Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8236–8240, August 1996
Biochemistry

Mammalian Sug1 and c-Fos in the nuclear 26S proteasome

(transcription/leucine zipper)

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Contributed by Daniel Nathans, May 6, 1996

ABSTRACT **In a search for regulatory proteins that interact with the leucine zipper motif of c-Fos in the yeast two-hybrid screen, we have identified a protein (FZA-B) that has extensive sequence similarity to SUG1 of *Saccharomyces cerevisiae*. Here we show that FZA-B can functionally substitute for SUG1 in yeast and that FZA-B interacts with Fos proteins *in vitro* through their leucine zippers. In rat liver and in HeLa cells, FZA-B is present in the 26S proteasome complex, as is c-Fos. Immobilized antibody raised against an FZA-B-specific peptide depleted peptidase activity, proteasomal proteins, FZA-B, and c-Fos from a 26S proteasome preparation. FZA-B is found predominantly in the nuclear fraction of COS cells expressing an FZA-B transgene and in the nuclear 26S proteasome of HeLa cells. We conclude that FZA-B is the mammalian homolog of SUG1 (mSug1) and that it is present in the nuclear 26S proteasome of cells. Our results suggest that mSug1 may be involved in the degradation of c-Fos and other transcription factors.**

The regulation of cellular transcription factors, including biosynthesis, activity, and degradation, occurs at several levels. A number of regulatory steps involve specific interactions of the transcription factor with other proteins. For this reason, in studying the regulation of the activities of the AP-1 family of basic region–leucine zipper (bZIP) transcription factors, we and others set out to identify proteins that interact with the AP1 components Jun and Fos (1, 2). Among the proteins that interact with the leucine zipper of c-Fos, we identified one (named FZA-B) that is closely related in amino acid sequence to SUG1 of *Saccharomyces cerevisiae* (3, 4) and is the subject of this report.

SUG1 was originally identified as a suppressor (in mutant form) of a mutant allele of the transcription factor GAL4 (4). Later SUG1 was found to be a component of a multiprotein mediator of transcription factor-dependent transcription by RNA polymerase II (pol II) holoenzyme from *S. cerevisiae* (5). For this reason, we began our analysis of FZA-B with the expectation that FZA-B and c-Fos were components of a multiprotein mammalian transcription mediator that interacted with an RNA pol II complex to effect Fos-dependent transcription. In searching for such a complex in mammalian cells, we found that FZA-B is a component of the nuclear 26S proteasome. Similar findings regarding yeast SUG1 were recently reported (6).

MATERIALS AND METHODS

Plasmids. Plasmids were constructed and used in the two-hybrid yeast assay as described (1, 7). For complementation experiments, FZA-B and FZA-C (7) were cloned as Gal4 fusions in pPC62 (1). Mammalian plasmids expressing FZA-B were constructed by cloning FZA-B into the vector pYN3215, which was provided by Y. Nakabeppu (Kyushu University, Fukuoka, Japan).

Preparation of Extracts. For preparation of liver whole cell lysate, the livers of decapitated rats were rinsed in buffer A (20 mM Hepes-potassium, pH 7.6/10 mM KCl/2 mM MgCl/1 mM EDTA/10% glycerol/1 mM DTT) plus 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml chymostatin, 1 μg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; Sigma), and 10 μg/ml pepstatin, and minced and suspended in buffer A containing 100 mM KCl (buffer B). The tissues were then homogenized with a Dounce homogenizer and gentle sonication and spun twice at 28,000 rpm for 1 hr. The clear supernatant was removed and stored at −80°C. Preparation of HeLa cytoplasmic and nuclear extracts was performed essentially as described (8).

***In Vitro* Binding Assays.** Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* transformed with pGEX plasmid constructs (Pharmacia), solubilized in urea, and purified (9). About 2 μg of GST or GST fusion protein was used to perform binding assays as described (10). ^{35}S-labeled proteins were *in vitro* translation products.

