SII AND ARREST IN *SACCHAROMYCES CEREVISIAE*

by

MATHEW GREGORY MILLER

A THESIS

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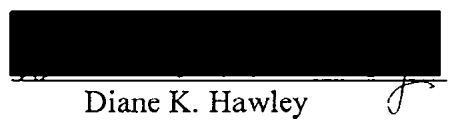
Approved : 

Diane K. Hawley

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Diane K. Hawley

In the absence of the elongation factor SII, RNA polymerase II halts transcription at certain sequences on the DNA template called arrest sequences; however, arrest is reversible in the presence SII. It is tempting to speculate that the cellular function of SII is to rescue arrested complexes formed *in vivo,* but the *in vivo* role of SII remains unestablished.

Thc subject of thesis is the *in vivo* investigation of SII and arrest phenomenon in *Saccharomyces cerevisiae*. To search for speculated SII homologues in yeast, we constructed a strain deleted of the SII gene and performed a suppressor screen of the SII deletion. We found no homologues of SII in *S. cerevisiae*, but we have found two suppressors containing the RRM binding motif that may elucidate the *in vivo* role of SII.

We have also directly searched for arrest *in vivo* by measuring expression of a reporter gene downstream of arrest sequences. Experiments directly analyzing the RNA products of transcription may soon provide a more complete picture of arrest in *S. cerevisiae*.

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I. INTRODUCTION

The field of biology has long engaged in studying nature's diversity, but in the recent history of science, discoveries have underscored the striking molecular and chemical order underlying this diversity. The landmark discovery of the structure of DNA by Watson and Crick in 1953 was another advance in this direction, and it confirmed the suspicions of many that DNA was the material responsible for genetic inheritance in nearly every organism. It also provided an explanation for how an organism's DNA could faithfully transmit information to descendants. Currently, one of the largest areas of biological research is concerned with understanding the mechanisms responsible for correct expression of the genetic information encoded by an organism's DNA. Ensuring correct expression through gene regulation is necessary for all life functions, including an organism's growth, adaptation to changing environments, and defense against infection. Because gene expression and gene regulation are the fundamental biological processes, research in these areas provides insights into cancer, HIV, and other human diseases. It is also true that many mechanisms of gene expression and regulation are conserved throughout nature. For this reason, biochemical paradigms discovered in bacteria like *Escherichia coli*, or in the yeast *Saccharomyces cerevisiae* provide effective tools for understanding problems in more complex animals like humans.

The subjects of this thesis are the characterization of a protein called SII and whether it affects transcription elongation in the budding yeast *S. cerevisiae*. The thesis begins with a discussion of transcription and previous knowledge of SII before describing experiments and results pertaining to its subjects.

Transcription

Inherent in the structure of the deoxyribonucleic acid (DNA) molecule are the capacities to guide its own replication and to store information, the two major biological functions of the molecule. DNA, like RNA and proteins, belongs to a class of molecules called polymers. These are large molecules composed of multiple smaller parts of similar structure called monomers. Polymers are important in nature because their structure allows information to be encoded within the molecule, and they allow the synthesis of a new molecule to be directed by the structure of another molecule in an ordered and predetermined manner. Polymers can be very complex molecules, but organisms can synthesize them from smaller and simpler components, analogous to how an entire novel may be composed of characters in the alphabet. A single DNA molecule may contain millions of monomers arranged contiguously as beads on a string, but it is composed of only four different types of monomers called nucleotides. These nucleotides contain the bases adenine, cytosine, guanine, and thymine and are named for their bases. The sequence of these four bases as they appear on a strand of DNA is sufficient to encode genetic information. An important characteristic of the bases is that, because of their physical properties, adenine will bond to thymine and cytosine will bond to guanine

through noncovalent annealing interactions. These interactions cause two complementary individual strands of DNA to pair and form the characteristic double-stranded structure of DNA.

When a gene exerts its influence over the organism, we say that it is expressed. Most genes in an organism contain sequence information for the synthesis of a protein molecule, but this information must first be transferred from the DNA to a related polymer called RNA. The process of *transcription* synthesizes a single-stranded RNA molecule with a sequence *complementary* to the strand of DNA used as the template.

During the initiation stage of transcription, enzymes cause the two strands of DNA to separate, and a large enzyme called the RNA Polymerase binds to the DNA. On the polymerase, individual RNA nucleotides pair with complementary bases on the DNA just as complementary strands of DNA would interact. The initiation phase of transcription concludes once the polymerase subunits have combined to form the polymerase *holoenzyme* and the first few RNA nucleotides have incorporated. The initiation stage is significant for gene regulation because it is a necessary step in gene expression, and various DNA or protein factors influence the probability that the holoenzyme will assemble to start transcription of a given gene.

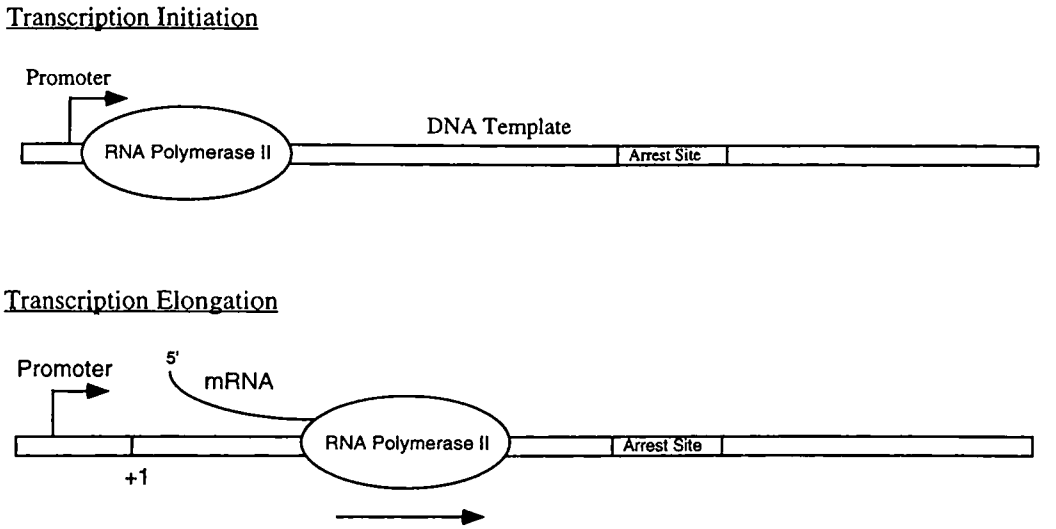

Figure 1. Transcription initiation and elongation. The polymerase assembles at the promoter and then synthesizes an RNA molecule while translocating along the DNA template.

In the elongation phase of transcription, the polymerase creates a long RNA molecule by catalyzing a chemical reaction that joins two consecutive RNA nucleotides together. This happens in a directional fashion because of qualities inherent in the RNA and DNA molecules; the beginning of the molecule is the 5' end, and the end of the molecule is the 3' end. After each successive nucleotide is joined to the 3' end of the growing RNA chain, the polymerase *translocates* along the DNA to the next position so that the reaction may continue. The final RNA product may itself act as an important cellular component, or it may contain information needed to synthesize protein.

Arrest and SII rescue

There are two ways in which investigators may solve biochemical problems. In order to characterize the molecular details of a biochemical reaction, *in vitro* experimentation- observation of chemical reactions between biological molecules outside of the organism- is necessary. On the other hand, in order to verify the natural significance of some phenomenon observed or postulated *in vitro, in vivo* experimentation must be performed, whereby researchers observe the biochemistry directly inside the organism. Researchers already understand much about the major subjects of this thesis, arrest and rescue, from *in vitro* experimentation.

In vitro transcription reactions under defined conditions reveal that certain DNA sequences cause the elongating RNA polymerase to stall on the DNA template and cease the polymerization of the attached RNA molecule. These sequences have been categorized as arrest sequences, and they have significant implications for the study of gene expression. Arrest sites are distinct from termination sites previously identified because the RNA polymerase stops at arrest sites without releasing the RNA chain.

One example, the human adenovirus major late (AdML) arrest site, had been well characterized *in vitro* before the commencement of this project. At the AdML arrest site, the elongating polymerase can arrest with greater than 90% efficiency (Holtz, 1995), but only in the absence of the protein SII. This efficiency is influenced by the concentration of the next nucleotide to be incorporated after the arrest position (Wiest and Hawley,

1990). These discoveries are particularly interesting because arrest is intrinsically associated with the polymerase, and does not require other *trans-acting* proteins.

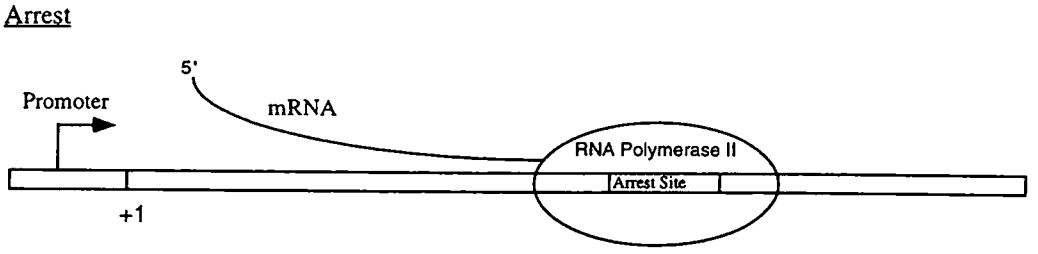

Figure 2. At the arrest site, the polymerase ceases to transcribe, yet remains attached to the DNA template and does not release the nascent RNA.

