCP1*. The *ANB1* gene is repressed at the transcriptional level by DNA-binding protein, Rox1p. In this strain, *ROX1* expression is driven by a heterologous promoter (derived from *CUP1*) which has a binding site for Ace1p (not shown in diagram). Addition of Cu^{++} to the growth medium results in Ace1p-dependent activation of *ROX1*, which in turn, stimulates the production of Rox1p, which represses the transcription of *pANB1-FCP1*. Degradation of pre-existing Fcp1p is achieved by introducing, along with *pANB1*, additional 5'-end codons to *FCP1* that encode ubiquitin and arginine (UB-R). Fcp1p that is tagged with UB-R is stable, however, Ubr1p, an enzyme involved in targeting proteins for degradation, recognizes this tag as a signal for protein degradation. Like *ROX1*, *UBR1* in this strain is also expressed from a heterologous Cu^{++}-inducible promoter. Hence, addition of Cu^{++} to the growth medium inhibits transcription of *FCP1* and degrades Fcp1p, resulting in rapid depletion of the protein. (B) pDJ80 (*RPO21-lacZ*; 2μm *URA3*) was introduced into strain YF2353. Cells were grown to log phase and the culture was split. Half of the cultures were brought to 0.5 mM $CuSO_4$ at time 0 (closed symbols) and the other half were not (open symbols). The growth rate of two independent cultures under each condition were monitored by measuring the optical density (600 nm) at the indicated time intervals (square symbols). The β-galactosidase activity (Miller units) of one culture at each indicated time was also measured (round symbols).

A



RPO21-lacZ reporter gene on 2μ plasmid

B



Open symbols (□, ○) Minus Cu++

Closed symbols (■, ●) Plus Cu++

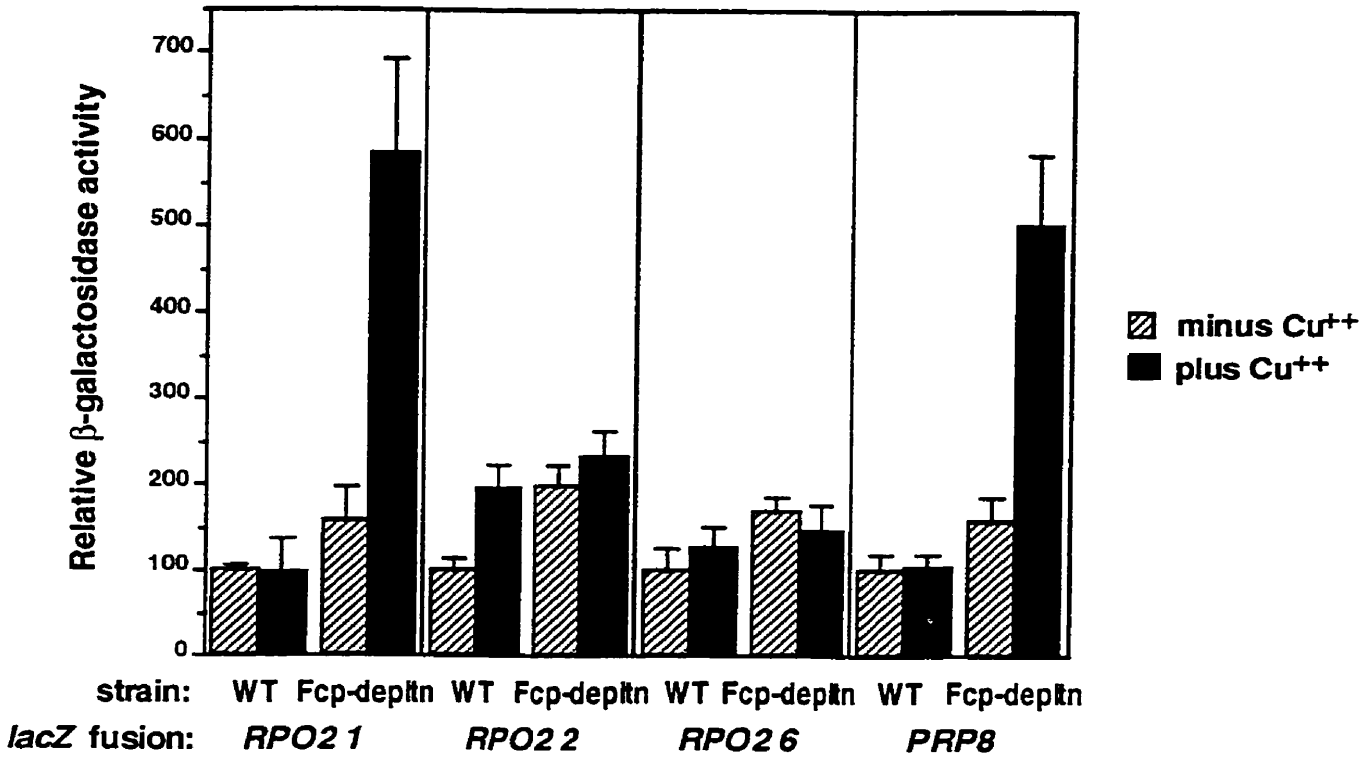
The effect of depletion of Fcp1p on the expression of various *lacZ*-fusion genes. I tested further the effect of the depletion of Fcp1p on the expression of *RPO21-lacZ* (Figure 4.6A). A high-copy plasmid (pDJ80) containing this reporter gene was introduced into YF2353 (Fcp1p-depletion strain) and into YF2352, a strain congenic with YF2353, except that *FCP1* is in its wild-type form and is not affected by the addition of Cu^{++} to the growth medium (data not shown). The level of β-galactosidase was determined for these strains after growth for 5 hours in the absence or presence of 0.5 μM $CuSO_4$ (Figure 4.6A). I made the following observations; i) The expression of *RPO21-lacZ* (pDJ80) was almost 6-fold higher in the Fcp1p depletion strain (YF2353) grown in the presence of Cu^{++} compared to the wild-strain (YF2352) grown under the same conditions. ii) *RPO21-lacZ* expression was the same in the wild-type strain either in the presence or absence of Cu^{++}. This indicates that Cu^{++} itself is not affecting *RPO21-lacZ* expression. iii) The expression of *RPO21-lacZ* is slightly higher (~1.5-fold) in YF2353 compared to Y2352 when both strains are grown in the absence of added Cu^{++}. This increase is likely due to the modifications in YF2353 of *FCP1* (addition of a ubiquitin moiety and expression from the *ANB1* promoter) that compromises mildly the activity of Fcp1p. Taken together, these data suggest that a depletion of Fcp1p results in an increase in the expression of *RPO21-lacZ*.

