Evidence That Proteolysis of Gal4 Cannot Explain the Transcriptional Effects of Proteasome ATPase Mutations*

Received for publication, December 4, 2000
Published, JBC Papers in Press, January 4, 2001, DOI 10.1074/jbc.M010889200

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The Gal system of *Saccharomyces cerevisiae* is a paradigm for eukaryotic gene regulation. Expression of genes required for growth on galactose is regulated by the transcriptional activator Gal4. The activation function of Gal4 has been localized to 34 amino acids near the C terminus of the protein. The *gal4D* allele of *GAL4* encodes a truncated protein in which only 14 amino acids of the activation domain remain. Expression of *GAL* genes is dramatically reduced in *gal4D* strains and these strains are unable to grow on galactose as the sole carbon source. Overexpression of gal4D partially relieves the defect in GAL gene expression and allows growth on galactose. A search for extragenic suppressors of *gal4D* identified recessive mutations in the *SUG1* and *SUG2* genes, which encode ATPases of the 19S regulatory complex of the proteasome. The proteasome is responsible for the ATP-dependent degradation of proteins marked for destruction by the ubiquitin system. It has been commonly assumed that effects of *SUG1* and *SUG2* mutations on transcription are explained by alterations in the proteolysis of gal4D protein. We have investigated this assumption. Surprisingly, we find that *SUG1* and *SUG2* alleles that are unable to suppress *gal4D* cause a larger increase in gal4D protein levels than do suppressing alleles. In addition, mutations in genes encoding subunits of the proteolytic 20S subcomplex of the proteasome increase the levels of gal4D protein but do not rescue its transcriptional activity. Therefore, an alteration in the proteolysis of gal4D by the proteasome cannot explain the effects of mutations in *SUG1* and *SUG2* on expression of *GAL* genes. These findings suggest that the 19S regulatory complex may play a more direct role in transcription.

In the yeast *Saccharomyces cerevisiae*, expression of genes required for the metabolism of galactose is controlled by the positive regulator Gal4 and the negative regulator Gal80. Gal4 is a transcriptional activator with an N-terminal DNA-binding domain and a C-terminal activation domain. Partial deletion of the activation domain of Gal4 in the *gal4D* allele leads to a dramatic loss in the ability to activate transcription of *GAL* genes. Using a reporter gene assay, the gal4D protein was found to activate transcription from the *GAL1/10* promoter to ~4% of the level driven by wild-type Gal4 (1). The gal4D protein does not activate the *GAL* genes sufficiently to allow growth on galactose as the sole carbon source (1). Recessive mutations in *SUG1* and *SUG2* have been identified that partially restore the ability of gal4D to activate transcription (1–3). In strains carrying the *sug1-1* or *sug2-1* alleles, reporter gene activity was restored to ~55% and 70%, respectively (1, 4). Both the *sug1-1* and *sug2-1* alleles allow *gal4D* strains to grow on galactose as the sole carbon source (1, 3). In the work reported here we have investigated the mechanism for this suppression.

*Sug1* (1) and Sug2 (3) are members of the ATPases Associated with diverse cellular Activities (AAA) family. Members of this family share a 230-amino acid conserved region known as the AAA module (5), which contains Walker A and B nucleotide-binding motifs (6). Both Sug1 and Sug2 are components of the 19S regulatory complex (regulatory particle) of the yeast 26S proteasome (3, 7, 8), along with four other AAA proteins (Ref. 9 and references therein). The finding that Sug1 and Sug2 are components of the proteasome suggested that *sug1-1* and *sug2-1* might suppress *gal4D* by stabilizing the mutant protein and allowing it to accumulate. Indeed, several authors have argued that the transcriptional phenotypes associated with mutations in ATPases of the proteasome are indirect and due to alterations in proteolysis (7, 10–14). Consistent with this possibility, high level overexpression of gal4D from a multicopy vector rescues the ability to activate a reporter gene to ~60%. 2