An equally interesting element of arrest is that arrest complexes formed *in vitro* are exceptionally stable and do not release the nascent mRNA from the polymerase or template. Although the arrested complex is stable, arrest observed *in vitro* is a reversible event. Arrested complexes may be "rescued" at the AdML and T1a arrest sites by the addition of a purified transcription elongation factor, SII (Wiest and Hawley, 1992; SivaRaman *et al.*, 1990). Upon addition of SII, transcription elongation resumes and allows synthesis of the full-length mRNA. It is known that cleavage at the 3' end of the mRNA is necessary for rescue of arrested complexes and that SII causes the stalled polymerase holoenzyme to move backwards on the DNA template.

One hypothesis for how cleavage results in read-through is based on the observation that the polymerase backs up along the DNA template. To the interest of many, the end of the mRNA attached to the polymerase is cleaved during SII rescue at the

AdML site such that the number of bases translocated backwards along the template corresponds to the amount of transcript cleaved (Wang and Hawley, 1993). It is possible that rescue occurs subsequent to the reversal of the polymerase on the template because the polymerase has a second opportunity to elongate through an arrest site that is not 100% efficient.

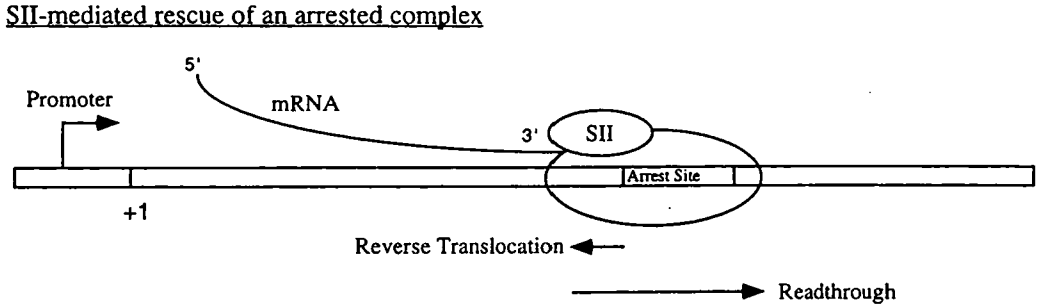

Figure 3. SII-mediated readthrough at arrest sites requires cleavage at the 3' end of the nascent transcript and backwards movement along the DNA template

Although SII can cause an arrested RNA polymerase to resume elongation *in vitro*, it is not known that SII serves the same function *in vivo*. There are several imaginable functions for arrest and rescue by SII within an organism. It has not escaped observation that coordinated reverse translocation and mRNA cleavage maintains the exact mRNA sequence after a complex is rescued, yet such fidelity would be necessary only if the integrity of the message were important. A phenomenon that might cause the polymerase to perform reversible pausing during a gene's transcription has the obvious potential to act as a regulatory mechanism for gene expression, in which case such fidelity could be beneficial. In addition, unpublished kinetic data (Thomas and Hawley),

combined with the observation that SII causes the polymerase to reverse along the template while excising the corresponding bases of the nascent mRNA, suggests that SII may be involved in a proofreading mechanism for RNA synthesis. Finally, a role of SII involvement in DNA repair could be imagined if repair machinery needs to reverse the RNA polymerase to gain access to concealed DNA aberrations. Each of these scenarios is possible, based on evidence gathered *in vitro*, but it is premature to claim that they function in nature without evidence *in vivo* that arrest actually occurs and these individual scenarios result.

In vivo evidence for arrest

Very little *in vivo* work with arrest phenomenon or the SII protein has been published to date. This is most significantly due to the difficulty of isolating the RNA products of arrested or prematurely terminated transcripts and a continued interest in the *in vitro* chemical and physical models of how arrest and rescue occur. However, some research has proceeded in the budding yeast *S. cerevisiae*. Geneticists commonly delete a gene in an organism to determine which functions are lost and are attributable to the gene of interest. This analysis had been published by the beginning of the project. An SII deletions in *S. cerevisiae* results in a viable organism, indicating that the gene is nonessential (Clark *et al.*, 1991); however, the SII deletion strain grows slowly compared to its parent strain under a variety of conditions. Furthermore, when grown in the presence of either mycophenolic acid (MPA) or 6-Azauracil (6-AU) at certain concentrations, the growth rate of the SII deletion strain is significantly reduced while

growth of the wild-type strain is only moderately affected. Both chemicals are enzyme inhibitors that ultimately cause decreased nucleotide pools in exposed cells (Sintchak *et al.*, 1996; Exinger and Lacroute, 1992). Lowered nucleotide concentrations inhibit elongation *in vivo* and increase the frequency of arrest *in vitro*, so it is hypothesized that arrest frequency increases *in vivo* due to this decreased nucleotide concentration, thereby conferring the slow growth *phenotype*. The reduction in growth on 6-AU may be restored, as expected, by inserting part of the SII gene into the yeast on a piece of DNA called a *plasmid*, suggesting that MPA and 6-AU possibly lead to increased frequency of arrest in the SII deletion strain (Nakanishi *et al.*, 1995).

SII Deletion Suppressor Screen

SII deletion is not lethal in *S. cerevisiae*, yet current *in vitro* evidence of arrest and SII behavior predict that loss of SII could potentially harm the cell. These considerations, taken with the fact that many organisms have more than a single copy of the same gene, suggest that an unidentified second copy, or *homologue*, of SII remains present in these strains and is responsible for their viability. Searching to determine the existence of an active SII homologue is a major subject of this thesis and was important for continuation of our work on arrest *in vivo*.

The suppressor screen is a powerful genetic technique which allows the researcher to recover homologues of a deleted gene along with other useful information about the organism. The screen begins with the deletion of the gene in question and the development of a screen which differentiates between the deletion strain and the wild-

type strain. Fragments of the organism's DNA carrying its own genes (a DNA "library") are *transformed* into the deleted strain, and the organism expresses the genes on the library-borne fragment in addition to its own genomic DNA. Expression of some fragments in the library may cause the transformed deletion strain to grow normally like the wild-type strain. In this case, the identity of the suppression fragment provides valuable information to the researcher.

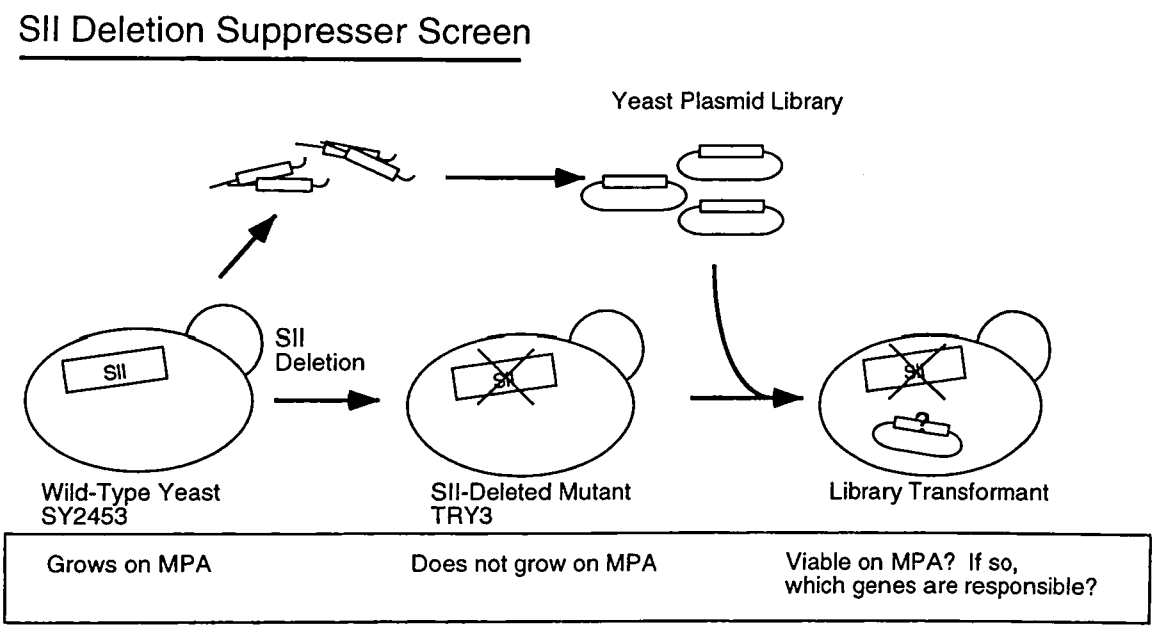

Figure 4. A SII Suppressor Screen Schematic. A SII-deleted yeast strain is transformed with a yeast plasmid library carrying gene fragments from the genome of the wild-type strain. If the growth characteristics of the library transformant are like those of the wild-type strain, then the gene fragment in the library plasmid may provide information about the deleted gene.

Our suppressor screen selects for genes which will compensate for the deletion of SII, so we expect to identify homologues of SII in yeast through this method, should they exist. These possible homologues present more than a curiosity for our research.