I repeated these experiments using *RPO22-lacZ* (pDJ81) (Figure 4.6A). The addition of Cu^{++} to the wild-type strain resulted in a small (2-fold) increase in the level of β-galactosidase activity. This amount of activity was also seen in YF2353 grown in the presence of Cu^{++}. These data suggest that the expression of *RPO22-lacZ* is not affected by the depletion of Fcp1p. This is similar to the observation that underproduction of Rpo21p does not affect the expression of *RPO22-lacZ* (Figure 4.2).

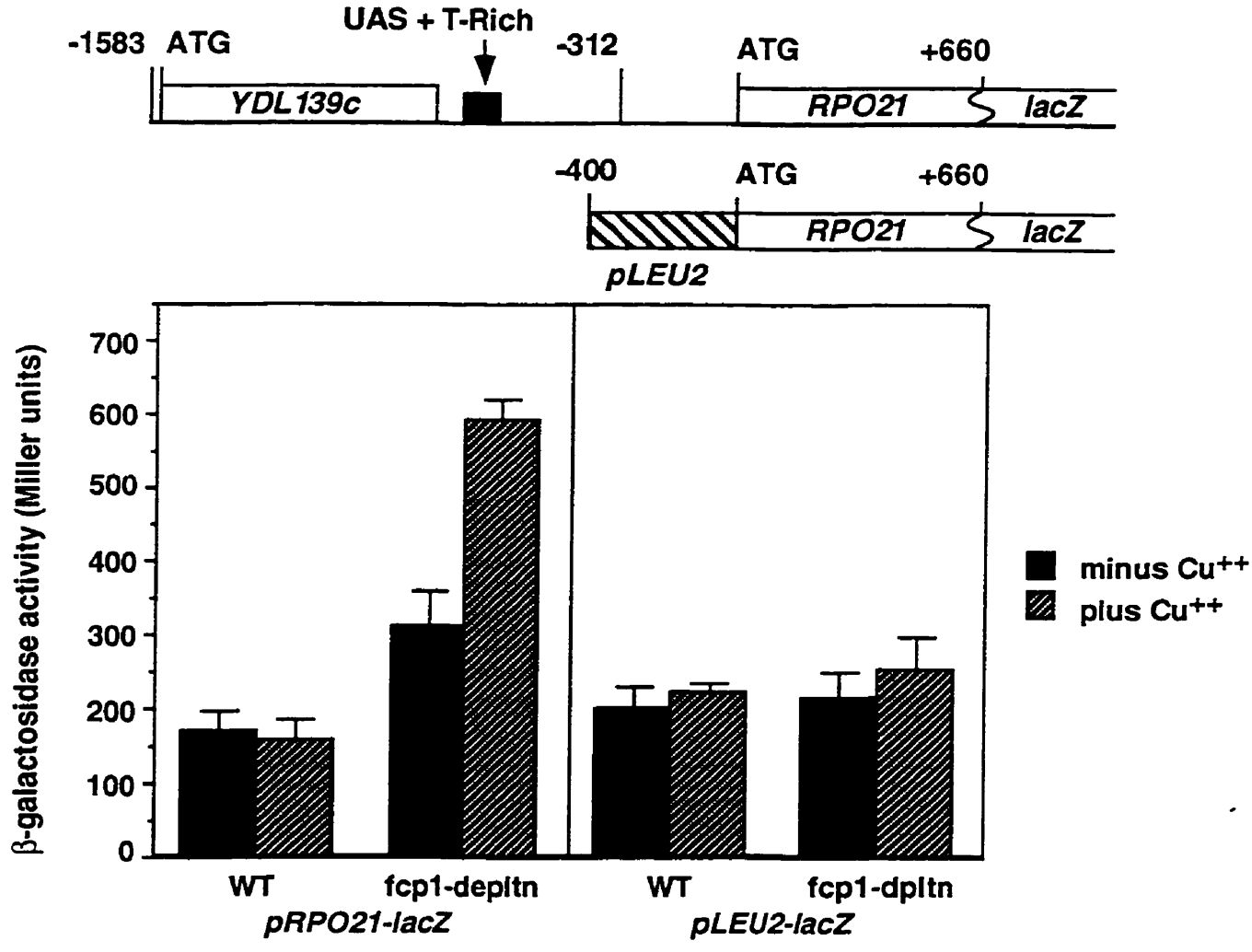
RPO26 encodes a subunit that is common to all three nuclear RNAPs in

Figure 4.6. The effect of the depletion of Fcp1p on the expression of *RPO21-*, *RPO22-*, *RPO26-* , *PRP8-*, and *LEU2-lacZ* fusion genes. (A) Levels of β-galactosidase activity were determined from YF2352 (wild-type; WT) or YF2353 (Fcp1p-depletion strain; Fcp-depltn) that contained 2μm *URA3* plasmids bearing either *RPO21-lacZ* (pDJ80), *RPO22-lacZ* (pDJ81), *RPO26-lacZ* (pSN318) or *PRP8-lacZ* (pDJ83). Strains were grown for 5 hours either in the absence or presence of 0.5 μM $CuSO_4$. β-galactosidase activities were normalized such that the activity in the wild-type strain in the absence of $CuSO_4$ was set at 100 units for each *lacZ*-fusion gene. (B) Levels of β-galactosidase activity (expressed in Miller units) were determined from YF2353 (wild-type; WT) or YF2353 (Fcp1p-depletion strain; Fcp-depltn) that contained 2μm *URA3* plasmids bearing either *RPO21-lacZ* (pDJ84; TOP, first line) or *LEU2-lacZ* (pDJ86; TOP, second line). Strains were grown for 5 hours either in the absence or presence of 0.5 μM $CuSO_4$.

A


Relative β-galactosidase activity
700
600
500
400
300
200
100
0
minus Cu++
plus Cu++
strain: WT Fcp-deplt WT Fcp-deplt WT Fcp-deplt WT Fcp-deplt
lacZ fusion: RPO2 1 RPO2 2 RPO2 6 PRP8

B


UAS + T-Rich
-1583 ATG
-312
ATG
+660
YDL139c
RPO21
lacZ
-400
ATG
+660
RPO21
lacZ
pLEU2
β-galactosidase activity (Miller units)
700
600
500
400
300
200
100
0
minus Cu++
plus Cu++
WT
fcp1-deplt
WT
fcp1-dplt
pRPO21-lacZ
pLEU2-lacZ

eukaryotes (Archambault, *et al.* 1990; Woychik and Young 1992). *RPO26-lacZ* expression was not affected by the depletion of Fcp1p (Figure 4.6A).

