Table of Contents

Examination of the Unconventional Role of the 19S Proteasome Subcomplex in RNA Polymerase II Transcription in *Saccharomyces cerevisiae*

Student L

Graduate Program in Molecular, Cellular & Developmental Biology (MCDB)

Candidacy Examination, July, 2002

Abstract

Conventionally, damaged and ubiquitinated proteins are subjected to degradation in the 26S proteasome in eukaryotes. However, several observations have indicated that the 19S subcomplex of the 26S proteasome may play a non-proteolytic role in RNA polymerase II (Pol II) transcription[1]: (1) Ubiquitination was thought to be required for activity of a viral transcription activator, VP16. (2) The transcription activation domains of several transcription activators including Myc overlap with the signal for ubiquitination. (3) Mutant alleles of yeast *SUG1* and *SUG2*, two essential ATPases of the 19S proteasome subcomplex, could suppress the deficiencies of transcription activator and transcription elongation factor mutants. (4) Sug1p and Sug2p were found to directly bind to the activation domains of transcription activators such as Gal4p. (5) The 19S subcomplex coprecipitated with a yeast transcription elongation factor. (6) An anti-Sug1p antibody was reported to block transcription *in vitro* and addition of the purified 19S subcomplex restored the lost transcription activity. (7) A recent report[2] identified a novel complex, APIS (AAA proteins independent of 20S), originated from the 19S subcomplex. The APIS complex, which contains six ATPases including Sug1p and Sug2p, was recruited to actively transcribed genes through Gal4p in *Saccharomyces cerevisiae*. Together, these intriguing genetic and biochemical discoveries strongly suggest that the APIS complex of the 19S proteasome subcomplex is involved in RNA Pol II transcription in yeast.

Although present observations suggest that the APIS complex plays a role in RNA Pol II transcription, it remains to be shown: (1) whether the recruitment of the APIS complex to the promoter by Gal4p is a pre-requisite for transcriptional activation of *GAL* genes and (2) in which steps of transcription complex assembly the APIS complex is involved.

Specific Aims

I. **To test the hypothesis that the recruitment of the APIS complex to the promoter is essential for transcription activation of *GAL* genes.**

II. **To test in which step(s) of transcription complex assembly on the *GAL1* promoter the APIS complex is involved.**

Background and Significance

Significance of the proposed work. This proposal seeks to investigate the unconventional roles of a subcomplex of the 19S regulatory particle of the proteasome, the APIS complex (AAA proteins independent of 20S), in transcription activation in *Saccharomyces cerevisiae*. In addition to the traditional role in cellular protein degradation[3-5], several genetic and biochemical data indicate that the APIS complex also participates in transcription regulation of *GAL* genes[1, 2,6-12]. Interestingly, the involvement of the APIS complex in transcription activation is independent of protein proteolysis[13]. However, there is no direct evidence that shows whether the APIS complex is a pre-requisite for the Gal4p-mediated activation of *GAL* genes transcription. It is also not yet known precisely what the APIS complex contributes in Gal4p-mediated transcription activation. To understand more insight of the involvement of this proteasome subcomplex, the APIS complex, in transcription regulation, I propose to address whether the APIS complex is a novel essential transcription factor in Gal4p-mediated transcription activation. In addition, analysis of in which aspects the APIS complex is involved in Gal4p-mediated assembly of transcription factors on *GAL* promoters will provide more understanding of Gal4p-mediated transcription activation. Moreover, the mechanism of how the regulatory 19S particle regulates protein degradation is still poorly understood presently[3]. Investigation of the transcriptional role of the APIS complex will also help to study how the ATPase components of

the 19S particle conduct regulation of protein degradation from a different angle. The observed intriguing phenomenon - complexes that can be involved in fundamentally different biological processes is speculated by responding to different cellular signals[1]. In this case, the APIS complex functions independently of the 26S proteasome and is involved in transcription activation rather than Gal4p degradation[2]. In this regard, this proposal will provide a basis for future researches into which cellular signals induce the APIS complex to act independently of the 26S proteasome.

The 26S proteasome. The 26S proteasome is a 2.4 MDa protein complex responsible for the degradation of damaged and polyubiquitinated proteins in eukaryotic cells[3, 9,14.] This huge protease complex is constituted of two subcomplexes (fig.1): the 20S barrel-like particle that forms the proteolytic cavity, and the regulatory 19S particle that recognizes polyubiquitinated proteins. The 20 S core subcomplex is comprised of 28 protein subunits which are arranged as an $(\alpha 1 \ldots \alpha 7, \beta 1 \ldots \beta 7)_2$ complex in four stacked rings with protease activity sites toward the inner surface[15]. The 19S subcomplex is composed of at least 18 proteins, including six highly related AAA (ATPases associated with various cellular activities) family ATPases, and can be further separated into two complexes, base (contains the six ATPases) and lid[4,5]. Unlike the known crystal structure of the *Thermoplasma* 20S particle[16] which has a 1.3nM pore at each end of the 20S barrel, the crystal structure of the yeast 20S particle shows no opening into the proteolytic cavity at the ends of the barrel; the ends appear to be covered by overlapping loops of the α-type subunits[15]. Therefore, the regulatory 19S particle is thought to regulate the opening of the 20S cavity by ATP hydrolysis[4]. Additionally, the ATP-dependent 19S subcomplex is also thought to provide substrate recognition, unfolding, translocation, and the regulation of the 20S-mediated

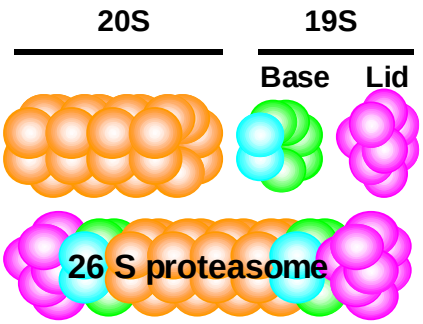

Fig1. The 26S proteasome. The APIS complex is originated from the base of the 19S subcomplex. (Adapted from reference 1)

proteolysis[17]. Several studies have shown that the 19S regulatory particle has protein chaperon activity[18] *in vitro* and that the six ATPases have no redundant functions in protein degradation regulation[19-22]. A recent report[2] identified a novel ATPase complex, the APIS complex, from the base of the 19S subcomplex and found that it was recruited to the promoters of *GAL* genes through Gal4p, a transcription activator which activates *GAL* genes in response to galactose in *Saccharomyces cerevisiae*. It is speculated that this subcomplex of the regulatory 19S complex may remodel transcription factors when recruited to promoters by hydrolyzing ATP[1].

Subcellular localization of 26S proteasome and the APIS complex. Unlike metazoans which have their proteasomes in both the cytoplasm and the nucleus, the subcellular localization the 26S proteasome of *Saccharomyces cerevisiae* has been reported differently. Johnston and colleagues found that the 26S proteasome almost exclusively localizes in the nucleus throughout the cell cycle by indirect immunofluorescent staining of epitope-tagged components of both the 19S and the 20S subcomplexes[23]. Kloetzel and colleagues observed that the proteasome localizes to the nuclear envelope-ER network by observing green fluorescent protein (GFP)-tagged components of the 20S and the 19S complexes[24]. The differences of the localization of the 26S proteasome may simply resulted from the different methods of epitope tagging. Although the observations were not unified, there is a common cellular compartment, the nucleus, where the proteasome exists, and therefore it supports the idea that the APIS complex may play a different role from protein degradation in the nucleus.