Deletion of SII and all homologues is necessary to determine if SII is an essential gene, and may be necessary for direct observation of arrest and SII-mediated rescue later in our research. The screen might also allow us to learn about the process of arrest *in vivo* if we find that certain elements of the transcription machinery or other novel genes suppress the SII deletion.

In vivo Arrest

It is widely speculated that the function of SII *in vivo* is to rescue arrested complexes; however, not even the phenomenon of arrest has been observed *in vivo*. Although the reduced growth of strains deleted of SII certainly indicate that SII has a significant function within the organism, it is premature to assert that SII rescues arrested complexes in natural situations. This thesis focuses on the direct *in vivo* observation of arrest and the effects of SII on arrest in *S. cerevisiae*.

We observe the activity of arrest sequences in yeast by observing *in vivo* expression of a reporter gene downstream from known *in vitro* arrest sites. The reporter gene is an easily observed protein encoded by the mRNA transcribed downstream of the arrest site. Our inducible *promoter*-reporter constructs with intervening arrest sequences and our SII deletion strain allow us to determine the activity of an arrest site in the absence of SII.

CHAPTER II: DELETION OF SII IN *S. CEREVISIAE*

Introduction

The budding yeast *S. cerevisiae* is widely used as a model eukaryotic organism because it has significant advantages over other eukaryotic cells and organisms. Yeast grow quickly and can be efficiently transformed with foreign DNA for ease in plasmid expression and identification. Yeast also have genetic advantages that allow for easy gene deletions and selections.

The deletion of SII from a model organism and initial observations of the new strain were vital to the remainder of the project. For a variety of reasons, we chose *S. cerevisiae* as our model *in vivo* system. Our promoter-reporter arrest observations require that we choose a model system which can easily express a reporter, and the suppressor screen requires manipulation of genetic material on a scale only practical with *S. cerevisiae*. An additional advantage to yeast is that purified yeast polymerase II arrests at the T1a arrest site *in vitro* (Cristie *et al.*, 1994), so a yeast system allows for comparison with previous *in vitro* experiments.

We confirmed the deletion of SII from *S. cerevisiae* primarily by direct means, but we also verified that the phenotype of our deletion strain similar to the slow growth phenotype of the SII deletion strain reported by Clark *et al.* (1991). We observed that this

phenotype was exaggerated in the presence of both 50 μg/ml mycophenolic acid (MPA) and 100μg/ml 6-azauracil (6-AU) as reported earlier by Exinger and Lacroute (1992).

Our SII deletion provides the opportunity to test a proposed model of SII function *in vivo*. SII has been observed*in vitro* to enable transcription at arrest sites, but the capacity to move backwards on the DNA template may also be significant in the process of DNA repair. Studies have shown that an elongating *E. coli* RNA polymerase halts upon encountering certain aberrations in the DNA strand (Selby *et al.*,1990). It is additionally possible that a stalled yeast RNA polymerase must be removed from the vicinity of the lesion to allow DNA repair machinery to enter the site during a process referred to as transcription-coupled repair. The stalled polymerase could leave the mutation site either by dissociating from the template or by moving backward along the template. SII, as an elongation factor, could potentially participate in either of these proposed events. To test this idea, we chose to compare the mutation sensitivity of our SII deletion strain with the isogenic wild type strain by exposing both strains to ultraviolet radiation.

Methods and Materials

We deleted the yeast SII gene in the yeast strain SY2453 because the strain contains several convenient and reliable auxotrophic markers, including *ura3*$^-$, *leu2*$^-$, and *his3*$^-$. We accomplished the deletion via the standard two-step disruption method because it has the advantage over the more simple one-step disruption process of maintaining the genetic marker.

The sequence of the yeast SII gene has been reported by different labs under two different names in the DNA databases, PPR2 and DST1, and was identified as SII by Ruet *et al.* (1990). The sequence of SII and its flanking regions was determined by combining these sequences and those of the nearby RNA15 gene to form a contiguous sequence of *PPR2* and its flanking regions. Flanking regions of PPR2 were subcloned and amplified by the Polymerase Chain Reaction (PCR) from a genomic preparation of SY2453 using Pfu polymerase. In order to maximize probability of mitotic recombination upon vector transformation, the upstream *PPR2* flanking sequence on the deletion plasmid had 532 bp homology with the 5' flanking region of *PPR2*, overlapping the open reading frame by 159 bp. The 3' flanking sequence used for disruption contained 426 bp of homology with 133 bp of overlap into the *PPR2* ORF. These upstream and downstream PCR fragments were designed with Kpn/ BamHI and BamHI/ SacI overhangs, respectively, and ligated with the vector pRS306 in the polylinker region to create the deletion plasmid pRS306-ΔSII. Ligations were transformed into E. coli strain DH5α for amplification, and proper insertion of the PCR fragments into the vector for multiple *E. coli* transformants was verified by restriction digest.

The pRS306-ΔSII construct was cut at the unique Bcl I restriction site in the upstream flanking sequence and transformed into SY2453 on -Ura medium to select for specific integration of the cut plasmid into the genome at the *PPR2* site. A single integration colony appeared, and a genomic DNA preparation was made from this transformant. Genomic integration of the plasmid was confirmed by PCR amplification using diagnostic primers hybridizing to regions inside and outside the homologous regions from the plasmid.

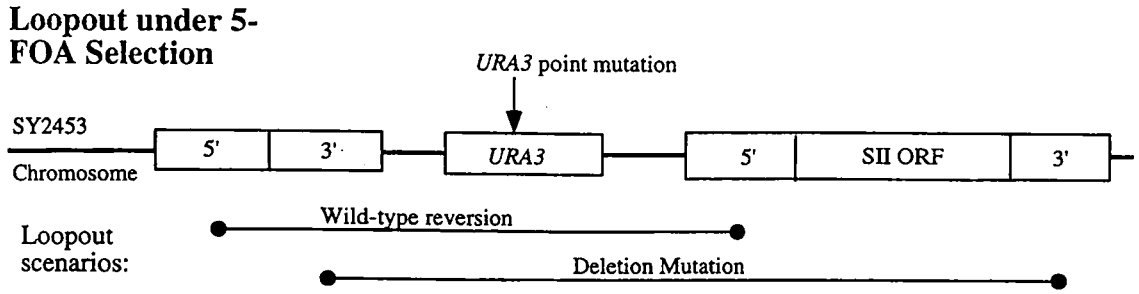

Figure 5. With the deletion plasmid integrated, selection against *URA3* results in three possible genetic consequences.

We selected for loopout of the deletion construct by streaking the integrated colony onto media containing 5-fluororutic acid (5-FOA) to select against *URA3*. Barring the unlikely event of a spontaneous point mutation in the integrated *URA3* gene, selection against *URA3* will result in yeast that have undergone recombination between the two copies of sequences present either upstream or downstream of *PPR2*. Recombination between upstream regions will result in a wild-type chromosomal loss of the deletion plasmid while recombination in the downstream region will result in the deletion of *PPR2*. Colonies growing on the selective media were therefore tested for reversion or SII deletion by PCR amplification using diagnostic primers.

The resulting mutant *ppr2* strain was dubbed TRY3. TRY3 grew more slowly on minimal media (SD + Ade) than its isogenic *PPR2* strain, in accordance with the observations of Clark *et al* (1991).

Growth of these strains was compared by analysis of their doubling times in log phase growth. Yeast cultures in synthetic glucose (SD) or rich media (YPD) were innoculated with dilute suspensions of either TRY3 or SY2453, and samples of these

cultures were grown at 30°C and 37°C. Growth of these cultures was observed by measuring scattering at 600nm with a Beckman DU-40 spectrophotometer over a 13 hour timecourse. Cultures with density above OD_{600}=0.300 were diluted to obtain accurate readings with the instrument. The natural log of cell density was plotted against time, and exponential growth constants were determined by linear regression of the linear portion of these graphs. The growth constant is equal to the slope of this growth, and doubling times were calculated from the relationship $t_d=\ln(2)/k_g$.

Ultraviolet sensitivity of TRY3 and SY2453 were determined by exposing plated cells to varying degrees of 254nm radiation in a Fischer Biotech FB-UVXL-1000 U.V. Crosslinker. Cultures of TRY3 and SY2453 were grown at 30°C in YPD into the log phase. Approximately 10^3 cells were plated onto YPD media, with the same amount of cells per plate, and were allowed to dry thoroughly before being exposed to varying degrees of radiation. Colonies appearing after two to three days were counted and tabulated.

Results and Discussion

PCR screening and growth phenotypes, taken with the previously reported phenotype of the SII deletion, confirmed that PPR2 was deleted from the strain TRY3.

Because growth phenotypes are the basis of our suppressor screen, we chose to quantitatively measure the doubling times of SY2453 and TRY3 at different temperatures and in different media. We wanted to determine if different temperature or media

conditions could help us differentiate more easily between the wild type and SII deletion strains.