Unexpectedly, the expression of *PRP8-lacZ* was 5-fold higher in YF2353 (Fcp1p depletion strain) compared to YF2352 (wild-type) when both strains were grown in the presence of Cu^{++} (Figure 4.6A). A similar increase was also observed for the steady-state level of Prp8p (M. Kobor, personal communication). As was the case with *RPO21-lacZ*, *PRP8-lacZ* is expressed slightly higher in the Fcp1-depletion strain compared to the wild-type strain when both strains are grown in the absence of added Cu^{++}. Therefore, unlike the situation when Rpo21p is underproduced, the expression of *PRP8 -lacZ* increases when Fcp1p is depleted.

The effect of depletion of Fcp1p on the expression of a *RPO21-lacZ* fusion gene with a substituted upstream region. In this study, reporter genes were constructed by fusing the upstream region of a gene with part of the ORF of that gene to the coding region of *lacZ*. Regulation of the reporter gene may, therefore, occur at the level of the transcriptional initiation rate, promoter clearance (but not the regulation of 3'-end processing), mRNA transport, translation, and even the rate of protein degradation, since a small part of the N-terminus of the protein is fused to β-galactosidase. In order to determine if the sequences upstream of the initiator codon of *RPO21-lacZ* were necessary for the increase in expression that occurs when Fcp1p is depleted, I constructed two *lacZ* reporter plasmids. The first (pDJ84) contained sequences of *RPO21* from -1583 to +660 fused to *lacZ*. The second plasmid (pDJ86) was identical to pDJ84 except that *RPO21* sequences from -1583 to -1 were replaced with sequences spanning -400 to -1 of the upstream region of *LEU2*. These plasmids were introduced into YF2352 (wild-type) and YF2353 (Fcp1p-depletion strain). The resulting strains were grown for 5 hours in the absence or presence of added Cu^{++} and β-galactosidase activities were determined (Figure 4.6B). The strains that

contained pDJ84 (*RPO21-lacZ*) had similar amounts of β-galactosidase as the same strains containing another *RPO21-lacZ* fusion gene (pDJ80) (Figure 4.6A). The latter reporter gene has only 171 bp of *RPO21* ORF. In contrast, the β-galactosidase activity of YF2353 that contains *pLEU2-RPO21*(+1 to +660)-*lacZ* was not significantly higher than the wild-type strain (YF2352) either in the absence or presence of added Cu^{++} (Figure 4.6B). I concluded the following; i) Sequences of *RPO21* upstream of +1 are necessary for the increase in the expression of *RPO21-lacZ* that occurs when Fcp1p is depleted. ii) Sequences between +1 and +660 are not sufficient for this response. iii) The response does not involve the stabilization of the Rpo21p-β-galactosidase fusion protein that is expressed from the reporter gene. Hence, DNA or RNA elements encoded in sequences upstream of the *RPO21* initiator ATG are implicated as controlling elements that mediate the increase in expression of *RPO21-lacZ* in response to the depletion of Fcp1p.

DISCUSSION

In this study I report mechanisms that increase the activity of sequences that control the expression of *RPO21* when either the level of Rpo21p is reduced, or Fcp1p is depleted. These mechanisms may be an important part of a cellular homeostatic response which maintains an optimal amount of RNAPII in the cell. The response to the underproduction of Rpo21p is specific to *RPO21*, since underproduction of Rpo21p does not affect the expression of *RPO22-lacZ*, a reporter gene controlled by the regulatory sequences of the gene encoding the second-largest subunit of RNAPII. The response of *RPO21-lacZ* and *PRP8-lacZ* to the depletion of Fcp1p is specific to these genes, since depletion of Fcp1p does not affect the expression of *RPO22-lacZ* or *RPO26-lacZ* (Figure 4.6A).

Similar regulatory mechanisms have been identified in *E. coli*, *C. elegans* and mammalian cells (reviewed in CHAPTER 1). In *E. coli*, feedback regulation affects the