The APIS complex. The APIS complex, which is comprised of six highly related AAA family proteins, was first identified from the base of the 19S regulatory subcomplex[2](fig. 2). Genetic data first indicated that two components of the APIS complex, Sug1p/Rpt6p[2, 6,9] and Sug2p/Rpt4p[11, 25], interact with Gal4p. Both mutant alleles of *SUG1* and *SUG2* were able to suppress a *GAL4* mutant allele (gal4^{D}) in which C-terminal-residues (854-881) are deleted and could restore the transcription activity of *GAL* genes to 65% of wild-type in the presence of galactose[6]. Although there is a slight gal4^{D}p protein degradation defect in the *sug1* and *sug2* mutant strains, the suppressor phenotype was not observed in other *sug1* and *sug2* mutant strains which have an equal degree of gal4^{D}p protein degradation. This result suggested that the interaction of Sug1p, Sug2p and Gal4p is independent of protein degradation[13]. Sug1p was found co-precipitated with TATA-binding protein and Gal4p subsequently[7]. In an *in vitro* binding assay, Sug1p and Sug2p were then found to be able to directly bind to the activation domain of Gal4p[7]. Next, the APIS complex was identified associated with the promoter of *GAL* genes by chromatin immunoprecipitation assays under inducing conditions[2]. In the gal4^{D} mutation strain, however, this association was not found. This result indicated that the recruitment of the APIS complex to promoters of actively transcribed *GAL* genes is through wild-type Gal4p. Moreover, the APIS complex which contains the gal4^{D} -suppressor sug1p or sug2p was found re-associate with promoters of *GAL* genes in the gal4^{D} mutation strain. Interestingly,

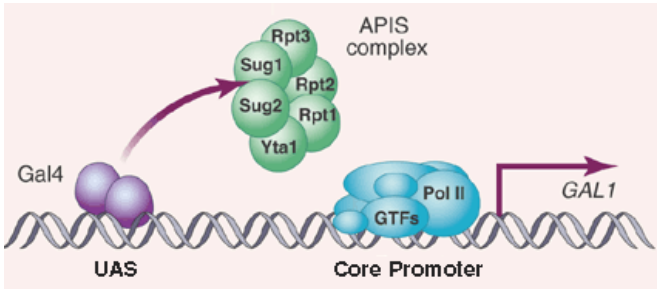

Fig. 2. The APIS complex, which is composed of six ATPases, is recruited to the upstream activation sequence (UAS) by the transcription activator, Gal4p, under inducing conditions[1]. Sug1p/Rpt6p and Sug2p/Rpt4p are the only two components of the APIS complex known to directly interact with Gal4p.

no components of the 20S core complex of the proteasome were identified in this experiment, suggesting that the association of the APIS complex to promoters of *GAL1* and *GAL10* upon induction is independent of proteolysis. Taken together, the APIS complex is believed to be involved in at least transcription of *GAL* genes and is independent of protein degradation. Since the APIS complex contains six ATPases, it is speculated that when recruited to the promoter, it may function in unfolding or restructuring protein components of the transcription machinery and result in transcription activation and elongation by hydrolyzing ATP. However, whether this complex is a pre-requisite of transcription activation is still unknown. Toward this end, this proposal mainly focuses on disruption of the interaction of Gal4p and the APIS complex by isolate appropriate *SUG1* and *SUG2* mutants to examine whether the APIS complex is an essential component for transcription activation of *GAL* genes.

Additional evidence for a role of the APIS complex in transcription regulation. Several lines of evidence from other aspects also indicate that the APIS complex is involved in transcription regulation. First, studies of the 19S subcomplex found that a yeast transcription elongation factor, Cdc68p, could be coprecipitated with the complex[8, 12]. Depletion of the regulatory 19S subcomplex from *in vitro* transcription reactions using an anti-Sug1p antibody was found to inhibit transcription elongation. When purified 19S subcomplex was provided to the system, transcription was restored. Second, a component of a transcription factor TFIIH was identified to directly interact with Sug1p[26]. Third, some viral transcription activators, such as VP16[27], were thought to require ubiquitination for their activities. Fourth, interaction between VP16 and Sug1p was also found in yeasts[7]. Finally, ubiquitination signals and transcription activation domains were found overlap in several transcription activators, including Myc; suggesting that

there is correlation of ubiquitination and transcription activities of transcription activators[28]. Together, these intriguing genetic and biochemical discoveries strongly suggest that the 19S proteasome subcomplex is involved in RNA Pol II transcription in yeast.

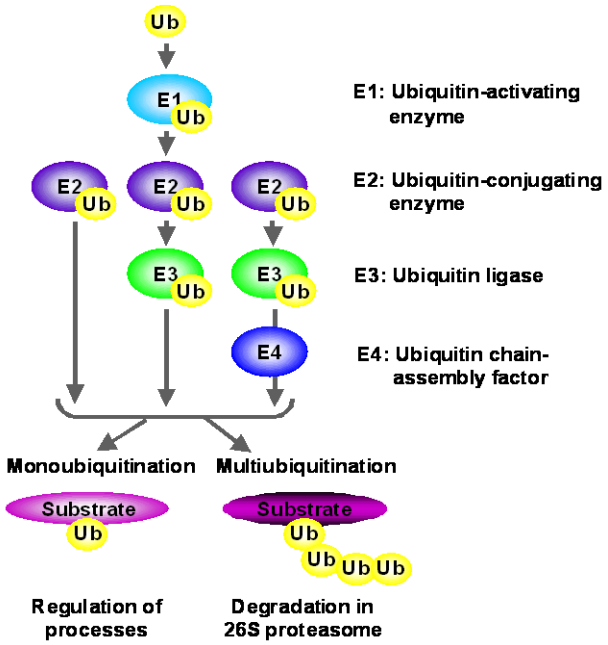

Fig. 3. Current view of ubiquitin-mediated proteolysis and regulation of cellular processes. (Adapted from reference 29)

Ubiquitin-mediated protein degradation. In ubiquitin (Ub)-mediated proteolysis, Ub is covalently linked to proteins that are subjected to degradation in the 26S proteasome through a cascade of enzymatic transfer (fig. 3)[29]. First, the C-terminus of Ub is covalently linked to an active-site cysteine in the E1, Ub-activating enzyme; this reaction is catalyzed by E1 itself. Next, Ub is transferred to an active-site cysteine in the E2, Ub-conjugating enzyme, and then to E3, Ub ligase. Finally, E3 will catalyze the covalent attachment of Ub to a lysine residue in the target protein. Presently there are two types of ubiquitination identified. The first one is the classical multiubiquitination of protein degradation in the 26S proteasome. Proteins which are targeted to degradation have to have at least four Ubs to be recognized. The second type is monoubiquitination, which is used for regulation of many cellular processes including endocytosis and the localization of proteins in the nucleus[30]. More recently, Ub was observed to be involved in many aspects of RNA polymerase II trasncription[31]. First, several transcription activators were found to require proteolytic processing from their inactive precursors via Ub-dependent protein degradation in order to

become active[32]. Second, based on a genetic interaction, proteolysis independent regulation of a yeast transcription factor, Met4p, by E3 was reported[33]. Third, Ub was found to be involved in function of transcription activation domain (TAD) of transcript activators[34]. Specifically, it was found that ubiquitination of TAD is required for its activation and also for their degradation. This "suicide" mechanism suggested that stronger transcription activators have a more rapid protein degradation rate that is probably the mechanism to regulate those strong transcription activators[35]. Fourth, components of the Pol II general transcription activation machinery may signal the ubiquitination of transcription activation. These components are thought to have activities similar to E3.[36, 37]

Although the 19S regulatory subcomplex is thought to recognize the multiubiquitinated protein substrates, we cannot rule out the possibility that some subunits of it, i.e. the APIS complex, may also recognize monoubiquitinated protein. Therefore it is possible that the recruitment of the APIS complex to the actively transcribed gene is signaled by monoubiquitinated transcription activators, e.g. Gal4p. It is noteworthy that many of these emerging roles of Ub in the regulation of Pol II transcription are independent of protein degradation[31].

Investigation of the unconventional role of the APIS complex in RNA Pol II transcription.