Gel Filtration. A Sephacryl S-400 column (2.5 × 100 cm) was equlibriated with buffer B plus protease inhibitors and loaded with 1 ml of lysate. Fractions of 6.3 ml were collected at a flow rate of 0.4 ml/min and analyzed. For small scale analysis, lysates were loaded onto a fast protein liquid chromatography Superose 6 gel filtration column equilibriated with buffer B plus phenylmethylsulfonyl fluoride at a flow rate of 0.2 ml/min, and 0.5 ml fractions were collected and analyzed. Proteasomal activities were assayed using the fluorogenic substrate Suc-LLVY-AMC (7-amido-4-methylcoumarin) (Bachem) as described (11).

Immunological Analysis. Antiserum against the 26S proteasome was generously provided by M. Rechsteiner (University of Utah, Salt Lake City). Anti-FZA-B antibody was generated against full-length FZA-B fused to GST. Affinity purified anti-peptide antibody was generated against the C-terminal peptide of FZA-B containing residues 390 to 406 of FZA-B (see Fig. 1) (with one additional cysteine residue at its N terminus) coupled to keyhole limpet hemocyanin. Affinity-purified antibody against a His–FZA-B fusion polypeptide containing residues 1 to 41 of FZA-B was also prepared. For affinity purification of proteasomes, anti-peptide antibody columns were prepared by coupling the purified peptide antibodies to agarose beads using a CarboLink kit (Pierce). The affinity matrices were washed with 0.1 M glycine·HCl (pH 2.8) and equilibriated with buffer B. S-400 fractions from cell lysates were pooled and applied to the antibody columns. The columns were then extensively washed and bound proteins eluted with glycine·HCl. Immunoprecipitation experiments were performed essentially as described (12). Western blot

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Abbreviations: GST, glutathione *S*-transferase; bZIP, basic region–leucine zipper motif; pol II, polymerase II; m, mammalian.

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analyses were performed and developed using an Enhanced Chemiluminescence kit (Amersham).

RESULTS

Identification of a Protein That Interacts with c-Fos as the Murine Homolog of SUG1. Several polypeptides that interacted with the bZIP of c-Fos were detected in a yeast two-hybrid screen of a mouse embryo cDNA library (7). Partial sequencing of the isolates indicated that most of them encode bZIP proteins known to dimerize with c-Fos or other proteins that form coiled-coil structures. The amino acid sequence of one isolate (named FZA-B) derived from full-length cDNA is closely related to a large, highly conserved ATPase family (13). Fig. 1 shows the predicted amino acid sequence of FZA-B and its sequence comparison with two members of this gene family, *S. cerevisiae* SUG1 (3, 4) and the related *Dictyostelium* protein TBP10 (14). FZA-B has 74% amino acid sequence identity to SUG1 and 77% sequence identity to dTBP10. FZA-B also has significant similarity to HIV Tat-binding proteins TBP1 (ref. 15, 41% identity), TBP7 (ref. 16, 38% identity), and MSS1 (ref. 17, 44% identity). FZA-B is nearly identical to the recently described proteins hTrip1 and human p45 (18, 19). The segment of FZA-B between amino acid residues 45 and 80 is predicted to form coiled-coil structures with high probability (20). This region is necessary and sufficient for the interaction with the bZIP region of c-Fos in the two-hybrid assay (data not shown).

To determine whether FZA-B interacts with c-Fos *in vitro*, immobilized GST–FZA-B fusion protein prepared in *E. coli* was used to bind c-Fos *in vitro* translation product. c-Fos bound to GST–FZA-B specifically with little or no specific binding to FZA-B containing either a partial deletion or leucine to proline changes in the predicted leucine zipper sequence; other members of the Fos family tested (Fos B and Fra-1) also bound to FZA-B, whereas c-Jun showed much weaker binding (Fig. 2 and data not shown). We conclude that FZA-B and c-Fos proteins interact via their leucine zippers *in vitro* as they do in the two-hybrid system.