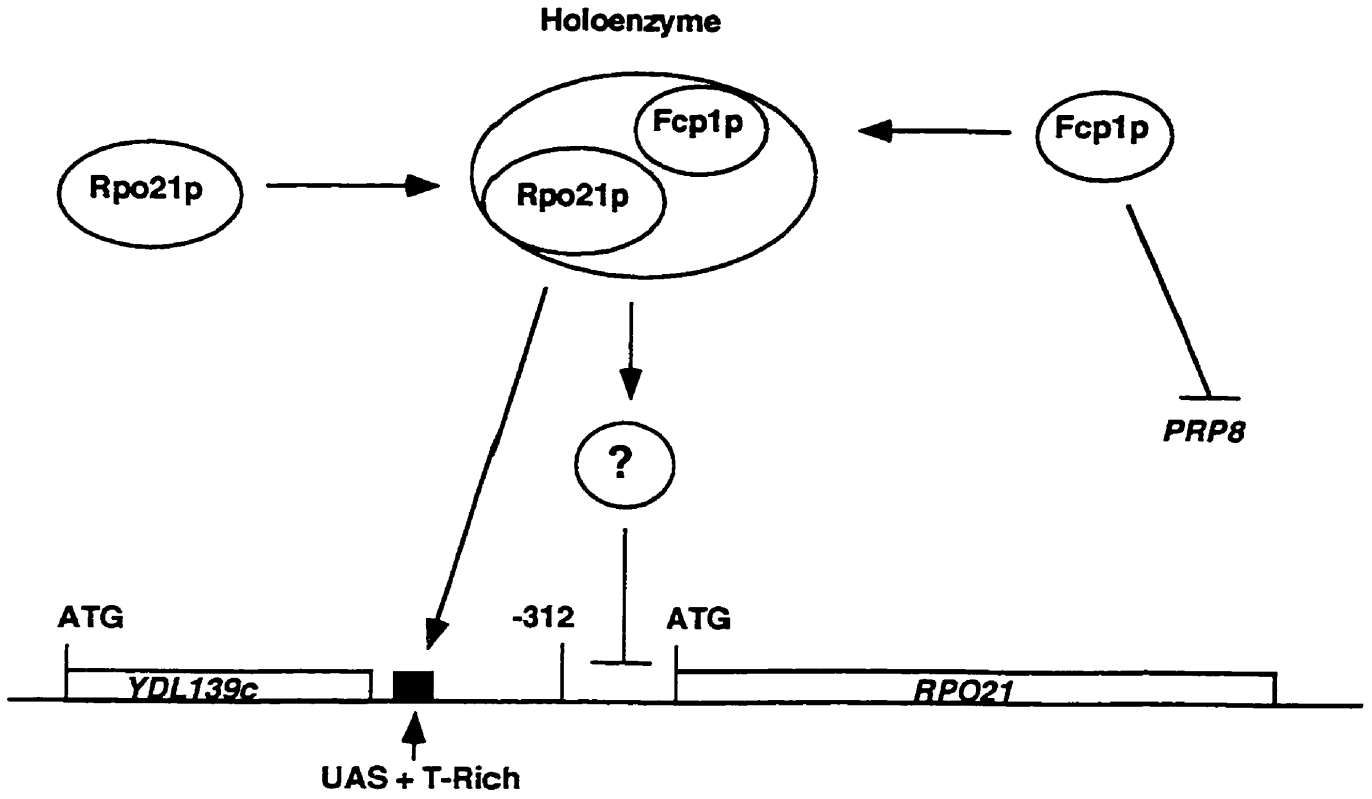
transcription of the genes (*rpoB* and *rpoC*) encoding the two largest subunits of RNAP (β' and β). The rate of transcriptional initiation at the promoters of the operon containing *rpoBC* and the rate of transcript termination at an attenuator that precedes *rpoBC* are influenced by the level of activity of RNAP in the cell (Downing and Dennis 1991; Dykxhoorn, *et al.* 1996; Steward and Linn 1992). A second mechanism, at the level of translation, represses the level of β' and β when these subunits are over-produced (Dennis and Fiil 1979; Dennis, *et al.* 1985; Dykxhoorn, *et al.* 1996). In *C. elegans*, the steady-state level of mRNA from the gene encoding the largest subunit of RNAPII is proportional to the copy number of the gene. The amount of the largest subunit, however, remains constant, regardless of the gene dosage (Dalley, *et al.* 1993). In rat and hamster cells, it was shown that the total amount of the largest subunit of RNAPII remains constant, even when some of the population of the largest subunit is selectively degraded (Crerar and Pearson 1977; Guialis, *et al.* 1977; Somers, *et al.* 1975b). In this case, the cells respond by increasing the rate of synthesis of the largest subunit. The synthesis rates of some other subunits also increase under the conditions tested (Guialis, *et al.* 1979).

At what level of synthesis is the expression of Rpo21p affected by feedback regulation? My results suggest that both transcriptional and post-transcriptional mechanisms are involved. In Figure 4.4, I showed that the 5-fold increase in the expression of *RPO21-lacZ* that results from the underproduction of Rpo21p is not reflected in a change in the steady-state level of *RPO21-lacZ* mRNA. This suggests that a post-transcriptional mechanism is involved. The story is, however, more complex. The removal of sequences upstream of -312 results in a ~3-fold *higher* response of *RPO21-lacZ* to the underproduction of Rpo21p (Figure 4.3A). Furthermore, when Rpo21p is underproduced, the activity of the UAS and T-rich region of *RPO21* is *reduced* 3-fold when they activate a heterologous promoter. These data suggest that the total 5-fold response of the full-length *RPO21* upstream region is

in fact the net result of a 15-fold activation from sequences downstream of -312 and a 3-fold repression by sequences upstream of -312. Since the repression component is likely to be mediated by the UAS and T-rich region, both of which are upstream of all of the transcriptional start-sites of *RPO21* (CHAPTER 1, Nonet, *et al.* 1987a), repression most likely operates at the level of transcription. The steady-state level of *RPO21-lacZ* mRNA was not, however, reduced by the underproduction of Rpo21p (Figure 4.4). Therefore, a second transcriptional response, caused by sequences downstream of -312, must counteract the repressing effect of the UAS and T-rich region. The overall result is an unchanged mRNA level. However, it can be inferred that both positive and negative transcriptional elements have a role in the response of *RPO21-lacZ* to the underproduction of Rpo21p.

What is the signal(s) that induces the increase in the expression of *RPO21-lacZ*? Figure 4.7 is a working model of how different factors may influence the expression of *RPO21*. Rpo21p is the largest subunit of the core RNAPII molecule. In order to initiate transcription, RNAPII is thought to form a large complex with other transcription factors. This complex is called the holoenzyme (Greenblatt 1997; Koleske and Young 1995). In mammalian cells, Fcp1p was identified as a component of the holoenzyme (Archambault, *et al.* 1998). Furthermore, Fcp1p is thought to be required to dephosphorylate the CTD, a necessary step for the formation of holoenzyme. Since both the underproduction of Rpo21p and the depletion of Fcp1p cause an increase in the expression of *RPO21-lacZ*, it is possible that the level of functional or complete holoenzyme may be the primary signal that leads to the feedback response (Figure 4.7). The holoenzyme has many potential catalytic activities, including RNA polymerization, kinase activity, phosphatase activity, acetyltranferase activity and helicase activity (Archambault, *et al.* 1998; Greenblatt 1997; Parvin and Young 1998). Any of these activities may be rate limiting for maintaining pathways (indicated by "?" in Figure 4.7) that repress the transcriptional or

Figure 4.7. A working model for the feedback regulation of *RPO21* expression. Rpo21p and Fcp1p are synthesized and become incorporated in the holoenzyme. The level of holoenzyme activity affects positively the activity of the UAS and T-rich region of *RPO21* such that a decrease in holoenzyme activity reduces the stimulatory action of these elements at the transcriptional level. A second part of the *RPO21* upstream region that includes sequences from -312 to +1 responds in the opposite way. A reduction in holoenzyme activity leads to increased expression of *RPO21*, either at the level of transcription, translation or both. The protein(s) that mediate these signals are indicated with a "?". Not shown are the additional possibilities that Rpo21p and Fcp1p repress or activate the expression of *RPO21* independently of the holoenzyme. Fcp1p also represses the expression of *PRP8*, but this mechanism may be independent of the mechanism(s) involved in the regulation of *RPO21* since a reduction of Rpo21p does not increase *PRP8* expression.