Present observations suggest that the APIS complex plays a role in RNA Pol II transcription. However, it first remains to be shown whether the recruitment of the APIS complex to the promoters by Gal4p is a pre-requisite for transcriptional activation of *GAL* genes. The idea is that if the APIS complex is essential for transcription activation, disruption of the interaction between it and Gal4p will cause *GAL* genes to not be expressed since it cannot be recruited to promoters by Gal4p. Gal4p is a well-known transcription activator and mediates the assembly of

many essential transcription complexes on *GAL* promoters. Instead of deleting the Sug1p/Sug2p interacting region(a.a. 854-881) of Gal4p, I will first map the Gal4p interacting domains of Sug1p or Sug2p and then isolate sug1p or sug2p mutations which cannot bind to Gal4p. Next, it will be determined whether *GAL* genes are able to be expressed in the absence of the APIS complex in the *sug1* or *sug2* mutant strains. The APIS complex binding region of Gal4p overlaps with binding regions for Gal80p (the negative regulation factor), and components of the SAGA complex and the Mediator. I will then determine whether the APIS complex is involved in the interactions of Gal4p and these complexes. By studying in which steps of transcription initiation, i.e. the preinitiation complex assembly, the APIS complex is involved, this proposal attempts to investigate the potential role of the APIS complex in transcription factor assembly on *GAL* promoters.

Research Design and Methods

Specific Aim #1: To test the hypothesis that the recruitment of the APIS complex to the promoter is essential for transcription activation of* GAL *genes.

Several observations indicated that the APIS complex is recruited to the promoters of *GAL* genes through Gal4p in the presence of galactose[1, 2.] In addition, Sug1p and Sug2p are the only components of the APIS complex known to directly interact with the activation domain of Gal4p[2]. To test the hypothesis that the recruitment of the APIS complex to the promoter is essential for transcription activation of *GAL* genes, I will first disrupt the interaction between Sug1p/Sug2p and Gal4p by identifying an appropriate sug1p and/or sug2p mutant which does not bind to Gal4p and then examine the transcription activities of *GAL* genes.

A. Identification of an appropriate sug1p and/or sug2p mutant which does not interact with Gal4p.

A-1. Mapping of the Gal4p-interacting regions of Sug1p and Sug2p.

To identify mutant Sug1p and Sug2p which cannot bind to Gal4p, I will first map the Gal4p-interaction domains of Sug1p and Sug2p. Serial deletion mutant derivatives of Sug1p and Sug2p from both the N-terminus and the C-terminus will be constructed as shown in figure 4. Three approaches will be used to determine whether these deletion mutant derivatives still interact with Gal4p *in vivo* and *in vitro*:

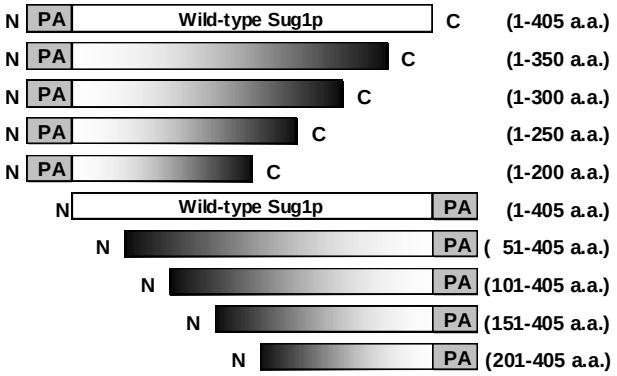

Fig. 4. Design of the sequential truncation of Sug1p. Serial deletion mutant derivatives of Sug1p from both the N-terminus and the C-terminus will be protein-A tagged at either the N-terminus or the C-terminus. Serial deletion mutant derivatives of Sug2p will be constructed by the similar design.

<u>*(a) The in vivo binding assay*</u>

To determine the physiological Gal4p-interacting region of Sug1p and Sug2p, this approach will be performed as following. Wild-type *SUG1* and *SUG2* and the serial deletion constructs will be fused with a protein-A tag at either the N-terminus or the C-terminus. Next, these clones will be transformed into a wild-type yeast strain and cultured in galactose medium. Yeast extract will then be prepared and incubate with IgG Sepharose beads. Protein-A Sug1p (or Sug2p) bound Gal4p will be detected by western blot analysis. The truncated sug1p and sug2p which have lost the Gal4p interacting region will show no or background-leveled Gal4p bound. One potential drawback to this approach is that these truncated sug1p or sug2p proteins may not be incorporated into the APIS complex. Therefore, a lack of interaction with Gal4p may be due to

the problem with the APIS complex, and may not reflect the true interaction between sug1p, sug2p and Gal4p. To test whether this is the case, several examinations will have to be performed. First it will be determined whether the truncated sug1p and sug2p can complement wild-type Sug1p and Sug2p. If so, wild-type alleles will be replaced by the truncated forms to ensure the truncated form is the only copy in the cell. Second, I will examine whether the protein A-tagged sug1p and sug2p can be incorporated in the proteasome by a proteasome co-purification assay. Third, the expression level and stability of the truncated sug1p and sug2p must be examined.

*(b) The **r**epressed **t**rans**a**ctivator (RTA) assay*

An alternative way to determine if the identified region(s) of Sug1p and Su2p truly interacts with Gal4p *in vivo* is the two-hybrid assay. However, Gal4p is a transactivator itself, it cannot be used as a bait in the conventional two-hybrid assay to identify interacting proteins of Gal4p. Therefore, a repressed transactivator (RTA)[38] assay will be conducted to investigate which deletion mutant derivatives of Sug1p and Sug2p have lost the Gal4p interacting domains *in vivo*. Figure 5 shows the strategy of the RTA assay and the predicted results. Briefly, the rationale of the RTA assay is to fuse Sug1p and Sug2p with the repressing domain (RD) of the N-terminal 200 residues of Tup1p which represses *GAL1* expression in glucose medium (fig. 5A). A *GAL1* promoter which contains a Gal4p binding site in the upstream activation sequence (UAS) is engineered upstream of a *URA3* reporter. The *URA3* product would confer 5-fluoorotic acid (5-FOA) into a toxic compound and therefore the cells with *URA3* expression will be dead on 5-FOA plates (fig. 5B). In the presence of galactose, RD-fused wild-type Sug1p and Sug2p interact with Gal4p and hence repress *URA3* expression, so cells cannot grow on uracil drop-out plate but grow on 5-FOA plates which selects for the Ura-minus phenotype generated by repression of the expression of

URA3 (fig. 5C). Because *GAL* genes can not be expressed in this case, raffinose would be added into the medium to be the carbon source. Truncated sug1p and sug2p which cannot bind to Gal4p will cause *URA3* expression and therefore cells will grow on uracil dropout plate and die on 5-FOA plates (fig. 5D). A potential drawback of this approach is that ND-fused wild-type Sug1p and Sug2p may not be incorporated into the APIS complex and therefore cannot interact with Gal4p.

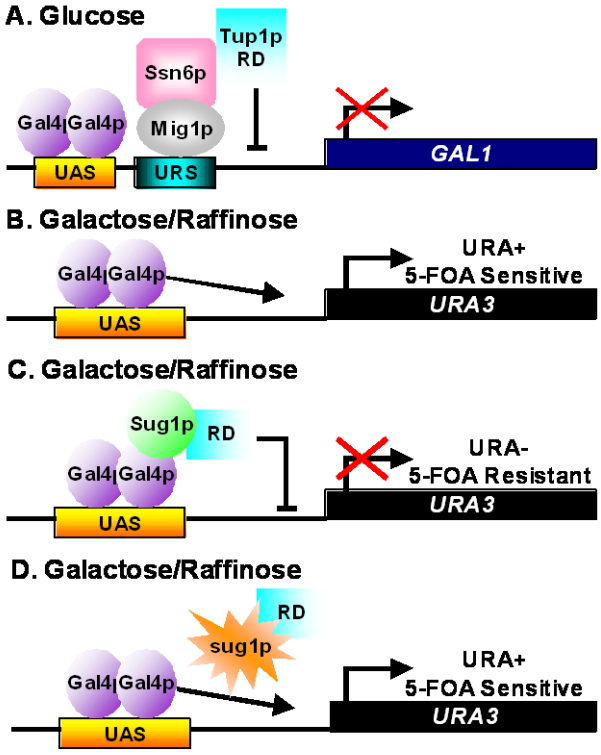

Fig. 5. Repressed transactivator (RTA) system. (A) Mig1p binds to the upstream repression sequence (URS) and recruits general repressors, Ssn6p and Tup1p in glucose medium. RD, repressing domain. (B) UAS is constructed upstream of *URA3* and Gal4p activates it in the presence of galactose and raffinose. (C) Tup1p-RD-fused Sug1p interacts with Gal4p and represses the expression of URA3 in galactose/raffinose medium. (D) Deletion mutant derivatives of Sug1p which cannot interact with Gal4p will cause *URA3* expression in galactose/raffinose medium.