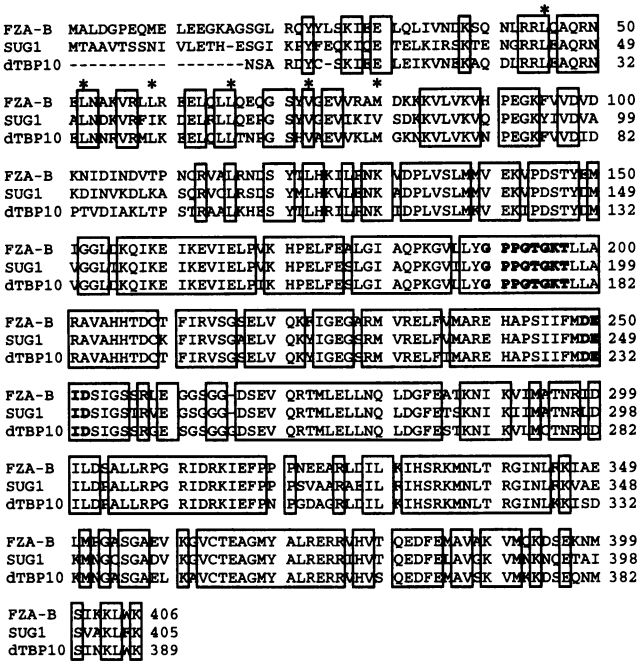

FIG. 1. Amino acid sequence of FZA-B and its alignment with *S. cerevisiae* SUG1 and *Dictyostelium* TBP10 (7). Identical amino acid residues are in boxes. Asterisks indicate heptad repeats of an amphipathic helix predicted to form a coiled-coil structure. Amino acid residues of a putative nucleotide binding site and ATPase motif are indicated in boldface type.

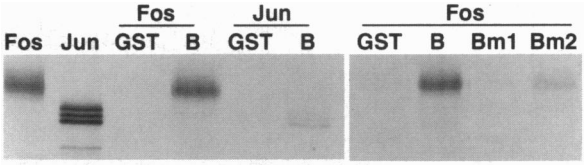

FIG. 2. Binding of FZA-B to c-Fos *in vitro*. *In vitro* translated and radiolabeled c-Fos or c-Jun was incubated with GST, GST-FZA-B (B), or GST-FZA-B mutants (Bm1, containing a deletion from residue 47 to residue 64; or Bm2, containing the point mutation L66P). Samples were then treated with glutathione-agarose beads, and the retained protein was analysed by gel electrophoresis. Input lanes represent 5% of total input in the binding reactions.

Sug1 Function of FZA-B in *S. cerevisiae*. To determine whether FZA-B has SUG1 function in yeast, we transformed a diploid strain of *S. cerevisiae* heterozygous for *sug1* [W303 ΔSug1 (*sug1*$^+$/*sug::ura3*)] (4) with a plasmid encoding full-length FZA-B fused to the Gal4 DNA-binding domain, as used in the two-hybrid assay, and scored the viability of haploid progeny of single spores (Fig. 3). The Gal4/FZA-B plasmid, but not a Gal4/FZA-C plasmid (a control plasmid), rescued the defective progeny, as indicated by the presence of three or four haploid colonies in the tetrad analysis. We conclude that FZA-B is the murine homolog of SUG1. Similar results with human Trip1 have been reported (18).

Cofractionation of FZA-B and c-Fos with the 26S Proteasome. Yeast SUG1 is a component of a multiprotein mediator of specific transcription (5). We wished to determine whether FZA-B is in a similar macromolecular complex in mammalian cells and, if so, whether c-Fos is associated with the complex. For this purpose, a soluble extract of rat liver was size-fractionated by gel filtration through Sephacryl S-400, and fractions were examined by electrophoresis and Western blot analysis for the presence of FZA-B using a polyclonal anti-FZA-B serum (Fig. 4). In the high molecular mass region of the eluate, antigen corresponding in electrophoretic mobility to ≈50 kDa was predominantly detected in fractions eluting before a 669 kDa standard. These fractions also contained 26S and 20S proteasomes, as determined both by activity assay and using anti-26S proteasome serum (21). FZA-B was also detected with two purified antibodies against either N-terminal or C-terminal peptides specific for FZA-B to probe the chromatographic fractions (data not shown). Therefore, the signals were not due only to the presence of related conserved ATPase domain-containing (CAD) proteins that crossreact with the anti-FZA-B serum. (Neither the N-terminal nor C-terminal peptides of FZA-B is present in other known proteasomal CAD proteins.) Anti-c-Fos antiserum detected a protein corresponding to ≈65 kDa that coeluted with the >669 K_d complex containing FZA-B antigen (Fig. 4). Similar results were obtained with an extract of HeLa cells (data not shown).