Holoenzyme
Fcp1p
Rpo21p
Rpo21p
Fcp1p
PRP8
?
ATG
-312
ATG
YDL139c
RPO21
UAS + T-Rich

post-transcriptional expression of *RPO21*. When holoenzyme levels are reduced, the pathway is no longer maintained in its repressing mode and *RPO21* expression is increased. On the other hand, the UAS and T-rich region of *RPO21* are affected in a positive manner by increased levels of holoenzyme. This may reflect the ability of this enhancer region to attract holoenzyme to the *RPO21* promoter. Reduced levels of holoenzyme may translate into less holoenzyme attracted by the enhancer.

This model does not exclude the possibility that Rpo21p (and Fcp1p) may have a more direct effect on the expression of *RPO21*. Models for the regulation of β' and β in *E. coli* include the idea that these proteins may bind directly to the mRNA of *rpoBC* and inhibit translation (Fukuda, *et al.* 1978; Peacock, *et al.* 1983). The largest subunit of RNAPII, on its own, is able to bind RNA (Gundelfinger 1983). It is possible that a specific interaction between *RPO21* mRNA and Rpo21p is important for regulation. There is no information on the ability of Fcp1p to bind to RNA.

Depletion of Fcp1p was found to increase the expression of *PRP8-lacZ* whereas underproduction of Rpo21p did not have the same effect. This suggests that Fcp1p may also be involved in an independent regulatory pathway that effects genes other than *RPO21*. It is not known what level of Prp8p synthesis is affected by the level of Fcp1p. Regulation of *RPO21* expression by the depletion of Fcp1p is mediated by DNA or RNA elements upstream of +1 rather than by protein stabilization. The next step in this study is to identify the DNA or RNA elements that mediate this regulation, and the proteins that bind to them.

CHAPTER 5

Future Directions

In this study, I have investigated the regulation of the expression of RNAPII in *Saccharomyces cerevisiae*. As an enzyme that is required for its own synthesis, RNAPII is, by definition, a positive regulator of its own synthesis. Although this is true for all of the essential proteins that are involved in the process of gene expression, RNAPII is of particular interest since it is the ultimate target of many regulatory pathways. Who watches the watchman? My data suggest that RNAPII is not only a positive factor for its own synthesis, but also a negative factor.

In this chapter I shall discuss the implications of the results I have obtained in relation to work that has been published and suggest approaches that may be taken for understanding the mechanisms that regulate the expression of RNAPII. In addition, I shall discuss experiments that could extend our understanding of the relationship between the amount of RNAPII in the cell and the normal expression of protein-encoding genes.

cis-acting elements in the upstream region of *RPO21*

In CHAPTER 3 I showed that the UAS of *RPO21* is the binding site for proteins (Reb1p and Abf1p) that are involved in transcription activation in yeast. Furthermore, these binding sites are important for the expression of *RPO21*. These elements are not, however, sufficient for *RPO21* expression. There must be additional element(s) that bind the RNAPII initiation complex. A thorough understanding of the transcriptional regulation of *RPO21* should include knowledge of the *cis*-elements that bind the pre-initiation complex, and hence, TFIID. Here, I describe experiments aimed at identifying the location of these elements.

As noted in CHAPTER 3, the upstream regulatory region of *RPO21* has more than one site for the initiation of transcription. The longest untranslated leader is 565 nucleotides (Nonet, *et al.* 1987a) and is devoid of the sequence ATG. This suggests that, although the leader is unusually long for a yeast mRNA, ribosomes are

unimpeded when scanning for the appropriate initiation codon to begin the synthesis of Rpo21p. There are at least five additional sites for the initiation of transcription, three mapping downstream of -312 (Nonet, *et al.* 1987a; J. Archambault, personal communication). The sites of initiation of transcription span a sequence of over 350 bp and are, therefore, probably too widely spaced to be associated with a single TATA-box element (Struhl 1989). This raises the possibility of multiple binding sites for TFIID in the upstream region of *RPO21*. The upstream region of *RPO21* is rich in thymidine and adenine residues, making it difficult to identify the TATA boxes simply by looking for the consensus sequence. For this reason, I suggest here more than one approach to identify the TATA-binding elements:

1. Introduce mutations into the *RPO21-lacZ* plasmid in candidate sites that closely match the consensus for TFIID-binding sites. These plasmids can be tested for loss of transcriptional start-sites *in vivo*. The start-sites could be identified by primer-extension analysis. Mutations that result in the loss of downstream start-sites would be good candidates for binding sites of TFIID.

2. Construct templates for testing transcription initiation *in vitro*. The best way to detect initiation at a particular start-site is to design the template such that the sequences *downstream* of the start-site of interest are devoid of guanine residues. This template could be added to a yeast extract that is competent to carry out transcription *in vitro* (Lue and Kornberg 1987). By adding to the *in vitro* transcription reaction only UTP, CTP and ATP, non-specific initiations would not be detected. If the template was able to generate a message that begins at the same nucleotide as is detected *in vivo*, then one could proceed to identify the sequences upstream of the start-site that bind TFIID. One approach would be to add purified TFIID directly to radioactively-labelled template DNA and separate DNA-protein complexes on a non-denaturing gel. These

complexes could be isolated and then treated with DNaseI in order to generate a series of DNA fragments. When the fragments are separated on a denaturing gel, and compared to naked DNA that had been treated in the same manner, a "DNaseI footprint" might be detected, which would identify sequences that are protected from digestion by TFIID. These sequences could be mutated to test if they are necessary for the start-site pattern that is observed *in vitro*. Finally the same mutations could be introduced into the plasmid, as described in 1, to confirm that the sequences are important for start-site selection *in vivo*.

If the protein-DNA complexes are difficult to isolate, one could modify the template at various positions such that it included photoreactive nucleotide analogs (Coulombe, *et al.* 1994). Radiolabelled template could be combined with purified TFIID and then treated with UV radiation. The nucleotide analog would cross-link TBP that is bound near it, allowing for the identification of sequences that bind the general transcription factor.

After all the transcriptional start-sites have been matched with binding sites for TFIID, some interesting questions can be investigated:

1. How does the UAS and T-rich region of *RPO21* influence the use of the various transcriptional start-sites? Is the use of all of the start-sites decreased equally when the UAS and T-rich region are mutated?

2. Does mutation of upstream binding sites for TFIID (which are likely to be downstream of the UAS) affect the use of TFIID-binding sites that are downstream and vice versa. This addresses the possible problem of the interference with each other of the various pre-initiation complexes.

It would be interesting to contrast this analysis with a similar one for the upstream region of *RPO22*. Although the UAS and T-rich region are similar for *RPO21*

and *RPO22* (CHAPTER 3), the start-sites of *RPO22* are confined to a smaller sequence(Sweetser, *et al.* 1987), suggesting they are formed by a single pre-initiation complex. These investigations would begin to address the question of why the *RPO21* upstream region has such a complex transcription-initiation pattern.