(c) The in vitro binding assay

Sug1p and Sug2p have already been shown to directly bind to GST-fused C-terminal activation domain (AD) of Gal4p *in vitro*[2, 7,11]. By using the established *in vitro* binding assay, deletion mutant derivatives of Sug1p and Sug2p will be synthesized *in vitro* and labeled with [^{35}S] methionine. The *in vitro* translation product will be incubated with GST-fused Gal4p AD, and the bound proteins will be detected by autoradiography. The potential limitation of this approach is that it may not reflect the physiological condition of protein interactions in living cells.

A-2. Identification of the sug1p and/or sug2p mutant.

The minimal Gal4p interacting region of Sug1p and Sug2p will first be deleted and tested if this form can complement wild-type *SUG1* and *SUG2* deletion. Since *SUG1* and *SUG2* are essential for yeast viability, the deleted forms may be lethal. In order to prepare for this, I will simultaneously generate mutations in this region using PCR mutagenesis. The RTA assay will be performed to screen the sug1p and sug2p which cannot interact with Gal4p. Because these mutants may be conditional, I will test for loss of interaction under varying conditions (i.e. high or low temperature). The desired sug1p and sug2p mutants which cannot bind to Gal4p will give URA+ and 5-FOA sensitive phenotypes (fig. 5D). Both sug1 and sug2 mutants will be introduced into a wild-type strain with chromosomal copies of *SUG1* and *SUG2* deleted by plasmid shuffling. Only the strains which cause no problems with cell viability and protein turnover (discussed in **A-4**) will be used to test the transcription activities of *GAL* genes (described in **B**). It is possible that either Sug1p or Sug2p alone are sufficient for the necessary interaction with Gal4p. If the interaction with Gal4p cannot be disrupt by either a sug1p or a sug2p mutation alone, a double mutant (sug1/sug2) strain will be generated and used for the examination of the transcription activities of *GAL* genes.

A-3. The alternative strategies for disrupting the interaction between Sug1p, Sug2p and Gal4p.

(a) Overexpression of the Gal4p interacting fragments. Gal4p-interacting regions of Sug1p and Sug2p will be constructed on a high-copy plasmid under a strong promoter, the GPD promoter, and transformed into a wild-type yeast strain. The Gal4p interacting fragments originated from Sug1p or Sug2p may cause a dominant-negative effect by competing with wild-type Sug1p and Sug2p for binding to Gal4p, and therefore disrupt the interaction between Gal4p and

Sug1p/Sug2p. The disruption of the interaction between Sug1p, Sug2p and gal4p will be determined by *in vivo* assay describe in B-1.

(b) Elimination of the putative ubiquitination site of Gal4p The strategy of this approach is based on the assumption that the interaction between Gal4p and the APIS complex is mediated by ubiquitination of Gal4p. Several lines of evidence indicate that the 19S regulatory subcomplex is responsible for the recognition of multiubiquitinated proteins[3]. Subunits of the APIS complex are also found directly bind to ubiquitinated substrates[22]. However, whether the interaction between Gal4p and the APIS complex is mediated by ubiquitination is not clear. To employ this approach, investigations of the nature of the modification of Gal4p by Ub have to be conducted. First, whether Gal4p is ubiquitinated before and after induction will be examined in the presence of a HA-Ub allele. If Gal4p will be modified by Ub, ubiquitination sites in Gal4p will next have to be determined. Then, ubiquitinated lysine residues will be changed to serine residues by site-directed mutagenesis. Finally, both the interaction between the APIS complex and Gal4p, and the transcription activities of *GAL* genes will be determined.

A-4. Examination of the cellular protein turnover in the isolated sug1/sug2 strain.

If the isolated *sug1/sug2* mutant strain has protein degradation problems, it may affect transcription indirectly and hence may lead to misinterpretation of the observed results. Therefore, cellular protein turnover will be examined in the identified *sug1/sug2* mutant strain. Two well-established approaches detailed below[19] will be used to determine the protein degradation activities of the 26S proteasome. In addition, I will also examine the stability of cellular proteins, including Gal4p.

(a) Protein turnover rate test

Two model substrates, Ub-Lys-βgalactosidase(βgal) and Ub-Pro-βgal, which are known targets with different types of modification. Each contains multi Ub chains, but they are degraded by different mechanisms via the 26S proteasome[39]. The amount of these substrates will be monitored at different time points after switching to galactose medium by pulse-chase analysis[19]. Briefly, substrates will be first labeled with [^{35}S] methionine and then immunoprecipitated from yeast extracts by anti-βgal antibodies and subsequently subjected to SDS-PAGE. The amount of the [^{35}S] labeled substrates will be detected by PhosphorImager analysis. Only the *sug1/sug2* mutants which have no or similar protein degradation defects when compared to the known suppressing *sug1* mutant strain will be used for following experiments.

(b) Peptidase activity assay

The other potential defect of the proteasome activity is from the improper assembly of the 26S holoenzyme. To address this question, I will examine the assembly state of the mutant proteasomes by the established peptidase activity assay[19]. The idea is that since there is no opening at the ends of the 20S subcomplex, proper assembly of the regulatory 19S subcomplex on the 20S subcomplex is required for the peptidase activity of the 20S subcomplex. Purified proteasome complexes will be subjected into non-denaturing PAGE. A fluorogenic peptide substrate, Suc-LLVY-AMC, will be used on the gel for visualizing the peptidase activity *in situ*.

(C) Examination of the stability of Gal4p. I will conduct a standard pulse-chase analysis to determine the stability of cellular proteins, including Gal4p. Briefly, labeled Gal4p will be recovered by denaturing immunoprecipitation, and detected by SDS-PAGE followed by autoradiography.

A-5. Examination of the recruitment of the APIS complex to the GAL1 promoter.

I will conduct the chromatin immunoprecipitation (ChIP) assay[40] (fig. 6) to determine whether the APIS complex which contains the desired sug1p/sug2p mutants is recruited to the *GAL1* promoter under inducing conditions *in vivo*. Yeast cells which contain the sug1p/sug2p mutants will be cultured in glucose medium and then switched to galactose medium. By using the same procedure as described in reference 2, yeast cells will first be cultured in galactose medium and formaldehyde will be used to crosslink DNA-binding proteins to DNA. Second, sonication will be conducted to shear the genomic DNA to smaller fragments with an averaged size about 300bp. Then the anti-Sug1p antibodies will be added into the extract to precipitate Sug1p-bound DNA fragments. Primers around the UAS region (**b** pair in fig. 6) will be used to amplify the UAS region by real-time PCR. As shown in figure 6, primer pairs (**a** and **c**) specific for other regions will be used as controls. The idea is that if the APIS complex has lost the ability to

