Recruitment of a 19S Proteasome Subcomplex to an Activated Promoter

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The 19S proteasome regulatory particle plays a critical role in cellular proteolysis. However, recent reports have demonstrated that 19S proteins play a nonproteolytic role in nucleotide excision repair and transcription elongation. We show by chromatin immunoprecipitation assays that proteins comprising the 19S complex are recruited to the *GAL1-10* promoter by the Gal4 transactivator upon induction with galactose. This recruited complex does not contain proteins from the 20S proteolytic particle and includes a subset of the 19S proteins. This subset is also specifically retained from an extract by the Gal4 activation domain. These data indicate that in vivo, the base of the 19S complex functions independently of the larger complex and plays a direct, nonproteolytic role in RNA polymerase II transcription.

The 26*S* proteasome is composed of a 20*S* proteolytic barrel and a 19*S* regulatory cap (*1*). The latter contains at least 18 proteins (*2*), including six highly related adenosine triphosphatases (ATPases) of the ATPases associated with various cellular activities (AAA) family (*3*). Five of these ATPases have been linked to transcription either biochemically or genetically (*4*–*7*). However, these observations have been presumed to reflect the indirect effects of proteasome-mediated proteolysis of transcription factors (*8*, *9*), and the 19*S* complex is generally thought solely to stimulate and regulate 20*S*-mediated proteolysis. However, recent biochemical studies have provided evidence that the 19*S* complex plays a nonproteolytic role in transcription elongation (*7*). This view was supported by the observation that certain mutations in *SUG1/RPT6* render yeast highly sensitive to 6-azauracil (*7*), a hallmark of a defect in elongation (*10*), but more direct in vivo evidence for a role of the 19*S* in transcription has been lacking. Here we provide evidence that a subset of the 19*S* complex is involved in transcription in vivo.

If the 19*S* complex is involved directly in transcription, it should be physically associated with the promoter region of a gene. To test this hypothesis, we examined the *GAL1-10* promoter of yeast by chromatin immunoprecipitation (ChIP) assays (*11*). As a positive control, we precipitated the promoter with antibodies against the regulator of the *GAL1-10* promoter, Gal4 (*12*). It is known that Gal4 protein is present on the promoter under both noninducing (raffinose) and inducing (galactose) conditions (*13*). A portion of the promoter encompassing the Gal4 binding sites was immunoprecipitated by antibodies to Gal4 under both conditions (Fig. 1A). Preimmune serum or antibodies raised against cyclophilin did not precipitate the promoter (Fig. 1) (*14*), nor was the promoter precipitated with the same anti-Gal4 antibodies against an extract from a strain deleted for *GAL4* (Fig. 1B). Amplification of an irrelevant portion of the yeast genome from chromosome VII showed no signal above background (*15*).

The *SUG1/RPT6* gene was originally identified genetically on the basis of mutations that suppressed defects in the COOH-terminal activation domain of Gal4 (*4*). Sug1/Rpt6 is one of the six highly conserved ATPases of the AAA class in the 19*S*. Anti-Sug1/Rpt6 antibodies precipitated little, if any, of the promoter region in raffinose medium (Fig. 1A). However, within 10 min of inducing the *GAL1-10* genes, Sug1/Rpt6 associated with the promoter. The same pattern of precipitation was also seen with antibodies raised against Sug2/Rpt4 (*14*) and Yta1/Rpt5 (Fig. 1A), two other ATPases of the 19*S* complex. These results indicate that the Sug1/Rpt6, Sug2/Rpt4, and Yta1/Rpt5 proteins are recruited to the *GAL1-10* promoter region rapidly upon induction of transcription. This association with the promoter is Gal4 dependent because little or no promoter was precipitated by anti-Sug1 antibodies from a Gal4-deletion strain (Fig. 1B).

In contrast, antibodies generated against the 20*S* complex did not precipitate the promoter in raffinose or galactose-grown cells (Fig. 1A). Antibodies raised against Rpn9, a non-ATPase 19*S* subunit, also did not precipitate the promoter after induction with galactose (Fig. 1A). Control experiments demonstrated the ability of the anti-20*S* and anti-Rpn9 antibodies to immunoprecipitate each of the corresponding proteins from an extract (*15*). Rpn9 is part of the so-called "lid" subcomplex of the 19*S* particle, which can be separated biochemically in high-salt buffers from the base (which includes the six ATPases, Rpn1, and Rpn2) (*16*, *17*). Both the 20*S* and lid components are present on the *GAL1* gene at later times after induction (*14*). These data therefore indicate that the 19*S* base is recruited to the promoter independently of the 20*S* and lid subcomponents.