One interpretation of the above results is that FZA-B is a component of proteasomes; another interpretation is that FZA-B is a component of an unrelated complex of similar size, perhaps a mammalian mediator of transcription analagous to

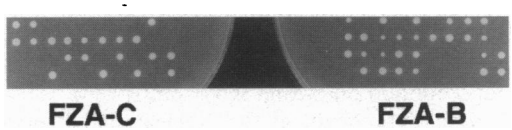

FIG. 3. Complementation of a lethal disruption of *Sug1* in yeast by FZA-B. In each vertical row are colonies derived from a single spore after transformation of *S. cerevisiae* W303 ΔSug1 (*sug1*$^+$/*sug::ura3*) (4) with a plasmid expressing either Gal4(DB)/FZA-B (FZA-B) or a control plasmid expressing GAL4(DB)/FZA-C (FZA-C), an unrelated Fos-interacting protein (7).

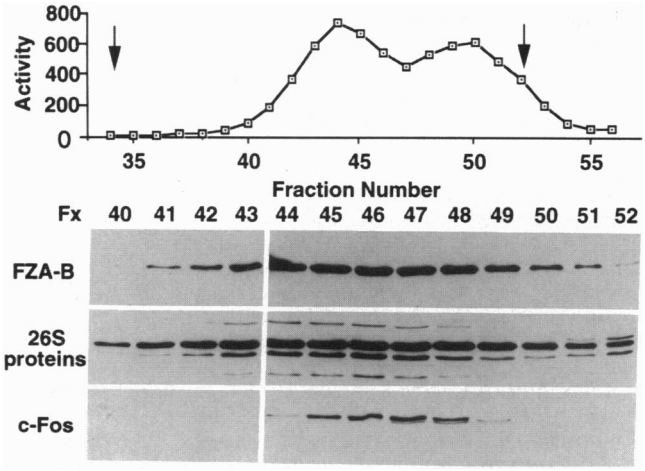

FIG. 4. Cofractionation of FZA-B and c-Fos with 26S proteasomes. Rat liver lysate was fractionated on a Sephacryl S-400 column, and fractions were collected and assayed for proteasome activity and immunologically detected FZA-B, c-Fos, and 26S proteasomal proteins. Arrows denote the elution positions of size markers: (*Left*) blue dextran (2×10^3 kDa) and (*Right*) thyroglobulin (669 kDa). Proteasomal activity is indicated as relative fluorescence units of the hydrolyzed substrate.

the yeast mediator. We carried out several different experiments to test the first possibility. Coelution of FZA-B and proteasomes was also observed upon fractionating the complex containing FZA-B on fast Q, hydroxyapatite, or heparin chromatogaphy (data not shown). When we assayed fractions eluting before 440 kDa for hydrolysis of a fluorescent peptide substrate, FZA-B was seen mainly in the fractions with the 26S proteasome activity, although some was also detected in the 20S peak, probably as a dissociated form (22–24) (Fig. 4). This inference is supported by the effect of ATP on the fractionation of the FZA-B-containing complex. In the presence of ATP, which is known to promote the association of 20S and 19S proteasomal subunits (25), the FZA-B peak shifted to coincide more precisely with that of the 26S proteasomal activity (data not shown).