Temperature Degrees C	Strain	Media	Exp. Growth Constant (1/hrs)	Doubling Time (hrs)
37	TRY3	YPD	0.39	1.77
37	TRY3	SD	0.41	1.68
37	SY2453	YPD	0.37	1.86
37	SY2453	SD	0.48	1.43
30	TRY3	YPD	0.34	2.04
30	TRY3	SD	0.43	1.63
30	SY2453	YPD	0.38	1.82
30	SY2453	SD	0.49	1.41

Table 1. Temperature and media dependence of TRY3 and SY2453 growth

Growth curves indicate that both strains double more quickly at 30°C than at 37°C, so we can speculate that the function of SII in the wild-type strain is not significantly sensitive to temperature in this range. Yeast generally grow more quickly in rich media, yet all strains doubled more rapidly in the minimal SD + Ade -Ura media than in rich YPD media. However, this is probably due to the genetics of the parent strain SY2453 or variability in the preparation of the media and does not affect future experiments because the strains are isogenic and only synthetic media is used for our experiments. Finally, we observed that, consistent with previous publications, the SII deletion strain grew more slowly than the wild-type strain. Thus, although we have not discovered significant new characteristics of the SII deletion strain, we have confirmed that the strain behaves as expected.

Ultraviolet irradiation causes one sort of DNA lesion that has been shown to halt an RNA elongating polymerase, the thymine dimer. However, we have observed that TRY3 is not more sensitive to ultraviolet radiation relative to SY2453, as measured by dose response curves measuring the viability of yeast after ultraviolet irradiation.

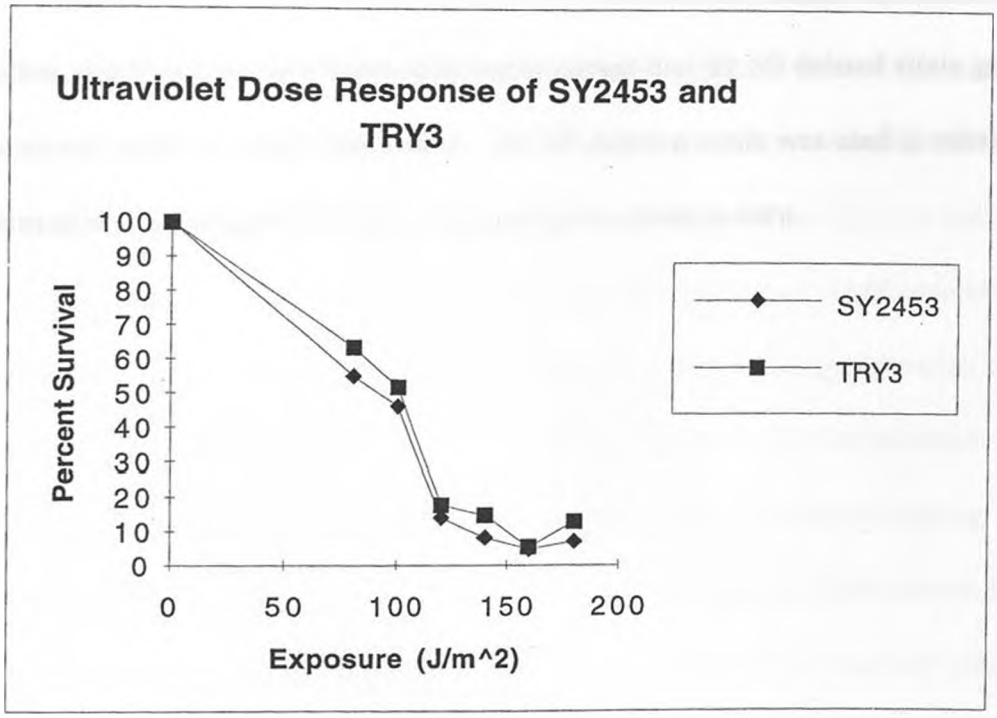

Figure 6. Ultraviolet Dose Response of SY2453 and TRY3. TRY3 and SY2453 are equivalently sensitive to Ultraviolet radiation at 254nm within statistical error.

The observed UV dose responses for SY2453 and TRY3, strains that differ only in the deletion of SII in TRY3, are equivalent within statistical error over the entire range tested. This experiment suggests that SII does not aid in the repair of ultraviolet damage,

and although SII involvement in repair of other DNA mutations caused by other sources is still possible, it is unlikely.

In the work described in this chapter, we performed and confirmed the deletion of SII in *S. cerevisiae* strain TRY3. The strain exhibited the same growth phenotypes relative to its parent strain and in the presence of MPA and 6-AU reported previously. We also quantified the response of both strains to temperature, media, and ultraviolet radiation, and found no significant differences except that the SII deleted strain grew more slowly under all conditions tested. The SII deletion strain was used in subsequent experiments to investigate the topic of transcription arrest *in vitro*.

CHAPTER III: AN SII DELETION SUPPRESSOR SCREEN

Introduction

One potential explanation for the viability of the SII mutant strain is that there is a functional homologue of SII somewhere in the yeast genome. SII has been shown *in vitro* to release RNA polymerase form an arrested state, and its role *in vivo* has been widely assumed to be related to this function. An arrested RNA polymerase could pose obstacles to further transcription of a gene if it did not terminate, thereby halting expression of a gene. Additionally, deletion of the SII gene could pose problems for the processes of RNA proofreading, DNA repair, DNA replication, or recombination events during meiosis. Therefore, deletion of the SII gene might be expected to be deleterious to yeast.

There is some evidence for the presence of SII homologues in yeast and other eukaryotic organisms. Southern hybridization, a technique used to observe sequence homology of DNA to a radiolabeled DNA probe, revealed that a probe containing SII sequence hybridized to multiple regions of the yeast genome under low stringency conditions (Archambault *et al.*, 1992). Although these sequences have not been identified, this result suggested that there might be genes in yeast that were related to the SII gene. Mice are known to have multiple forms of SII, expressed in a tissue-specific manner (Kanai *et al.*, 1991), suggesting that SII isoforms might exist in yeast. Although

this evidence is suggestive that SII homologues may exist in yeast, the presence of homologues is one of several ways to explain SII mutant viability.

The viability of the deletion strains may also be rationalized by considering that other proteins in the cell might partially compensate for loss of SII activity. When purified transcription elongation factor TFIIF, which increases that rate of transcription elongation *in vitro*, is included in *in vitro* reactions before the polymerase is allowed to elongate to the arrest site, the efficiency of arrest decreases (Price *et al.*, 1989). Other proteins are known to increase elongation rates *in vitro*, including pTEFb in Drosophila (Marshall and Price, 1995). The mammalian ELL, and elongin (SIII) proteins (Shilatifard *et al.*, 1992), possibly act similarly to TFIIF to minimize arrest frequencies *in vivo*. In fact, SIII shares significant homology with the amino terminus of SII (Aso *et al.*, 1995). Termination factors could potentially compensate for SII mutation as well, by allowing the cell to clear arrested complexes from the DNA template. Identification of these components of the transcription machinery as SII deletion suppressors would provide interesting information about the process of arrest *in vivo* and could lead to future genetic analysis.

The suppressor screen, because it directly selects for DNA elements able to suppress the SII deletion, is the natural genetic method to search for factors that compensate for the SII deletion and restore growth on the mycophenolic acid (MPA). Even if the deletion is not naturally suppressed by cellular elements at normal levels of expression, it is possible that over-expression of some genes by the strong and inducible Gal1 promoter on a library plasmid could suppress the deletion.

We have performed a partial suppressor screen of the SII deletion and discovered no homologues of SII in yeast. However, we have found several interesting suppressors and determined the genes responsible for suppression on the suppressor fragments by isolation of the suspected open reading frames (ORFs) involved. Two confirmed suppressor open reading frames are truncated RNA binding proteins retaining elements of the conserved RNA recognition motif (RRM) binding domain and may potentially provide insight into arrest phenomenon through future work.

Materials and Methods

The yeast genomic library used in our suppressor screen was constructed in the laboratory of Ronald Davis (Elledge *et al.*, 1991), and is based on the pYES-R plasmid. The library was constructed by physically shearing yeast genomic DNA into fragments of approximately 6000 bp in length and ligating them into a phage library for propagation. The average length of a genomic suppression fragment observed among true suppressors in our screen was slightly shorter: around 5000 bp. The vector contains sequences sufficient for propagation at high copy in *E. coli* and contains the CEN/ARS and *URA3* sequences for propagation and plasmid selection in yeast. The library plasmid also has an inducible Gal promoter for selective expression of genes fused next to it in the correct orientation. The vector is nearly 7 kb in length even without the genomic fragment. The sizes of the suppression plasmids caused noticeable but manageable difficulties in plasmid manipulations during the screen.

We estimated that to execute the suppressor screen to its completion, we should have to screen approximately 720,000 individual transformants. The yeast genome contains an estimated 6000 genes distributed over 12 million base pairs (Goffeau *et al.*, 1996). This gives 2 x $12x10^6$ =$24x10^6$ possible starting points for a given sequence when ligated to the Gal1 promoter on the library plasmid. In order to observe a particular sequence ligated within 100 bp of the 5' end of the fragment in a given plasmid, the probability is P(100)= $100/24x10^6$. In order to make the probability of not observing a given sequence within 100 bp of the promoter equal to 5%, we need to make n independent observations where n is described by: $P=[1-(100/24x10^6)]^n=0.05$. This method of estimation predicts that we will have a 95% chance of observing any given gene ligated to within 100 bp of the Gal1 promoter after screening n=720,000 colonies. Gene lengths, selection for certain ligations into the pYES-R vector, and irregularities in DNA shearing may all increase the number of observations necessary for a complete screen.