If suppression of *gal4D* is dependent on stabilization of an unstable protein, then levels of gal4D protein in the cell should correlate with suppression. To address this question, we made a set of congenic yeast strains carrying wild-type and mutant *SUG1* and *SUG2* alleles. We used these strains to compare levels of gal4D protein in strains that suppressed the transcriptional phenotype of gal4D and those that did not. In addition to comparing *sug1-1* and *sug2-1* to their respective wild-types, we also wished to evaluate other alleles of *SUG1* and *SUG2*. Alleles of *SUG1* have been identified independently by Xu et al. (15) as mutations that suppressed a temperature-sensitive allele of *CDC68*, but their interaction with gal4D has not been defined. We chose one of these alleles, *sug1-20*, to compare with *sug1-1*. We also wanted to compare *sug2-1* to an allele isolated in a different manner. However, until recently, no *SUG2* allele besides *sug2-1* was known. In 1988 McCusker and Haber (16) isolated mutants exhibiting both cycloheximide resistance and temperature-sensitive lethality (*cfl*). These strains have hypersensitivity to amino acid analogs and fail to arrest in G1 under some starvation conditions (17). After *SUG2* was cloned (3) it

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1 The abbreviations used are: AAA, ATPases Associated with diverse cellular Activities family; bp, base pair(s); PCR, polymerase chain reaction; 5-FOA, 5-Fluoro-orotic acid; PAGE, polyacrylamide gel electrophoresis; NER, nucleotide excision repair.

2 J. C. Swaffield and S. A. Johnston, unpublished data.
became clear from mapping data that crl13 was an allele of SUG2 (16). We have determined the mutation in the crl13 allele of SUG2, which we have designated sug2-13, and have compared its phenotype to that of sug2-1.

In this work, we have produced a set of congenic strains carrying wild-type and mutant alleles of both SUG1 and SUG2. Using these strains we have shown that there is allele specificity to suppression of gal4D. Mutations in alleles of SUG1 and SUG2 not selected for their ability to suppress gal4D do not do so. This has allowed us to explicitly test the hypothesis that the sug1-1 and sug2-1 mutations lead to alterations in the level of gal4D protein and that this accounts for rescue of the gal4D phenotype. We find that the levels of gal4D protein are higher in strains carrying sug1 and sug2 mutations that do not suppress gal4D than in strains carrying the sug1-1 and sug2-1 mutations. Therefore, changes in the proteolysis of gal4D by the proteasome cannot account for the transcriptional effects of the sug1-1 and sug2-1 mutations.

**EXPERIMENTAL PROCEDURES**

**Identification of the SUG2 Mutation in the crl13 Strain—** James Haber and John Mcfusker supplied us with Y55-297 (crl13) and its congenic wild-type Y55 (CRL13). The SUG2 gene from 85 bp before the start codon to 200 bp after the stop codon was amplified by PCR from both strains and cloned using the TA cloning kit to produce pSJ187 (crl13) and pSJR188 (CRL13). The sequence of the entire gene was then determined by fluorescence-automated sequencing. There were several silent polymorphisms as compared with the data base sequence present in the SGU2 alleles amplified from both the crl13 and wild-type strains. However, only one mutation in the crl13 strain resulted in an amino acid change. The codon for amino acid 231 was changed from CTA to CGA to create a L231R substitution. We designated this mutation sug2-13. The starting material for the production of these strains was pJS159, which contains the SUG1 locus as a XhoI/BamHI fragment. The sug1-1 and sug1-20 mutations were inserted into this plasmid by site-directed mutagenesis to produce pSJR171 and pSJR173, respectively. Fragments containing the wild-type and mutant genes were ligated into the vector pMTL-SUG1 and transformed into Sc500 (SUG1::URA3 pMTL-SUG1), which was complemented to W303. Gene replacement events were selected as described above. The SUG1 gene was amplified from these strains by PCR and completely sequenced. Sequencing of the genes confirmed that the desired mutations had been introduced and that no other mutations were present.

Sc654 contained the sug1-1 mutation, Sc658 contained the sug2-13 and sug2-1 mutations, and Sc607 was the congenic wild-type strain. GALA was deleted from these strains as described above to give Sc729 (sug1-1 GALA::HIS3), Sc733 (sug1-20 GALA::HIS3), and Sc727 (SUG1 GALA::HIS3), respectively.