To demonstrate more convincingly that FZA-B is a component of the 26S proteasome complex, the high molecular mass fractions containing proteasomal activity were adsorbed to a column containing covalently linked antibody against the C-terminal segment of FZA-B. The column was extensively washed and the retained proteins were eluted with acidic solution, separated by SDS/PAGE, and subjected to Western blot analysis. As shown in Fig. 5, together with FZA-B, the 26S proteasome subunits and c-Fos are largely retained by the column. We also determined whether the large subunit of RNA pol II was present in the affinity purified fraction; as shown in Fig. 5, some RNA pol II large subunit (RPB1) was also retained by the column. As a control, similar samples were passed through a column containing covalently linked antibody against an N-terminal peptide of FZA-B. In this case, FZA-B, 26S proteasome, c-Fos, and RNA pol II were all seen almost exclusively in the flowthrough fractions (data not shown). These results indicate that FZA-B is present in most, if not all, proteasomes, and that the C-terminal segment of FZA-B is accessible to antibody. At least a fraction of the proteasomes contain c-Fos and RNA pol II.

FZA-B as a Component of the Nuclear 26S Proteasome. Association of proteasomal FZA-B with c-Fos and RNA pol II raised the possibility that FZA-B may play a role in the regulation of transcription via proteasomal degradation of transcription factors and RNA polymerase. If that is the case, FZA-B is likely to be predominantly in the nucleus. To determine the subcellular localization of FZA-B, we transfected plasmids expressing FZA-B or a deletion form of FZA-B (ΔFZA-B) into COS cells, and cytoplasmic and nuclear proteins were analyzed by Western blotting for the presence of FZA-B antigen. Fig. 6*A* shows that FZA-B is almost exclusively in the nuclear fraction of COS cells whether or not they are expressing the transgene. We then examined the subcellular distribution of FZA-B-containing proteasomes. For this purpose, HeLa nuclear and cytoplasmic extracts were size-fractionated on a Superose 6 column, and fractions having proteasomal activity were subjected to Western blot analysis with anti-FZA-B antiserum. The cytoplasmic fractions contained very little FZA-B relative to hydrolytic activity of the

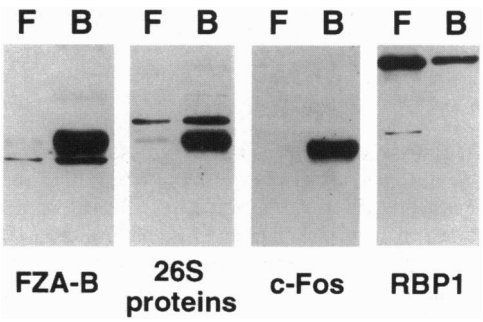

FIG. 5. Retention of FZA-B, 26S proteasomal proteins, c-Fos, and RNA pol II by immobilized antibody against a C-terminal peptide of FZA-B. S-400 fractions containing FZA-B from a rat liver lysate were pooled and loaded onto a column containing antibody against the C-terminal section of FZA-B. The column was then washed and retained proteins eluted. The proteins in the flowthrough (F) and bound and eluted fractions (B) were trichloroacetic acid-precipitated and subjected to electrophoresis, and immunoblotted for FZA-B, 26S proteasomal proteins, c-Fos, and RNA pol II large subunit.