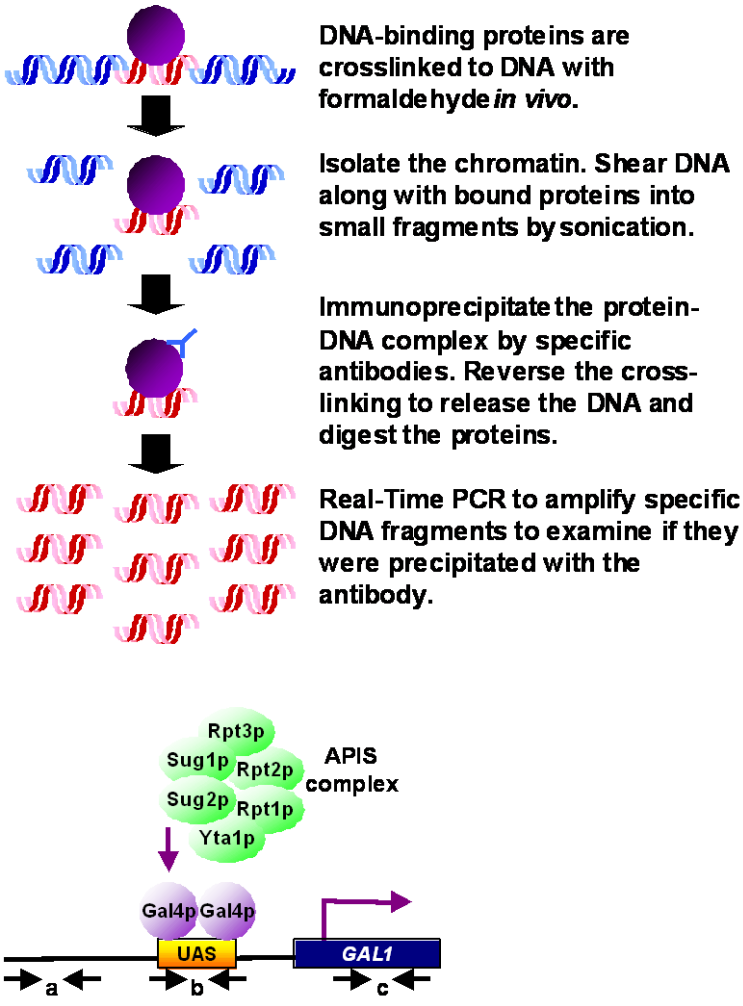

Fig. 6. The principle and the design of the chromatin immunoprecipitation assay(ChIP).

interact with Gal4p, there will be no DNA precipitated by anti-Sug1p antibodies and hence no PCR products. Figure 7 shows the ideal results of the ChIP assay of wild-type and desired *sug1/sug2* mutant strains. It is known that the APIS complex also associated throughout the entire *GAL1* region in the wild-type strain; it is speculated that this interaction is mediated by an elongation factor, Cdc68p. However, whether the association between the APIS complex and the entire *Gal1* region is dependent on the Gal4p interaction remains to be shown. If there are PCR products amplified by primer pair c in the *sug1/sug2* mutant strain, it can be conclude that the interaction between Gal4p and the APIS complex is not required for the recruitment of the APIS complex to the GAL open reading frame. If the opposite result is obtained, either the interaction between Gal4p and the APIS complex is important for the recruitment to the *GAL1* open reading frame or that the sug1p and sug2p have lost their interaction with the recruiting factors. Anti-Gal4p antibodies will be used as a positive control because Gal4p is known to bind to UAS under both non-inducing and inducing conditions[41]. If the hypothesis that the APIS complex is required for *GAL* genes expression is true, cells will die after switching to galactose medium. Therefore, examination of the association at different time points after induction will be conducted if necessary.

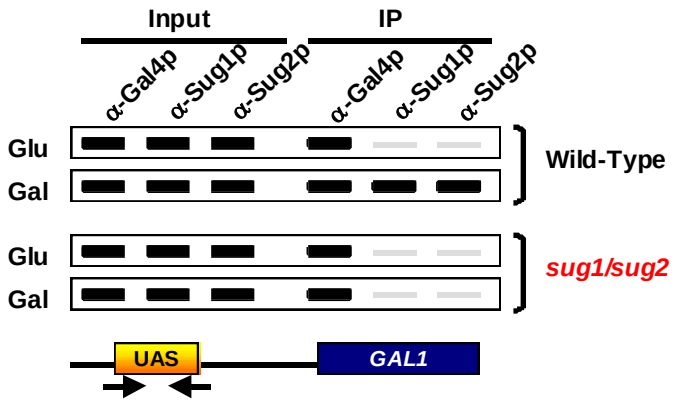

Fig.7. Idealized results of the ChIP assay of the association of wild-type and mutant sug1p or sug2p on UAS in glucose(Glu) and galactose(Gal) medium.

B. Examination of the transcription activities of GAL genes

The requirement of the APIS complex in the transcription of *GAL* genes will be examined from two aspects: (1) whether cell can survive in galactose medium, and (2) whether *GAL* genes can be transcribed in galactose medium.

B-1. Examination of cell viability and growth rate in galactose medium.

I will first test the viability and growth rate of the isolated sug1p/sug2p mutant strain in glucose, raffinose, and galactose medium. The prediction is that under repressing conditions (glucose) and non-repressing condition (raffinose), there will be no obvious growth defects of the sug1p/sug2p strains. Under inducing conditions (galactose), sug1p/sug2p mutant strains will grow much more slowly than wild-type strain if the APIS complex is important for *GAL* genes transcription. Otherwise, the sug1p/sug2p mutant strain will have no growth defect when switched to galactose medium. If the mutant strain can still grow in galactose medium but much more slowly than wild-type strain, it could be because the APIS complex is not essential but plays an important role for the transcription activation of *GAL* genes.

B-2. Examination of GAL genes transcripts in galactose medium.

I will examine the *GAL1* and *GAL10* transcripts produced under inducing conditions by Northern blot analysis with *GAL1* and *GAL10* specific probes. If the interaction of Gal4p and the APIS complex is required for the transcription of *GAL* genes, there will be no *GAL1* and *GAL10* transcripts detected in the sug1p/sug2p mutant strain under inducing conditions.

B-3. Examination of whether the wild-type Sug1p and Sug2p can restore the transcription activities of GAL genes in the isolated sug1/sug2 strain.

To test whether wild-type Sug1p and Sug2p can rescue the transcription activities of *GAL* genes, *SUG1* and *SUG2* will be overexpressed in the isolated *sug1/sug2* strain. *SUG1* and *SUG2* will be constructed on a high-copy plasmid under the GPD promoter and transformed into the isolated *sug1/sug2* strain. Examination of the transcription activities of *GAL* genes will be conducted as described in B-1 and B-2. The prediction is that by competing with sug1p and sug2p, wild-type Sug1p and Sug2p will cause the re-association of the APIS complex to Gal4p and hence restore the transcription activities of *GAL* genes.

Specific Aim #2: To test in which step(s) of transcription complex assembly[42] on the GAL1 promoter the APIS complex is involved.

Although the 19S complex activity was reported to be required after PIC formation, it is still unknown whether it also participates before or during preinitiation complex (PIC) formation[12]. Because the *sug1* and *sug2* mutations can suppress the *gal4*^{D} mutation, and the APIS complex is recruited to the *GAL* promoters via Gal4p, the APIS complex may participate in several events related to Gal4p-mediated transcription activation. To address this possibility, specific aim #2 is proposed to understand in which step(s) of gal4p-

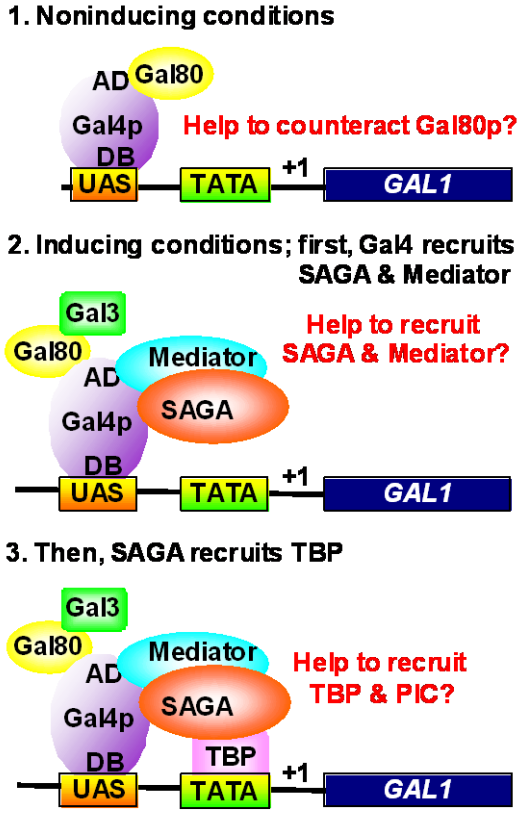

Fig. 8. Current model of transcription complex assembly on the *GAL1* promoter. (adapted and modified from reference 43) AD, activation domain; DB, DNA-binding domain; TBP, TATA-binding-protein.

mediated assembly of transcription complex on *GAL* promoters the APIS complex is involved. Figure 7 summarizes the current model[43, 44] of the three stages of Gal4p-mediated assembly of transcription complex on the *GAL1* promoter. I will determine in which stages the APIS complex is involved by addressing following hypotheses both *in vivo* and *in vitro*:

A. Examination of timing of the APIS complex recruitment to GAL promoters by Gal4p.

A-1. Determination of the kinetics of the transcription complex, including the APIS complex, the SAGA complex, the Mediator, and RNA polymerase II, assembly on the GAL1 promoter.