**Production of cdc68-1 Strains—** To test the interaction of SUG1 alleles and the cdc68-1 mutation, strains Sc729 (sug1-1), Sc733 (sug1-20), and Sc727 (SUG1) were transformed with YepDE681 (a multicopy plasmid carrying the URA3 marker and expressing cdc68-1). These strains were then transformed with the BamHI fragment of pBM10 (LEU2) to disrupt the chromosomal CDC68 gene. Transformants were selected on medium lacking leucine to select for integration events. To ensure that the chromosomal CDC68 gene was disrupted, transformants were tested for their ability to grow on medium containing 5-FOA. Because CDC68 is an essential gene, yeast that carry CDC68 only on the URA3 plasmid should not survive on 5-FOA. Accordingly, 5-FOA- clones were discarded. The remaining strains were deduced to have the chromosomal CDC68 locus deleted and to carry cdc68-1 on the multicopy plasmid. The congenic set of strains produced was Sc761 (SUG1 CDC68::LEU2 Yep352-cdc68-1), Sc766 (sug1-1 CDC68::LEU2 Yep352-cdc68-1), and Sc769 (sug1-20 CDC68::LEU2 Yep352-cdc68-1).

**Assay of Cycloheximide Resistance and Temperature Sensitivity—** Strains were grown in Yeast-extract-peptone-dextrose (21) to stationary phase. The A600 values of the cultures were normalized to 0.1 by dilution in water. Serial 10-fold dilutions were performed, and 10 µl of each dilution was spotted onto plates. Cycloheximide plates were prepared as described previously (16). Temperature sensitivity assays were done on YEPD plates. Cycloheximide plates and control plates were grown for 30 h at 30 °C. Plates were incubated for 4, 5, 6, and 7 days at 30 °C. Plates were also incubated for 4 days at 37 °C.

**Assay of gal4D Suppression—** Yeast strains were transformed with single-copy plasmids (derived from pSB32 expressing either gal4D (psBR261) or wild-type Gal4 (pSJR263), or with a multicopy plasmid (derived from Yep351 expressing gal4D (pSR365)). In each case the encoded proteins were tagged at their N termini with three tandem epitopes (Novagen), and the GALA gene was expressed from its own promoter. The transformed strains were grown to stationary phase in complete medium lacking leucine (21), to select for the plasmid, with raffinose as the carbon source. The use of raffinose ensured that there was no selection for suppressors of gal4D. Glucose was not used, because it represses the synthesis of Gal4. The cell suspensions were diluted to 1:10 and spotted to complete-leucine plates with glucose or galactose as the carbon source.

**Assay of Gal4 and gal4D Protein Levels—** Transformed strains were grown to stationary phase as above, then diluted into a larger volume of complete-leucine medium with raffinose as the carbon source. They were grown to an A600 of 0.8 (mid- to late-log for these strains), and 15 ml was harvested by centrifugation. The cells were washed once in ice-cold water, then suspended in 100 µl of 2× SDS loading buffer and snap-frozen in liquid nitrogen. At the same time the cells were harvested, another aliquot of the culture was diluted 1:10 with water containing 0.02% sodium azide. The A600 of these samples was used to calculate a volume of 2× SDS loading buffer to add to the frozen samples so that they had equal concentrations of cells. After the appropriate dilution, the cells suspended in SDS loading buffer were boiled for 10 min, they were spotted to remove the supernatant, and equal amounts of the supernatant were loaded onto an SDS-PAGE gel. A three-stage polyacrylamide gel (stacking 4%, top separating 10%, bottom separating 12.5%) was run in a Bio-Rad Protein apparatus overnight at 70 V with cooling by circulating water at 16 °C. The high percentage bottom stage of the gel was used to retard the low molecular mass cyclophilin (used as a leading control) during the long run re...
quired to separate Gal4 protein from a cross-reacting band with a similar electrophoretic mobility. Proteins were transferred to a nitrocellulose membrane (Millipore) in a Genie blotting apparatus (Idea Scientific) for 50 min at 24 V. The membranes were blotted with anti-T7 monoclonal antibody (Novagen) to detect Gal4 and with rabbit anti-cyclophilin antibody (a gift of K. Sykes). Membranes were developed with horseradish peroxidase-conjugated secondary antibodies and the Renaissance chemiluminescence reagent.