Regulation of *RPO21* expression

My investigation has begun to uncover important elements for the expression of *RPO21* and a feedback mechanism that may be important for maintaining the appropriate amount of the protein in the cell. However, nothing is known about the expression of *RPO21* when yeast cells are grown under different conditions. The *RPO21-lacZ* reporter gene would be an ideal probe for testing the expression of *RPO21* when cells are grown in media that vary the rate of growth. The response of *RPO21* expression to growth rate can then be compared with similar studies of the expression of ribosomal protein (r-protein) genes. The r-protein genes increase expression as the growth rate increases (Herruer, *et al.* 1987). This is due, at least in part, to UAS elements that bind either Abf1p or Rap1p (Gonçalves, *et al.* 1995; Herruer, *et al.* 1987; Klein and Struhl 1994; Neuman-Silberberg, *et al.* 1995). Thymidine-rich sequences that are near the UAS are also necessary for the regulation of expression by carbon source (Gonçalves, *et al.* 1995). This combination of an Abf1p-binding site and a T-rich region is clearly similar to the elements that are important for the expression of *RPO21* and *RPO22* (CHAPTER 3). If *RPO21* is indeed expressed in a manner that is similar to the r-protein genes, how is this global regulation managed? There is some indication for the involvement of the *RAS*/cyclic AMP pathway, which leads to activation of protein kinase A (Klein and Struhl 1994; Neuman-Silberberg, *et al.* 1995). The *RPO21-lacZ* reporter could be used in a screen for mutants that effect growth-dependent regulation. Mutants that fail to express *RPO21* at higher levels on good carbon sources might define a class of genes that are

important for the regulation of many genes in response to changes in the rate of growth.

The feedback phenomenon

One of the most exciting avenues of investigation generated from this study will be the exploration of the mechanism(s) controlling the feedback regulation of RNAPII synthesis. The description of feedback phenomena for RNAP synthesis in organisms ranging from *E. coli* to mammals has shown its universality. The identification of the same kind of regulation in yeast cells offers powerful genetic and biochemical tools to understand the mechanism.

I have shown that a reporter gene whose expression is regulated by *RPO21* control elements responds with an increase in expression when either the level of Rpo21p in the cell is reduced by 10-fold, or Fcp1p is depleted (CHAPTER 4). The next step in the investigation is to use mutational analysis of the upstream region of the reporter gene to identify sequences that are necessary for this response. I have already shown that sequences downstream of -312 are sufficient for the feedback response to underproduction of Rpo21p (CHAPTER 4). The following are questions that need to be addressed.

1. Are sequences downstream of -312 also sufficient for the response to depletion of Fcp1p?

2. My data suggest that part of the feedback response is an increase in transcription from sequences downstream of -312. This increase was not detected from a reporter with 1583 bp of upstream sequences because of the counteracting effect of sequences upstream of -312 (CHAPTER 4). Can an increase in the steady-state level of *RPO21-*

lacZ mRNA be detected using a reporter gene with only sequences downstream of -312?

3. By swapping sequences of *RPO21* for *LEU2* upstream of +1, I showed that stability of the Rpo21p-β-galactosidase protein was not a factor in the feedback response to a depletion of Fcp1p (CHAPTER 4). Is this also true for the feedback response to the underproduction of Rpo21p? Since the *pLEU2-RPO21* strain (YF1971) relies on the promoter of *LEU2*, it would be necessary to choose a different set of upstream sequences to switch with those upstream of +1 in the *RPO21-lacZ* reporter. As the sequences of the *SCB-CYC1* reporter do not respond to an underproduction of Rpo21p (CHAPTER 2), they may be appropriate for this experiment.

4. The converse of the experiment described in 3 would be to determine whether the sequences between -312 and +1 are sufficient to generate the feedback response to either underproduction of Rpo21p or depletion of Fcp1p.

5. If the sequences between -312 and +1 are sufficient for the feedback response, what is the nature of these sequences? This question would best be answered through random mutagenesis of the region. By screening for reporter genes unable to respond to the underproduction of Rpo21p or the depletion of Fcp1p, the specific sequence elements could be identified. Analysis of the reporter gene mRNA by primer extension would indicate whether the mutations were affecting transcriptional initiation or post-transcriptional mechanisms. It would be informative to determine if mutants were affected in the same way by underproduction of Rpo21p or depletion of Fcp1p.

6. When candidate feedback response elements are identified, they must be shown to behave as such in the context of another gene. If the element affects the use of

transcriptional-start sites, it should be introduced upstream of the TATA-box and start-sites of *CYC1-lacZ* to test if this gene will now respond to underproduction of Rpo21p or depletion of Fcp1p. If the element affects post-transcriptional processes, it should be cloned downstream of the start-sites of the *CYC1-lacZ* test gene and tested for its response. The nature of any post-transcriptional effect should be investigated further by determining how RNA element(s) from the *RPO21* leader sequence influence the translation of mRNAs that contain them. This may be examined by determining the distribution of the mRNA in a polysome profile. Translational derepression would be indicated by a shift of mRNA from association with monosomes to polysomes upon underproduction of Rpo21p. This shift should depend on the presence of the *RPO21* RNA regulatory element in the leader of mRNA that is tested.

7. When elements have been shown to be sufficient for regulation, they may then be used as tools to detect DNA-protein interactions (in the case of elements that affect transcription) or RNA-protein interactions (for elements that affect post-transcriptional expression).

8. Interacting proteins, once detected, could be purified by a variety of techniques (conventional chromatography, affinity chromatography, EMSA) and, when sufficiently pure, identified by mass spectrometry.

9. The identity of the interacting proteins would largely determine subsequent experiments but, in general, the gene(s) encoding the protein(s) could be mutated to see if it is important for the feedback response. Analysis of mutants of the regulatory proteins would continue with screens for other genes that are regulated by the protein (*e.g.*, in the case of proteins that regulate transcription, through the use of gene-chip technology; Lashkari, *et al.* 1997). The other major challenge is to link regulatory

proteins to the signals (underproduction of Rpo21p or depletion of Fcp1p) that elicit the response. Genetic screens and biochemical experiments focused on identifying interacting proteins will be useful in this search.