To determine whether recruitment of the 19*S* ATPases was restricted to the promoter, we repeated the ChIP assays using polymerase chain reaction (PCR) primers targeted to regions along the *GAL1* gene. Antibodies raised against Gal4 precipitated predominantly the

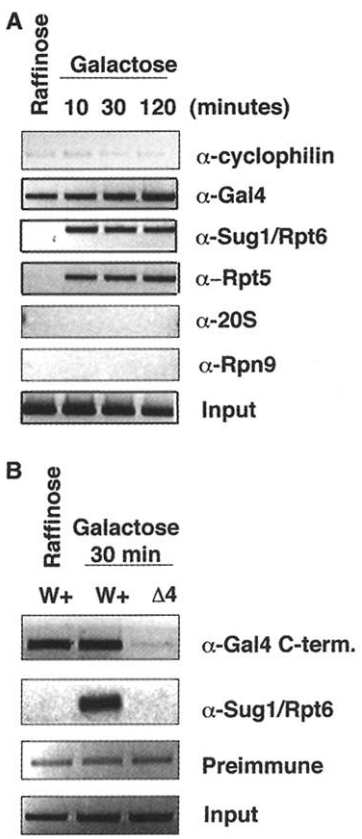

Fig. 1. Localization of proteasome proteins on the *GAL1-10* promoter under inducing conditions by ChIP assay. (**A**) Analysis of the level of *GAL1-10* DNA coimmunoprecipitated by the antibodies shown. The region of the *GAL1-10* promoter is designated on the diagram on Fig. 2. Serum with antibody to cyclophilin served as a negative control. (**B**) Gal4 dependence of Sug1/Rpt6 protein binding to the *GAL1-10* promoter. Wild-type (W+) or GAL4-deleted (Δ4) yeast were grown under conditions indicated in the figure and the resultant extracts were used for ChIP analysis. Preimmune serum was used to determine the level of background binding in this experiment. Various dilutions of chromatin from samples not subjected to immunoprecipitation were used to demonstrate that the samples were run in the linear range of the reaction (*14*).

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promoter region of the *GAL1* gene (Fig. 2). In contrast, using anti-Sug1/Rpt6 antibodies, we found that Sug1/Rpt6 protein was present throughout the length of the *GAL1* gene (Fig. 2, bottom panel). This is consistent with our previous report that the 19*S* ATPases are important for efficient RNA polymerase II elongation (*7*).

The Gal4 activation domain (AD) was previously shown to associate with Sug1/Rpt6 and other 19*S* ATPases when added to a crude extract (*5*) or an immunopurified 19*S* complex (*6*). This suggests that Gal4 directly recruits a fragment of the proteasome that includes the 19*S* ATPases and perhaps other proteins, but not the 20*S* proteolytic or 19*S* lid subunits. Therefore, we investigated whether the Gal4 AD retains each of the six 19*S* ATPases from an extract. As shown in Fig. 3A, approximately the same proportion of input protein was retained for all of these factors (Rpt1 to Rpt6). However, using antibodies raised against the 20*S* Pre1 (Fig. 3A), Pre5, or Pre6 (*14*) proteins, we found no evidence for retention of the 20*S* subunit. Furthermore, neither Rpn9 nor Rpn12, two 19*S* lid components, could be detected in the Gal4 AD–bound fraction (Fig. 3A). These results are not due to artifactual separation of the various subcomplexes during extract preparation because pull-down of the intact 26*S* proteasome was observed when the Gal4AD was replaced by the Rad23 ubiquitin-like domain, a known proteasome ligand (*18*). Thus, there is a correlation between the in vivo ChIP results and the in vitro Gal4 AD association assays.

Finally, we examined whether there was specificity in the interactions between the Gal4 AD and any of the six AAA ATPases. Whereas direct association of the Gal4 AD and in vitro–translated Sug1/Rpt6 and Sug2/Rpt4 has been reported previously (*5*, *6*), binding to the other yeast 19*S* ATPases has not been assessed. Each of these proteins was transcribed and translated in vitro (*19*, *20*) and assayed for their retention by the glutathione-*S*-transferase (GST)–Gal4 AD fusion protein. Only Sug1/Rtp6 and Sug2/Rtp4 bound the AD above background levels (Fig. 3B). This is important given that only Sug1/Rpt6 and Sug2/Rpt4 mutations arose in a selection for suppressors of a defect in the Gal4 AD (*4*). This correlation between the genetics and the specificity of binding in vitro strongly suggests that the AD-Sug1/Sug2 interactions are physiologically relevant.