The phenotypic differences between SY2453 and TRY3 are differentiable by eye, but do not provide sufficient resolution for a screening procedure in which 720,000 individual transformants are to be screened. Thus, we used the extremely slow growth of the SII deletion strain on the substrates mycophenolic acid (MPA) and 6-azauracil (6-AU) to differentiate suppressors from nonsuppressors in our screen.

In the screen, TRY3 samples transformed with the plasmid library were plated onto SD +Ade -Ura medium to compensate for the adenine deficiency of TRY3 inherited from SY2453 and to select for transformed colonies. Between 200 and 1800 colonies, depending on efficiency of transformation, were allowed to grow for approximately 48

hours and were then replica plated to plates containing SG +Ade -Ura + 50μg/ml MPA media to select for suppression of the SII phenotype. Colonies were allowed to grow from between four to eight days on the selective media before we picked putative suppressors.

Suppressor colonies grew significantly faster than surrounding colonies, as expected, and they appeared to suppress to different degrees. TRY3's adenine deficiency proved useful in differentiating suppressors because non suppressing colonies developed the red colored phenotype characteristic of adenine deficiency before the suppressing colonies developed the phenotype.

While the transformed colonies grow on + MPA media, they experience selective pressure to develop mutations which confer resistance to MPA. For this reason, it was likely that some of the putative suppressors grew, not because of the suppression fragment on the library plasmid, but due to a spontaneous mutation selected by growth conditions. The subjective nature of the selection based on growth phenotype from a replica plated colony also presents a source of error in the screen. To eliminate these undesirable candidate suppressors from our screen, we ensured that the genetic material conferring suppression was plasmid-borne.

Library plasmids were harvested from suppressing colonies and transformed into *E. coli* strain DH5α for amplification. Once the library plasmids were isolated from our 96 putatitve suppressors and amplified, they were transformed back into TRY3 and tested for growth phenotype by streaking and spot tests. In the streak test, a small amount of each newly transformed colony was streaked onto + 50 μg/ml MPA or 100 μg/ml 6-AU plates, and growth was observed after four to six days. In the spot test, about 20 μl of a

very dilute SD cell suspension was dropped onto + MPA or +6-AU media and observed after four to eight days of growth. Growth rates of the parent strain SY2453 and TRY3 carrying the null plasmid pSL1597 were used to control these experiments, and each of the colonies tested were scored on an arbitrary scale of one to five for growth rate between that of TRY3 and SY2453. We were then prepared to sequence and interpret the library plasmids found to confer suppression in this secondary screen

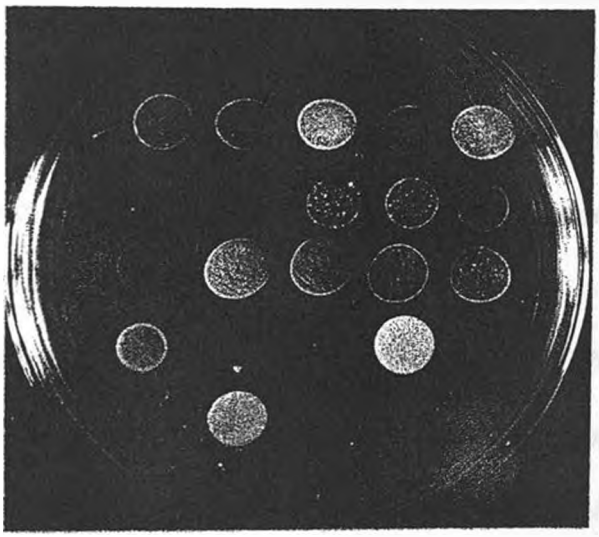

Figure 7. An example of a spot test used in the secondary screen to identify true plasmid-borne suppressors.

Nearly the entire yeast genome had been sequenced by the time we began to identify and characterize the 58 true suppression fragments found in the screen. In addition, the Stanford Genome Database provided access to sequences and search functions, allowing us to easily identify the sequence of entire suppression fragments. We sequenced close to 150 bp at the 5' end of every suppressor fragment with a primer annealing to the vector, and we used this sequence to probe the database. If it was

necessary to determine the 3' end of the suppression fragment, we used an alternative primer to obtain sequence information from the other end of the fragment.

Most of the suppression fragments contained multiple open reading frames (ORFs), so cloning techniques were used to determine the ORF responsible for suppression. Among these were SDS 87 and SDS 59. We expected that the truncated open reading frame fused to the gal promoter in SDS 87 was responsible for the weak suppression conferred by this plasmid, so sequential deletions at the 3' end of the plasmid were performed by restriction digest until only the truncated ORF fused to the Gal promoter remained intact. The resulting phenotype of this construct was one of considerable suppression, suppressing more than the original fragment but less than the Suppressors carrying IMPDH or PPR2.

SDS 59 contained an ORF known to code for the gene NOP4. This ORF was inserted in the opposite orientation from the Gal promoter in the library plasmid and was truncated on the Gal side of the suppression fragment. We expected that this ORF, expressed in its truncated form and driven by its own promoter in the suppression fragment, was responsible for conferring suppression. We subcloned this fragment by PCR into the vector pSL1597 downstream of the Gal1 promoter and engineered an ochre stop codon at the location of the protein's truncation in the library plasmid. We observed that this construct conferred weak suppression in transformed TRY3 strains, but suppression was more vigorous than on the original library suppressor plasmid.

Results and Discussion

We screened approximately 133,000 colonies for suppression of the SII deletion, well under the value of 720,000 suppressors we estimated is needed to guarantee 95% certainty of observing a given DNA sequence fused to within 100 bp of the Gal1 promoter. Out of these colonies, 94 were identified as putative suppressors in the primary screen, at a rate of one putative suppressor for every 1300 transformed colonies. Of 94 putative suppressors, 58 were confirmed to have plasmid-borne suppression for a recovery rate of 62%. Out of 58 suppressors, we recovered only one with the suppressing ORF inducible by the Gal1 promoter.

A suppressor screen benefits from reasonably stringent conditions so that not all library transformants appear to marginally suppress, because too many false suppressors identified in the primary screen would have to be tested in the secondary screen otherwise. At the same time, it is important that screening conditions are not so stringent as to eliminate weaker suppressors from the selection process. Our 62% suppression recovery rate was fairly constant throughout the screen and indicates that our screen is efficient, yet it is not so stringent as to eliminate weak suppressors as evidenced by the weakness of some of the suppressors we have detected. The recovery of the SII gene on suppressor SDS 1 from a minimal plate containing 50μg/ml MPA from approximately 1000 transformants confirmed the ability of our screen to select for active suppressor fragments from the library under growth conditions of 50μg/ml MPA.

We recovered eight suppressor fragments containing the gene PPR2 (SII), the most vigorous suppressors we have observed. The recovery of the deleted gene was expected and thus serves as an internal control for the suppressor screen, confirming that

the screen selects for DNA sequences that complement the deletion of PPR2. All eight SII suppressor fragments contained the SII ORF in the middle of the fragment or in the opposite orientation such that *PPR2*'s expression was not driven by the Gal1 promoter. We verified that the SII suppressors were expressed by their own promoters by observing that they suppressed equally well on SG and SD media, the latter of which would have repressed the Gal1 promoter. This observation raises an interesting issue. It is possible that overexpression of SII *in vivo* could be harmful to the organism. A simple future experiment would be to clone SII into the CEN/ARS plasmid pSL1597, directly under the control of the Gal1 promoter. It would also be interesting to observe the activity of the same arrangement on a higher copy 2μ plasmid.

We expected to recover suppressor fragments carrying genes coding for the enzyme IMP dehydrogenase (IMPDH) from our screen. MPA is known to be a potent uncompetitive, reversible inhibitor of IMPDH (Franklin and Crooke, 1969), and it had been speculated that the sensitivity of the SII mutant strain to MPA arises due to loss of activity in this enzyme. IMP Dehydrogenase catalyzes a reaction in the *de novo* nucleotide synthesis pathway (Crabtree and Henderson, 1971), thus MPA decreases *in vivo* nucleotide concentrations (Exinger and Lacroute, 1992). Interestingly, *in vitro* transcription demonstrates that the concentration of the next nucleotide to be incorporated affects frequency of arrest (Wiest and Hawley, 1990). Thus, it is reasonable to propose that the decreased *de novo* nucleotide synthesis is responsible for increased MPA sensitivity in SII mutants. If IMP dehydrogenase were expressed at higher levels in the cell by the suppressor fragment, it is possible that the *de novo* pathway would be less inhibited by IMP dehydrogenase inhibition.

We ultimately identified 41 true suppressor fragments carrying one of three redundant genes coding for IMP dehydrogenase. The recovery of IMPDH from our suppressor screen is important. Previously, the expectation that MPA affects SII mutants by lowering nucleotide concentrations was only speculation. IMPDH suppression of the SII deletion growth phenotype indicates that the effect of MPA on TRY3 growth is due to loss of IMPDH activity and decreased nucleotide concentrations rather than through some indirect mechanism. Our discovery provides support for the occurrence of arrest *in vivo* to the extent that it is consistent with lowered nucleotide concentrations causing an increased frequency of arrest *in vitro*.