RPO22 and the feedback response

My results indicate that the expression of *RPO22-lacZ* is not affected either by the underproduction of Rpo21p or by the depletion of Fcp1p (CHAPTER 4). At first glance, this may seem unusual, since Rpo22p is as important for the assembly of RNAPII as Rpo21p. When the amount RNAPII in the cell is reduced, why does the gene encoding the largest subunit of RNAPII respond with an increase in expression, while the gene encoding the second-largest subunit does not?

One possibility is that Rpo21p normally is synthesized at sub-stoichiometric amounts relative to Rpo22p. Therefore, the amount of Rpo21p might be rate-limiting for the assembly of RNAPII. Hence, an increase in the expression of *RPO21* would be the optimal response to a reduction in the amount of RNAPII in the cell. Most of the data concerning the amounts of RNAPII subunits focus on assembled RNA polymerase II molecules. The rates of synthesis of Rpo21p and Rpo22p have not been determined in yeast. It is known that some r-proteins, when expressed at levels greater than that required for assembly of ribosomes, are rapidly degraded (Maicas, *et al.* 1988). It is possible, therefore, that Rpo22p is synthesized at a higher rate than Rpo21p, but that unassembled subunits are targeted for degradation. There is some evidence for a relative abundance of the second-largest subunit of RNAPII in hamster cells (Guialis, *et al.* 1979). RNAPII was immunoprecipitated from cells that had been labelled with [^{35}S]methionine. Whereas radioactively-labelled largest subunit was detected in RNAPII from cells after 20 minutes of labelling, the second-largest subunit was not labelled. Only after a 30 minute chase period did RNAPII contain labelled

second-largest subunit (Guialis, *et al.* 1979). These data suggest that the reservoir of the second-largest subunit is in excess to that of the largest subunit.

In the course of our collaboration investigating the function of Fcp1p, Michael Kobor and I constructed a strain that combined the *pLEU2-RPO21* allele with the *FCP1*-shut off allele (YF2368; *MATa trp1-1 ura3-52 leu2::PET56 ade2 pLEU2-RPO21 fcp1::ANB-URLF-FCP1 TRP1*). Mr. Kobor showed that, when Fcp1p is depleted in this strain, the amount of Rpo21p does not increase as it did in the congenic strain in which *RPO21* expression is controlled by its own upstream sequences (M. Kobor, personal communication). This confirmed my results, shown in CHAPTER 4, which showed that, when Fcp1p is depleted, the increase in *RPO21-lacZ* expression was not dependent on the stability of Rpo21p-β-galactosidase, but rather on DNA or RNA elements that control the expression of the reporter gene. Mr. Kobor also examined the amount of other subunits of RNAPII in this strain. He noted that the amount of the fourth-largest subunit decreased (about 2-fold) while the amount of the second-largest subunit *increased* (about 3-fold). The implication was that in a cell depleted for Fcp1p, in which the synthesis of Rpo21p cannot increase (*i.e.*, no feedback response), there will be an increase in the amount of the second-largest subunit. Note that this experiment was carried out with cells grown in the absence of leucine, indicating that cells still contained a normal amount of Rpo21p. Therefore, when the diminution of RNAPII holoenzyme assembly is drastic enough (by depletion of Fcp1p and the elimination of the normal feedback response of *RPO21*), the expression of *RPO22* is now subject to a feedback mechanism. Experiments for following up on this discovery include:

1. Testing the effect of the depletion of Fcp1p (in a strain with a restriction of the *RPO21* feedback response) on the expression of *RPO22-lacZ*.

2. If there is an increase in expression, determine the level at which the response is operating (transcriptional or post-transcriptional).

3. Identify sequence elements that mediate the response and compare to those found in *RPO21*.

4. Test *RPO21-lacZ* for further increase in expression in this strain.

5. Combine the depletion of Fcp1p with the underproduction of Rpo21p by also growing the strain in the presence of leucine, isoleucine and threonine. Does depletion of Fcp1p in combination with a reduction in Rpo21p lead to an increase in the feedback response of *RPO21*, *RPO22* or both?

The stage is set for discovering mechanisms that control an evolutionarily conserved response to reduced levels of RNAPII activity in the cell. It seems likely that interesting answers to questions concerning these mechanisms can be attained with well-established molecular-biological techniques.

Applications of the *pLEU2-RPO21* strain (YF1971)

In collaboration with Jacques Archambault, I have shown that the underproduction of Rpo21p results in cells acquiring growth phenotypes that are characteristic of strains with mutations in the structural gene encoding Rpo21p (CHAPTER 2). These phenotypes include slow growth, temperature sensitivity and inositol auxotrophy. The expression of some genes is affected more strongly than

others by a 10-fold decrease in the amount of Rpo21p. In this section I outline two general approaches for further investigation of these results.

1. Studies of the expression of genes

Gene-chip technology offers the possibility of monitoring the difference between two yeast strains of the level of steady-state mRNA of every yeast gene (DeRisi, *et al.* 1997; Lashkari, *et al.* 1997). Holstege *et al.* (1998) have already used this technology to demonstrate the importance of *RPO21* for the expression of all protein-encoding genes (Holstege, *et al.* 1998). I propose using this technology to test the effect, on the expression of all yeast genes, of a 10-fold decrease in the level of Rpo21p. Cultures of YF1971 (*pLEU2-RPO21*), grown in the absence and presence of leucine, isoleucine and threonine, would be used to prepare mRNA. These two batches of mRNA would be converted to cDNA, tagged with two different chromophores, and then simultaneously hybridized to a chip containing an ordered array of DNA probes for every open reading frame from *S. cerevisiae*. The relative intensity of the chromophores at each spot would indicate the difference in the level of expression of each gene.

The information obtained would be a guide to genes that are particularly sensitive to the level of RNAPII in the cell. A comparison of the promoter structure of these genes would give clues to what makes some genes hypersensitive to RNAPII levels, while others are not. Furthermore, it may be useful to look for patterns in the genes that are in different categories of sensitivity. Are genes with well-defined TATA-boxes more or less sensitive to the level of RNAPII in the cell? Do any common sequence elements lend genes resistance to a decrease in the amount of RNAPII?

It would be interesting to distinguish genes that are directly sensitive to the amount of RNAPII in the cell from those that may be affected only by the decrease in the expression of another gene. It is unlikely, however, that the *pLEU2-RPO21* strain

would be useful for these kind of kinetic analyses, since the depletion of RNAPII would likely be too slow to offer insight.