RESULTS

Characterization of the crl13 Mutations and Production of Congenic Strains Carrying Different sug2 Alleles—Despite the fact that mutations in SUG1 have been isolated in multiple different screens (1, 15, 22), until recently no mutant alleles of SUG2 besides sug2-1 were known. We wanted to investigate the allele specificity of gal4D suppression by alleles of SUG2. Therefore, we decided to identify the mutation in the crl13 allele of SUG2. The SUG2 open reading frames were amplified from the crl13 strain and its parental wild-type strain and were completely sequenced. Both of these strains were kindly provided by J. E Haber and J. H. McCusker. Two nucleotide changes were found in the SUG2 gene from the crl13 strain. Only one of these resulted in an amino acid change, substituting an arginine for a leucine at position 231. We will refer to this novel sug2 allele as sug2-13. The mutation in sug2-1 results in a substitution of glycine 300 by a lysine (3). Unlike the G300K substitution encoded in sug2-1, the L231R substitution encoded in sug2-13 is within the Walker A nucleotide-binding motif of Sug2 (6, 23).

We produced a set of congenic strains carrying either the sug2-1, sug2-13, or wild-type SUG2 alleles. These strains were produced by a two-step gene replacement strategy so that the mutant sug2 alleles were integrated at their native chromosomal loci (see “Experimental Procedures”). We verified that Sug2 protein was expressed at identical levels in each of these strains, demonstrating that neither of the mutant sug2 alleles destabilized the Sug2 protein (data not shown). We also tested the sub-cellular localization of Sug2 and found that it was primarily nuclear in each strain as it is in wild-type yeast (data not shown) (24). So that the interaction of the sug2 alleles with different GAL4 variants could be tested, we deleted the GAL4 gene from each of these strains (see “Experimental Procedures”).

Temperature Sensitivity and Cycloheximide Resistance of sug2 Mutant Strains—The crl mutants were selected for temperature sensitivity and cycloheximide resistance. Therefore, the set of congenic strains we produced were tested for temperature sensitivity at 37 °C. These results, presented in Fig. 1A, are consistent with earlier reports. The sug2-13 strain is extremely temperature-sensitive just as reported for the crl13 strain (16). In contrast, sug2-1 does not cause temperature sensitivity, consistent with previous results (3). The other reported phenotype of the crl13 strain was resistance to cycloheximide. Therefore, we tested the panel of congenic strains for this phenotype, but found no evidence for cycloheximide resistance in any one sug2 strain (Fig. 1A). Indeed, both sug2 strains may be more sensitive to cycloheximide than the congenic wild-type strain. This was not surprising, as McCusker and Haber previously found that cycloheximide resistance did not segregate 2:2 when the crl13 strain was crossed into a different genetic background, the commonly used S288c strain (16). In fact, only a very small proportion of segregants from this cross displayed cycloheximide resistance. The authors concluded that there must be at least two suppressors of cycloheximide resistance in S288c, perhaps due to differences in permeability of the drug (16). It seems likely that the W303 background of our congenic strains also contains such suppressors. In contrast, temperature sensitivity segregated 2:2 when the crl13 mutation was crossed into the S288c background (16), consistent with our finding of temperature sensitivity in the W303 background.

Suppression of gal4D by sug2 Alleles—To test the suppression of gal4D by sug2 alleles we transformed each strain with a single-copy, centromeric vector carrying the gal4D gene. As controls, each strain was also transformed with a multicopy, 2 μm plasmid that overexpresses gal4D and a centromeric plasmid expressing wild-type GAL4. In each case the expressed Gal4 protein was epitope-tagged with three tandem copies of the T7 epitope tag at its N terminus to facilitate immunologic detection. Because the chromosomal copy of GAL4 had been deleted in each of these strains, the epitope-tagged proteins expressed from the plasmids were the only source of Gal4 activity in these strains.