A large number of IMPDH suppressor sequences gave us difficulties in the beginning of our screen. Sequencing from a primer annealing to the bottom strand in the Gal promoter region on the vector resulted in sequences containing only A and C residues. These sequences are characteristic of DNA in the telomeres which contain several repeated motifs, so they cannot be used to probe the database. We briefly entertained the idea that these extra telomeric sequences could be responsible for titrating certain proteins away from the organism's chromosomes, but another problem presented itself: telomeres had only ligated into the library plasmid in one orientation. No G or T residues were observed directly fused to the Gal promoter. To solve the problem, we sequenced some of the "telomeric" suppression fragments from primers annealing to the other side of the vector, and we found that an IMPDH gene was present on the far ends of these fragments. The presence of an IMPDH gene in each of our suppressor fragments containing telomeric sequences was confirmed by PCR using primers internal to IMPDH.

Several open reading frames coding for hypothetical membrane proteins or proteins related to drug resistance were found on suppression fragments conferring the suppressor phenotypes, but this background contamination is hard to avoid in a screen based on drug sensitivity.

Three unique suppression fragments turned out to be the major findings of the suppressor screen. All three suppressors, SDS 59, SDS 87, and SDS 97, contain truncated open reading frames with sequences homologous to RNA recognition motifs (RRMs). We confirmed that the suppression on fragments SDS 59 and SDS 87 were due to the suspected open reading frames by cloning the ORF in SDS 59 under control of the Gal1 promoter and deleting other ORFs on the suppression plasmid for SDS87, as described in materials and methods.

The ORF in SDS 59 codes for a truncated version of the gene *NOP4*, an RNA binding protein involved in ribosomal RNA processing (Sun and Woolford, 1994) that belongs to the RRM family of RNA binding proteins. The RRM motif, also referred to as the CS-RBD or RNP motif, is a very common motif comprised of family members able to bind both single and double-stranded RNA. The hallmark of the RRM motif is a pair of two conserved sequences, the RNP1 octamer and the somewhat less conserved RNP2 hexamer (reviewed in Dreyfuss *et al.*, 1993). Wild type *NOP4* contains two perfect RRMs flanking an imperfect RRM which lacks the characteristic RNP1 and RNP2 binding motifs, and finally a fourth incomplete RRM. In the truncated form present in SDS 59, the imperfect binding domain and the first consensus binding domain remain intact, the second consensus domain is truncated, and the incomplete RRM and nuclear localization sequences further towards the C-terminus are absent from the suppression

fragment. It is reasonable to expect that the truncated *NOP4* has lost its wild-type functionality and assumed a new function when suppressing SII.

SDS 87 contains the C-terminus of the as-yet uncharacterized open reading frame YJRO98C. The open reading frame predicted for translation in the suppressor fragment is driven by the Gal promoter and codes for 97 amino acids with 27% identity to NOP4 over 80 of these amino acids. Furthermore, the protein contains a semi-conserved RNP1 domain with two amino acid variations from the RNP1 conserved sequence octamer. The RNP1 domain is one of two elements conserved in the RRMs present in *NOP4*. In light of the fact that the RRM-containing *NOP4* truncation confers suppression, it is reasonable to hypothesize that the small ORF on SDS87 confers suppression by binding RNA. We have identified an additional small ORF on SDS 97 that has potential RNA binding capability, although we have not confirmed this ORF to be responsible for suppression of that fragment.

Previous *in vitro* experimentation provides insight into a possible mode of action by which these RNA binding suppressor fragments compensate for the SII mutation. The adenovirus major late arrest site causes efficient arrest *in vitro* under normal conditions; however, addition of oligonucleotides complementary to the nascent mRNA just upstream of the arrest site prevents arrest. It is thought that arrest occurs by retraction of the nascent transcript into the interior of the polymerase and that hybridization of oligonucleotides to the mRNA prevents this process from occurring (Reeder and Hawley, 1996). The polymerase then continues elongation without arresting. The RNA binding domains present on our unique suppressors may bind nonspecifically or specifically to the

nascent mRNAs upstream of arresting polymerases on the yeast genome, suppressing the SII deletion by preventing backward polymerase movement and, hence, arrest.

A final and important conclusion from this screen is that homologues of SII probably do not exist in TRY3. If an SII homologue exists which is able to suppress the SII deletion equivalently to *PPR2* in the primary screen, then the probability of selecting eight PPR2 genes and no homologues is $(0.5)^8 = 0.39\%$. This assumes that the genes are equally represented in the library and that there is no selection for transformation of either clone during transformations for the primary screen, but these assumptions are fairly reasonable. However, we have only isolated SII under its own promoter, so it is probable that we would not have not observed any putative SII homologue under Gal1 regulation. Therefore, our statistics do not rule out the existence of a homologue if it were expressed at very low levels by its native promoter. In this case, however, the *in vivo* concentration of the SII homologue should not be significant, so TRY3 would not contain noticeable SII activity. Having established the absence of significant SII activity in TRY3, we now proceed to observe arrest *in vivo*.

CHAPTER IV: OBSERVATION OF ARREST AND SII *IN VIVO*

Introduction

A number of genes transcribed by pol II *in vivo* have been found to contain sequences that efficiently block elongation by pol II *in vitro* (Spencer and Groudine, 1990; Krumm *et al.*, 1993). We used our SII deletion strain and its isogenic parent to test whether we could observe arrest at two of these sequences *in vivo*.

To accomplish this task, we followed expression of the β-galactosidase reporter gene downstream of arrest sites on templates driven by strong inducible promoters. We have chosen the Fus1 and Gal1 promoters for this purpose and have begun with the wild-type AdML and T1a arrest sites for our intervening sequences. We compared expression from these constructs relative to expression from the corresponding construct without intervening sequence to determine the degrees of arrest.

Materials and Methods

The constructs used in these experiments are based on the pTYP1 plasmid. The plasmid was constructed from the pYLZ family (Hermann et al., 1992) and contains several convenient restriction sites within a polylinker region. The lacZ reporter gene is fused just downstream of this polylinker region and efficient translation is ensured by a

His 4 translation initiator just upstream of the initiating AUG. pTYP1 may be easily converted from a 2μ plasmid to a CEN/ARS plasmid.

The Fus1 and Gal1 promoters were cloned into pTYP1 for construction of the pFus and pGal plasmids. Both the Gal1 and Fus1 promoters were subcloned via PCR, and appropriate restriction sites were incorporated onto the end of the PCR primers so that the promoter fragments could be ligated between the EcoRI and Bgl II sites in the polylinker region of pTYP1. The Fus1 promoter region from -261 to -7 with respect to the translation start of Fus1was amplified from pSL553, and the Gal1 promoter from -422 to +42 with respect to the Gal1 transcription start site was amplified from pSL1597. Arrest sequences were cloned between the Xho I and Hind III sites on pFus and pGal by hybridization and ligation of oligonucleotides coding for the arrest sites in appropriate plasmids.

β-Galactosidase assays were performed as in Jarvis *et al.* (Jarvis *et al.*, 1988), except for minor modifications. pFus cultures were induced by the addition of 1ml YEPD with 2.5 μg/ml or 1.25 μg/ml α-factor, and pGal cultures were induced or repressed by 48 hours of growth in SG or SD media. All cultures were grown at 30°C. Reactions were performed in volumes smaller than in Jarvis *et al.*, so a correction factor of (1.5 mls/2.0 mls) compensates for the increased relative concentration of cell suspension in the assay and allows us to report absolute values in modified Miller Units as in Jarvis *et al.* Optical densities were measured using a Beckman DU-40 Spectrophotometer.

The corrected units of β-galactosidase activity are calculated as follows.

$$\text{Units} = \frac{1000 \times OD_{420} \times d}{t \times v \times OD_{600}} \times \left(\frac{1.5}{2.0}\right)$$

In the above calculation, d is the dilution factor of resuspended cells in the assay (vol cells/total volume), t is time in minutes, v is volume of cells added to the reaction in mls, OD_{420} is the density of the reaction supernatant, and OD_{600} is the density of the cells added to the assay.

Experiments and Results

Time-Independent Decrease in β-galactosidase Expression

We measured an induction timecourse of β-galactosidase activity for the pFus, pFus-AdAr, and pFus-T1a constructs in our first experiments to observe the effects of the AdAr and T1a arrest sites *in vivo*. Expression was monitored for a 3.5 hour period, but was expected to decrease after 2.5 hours because induction with α-factor halts the cell cycle of MATa cells such as TRY3.