2. Genetic pathways

Genetic screens have been useful for uncovering families of genes involved in related pathways. Screens for *sit*, *spt* and *swi* mutants are examples of screens that uncovered genes involved in transcription (Arndt, *et al.* 1989; Winston and Carlson 1992). YF1971 could be used to uncover a family of genes that interact with *RPO21*. Two general approaches could be taken:

Genetic suppressors

A selection for mutants that suppress the temperature-sensitive phenotype of YF1971 grown in the presence of leucine, isoleucine and threonine might reveal genes that normally have a negative influence on the expression of many genes. It will be necessary to separate trivial mutants that derepress the *LEU2* promoter and, therefore, allow a higher level of expression of *RPO21*. This could be achieved by using a *LEU2-lacZ* reporter gene. Interesting suppressor mutants could be identified using standard genetic techniques that include testing the suppressors for their own phenotype and cloning by complementation. Mutants may also be identified by genetic mapping using a variety of tester strains with well-spaced genetic markers.

Synthetic lethals

A screen for genes that are synthetically lethal with a 10-fold decrease in the amount of Rpo21p could be carried out as follows. (i) Mutagenize YF1971 with an appropriate mutagen. (ii) Plate out mutagenized cells on medium lacking leucine such that they form separate colonies. (iii) Patch cells from colonies onto a master plate lacking leucine and onto a test plate that contains leucine. (iv) Select candidates from the

master plate that correspond to patches that do not grow on the test plate. Clone the genes using standard genetic techniques that include complementation of phenotypes and genetic mapping.

The *pLEU2-RPO21* strain has already been used to test its effect on mutations of genes with related functions. I combined genetically the *pLEU2-RPO21* allele with mutations in *FCP1*. The result was that underproduction of Rpo21p was synthetically lethal with three different mutations in *FCP1* (D. Jansma and M. Kobor, unpublished results). Among the *FCP1* alleles that I tested was one with a deletion that results in an N-terminal truncation. In an otherwise wild-type background, or in the *pLEU2-RPO21* strain grown in the absence of leucine, this allele has no growth defect when grown under optimal yeast growth conditions (30° C, rich medium). This suggests that underproduction of Rpo21p may be a sensitive tool for assessing whether genes have functions related to transcription. The screen also may uncover previously unidentified genes that are important for the transcription of protein-coding genes.

Recently, YF1971 was used by Thomas *et al.* (1997) to test the effect *in vivo* of a short RNA molecule that inhibits RNAPII activity *in vitro*. Whereas over-expression of the RNA molecule in a wild-type strain had no effect on cell growth, over-expression in YF1971 grown on leucine medium resulted in a marked reduction in the rate of growth (Thomas, *et al.* 1997). Hence, a reduction in the amount of RNAPII in the cell was able to sensitize the cell to an inhibitor of RNAPII.

Johnson Wong, a graduate student in the laboratory of James Ingles, has demonstrated that underproduction of Rpo21p has no effect on the viability of cells exposed to UV radiation. When he combined the *pLEU2-RPO21* allele with mutations in genes involved in DNA repair, he saw an increase in the sensitivity of these cells to UV radiation (J. Wong and C. J. Ingles, personal communication). Therefore, underproduction of Rpo21p increases the sensitivity of cells to environmental insults

when combined with mutants already defective in the pathway. This may point to a relationship between transcription and DNA repair.

In conclusion, my analysis of the expression of RNAPII subunits and the effects of the underproduction of the largest subunit have increased our understanding of both the role of RNAPII in the cell and the mechanisms that regulate the production of this centrally important enzyme. Further investigations may uncover well-conserved processes that may be linked in interesting ways with molecular processes that have already been studied, or present new interactions that had hitherto remained undetected.

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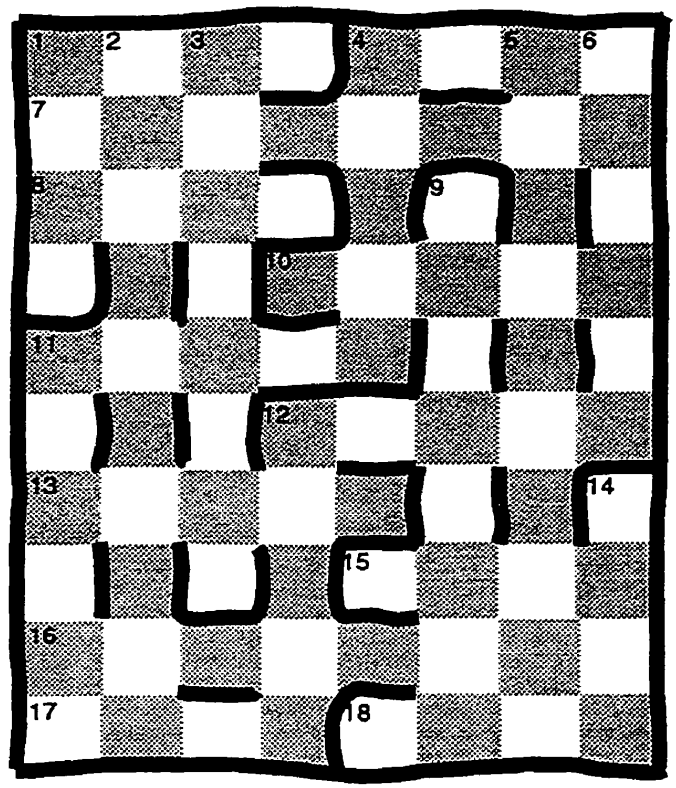
APPENDIX

A Cryptic Diversion



Across

1. Finishes five prime parts (4)

4. Race around a piece of land (4)

7. Dominatrix in back, held by drug dealer (8)

8. What the little engine thinks are inside pelicans (1, 3)

10. Crunch puts back one inside (5)

11. Gosh! See unending Canadian birds (5)

12. Rants about codon binders (5)

13. Rosie chewed up a willow shoot (5)

15. Atlas I assume holds continent (4)

16. It and I do crazy summation (8)

17. Guide boy holds direction (4)

18. I need, initially, north-south taverns (4)

Down

1. "Energy and speed of light grab researcher." Great story! (4)

2. "Drop the bomb on lion's flank", reported sugar bound to base (10)

3. James and Francis had this idea and turned back to history's beginning, before the "Spanish Nine" (3, 5)

4. Snake that is this easy? (2, 3)

5. Haul back in heavens and earth without opening orchestration (10)

6. Blows wild. P.S. True (6)

9. Saint, and evil substitutes (6, 2)

11. Worldwide goal: LB distributed (6)

12. Lukewarm tea is unfinished upon backward decline (5)

14. Terminates about poles (4)