Cultures were grown to stationary phase in selective medium with raffinose as the carbon source. After the densities of the cultures were normalized, serial dilutions were performed and the diluted cells were spotted to selective plates with either glucose or galactose as the carbon source. Fig. 2A shows that each of the strains grew well on glucose medium regardless of which plasmid they contained. This is expected, because Gal4 is dispensable for growth on glucose. As expected, each of the strains could grow on galactose medium when transformed with the wild-type GAL4 plasmid or the plasmid overexpressing gal4D. However, only the sug2-1 strain could grow when transformed with gal4D on a single-copy plasmid. The finding that sug2-13 does not suppress gal4D demonstrates allele specificity in suppression of gal4D by SUG2 alleles.

Levels of gal4D in sug2 Mutant Strains—To directly test the hypothesis that defective protein degradation by proteasomes allows gal4D protein accumulation and that this is responsible for suppression of gal4D by sug2-1, we determined the levels of the gal4D and Gal4 proteins in each strain. Strains were grown to mid- to late-log phase and harvested quickly. Equal numbers of cells were boiled directly in 2× SDS loading buffer. The samples were centrifuged briefly to remove cell debris and then separated by SDS-PAGE. Western blots were performed with anti-T7 epitope antibodies for detection of gal4D and Gal4 proteins and with anti-cyclophilin antibodies as an internal
Multicopy vector alone (vector) formed with GAL4 epitope tag. Proteins were tagged at the N terminus with three copies of the T7 source, but the single-copy vector to promote growth on galactose as the sole carbon source. The glucose and galactose plates 10-fold dilutions were spotted onto selective plates with either glucose or galactose as the sole carbon source. The glucose and galactose plates were incubated at 30 °C for 2 or 3 days, respectively. B, the sug2-13 mutation leads to a large increase in Gal4 and gal4D protein levels, whereas the sug2-1 mutation has a smaller effect. Yeast strains were grown in liquid medium, harvested from log-phase cultures, and boiled directly in 2× SDS-PAGE loading buffer. The extracts were separated on a long SDS-polyacrylamide gel and analyzed by Western blotting. The Gal4 and gal4D proteins were detected with a monoclonal antibody against the T7 epitope tag. The blots were also probed with anti-cyclophilin antibodies to verify equivalent loading. The lower band seen on the cyclophilin blot is presumed to be a cyclophilin degradation intermediate and is more abundant in strains with mutations in proteasome subunits (see also Figs. 4B and 5B).

**Fig. 2.** Suppression of gal4D is specific for the sug2-1 allele and does not positively correlate with levels of gal4D protein. Congenic Gal4 strains containing different alleles of sug2 were transformed with gal4D on either a single-copy vector (4D) or a multicopy vector (mc 4D), with wild-type GAL4 on a single-copy vector (4), or with multicopy vector alone (vector or -). In each case the Gal4 and Gal4 proteins were tagged at the N terminus with three copies of the T7 epitope tag. A, the sug2-1 mutation allows gal4D expressed from a single-copy vector to promote growth on galactose as the sole carbon source, but the sug2-13 mutation does not. Wild-type Gal4 and overexpressed gal4D allow growth on galactose in all strain backgrounds. Yeast strains were grown in liquid culture to log phase, and serial 10-fold dilutions were spotted onto selective plates with either glucose or galactose as the sole carbon source. The glucose and galactose plates were incubated at 30 °C for 2 or 3 days, respectively. B, the sug2-13 mutation leads to a large increase in Gal4 and gal4D protein levels, whereas the sug2-1 mutation has a smaller effect. Yeast strains were grown in liquid medium, harvested from log-phase cultures, and boiled directly in 2× SDS-PAGE loading buffer. The extracts were separated on a long SDS-polyacrylamide gel and analyzed by Western blotting. The Gal4 and gal4D proteins were detected with a monoclonal antibody against the T7 epitope tag. The blots were also probed with anti-cyclophilin antibodies to verify equivalent loading. The lower band seen on the cyclophilin blot is presumed to be a cyclophilin degradation intermediate and is more abundant in strains with mutations in proteasome subunits (see also Figs. 4B and 5B).