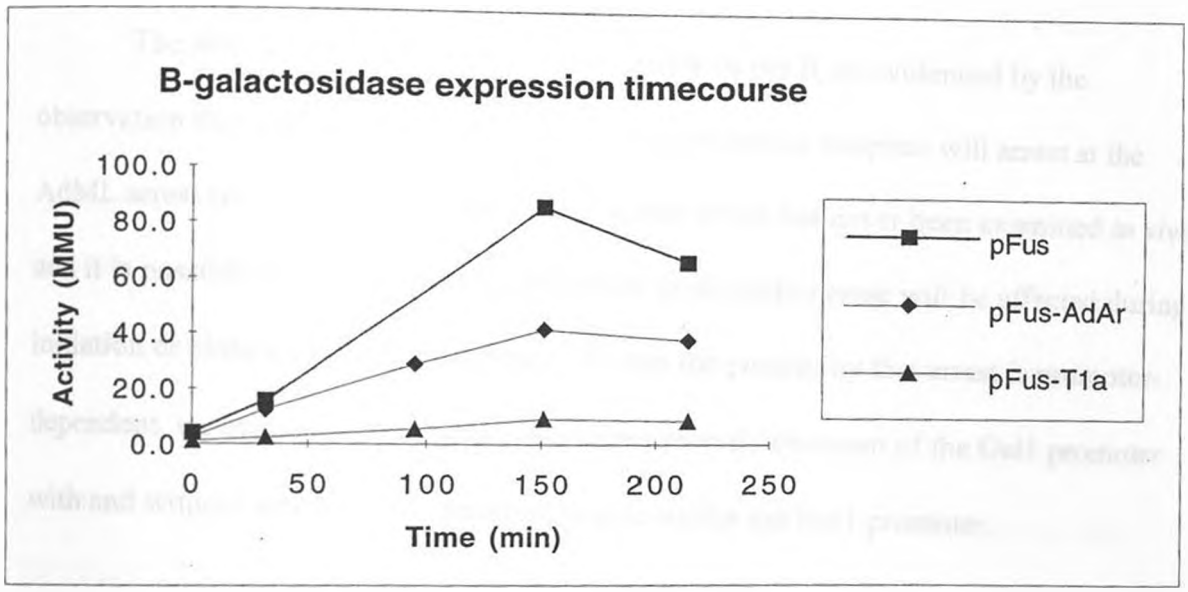

Figure 8. Expression of β-galactosidase downstream from the AdMl and T1a arrest sites in pFus-AdAr and pFus-T1a is decreased from pFus expression levels in a time-independent manner. Basal expression of these promoters is indicated at t=0, and all other timepoints have been induced with 2.5 μg/ml α-factor.

We observed significantly less β-galactosidase expression in the pFus-AdAr and pFus-T1a constructs than in the pFus1 construct, and the similarity between all curve shapes further indicates that this decrease in expression is time-independent. The results suggest that arrest occurs *in vivo* on the pFus-AdAr and pFus-T1a constructs, provided that we can eliminate other possible reasons for the inserted AdML and T1a sequences to decrease β-galactosidase expression.

Promoter-Independent Decrease of β-galactosidase expression

The ability to arrest is an intrinsic property of pol II, as evidenced by the observation that purified pol II transcribing a promoterless template will arrest at the AdML arrest site. (Wiest *et al.*, 1992). However, arrest has never been examined *in vivo*, and it is possible that the elongation efficiency of the polymerase will be affected during initiation or elongation by the promoter. To test the possibility that arrest is promoter-dependent, we evaluated β-galactosidase expression downstream of the Gal1 promoter with and without arrest sites in addition to activity for the Fus1 promoter.

Promoter Family:	pGal/pFus (MMU)	pGal/pFus-T1a (MMU)	Fractional Activity
pGal	259.6	28.1	0.108 ± 0.031
pFus	67.1	8.2	0.122 ± 0.034

Table 2. Promoter-independent reduction of β-galactosidase expression. All observations are made in TRY3 transformants of the plasmids indicated. PFus transformants were induced with 1.25 μg/ml α-factor and induced levels of β-galacotsidase were measured after 150 min while pGal transformants were induced by 48 hour growth in SG media.

We found that β-galactosidase expression downstream of an arrest sequence in the pGal-derived constructs pGal-AdAr and pGal-T1a is decreased from the levels expressed by pGal by the same relative amount for a given arrest site. Thus, the observed decrease in gene expression downstream of the Gal1 and Fus1 promoters is promoter-independent. Given this evidence, it is possible but unlikely that either promoter affects arrest

frequency downstream because both promoters would have to influence arrest equally for this to be the case.

Relative β-galactosidase Expression is Strain-Independent.

Although we expect that arrest occurs in both TRY3 and SY2453, we expect that SII activity in SY2453 should rescue arrested complexes, thereby increasing expression of β-galactosidase downstream of the AdML and T1a arrest sites in our arrest constructs from levels expressed in TRY3, which does not contain SII activity. We measured β-galactosidase expression from both pGal and pFus arrest constructs transformed into TRY3 and SY2453.

Strain:	pFus-T1a Fractional activity	pFus-AdAr Fractional activity
TRY3	0.122 ± 0.034	0.497 ± 0.051
SY2453	0.127 ± 0.059	0.537 ± 0.038

Table 3. Strain-independent reduction of β-galactosidase expression. pFus, pFus-T1a and pFus-AdAr cultures were induced with 1.25 μg/ml α-factor, and β-galactosidase expression was measured after 150 min. Fractional activity is the ratio of the arrest construct's activity to pFus activity in a given strain.

We observed in the experiments above that relative β-galactosidase expression in the pFus and pGal constructs is independent of strain. Reduction of β-galactosidase expression on the pFus and pGal arrest constructs was equal in TRY 3 and SY2453,

contrary to our expectation that the absence of SII activity in TRY3 would decrease expression of β-galactosidase downstream of arrest sites to a greater degree. One possible explanation for this observation is that decreased β-galactosidase expression in the constructs containing arrest sites is not due to arrest, because SII is not involved. It is also possible that arrest does occur, but SII does not function *in vivo* to rescue arrested complexes. Alternatively, arrest may occur in both strains, but arrested complexes are quickly terminated before SII has the opportunity to act.

We also observed in the experiments above (data not shown) that TRY3 expressed β-galactosidase at higher absolute levels for a given construct, regardless of the construct. This finding is consistent with observed kinetic data showing that SII slows transcription by RNA polymerase II *in vitro*.

Arrest site mutations do not restore β-galactosidase expression

An essential control for this project is to demonstrate that mutations known to abolish activity of arrest sites *in vitro* prevent the reduction of β-galactosidase activity *in vivo*. We observed the effects of two T1a arrest site mutants, a dual point mutant and a sequence inversion, identified by Kerpolla and Kane as sites no longer conferring significant arrest activity *in vitro* (Kerpolla and Kane, 1990). We measured the effects of these mutant sites on β-galactosidase activity in the pFus constructs to provide negative controls for our previous experiments.

Strain	Fraction of pFus activity	
	pFus-T1aMUT	pFus-T1aINV
TRY3	0.065 ± 0.030	0.234 ± 0.055
SY2453	0.163 ± 0.023	0.309 ± 0.053

Table 4. Decreased β-galactosidase expression in mutant arrest constructs. pFus, pFus-T1aMUT and pFus-T1aINV cultures were induced with 1.25 μg/ml α-factor, and β-galactosidase expression was measured after 150 min.

Contrary to our expectation, we observed that even the mutant arrest sites significantly reduced downstream β-galactosidase expression in the pFus context. Assuming that arrest did not occur at these mutant sites *in vivo*, as is predicted by their reported *in vitro* behavior, this result suggests that the nucleotide sequence of the leader is responsible for decreased expression in both the consensus and mutant sequences in a manner not connected with arrest phenomenon. However, we cannot prove that these mutant sites did not cause arrest *in vivo*. Runs of consecutive T residues are associated with most known arrest sites and are present in the wild-type T1a site, but it is possible that, *in vivo*, a run of A residues is sufficient to cause arrest. The same argument can be made for the double point mutant.

To address this problem, we constructed two more negative controls which we expect have no potential for arrest, yet which maintain the length of the mRNA leader. We replaced the T runs in both the AdML and T1a arrest sites to random sequences of other residues to produce the pFus-AdArGC and pFus-T1a constucts and repeated the above experiments with the second set of negative controls.

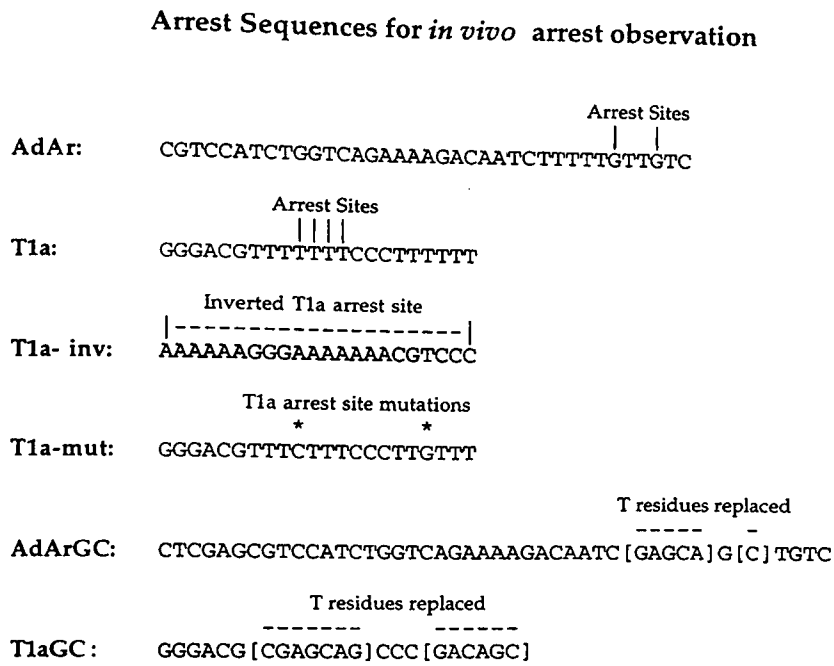

Figure 9. Arrest sequences used for *in vivo* arrest observation

	Fraction of pFus activity
pFus-AdAr	0.501 ± 0.100
pFus-T1a	0.087 ± 0.010
pFus-AdArGC	0.340 ± 0.057
pFus-T1aGC	0.023 ± 0.004

Table 5. Decreased β-galactosidase expression in alternative mutant arrest constructs. pFus, pFus-AdArGC and pFus-T1aGC cultures were induced with 1.25 μg/ml α-factor, and β-galactosidase expression was measured after 150 min. All observations are made in TRY3.