If the APIS complex is a pre-requisite for *GAL* genes transcription activation, it must be recruited to *GAL* promoters before transcription can take place. Since the suppressor sug1-1p is known to re-associate with the *GAL* promoters and to suppress the C-terminal AD deleted $gal4^{D}$ mutation, it is very possible that the APIS complex participates in early events of Gal4p-mediated transcription activation. Green and colleagues have successfully determined the stepwise pathway of transcription complex formation, including Gal4p, the SAGA complex, and RNA polymerase II, assembly on the *GAL1* promoter[43]. According to their results, the recruitment of Gal4p to the UAS was first detectable after switching to galactose medium. They can also clearly determine the subsequent binding order of the SAGA complex, then TBP and finally RNA polymerase. To determine when the APIS complex is recruited to *GAL* promoters by Gal4p, I will examine the association order of these known transcription factors, including the SAGA complex, the Mediator, TATA-bind protein, on the UAS by employing this established system. Briefly, wild-type yeast will be culture in glucose medium before switching to galactose medium. After switching to galactose medium, the ChIP assay will be conducted at different time points. The result of when the APIS complex joins the transcription complex may provide a clue to the identity of the targets of the APIS complex.

A-2. Determination whether the APIS complex is recruited in the GAL1 promoter in different strains containing mutant transcription complexes.

In addition to examining if the stepwise pathway identified above truly reflects a series of obligatory steps, the association of the APIS complex and the UAS will be examined in available

yeast strains bearing mutations in Gal4p, the SAGA complex, TBP, or RNA polymerase II. In theory, *GAL4* deleted strains will fail to recruit the APIS complex, the SAGA complex, and RNA polymerase II to the promoter. The SAGA deleted strains will fail to recruit TBP, RNA polymerase II but not Gal4p. However, the RNA polymerase II mutant strains will not fail to recruit these transcription complexes. This approach will determine whether the recruitment of the APIS complex is also mediated by other transcription complex.

B. Examination in which steps the transcription complex assembly are affected when the APIS complex cannot bind to Gal4p.

B-1. Test whether the APIS complex facilitates to counteract Gal80p.

Gal80p which binds to the activation domain of Gal4p is the negative regulator of Gal4p under repressing conditions, i.e. in glucose medium[45]. Gal3p interacts with Gal80p in galactose medium via a complex pathway leading to Gal4p activation. Since Gal80p and the APIS complex both bind to the C-terminal activation domain of Gal4p, it is possible that the APIS complex functions in displacing Gal80p from the activation domain of Gal4p under inducing conditions. To test this hypothesis, *GAL80* (nonessential) will be deleted in the isolated *sug1/sug2* mutant strain in which the APIS complex does not interact with Gal4p. Then the requirement for the APIS complex in *GAL1* transcription activation will be examined under inducing conditions as described in specific aim #I (B). If the APIS complex is required to displace Gal80p from the Gal4p activation domain, the APIS complex will no longer be required for *GAL* genes transcription in the absence of Gal80p. Therefore *GAL* genes will be constitutively expressed in the *sug1/sug2/gal80Δ* strain in either glucose medium or galactose medium. If the APIS complex does not participate in this step of Gal4p activation, disruption of

the interaction of the APIS complex and Gal4p will still affect the transcription of *GAL* genes (if the APIS complex is essential for *GAL* genes transcription) in the absence of Gal80p.

B-2. Test whether the APIS complex facilitates recruitment of the SAGA and the Mediator complexes.

The SAGA complex, which has at least 14 subunits including many chromatin remodeling enzymes, is identified as an essential coactivator of Gal4p[43, 44]. The Mediator is an essential, approximately 20-protein complex which transduces signals from the activators and the repressors to the core transcriptional machinery[42]. Several components of both the SAGA complex and Mediator have been shown to directly bind to the activation domain of Gal4p although it is not clear which complex interacts with Gal4p first. I will examine whether the APIS complex participates in facilitating the recruitment of the SAGA or Mediator complexes by Gal4p in the isolated *sug1/sug2* mutant strain. I will perform the well established ChIP analysis by using antibodies raised against the SAGA-specific or the Mediator-specific components (Spt3p and Srb4p, respectively) to test whether the SAGA complex and the Mediator can be recruited to the promoter when the APIS complex does not interact with Gal4p under inducing conditions. If the APIS complex is involved in recruitment of the SAGA complex or the Mediator, the SAGA complex or the Mediator will not be recruited to the promoter in the presence of the APIS complex in *GAL* promoters. Therefore anti-SAGA or anti-

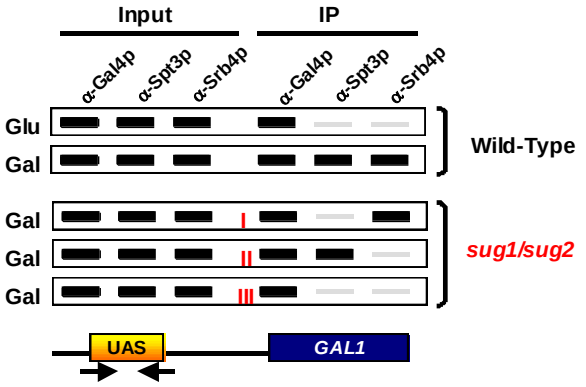

Fig. 9. Idealized results of the ChIP assay. Gal4p which is known to stay in the UAS in both glucose(Glu) and galactose(Gal) medium serves as the positive control. Spt3p is one of the components exclusively exists in the SAGA complex. Srb4p is one of the subunits of Mediator. In the *sug1/sug2* mutant strain, the APIS complex is not able to be recruited to the promoter via Gal4p and maybe will result in failing to recruit the SAGA complex(I), Mediator(II) or both(III)

Mediator antibodies will not precipitate their bound DNA fragments and hence no PCR products will be observed. Figure 9 shows the potential results of this assay.

Gal1p and Srb4p, which are components of the SAGA complex and the Mediator respectively, were reported to co-immunoprecipitate with Gal4p from crude cell extracts. An alternative method to determine whether the recruitment of the SAGA complex and the Mediator is affected in the isolated sug1/sug2 mutant strain is to examine whether these components of the SAGA complex and the Mediator can be co-precipitated with Gal4p under inducing condition.

B-3. Test whether the APIS complex plays a role in recruitment of TATA-binding-protein (TBP) and other PIC components.

Although components of the SAGA complex and the Mediator have been shown to regulate the recruitment of TBP and other PIC components, including several general transcription factors and RNA polymerase II holoenzyme, several reports have indicated that Gal4p and Sug1p also interact with TBP and components of PIC[3, 4,43-47]. To test whether the APIS complex is involved in the recruitment of TBP and other PIC components, a similar approach as described above will be performed in the mutant *sug1/sug2* strain. Briefly, a ChIP assay will be performed under inducing conditions by using anti-TBP antibodies and followed by real-time PCR amplification with TATA-box specific primer pairs.