Loading control. The levels of both the Gal4 and gal4D proteins were slightly increased compared with wild-type in the sug2-1 strain as shown in Fig. 2B. Surprisingly, the levels of Gal4 and gal4D were more dramatically increased in the sug2-13 strain. Similar results were obtained in three separate experiments. These data show that suppression of gal4D does not positively correlate with the levels of gal4D protein in the sug2 mutant strains. Of note, the dramatically increased levels of gal4D seen in the sug2-13 strain are still much lower (~20-fold) than those seen in strains carrying the plasmid overexpressing gal4D (Fig. 2B). As previously shown, overexpression of gal4D on a multicopy plasmid (~50-fold overexpression) conditions 60% of wild-type activation as assayed by β-galactosidase assays (8). The threshold for growth on plates is ~20% of wild-type activity. Therefore, assuming a linear response of activation to activator levels, the lack of complementation in the sug2-13 strain is not surprising. We conclude that stabilization of gal4D protein due to a defect in proteasomal proteolysis cannot explain suppression of gal4D by sug2-1.

**Temperature Sensitivity and Suppression of cdc68-1 by sug1 Mutant Alleles**—The sug1-1 and sug1-20 alleles of SUG1 have been previously characterized. As summarized in the introduction, sug1-1 suppresses the mutant phenotype of gal4D (1). The sug1-20 allele, in contrast, was identified as a suppressor of the transcription factor cdc68-1 (15). Both alleles have been found to confer temperature sensitivity at 37 °C. Consistent with this, we found that both the sug1-1 and sug1-20 alleles conferred temperature sensitivity in the W303 background (Fig. 3A). To test suppression of cdc68-1 by these sug1 alleles in the W303 background, a plasmid expressing cdc68-1 was transformed into each strain of the congenic set. The chromosomal CDC68 gene was then deleted, and the strains were characterized as described under “Experimental Procedures.” These strains were then tested for temperature sensitivity (Fig. 3B). Consistent with the findings of Xu et al. (15), the sug1-20 allele suppresses the temperature sensitivity of cdc68-1 at the intermediate temperature of 35 °C. In contrast, sug1-1 does not. Therefore, the allele specificity of cdc68-1 suppression noted by Xu et al. is reproduced in the W303 background.

Suppression of gal4D by sug1 Alleles—The ability of mutations in sug1 to suppress the transcriptional phenotype of gal4D was assayed in the same way as for sug2 alleles. Wild-type, sug1-1, and sug1-20 strains from which GAL4 had been deleted were transformed with a centromeric vector carrying the gal4D gene, a multicopy, 2μ plasmid that overexpresses gal4D, or a centromeric vector expressing wild-type GAL4. The Gal4 proteins produced were epitope-tagged with three tandem copies of the T7 epitope tag as previously described. The epitope-tagged proteins expressed from the plasmids were the only source of Gal4 activity in the cell. Spotting tests for growth on glucose and galactose were performed as described above. As shown in Fig. 4A, each strain was able to grow on glucose and galactose when either wild-type Gal4 was expressed at normal levels or gal4D was overexpressed. In contrast, gal4D was unable to support growth in the wild-type and sug1-20 backgrounds. Only the strain carrying the sug1-1 mutation was able to grow on galactose when gal4D expressed at wild-type levels was the activator. Therefore, there is allele specificity in the suppression of gal4D by sug1 alleles.