To more specifically address the physiological relevance of the interaction between the 19*S* and Gal4 on DNA, we examined whether mutations in *SUG2* that do or do not suppress a defect in the Gal4 AD also differentially affected the association of the 19*S* with the promoter in vivo. A partial deletion of the Gal4 AD (4D) that yields a galactose-minus phenotype also abolished the association of the Sug1 protein with the promoter (Fig. 4). However, when this *gal4D* mutation was combined with the *sug2-1* mutation that restores the ability to grow on galactose, the association of the Sug1 protein with the promoter was restored (Fig. 4). In contrast, another mutation in *SUG2*, *sug2-13*, that does not suppress the *gal4D* defect did not restore Sug1 protein to the promoter (Fig. 4). This strict correlation of mutations in *Sug1* and *Sug2* with gene expression and recruitment of the Sug proteins to the promoter, especially when considered together with the biochemical associations demonstrated in Fig. 3, imply that the Sug proteins play a functional role in gene expression.

We previously demonstrated a nonproteolytic role for the 19*S* proteins in elongation in vitro (*7*). The observation of a rapid, activator-dependent association of at least three of the 19*S* ATPases with the *GAL1* promoter reported here suggests a functional role in the early events of transcription in vivo as well. The finding that Sug1/Rpt6 protein also associates throughout the length of the gene is consistent with its proposed role in elongation.

The results reported here demonstrate that the Gal4 AD binds only a subset of proteasomal proteins in vitro and that this subset appears to be the same one that is recruited to the *GAL1* promoter in vivo. The 19*S* and 20*S* complexes

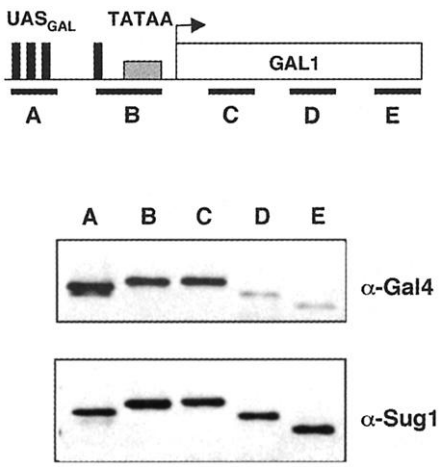

Fig. 2. The Sug1/Rpt6 protein is present throughout the length of the *GAL1* gene after induction. (**Bottom**) ChIP assays were performed as in Fig. 1A, and the results displayed are those obtained from yeast cultures induced in galactose for 10 min. (**Top**) Each of the regions measured is represented by the horizontal bar below the diagram.

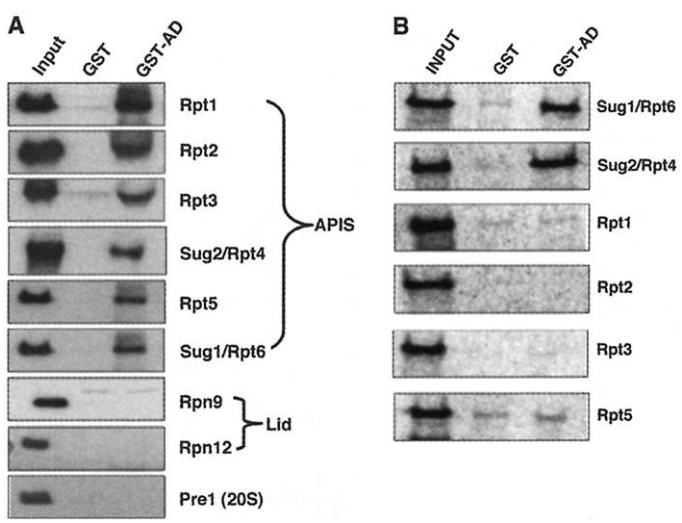

Fig. 3. The Gal4 activation domain retains the 19*S* ATPases, but not 20*S* or lid components from a crude yeast extract. Extracts were incubated with immobilized GST–Gal4 AD (*10*) or, as a control, GST. After precipitation and washing, the retained proteins were analyzed by polyacrylamide gel electrophoresis and Western blotting with the antibodies indicated. (**A**) All six AAA ATPases in the 19*S* complex were detected in the AD-bound fraction. Pre1, a 20*S* component, was not retained by the AD. In addition, proteins found in the lid subcomplex (Rpn9 and Rpn12) were also not retained by the Gal4 AD. (**B**) The Gal4 AD binds Sug1/Rpt6 and Sug2/Rpt4 directly. Each of the 19*S* ATPases was translated in vitro in the presence of radiolabeled methionine. The translation mixtures were then incubated with immobilized GST–Gal4 AD fusion protein or GST, and the retained proteins were analyzed by autoradiography.