C. Employment of a strain containing the ATPase activity-deficient APIS complex.

A different approach to fulfill the identification of the unconventional role of the APIS complex in *GAL* genes transcription activation is to abolish its ATPase activities. The APIS complex contains six ATPases, including Sug1p, Sug2p, Rpt1p, Rpt2p, Rpt3p, and Yta1p[14]. It has been suggested that they have non-redundant functions in regulation of protein degradation when associated with the 26S proteasome. Point mutations of lysine to arginine on the conserved

ATPase active site of either of Sug1p, Sug2p, Rpt2p, and Rpt3p are lethal for yeast cells[19]. Although it is very possible that Sug1p, Sug2p, and maybe other components of the APIS complex function in *GAL* genes activation by hydrolyzing ATP, no current evidence supports this idea. Several approaches described below will be used to abolish the ATPase activity of Sug1p, Sug2p, and other components of the APIS complex if necessary. Then the Gal4p-mediated transcription will be determined.

C-1. Construction of the ATPase deficient APIS complex by NEM method.

Johnston and colleagues have shown the selective inhibition of the individual proteasome ATPase activities using the cysteine-selective alkylating agent *N*-ethylmaleimide (NEM). I will use the established *sug1* or *sug2* mutant strain which has a threonine-to-cysteine mutation in the ATP binding site and hence will be sensitive to NEM. These will then be used to examine if transcription of the template will be affected in the established *in vitro* transcription assay[21]. Figure 10 shows the strategy for identification of the steps of transcription factor assembly on the promoter the APIS complex is involved in. First, the APIS complex will be depleted from the *gal4Δ/gal80Δ* yeast whole cell extracts (WCE) by anti-Sug1p and anti-Sug2p antibodies. Next, purified NEM-sensitive 19S complex which is treated with or without NEM will be added back

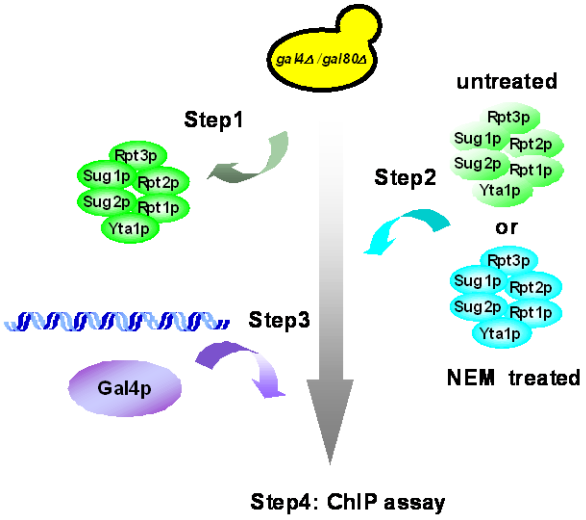

Fig. 10. Design of the *in vitro* assay by using NEM treated or untreated 19S complexes. Step 1, the cellular APIS complex is depleted by anti-Sug1p and anti-Sug2p antibodies from yeast whole cell extract(WCE). Step 2, NEM treated or untreated 19S complex is added back into the extract. Step 3, the template DNA and activator Gal4 are added into the WCE to let SAGA, Mediator and PIC assembly. ChIP assay will then be conducted to examine the association of different complexes

into the *in vitro* transcription system. Finally, the template DNA (*GAL1*) and the activator (Gal4p) will be added into the reactions. Two examinations will be conducted: (1) determination of whether the PIC can form on the template DNA, and (2) determination of whether transcription can take place. According to the known conditions, the PIC will be formed within 40 minutes. ChIP assays using different antibodies against different proteins including components of the SAGA complex, Mediator and PIC will be conducted to examine if the NEM sensitive19S complex causes any of them to be unable to be recruited to the template DNA at this stage. Next, [^{32}P] labeled UTP and other NTPs will be added to reactions. Resulting transcripts will be detected by autoradiography. The prediction is that NEM treated 19S subcomplex will abolish transcription activities. To determine whether the individual ATPases in the APIS complex confer the same degree of ATPase activity in transcription, the ATPase activities of all ATPases of the APIS complex will be abolished at the same time. One limitation of this assay is that the activity of the APIS complex in counteracting Gal80p may not be able to be determined.

C-2. Construction of the ATPase deficient APIS complex by ATP analog method.

An alternative method to abolish the ATPase activity of the APIS complex without mutating the ATPases is to employ an ATP analog, e.g. a non-hydrolizable ATP analog or a photoreactive ATP analog crosslinker[48-50]. After crosslinking of the purified 19S subcomplex with the ATP analog crosslinker, the ATPases will be stuck in its ATP-bound form and will not be able to hydrolyze it. A similar approach as described above would be performed by using the wild-type APIS complex treated with or without the ATP analogs and then its transcription activation activities will be tested. The advantage of this approach is that the wild-type APIS complex will be used and perhaps reflects more closely its real function in physiologic stages.

In summary, in specific aim #1, I anticipate to first map a Gal4p interacting region(s) of Sug1p and Sug2. Next, I will isolate an appropriate sug1 or sug2 mutant which has lost the Gal4p binding activity and hence the APIS complex cannot interact with Gal4p under inducing conditions. Finally, I will determine if GAL genes can transcribed when the APIS complex is not recruited to GAL promoters via Gal4p. In specific aim #2, I anticipate to determine in which step(s) of transcription complex assembly on the GAL1 promoter the APIS complex is involved by employing the isolated sug1/sug2 mutant strain which cannot interact with Gal4p and the mutant APIS complex which has no ATPase activity.

References

1. Ottosen, S., Herrera, F.J., and Triezenberg, S.J. (2002) Proteasome parts at gene promoters. *Science* **296,** 479-481
2. Gonzalez, F., Delahodde, A., Kodadek, T., and Johnston, S.A. (2002) Recruitment of a 19S proteasome subcomplex to and activated promoter. *Science* **296,** 548-550
3. Goldberg, A., (1995) Functions of the proteasome: the lysis at the end of the tunnel. *Science* **268,** 522-523
4. Glickman, M.H., Rubin, D.M., Fried, V.A., and Finley, D. (1998) The regulatory particle of the *Saccharomyces cerevisiae* proteasome. *Mol. Cell. Biol.* **18,** 3149-3162
5. Fu, H., Reis, N., Lee, Y., Glickman, M.H., and vierstra, R.D. (2001) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP signalosome. *EMBO J.* **20,** 7096-7107
6. Swaffield, J.C., Bromberg, J.F., and Johnston, S.A. (1992) Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4. *Nature* **357,** 698-700
7. Swaffield, J.C., Melcher, K., and Johnston, S.A. (1995) A highly conserved ATPase protein as a mediator between acidic activation domains and the TATA-binding protein. *Nature* **374,** 88-91

8. Xu, Q., Singer, R.A., and Johnston, G.C. (1995) Sug1 modulates yeast transcription activation by Cdc68. *Mol. Cell. Biol.* **15,** 6025-6035

9. Rubin, D.M., Coux, O., Wefes, I., Hengartner, C., Young, R.A., Goldberg, A.L., and Finley, D. (1996) Identification of the gal4 suppressor Sug1 as a subunit of the yeast 26S proteasome. *Nature* **379,** 655-657

10. Fraser, R.A., Rossignol, M., Heard, D.J., Egly, J.-M., and Chambon, P. (1997) SUG1, a putative transcriptional mediator and subunit of the PA700 proteasome regulatory complex, is a DNA helicase. *J. Biol. Chem.* **272**, 7122-7126

11. Chang, C., Gonzalez, F., Rothermel, B., Sun, L., Johnston, S.A., and Kodadek, T. (2001) The Gal4 activation domain binds Sug2 protein, a proteasome component, *in vivo* and *in vitro*. *J. Biol. Chem.* **276**, 30956-30963

12. Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S.A. (2001) The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. *Molecular Cell* **7,** 981-991

13. Russell, S.J. and Johnston, S.A. (2001) Evidence that proteolysis of Gal4 cannot explain the transcriptional effects of proteasome ATPase mutations. *J. Biol. Chem.* **276**, 9825-9831

14. Finley, D., Tanaka, K., Mann, C., Feldmann, H., Hochstrasser, M., Vierstra, R., Johnston, S., Hampton, R., Haber, J., Mccusker, J., Silver, P., Frontali, L., Thorsness, P., Varshavsky, A., Byers, B., Madura, K., Reed, S.I., Wolf, D., Jentsch, S., Sommer, T., Baumeister, W., Goldberg, A., Fried, V., Rubin, D.M., Glickman, M.H., and Toh-E., A. (1998) Unified nomenclature for subunits of the *Saccharomyces cerevisiae* proteasome regulatory particle. *Trends. Biochem. Sci.* **23,** 244-245

15. Groll, M., Ditzel, L., Loewe, J., Stock, D., Bochtler, M., Bartunik, H.D., and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4A resolution. *Nature* **386,** 463-471

16. Seemuller E, Lupas A, Stock D, Lowe J, Huber R, Baumeister W. (1995) Proteasome from Thermoplasma acidophilum: a threonine protease. *Science* **268,** 579-82

17. Strickland, E., Hakala, K., Thomas, P.J., and DeMartino, G.N. (2000) Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26S proteasome. *J. Biol. Chem.* **275**, 5565-5572

18. Braun, B.C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.-M., Finley, D., and Schmidt, M.(1999) The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nature Cell Biology* **1,** 221-226
19. Rubin, D.M., Glickman, M.H., Larsen, C.N., Dhruvakumar, S., and Finley, D. (1998) Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. *EMBO J.* **17,** 4909-4919
20. Leonhard, K., Stiegler A., Neupert, W., and Langer, T. (1999) Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. *Nature* **398,** 348-351
21. Russell, S.J., Gonzalez, F., Joshua-Tor, L., and Johnston, S.A. (2001) Selective chemical inactivation of AAA proteins reveals distinct functions of proteasomal ATPases. *Chem. Bio.* **8,** 941-950
22. Lam, Y.A., Lawson, T.G., Velayutham, M, Zweier, J.L., and Pickart, C.M. (2002) A proteasomal ATPase subunit recognizes the polyubiquitin degradation siganl. *Nature* **416,** 763-767
23. Russell, S.J., Steger, K.A., and Johnston, S.A. (1999) Subcellular localization, stoichiometry, and protein levels of 26S proteasome subunits in yeast. *J. Biol. Chem.* **274**, 21943-21952
24. Enenkel, C., Lehmann, A., and Kloetzel, P.-M. (1998) Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast. *EMBO J.* **17,** 6144-6154
25. Russell, S.J., Sathyanarayana, U.G., and Johnston, S.A. (1996) Isolation and characterization of SUG2. *J. Biol. Chem.* **271**, 32810-32817
26. Weeda, G., Rossignol. M., Fraser, R.A., Winkler, G.S., Vermeulen, W.W., van't Veer, L.J., Ma, L., Hoeijmakers, J.H.J., and Egly, J.-M. (1997) The XPB subunit of repair/transcription factor TFIIH directly interacts with SUG1, a subunit of the 26S proteasome and putative transcription actor. *Nucleic Acids Res.* **25,** 2274-2283
27. Triezenberg, S.J., Kingsbury, R.C., and McKnight, S.L. (1988) Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* **2,** 718-729

28. Simone E. Salghetti, Masafumi Muratani, Herman Wijnen, Bruce Futcher, and William P. Tansey. (2000) Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc. Natl. Acad. Sci.* **97,** 3118-3123
29. Jesenberger V and Jentsch S. (2002) Deadly encounter: ubiquitin meets apoptosis. *Nat Rev Mol Cell Biol.* **3,**12-21
30. Finley, D. (2001) An alternative to destruction. *Nature* **412,** 283-286
31. Conaway, R.C., Brower, C.S., and Conaway J.W. (2002) Emerging roles of ubiquitin in transcription regulation. *Science* **296,** 1254-1258
32. Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation Meets Ubiquitination: The Control of NF-B Activity. *Annu. Rev. Immunol.* **18,** 621-663
33. Salghetti, S.E., Caudy, A.A., Chenoweth, J.G., and Tansey, W.P. Regulation of transcriptional activation domain function by ubiquitin. *Science*, **293,** 1651-1653
34. David M. Lonard, Zafar Nawaz, Carolyn L. Smith, and Bert W. Omalley. (2000) The 26S Proteasome Is Required for Estrogen Receptor-α and Coactivator Turnover and for Efficient Estrogen Receptor-α Transactivation. *Molecular Cell* **5,** 939
35. Molinari, E., Gilman, M., and Natesan, S. (1999) Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency *in vivo*. *EMBO J.* **18,** 6439-6447
36. Meimoun, A., Holtzman, T., Weissman, Z., McBride, H.J., Stillman, D.J., Fink, G.R. and Kornitzer, D. (2000) Degradation of the Transcription Factor Gcn4 Requires the Kinase Pho85 and the SCFCDC4 Ubiquitin-Ligase Complex. *Mol. Biol. Cell* **11,** 915-927
37. Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev.* **15,** 1078-1092
38. Hirst, M, Ho, C., Sabourin, L., Rudnicki, M., Penn, L., and Sadowski, I. (2001) A two-hybrid system for transactivator bait protins. *Proc. Natl. Acad. Sci. USA* **98,** 8726-8731
39. Varshavsky, A. (1992) The N-end rule. *Cell* **69,** 725-735
40. Dedon, P.C., Soults, J.A., Allis, C.D., and Gorovsky, M.A. (1991) A smiplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions. *Analytical Biochemistry* **197,** 83-90

41. Leuther, K.K. and Johnston, S.A. (1992) Nondissociation of GAL4 and GAL80 in vivo after galactose induction. *Science* **256,** 1333-1335

42. Woychik, N.A. and Hampsey, M. (2002) The RNA polymerase II machinery: structure illuminates function. *Cell* **108,** 453-463

43. Bhaumik, S.R. and Green, M.R. (2001) SAGA is an essential in vivo target of the yeast acidic activator Gal4p. *Genes & Dev.* **15,** 1935-1945

44. Larschan E. and Winston, F. (2002) The S. cerevisiae SAGA complex functions in vivo as a coactivator for transcriptional activation by Gal4. *Genes & Dev*. **15,** 1946-1956

45. Lohr, D., Venkov, P., and Zlatanova, J. (1995) Transcriptional regulation in the yeast GAL gene family: a complex genetic network. *FASEB* **9,** 777-787

46. Wu, Y., Reece, RJ, and Ptashne, M. (1996) Quantitation of putative activator-target affinities predicts transcriptional activating potentials. *EMBO J.* **15,** 3951-3963

47. Xie, Y., Denison, C., Yang, S.-H., Fancy, D.A., and Kodadek, T. (2000) Biochemical Characterization of the TATA-binding Protein-Gal4 Activation Domain Complex. *J. Biol. Chem.* **275,** 31914-31920

48. Laethem, R. and Zull, J.E. (1990) Characterization of the interaction of parathyroid hormone with the mitochondrial ATPase. *Arch. Biochem. Biophysics* **282,** 161-169

49. Boehlein, S.K., Walworth, E.S., and Schuster, S.M. (1997) Identification of cycteine-523 in the aspartate binding site of *Escherichia coli* asparagine synthetase B. *Biochemistry* **36,** 10168-10177

50. Doronin, S., Murray, L., Dessauer, C.W., and Johnson, R.A. (1999) Covalent labeling of adenylyl cyclase cytosolic domains with γ-methylimidazole-2',5'-dideoxy-[γ-^{32}-P]3'ATP-and the mechanism for p-site-mediated inhibition. *J. Biol. Chem.* **274,** 34745-34750