**Levels of gal4D in sug1 Mutant Strains**—If a defect in protein degradation by the proteasome is responsible for accumulation of gal4D and growth of strains expressing ga4D on galactose, we would expect gal4D levels to be higher in the sug1-1 background than the wild-type and sug1-20 strains. To test this hypothesis, the levels of the gal4D and Gal4 proteins in each strain were determined by Western blot as described above. The levels of both the Gal4 and gal4D proteins were elevated in the sug1-1 strain compared with wild-type strain (Fig. 4B). However, the level of gal4D protein was more dramatically elevated in the sug1-20 background. Similar results were obtained in three separate experiments. Therefore, suppression of gal4D does not positively correlate with the levels of gal4D in the sug1 mutant strains. Although the levels of gal4D are increased in the sug1-20 strain, they are not as high as in the gal4D-overexpressing strain. For the same reasons noted above regarding the sug2-13 strain, the lack of gal4D complementation in the sug1-20 strain is not surprising. We conclude that stabilization of gal4D due to a defect in proteasomal proteolysis cannot explain suppression of gal4D by sug1-1. This is entirely consistent with the results obtained for sug2-1 (Fig. 2).
above suggest that a change in the proteolytic function of the proteasome is not responsible for the suppression of gal4D by sug1-1 and sug2-2. Both Sug1 and Sug2 are components of the 19S regulatory complex of the proteasome. As an additional test of this hypothesis, we examined the levels of gal4D and growth on galactose in strains carrying mutations in subunits of the 20S proteasome. Strains carrying mutations in 20S proteasome subunits have been previously described. WCG4-11/22a (pre1-1 pre2-2 pre1-1 pre4-1) and yH129/14 (pre1-1 pre4-1) each have mutations in two 20S subunits (19, 20). They both have severe defects in protein degradation by the proteasome. We deleted the chromosomal copy of GAL4 from each of these strains and from the congenic wild-type strain WCG4a. We then examined the levels of Gal4 and gal4D proteins expressed from centromeric vectors in the strains produced. As shown in Fig. 5A the levels of both gal4D and Gal4 were significantly increased in both 20S mutant strains when compared with the wild-type. They were not, however, increased to the level of gal4D expressed from a multicopy (2μm) vector. These strains were also assayed for the ability to grow on galactose as the sole carbon source. As shown in Fig. 5B, all three strains were able to grow on glucose and galactose when transformed with a plasmid, all three strains were able to grow on galactose as the sole carbon source. Therefore, even though sug1-1 sug2-1 cannot be explained by alterations in proteasome-mediated proteolysis of gal4D protein.

**DISCUSSION**

The finding that Sug1 and Sug2 are components of the proteasome suggested a simple explanation for the suppression of gal4D by sug1-1 and sug2-2: A defect in degradation by the proteasome in sug mutant strains might lead to an accumulation of gal4D protein. Although gal4D lacks much of its activation domain, at high levels it might activate sufficient transcription to allow growth on galactose. This argument was given credence by the finding that high level overexpression of gal4D from a high-copy-number plasmid does partly rescue its growth on galactose phenotype. Likewise, sug1-20 stabilizes gal4D more than sug1-1, but only sug1-1 suppresses gal4D. These results imply that inefficient proteolysis is responsible for the suppression of gal4D by sug1-1 and sug2-1. The proteolytic target resistant for the phenotype. Reinforcing this conclusion, strains that have very severe defects in proteolysis due to mutations in 20S subunits are not able to grow on galactose when gal4D is the activator (Fig. 5B).

We propose two possible explanations for these data. The rescue of gal4D by sug1-1 and sug2-1 may be due to altered proteolysis of an as yet unidentified transcription factor, which enhances the weak activity of gal4D protein. This model would imply that this putative factor is stabilized by some mutations in SUG1 and SUG2, but not by others. Furthermore, this putative factor would have to be degraded normally in the 20S double mutant strain, even though they have a severe defect in general degradation of proteins by the proteasome. Though we feel that this explanation is unlikely, we cannot rule it out. Such a putative factor could be identified by transforming a gal4D strain with a multicopy library derived from a ΔGAL4 strain. Clones allowing growth on galactose could then be selected from the library. Overexpression of this putative factor should suppress gal4D just as its stabilization by sug1-1 or sug2-1 does.

An alternate explanation is that the 26S proteasome or its 19S regulatory subunit may be involved in the transcriptional process in a nonproteolytic fashion. It has long been speculated that proteasomal ATPases are responsible for unfolding substrate proteins so that they can be threaded into the proteolytic chamber of the proteasome (25–27). We have suggested, by analogy to activities of the structurally and functionally related CipX ATPase in *Escherichia coli* (28, 29), that the 19S ATPases might also have a “chaperone-like” role in rearranging protein complexes without degrading them (30). This proposal was prompted by our findings suggesting that proteasomal ATPases were important for nucleotide excision repair (NER) in yeast, but that the proteolytic activity of the proteasome was not (30). Specifically, we found that mutations in SUG1 resulted in sensitivity to ultraviolet light in *vivo* and that antibodies to Sug1 inhibited NER in *vitro*. Surprisingly, mutations in 20S proteasome subunits do not lead to sensitivity to ultraviolet light and the potent proteasome inhibitor lactacystin has no effect on NER in *vitro* (30). Consistent with a nonproteolytic role for proteasomal ATPases, the 19S regulatory complex of the proteasome has now been shown to possess classically defined chaperone activity using model substrates (31). Of note, the folding substrate was not degraded, even in the pres-
A, the were tagged at the N terminus with three copies of the T7 epitope tag. In each case the gal4D and Gal4 proteins were tagged at the N terminus with three copies of the T7 epitope tag. A, the sug1-1 mutation allows gal4D expressed from a single-copy plasmid to promote growth on galactose as the sole carbon source, but the sug1-20 mutation does not. Wild-type Gal4 and overexpressed gal4D allow growth on galactose in all strain backgrounds. Yeast strains were grown in liquid culture to log phase, and serial 10-fold dilutions were spotted onto selective plates with either glucose or galactose as the sole carbon source. The glucose and galactose plates were incubated at 30 °C for 2 or 3 days, respectively. B, the sug1-20 mutation leads to a larger increase in Gal4 and gal4D protein levels than the sug1-1 mutation. Cell extracts were prepared and assayed for epitope-tagged Gal4 and gal4D proteins and for cyclophilin protein as a loading control, as described in Fig. 2.