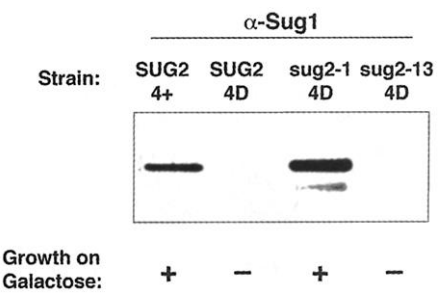

Fig 4. Mutations in Sug2 correlate transcriptional phenotype and association of the 19*S* with the *GAL1-10* promoter. ChIP assays were performed as in Fig. 1A in four different strains: *SUG2 GAL4* (4+), *SUG2 gal4D*(*4D*), *sug2-1 gal4D*, and *sug2-13 gal4D*. The gal4D protein is missing 28 amino acids of the AD and in a *SUG2* background does not grow on galactose. The *sug2-1* mutation was selected for its ability to suppress the *gal4D* mutation and restore growth on galactose. The *sug2-13* mutation was selected under different criteria and does not suppress the *gal4D* phenotype. ChIP assays were performed after formaldehyde treatment 30 min after galactose was added to the medium, and with antibody to Sug1 protein.

can be separated biochemically, and the former can be further subdivided into base, which contains Rpts1-6, Rpn1, and Rpn2 and exhibits chaperonin activity (*21*, *22*), and lid (*16*, *17*). However, there has been no previous evidence that these subspecies represent physiologically relevant complexes. Our results suggest that there is at least one discrete subcomplex of the 19*S* and that it functions independently of other proteasome subunits in Gal4-mediated transcription. This species, which we call the APIS (AAA proteins independent of 20*S*) complex, clearly includes the six 19*S* ATPases (Rpt1 to Rpt6) and perhaps other proteins. The precise composition of the APIS complex, and whether it corresponds to the biochemically defined base, remains to be elucidated.

There has been increasing evidence of a link between ubiquitylation and transcription (*23*–*26*). Recent work by Tansey and colleagues suggests a mechanism by which they might be linked temporally. They found that for the artificial LexA-VP16 activator, ubiquitylation of the activator is required for the activator to function in yeast (*26*). Importantly, linkage of a single ubiquitin molecule to the activator was shown to lead to activation, but did not signal proteolytic turnover. This suggests that it is ubiquitylation per se, and not ubiquitin-linked proteolysis, that is crucial for activator function. Whereas the Gal4 AD alone is capable of binding the APIS complex (Fig. 3), an attached monoubiquitin might enhance this interaction or modulate the activity of the AD-bound complex in a way that is important for transcription to proceed. After induction, the ubiquitin chain on the activator would grow, possibly signaling a switch in activator association from the APIS complex to the full 26*S* proteasome. The time required for the ubiquitin chain to reach the minimum size needed to signal proteasome-mediated degradation (*27*) would be used by Gal4 to drive high-level transcription. But after that time, the activator would be subject to degradation, thus placing a "governor" on gene expression. The critical element of this model is that mono- and polyubiquitin chains are fundamentally different modifications that signal different intermolecular interactions. Although speculative, we believe that this model is useful in potentially linking a number of notable recent findings and in providing a number of readily testable hypotheses.

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A System for Stable Expression of Short Interfering RNAs in Mammalian Cells

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Mammalian genetic approaches to study gene function have been hampered by the lack of tools to generate stable loss-of-function phenotypes efficiently. We report here a new vector system, named pSUPER, which directs the synthesis of small interfering RNAs (siRNAs) in mammalian cells. We show that siRNA expression mediated by this vector causes efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time. Therefore, the pSUPER vector constitutes a new and powerful system to analyze gene function in a variety of mammalian cell types.

In several organisms, introduction of double-stranded RNA has proven to be a powerful tool to suppress gene expression through a process known as RNA interference (*1*). However, in most mammalian cells this provokes a strong cytotoxic response (*2*). This non-specific effect can be circumvented by use of synthetic short [21- to 22-nucleotide(nt) interfering RNAs (siRNAs)], which can mediate strong and specific suppression of gene expression (*3*). However, this reduction in gene expression is transient, which severely restricts its applications. To overcome this limitation, we designed a mammalian expression vector that directs the synthesis of siRNA-like transcripts [pSUPER, suppression of endogenous RNA, Fig. 1A and Supplementary fig. 1C (*4*)]. We used the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). Most important, the cleavage of the transcript at the termination site is after the second uridine (*5*) yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides (nt) (Fig. 1A). We designed the gene-specific insert such that it specifies a 19-nt sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 19-nt sequence. The resulting transcript is predicted to fold back on itself to form a 19–base pair stem-loop structure, resembling that of *C. elegans Let-7* (Fig. 1A).

We used the pSUPER vector to suppress the endogenous *CDH1* gene, an activator of

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