We observed that the second pair of mutant constructs, pFus-AdArGC and pFus-T1aGC reduced downstream expression of β-galactosidase to an even greater degree than the wild-type arrest sites. Like the previous mutant sites, these new sequences suggest that decreased downstream gene expression is due to an event other than arrest.

Discussion

We have shown in these experiments that arrest sequences reduce downstream gene expression in the context of our pFus and pGal constructs, but we have also observed the same reduction with mutant arrest sequences which we do not expect to cause arrest *in vivo*.

One possible explanation for the uniform activity of all our intervening arrest sequences is that they affect translation efficiency instead of, or in addition to, transcription efficiency. A problem inherent in our present method of conducting experiments is that β-galactosidase must go through both the transcription and translation phases of gene expression before being observed. Arrest is exclusively a transcription phenomenon, and the translation step in β-galactosidase expression confounds the interpretation of our results. We cannot simultaneously abolish arrest activity of the intervening sequences and ensure that translation is not affected.

However, the possibility that decreased β-galactosidase is caused by the arrest sequences and mutants inhibiting translation is unlikely. Changes in the mRNA leader length over a range of 39 to 115 nucleotides in the *HIS4* gene do not significantly affect efficiency of translation in yeast (Cigan *et al.*, 1988). Although secondary structures in the mRNA do significantly effect translation efficiency (Pelletier and Sonenberg, 1985), we do not predict significant potential for secondary structure formation in any of our mutant arrest site constructs. It would therefore be unexpected if these intervening

sequences reduce β-galactosidase expression by affecting efficiency of translation. However, we cannot rule out this possibility.

An alternative way of observing arrest *in vivo* that avoids the potential contribution of translation is to directly analyze the levels of mRNA produced by means of Northern blot analysis. The results may be quantitated just as those in our β-galactosidase assays, and we would use the same constructs as before to observe more directly the transcriptional effect of our arrest sites.

CHAPTER V: CONCLUSION

Our suppressor screen addressed the question of the presence of SII homologues in *S. cerevisiae*. The screen has not yielded homologues of SII, although we cannot rule out the possibility that a homologue conferring weak suppression, possibly due to a weak intrinsic activity or steady-state concentration, is present in the *S. cerevisiae* genome. The screen does, however, strongly suggest that no homologue exists in TRY3 that is competent to suppress SII deletion when expressed under its own promoter.

We have identified two novel suppressors of SII deletion containing varying degrees of homology to the RRM class of RNA binding domains that may provide us with information about the function of SII *in vivo* through future experimentation. Mutation of the RRM domains in both ORFS from SDS 59 and SDS 87 would demonstrate that the RRM is required for the suppressing activity, provided that the resulting constructs did not suppress. Both of these suppressors are weak, and it may prove useful to attempt to boost the suppression signal by engineering nuclear targeting sequences into the coding regions or cloning them onto 2μ plasmids downstream of the Gal1 promoter. It is reasonable to assume that these new proteins either fold poorly or are degraded at high frequency because of their truncation , and these problems could be addressed by mutagenesis to some degree.

Provided that the RRM domains are responsible for suppression *in vivo*, it would be interesting to identify the physical and chemical basis for their suppression through*in*

vitro experimentation. The RRM-containing proteins could be purified for use in *in vitro* experiments to answer these questions and possibly elucidate their function *in vivo.*

We have observed a reduction of β-galactosidase activity downstream of sequences known to cause arrest *in vitro*, but we cannot conclude from our experiments whether arrest or an unrelated translation effect is the cause of this decrease *in vivo*. The observed relative decrease in β-galactosidase expression downstream of arrest sites is both promoter- and strain-independent. Although we expected a more pronounced decrease of β-galactosidase expression downstream from arrest sites when expression is observed in the absence of SII, our finding does not necessarily indicate that arrested complexes do not form *in vivo*. However, if arrest does occur *in vivo,* this result suggests that arrested complexes are not affected by SII.

More experimentation is necessary to determine if arrest occurs *in vivo*. Analysis of arrest site mutants suggests that the reduction in β-galactosidase activity downstream of our intervening sequences is unrelated to arrest, yet the reduction is not easily explainable. It is improbable based on current knowledge of sequences affecting translation efficiency that translation causes the observed reduction in β-galactosidase activity, but we must rule out this possibility. This will be accomplished by direct analysis of the mRNA products using the Northern blot technique.

Provided that we find more evidence of arrest in *S.cerevisiae* through direct analysis of the mRNAs produced, we will proceed to investigate the arrest phenomenon *in vivo*. We will map the 3' ends of the RNA products to confirm that a block to transcription occurs at the arrest site and compare the arrest position found *in vivo* to the 3' ends of the RNAs associated with arrested complexes *in vitro*. To demonstrate that an

arrested polymerase actually resides at an arrest site *in vivo*, we will perform $KMnO_4$ DNA footprinting at the arrest sites.

The results presented in this thesis are far from providing a complete picture of arrest *in vivo*, but they represent the first attempt we know of to characterize the proteins involved in RNA polymerase II elongation through arrest sites and the first attempts to observe nucleic acid sequences blocking pol II elongation at arrest sites in natural systems. The questions of whether or not arrest occurs in *S. cerevisiae* and the function of SII *in vivo* remain unanswered, but we have many avenues left for exploration.

GLOSSARY

Complementary: Complementary bases in both RNA and DNA exhibit particular intermolecular interactions which cause two bases to stick to each other. In DNA, adenine interacts with thymine, and guanine interacts with cytosine.

Holoenzyme: The holoenzyme is the finished assembly of several core subunits of the RNA polymerase complexed with several transcription factors.

Homologue: Genetic homologues are similar on the DNA or protein level and may have similar functions as a result.

In vitro: Experiments performed *in vitro* are observations of biochemical events outside of the organism.

In vivo: Experiments performed *in vivo* are direct observations of living systems.

Plasmid: A plasmid is a circular molecule of double-stranded DNA. The plasmids used in these experiments are shuttle plasmids, meaning they have DNA sequences that allow for replication in *S. cerevisiae* and *E. coli*.

Phenotype: The phenotype is a physically observable characteristic of an organism.

Proofreading: During transcription, it is possible that the wrong base will enter the pairing site in the RNA polymerase holoenyzme. Proofreading mechanisms correct these mismatches, and have been discovered for DNA polymerases.

Promoter: A promoter is a sequence on the DNA template, usually near the transcription start site, that controls formation of the polymerase holoenzyme.

Suppressor: In the context of our screen, a suppressor is a yeast gene capable of overcoming the growth inhibition by MPA when transformed into the SII-deleted strain.

Trans-acting: Trans-acting factors are diffusable molecules capable of influencing genetic events.

Transcription: Transcription is the process of converting genetic information in the form of DNA into RNA. In this process, all genetic information is conserved because the RNA produced is exactly complementary to the region of the DNA transcribed.

Transformation: Transformation is the introduction of new genetic material into an organism. In these experiments, yeast and *E. coli* are routinely transformed with plasmids.

Translocation: Translocation is the movement of the RNA polymerase from one position on the template to the next position downstream.

SII Deletion Suppresser Screen Summary

SDS index:	Sequencing: Gal primer	Lac primer	Identity
1	X		SII
3	X	X	Telomere
5	X	X	Telomere
6	X	X	Telomere
8	X		IMPDH
9	X		IMPDH
10	X		Telomere
12	X	X	Telomere
13	X		IMPDH
15	X		IMPDH
17	Digest*		SII
19	Digest*		SII
20	X		Telomere
21	X		IMPDH
24	X		IMPDH
26	X		Telomere
27	X		SII
28	X		IMPDH
29	X		Telomere
30	X	X	Unique
31	X		Telomere
34	X		IMPDH
36	X		Telomere
39	X		SII
40	X	X	Unique
41	X		IMPDH
43	X		Telomere
44	X		IMPDH
45	X		Telomere
46	X		Telomere
50	X		Telomere
51	X		IMPDH
54	X		Telomere
55	X		Telomere
56	X		Telomere
57	X		Unique
58	X		IMPDH
59	X	X	Unique
60	X	X	IMPDH
61	X		SII
64	X	X	IMPDH
67	X		Telomere
71	X		Telomere
72	X		SII
74	X		IMPDH
75	X		IMPDH
76	X		Telomere
77	X		IMPDH
80	X		Unique
82	X		Telomere
85	X		SII
86	X	X	Unique
87	X	X	Unique
88	X		Telomere
89	X		IMPDH
94	X		Unidentified
96	X		Telomere
97	X		Unique

* Entries marked Telomere contain IMP dehydrogenase

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