Consistent with a nonproteolytic role for the proteasome in transcription, antibodies to Sug1 inhibit yeast in vitro transcription, although control antibodies do not. In this same in vitro transcription system, complete inhibition of proteasome peptidase activity by lactacystin has no effect on transcription. These findings are consistent with a role for Sug1 in transcription that is not dependent on alterations in the proteolytic activity of the proteasome.

In contrast to our results, Molinari et al. (32) have reported a different connection between the proteasome and transcriptional activators. They have shown that, at least for chimeric activators in mammalian cells, impairment of transcription through mutations of DNA-binding or activation domains leads to an increase in steady-state levels of the activator. The implication is that transcriptionally engaged activators are preferentially degraded by the proteasome. In contrast, we show that levels of the crippled gal4D activator are less than those of wild-type Gal4. Although production of more gal4D leads to more transcriptional activity, selected mutations in SUG1 and SUG2 can rescue gal4D in a way that cannot be explained by a simple increase in activator levels. The implication of our results is that components of the 19S regulatory complex may have nonproteolytic roles in transcription. We feel our results and those of Molinari et al. are probably manifestations of the proteasome having more than one role in transcription. In this regard, we have recently found that the 19S subcomplex of the proteasome functions in transcription elongation in yeast independent of its proteolytic activity.
Another conclusion from the results we report here is that mutations in the SUG1 and SUG2 genes can produce very distinctive phenotypes. It had previously been reported that the sug1-3/cim3 allele did not suppress the gal4D transcription phenotype (1), although it does suppress the cell cycle defect of cdc28N-1 (22). Nor did the sug1-1 allele suppress the cdc68-1 defect (which is suppressed by sug1-20) even though CDC68, like GAL4, is a transcription factor (15). We now report that neither the sug1-20 nor the sug2-13 allele can suppress the gal4D phenotype. It appears that each SUG1 and SUG2 allele selected has very specific phenotypes. This allele specificity argues against a common pathway, such as proteolysis, as an explanation for all of the identified phenotypes. It further implies that the 19S regulatory complex may have complex regulatory functions beyond regulating the time and manner of protein degradation by the 20S proteasome.

Clearly, more work is required to define the function of the proteasome in transcription and DNA repair. The work presented here gives strong evidence that alterations in proteolysis cannot explain the transcription phenotypes of sug1-1 and sug2-1. As such, it clears the way for focusing on the exploration of alternative models and provides a second example of the nonproteolytic role of the proteasome, further expanding the functional repertoire of this complex protein machine.

Acknowledgments—We thank James Haber and John McCusker for bringing the identity of SUG2 with CRL13 to our attention and for supplying yeast strains; Kathryn Sykes for anti-cyclophilin antiserum; Gerry Johnston for plasmids and strains; Dieter Wolf, Wolfgang Hilt, and Wolfgang Heinemeyer for strains; and Xiang Chen and Eunice Webb for invaluable technical assistance. We thank the Johnston Laboratory for helpful discussions.

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J. Biol. Chem. 2001, 276:9825-9831.
doi: 10.1074/jbc.M010889200 originally published online January 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010889200

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