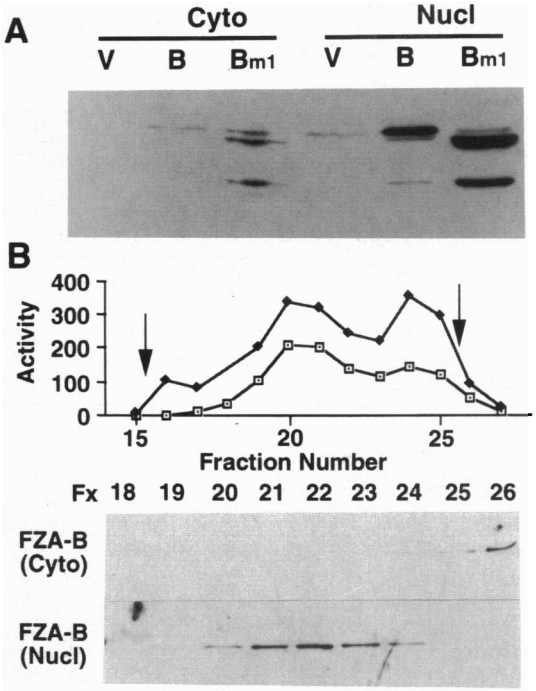

FIG. 6. Presence of FZA-B in nuclear versus cytoplasmic proteasomes. (*A*) Plasmids expressing FZA-B (B) and an FZA-B deletion mutant (Bm1 as in Fig. 2) were transfected into COS cells; cytoplasmic and nuclear proteins were analyzed for the presence of FZA-B (V, vector alone). (*B*) HeLa nuclear and cytoplasmic extracts were size-fractionated on a Superose 6 column, and fractions were assayed for proteasomal activity (solid symbols, cytoplasm; open symbols, nucleus) and the presence of FZA-B. Arrows and activity are as in Fig. 4.

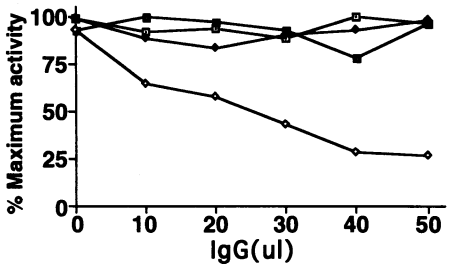

FIG. 7. Depletion of 26S proteasomal activity with antibody against the C-terminal section of FZA-B. HeLa nuclear lysate was fractionated on a Sephacryl S-400 column, and fractions corresponding to 26S or 20S proteasome activities were incubated with increasing concentrations of preimmune IgG (■, 26S; □, 20S) or affinity purified C terminal peptide antibodies (◇, 26S; ◆, 20S). Immune complexes were pelleted, and hydrolytic activity in the supernatant was determined. The results are expressed as percent of maximal hydrolytic activity left in the supernatant after immunodepletion.

proteasome compared with the nuclear fraction (Fig. 6*B*). Stable association of FZA-B with the nuclear 26S proteasome was further demonstrated by immunoprecipitation experiments using antibody against the C-terminal peptide of FZA-B to deplete the hydrolytic activity from the fractions containing 26S proteasome (Fig. 7). For this purpose, fractions from the heavy side of the 26S activity peak were used. Depletion of 26S proteasome hydrolytic activity was dependent on the concentration of antibody. As seen in Fig. 7, hydrolytic activity of the 20S subunit (taken from the light side of the 20S peak) was not affected by the anti-peptide antibody, and preimmune IgG was inactive with either the 26S or the 20S fractions. We conclude that FZA-B is an integral component of the nuclear 26S proteasome.

DISCUSSION

In this report, we describe the identification by means of the yeast two-hybrid screen (1, 26) of a highly conserved murine homolog of *S. cerevisiae* SUG1 that interacts with the bZIP segment of c-Fos. The interaction occurs via a predicted leucine zipper of mammalian Sug1 (mSug1), presumably resulting in the formation of a coiled-coil interface between the two proteins. Other Fos family members also interact, but Jun shows only a weak interaction. Earlier reports describe the identification of murine or human Sug1 based on its interaction with transcription factors of the nuclear receptor superfamily (18, 27). Mammalian Sug1 is thus capable of interacting with transcription factors of diverse structure.

Based on initial genetic analysis, it appeared that SUG1 played a role in regulating the activity of yeast transcription factor GAL4 (4). This inference was strengthened by the finding that SUG1 is a component of a multiprotein mediator of specific transcription by an RNA pol II holoenzyme derived from *S. cerevisiae* (5). Our identification of a mammalian homolog of SUG1 that interacts with c-Fos (28, 29) suggested the possibility that Sug1 is a connecting link between transcription factors and an RNA pol II complex. In testing this possibility, we have found that mSug1 is an integral component of the nuclear 26S proteasome. The critical evidence for this conclusion is: (*i*) that antibody to a C-terminal, Sug1-specific peptide depleted most of the proteasomal activity from a 26S nuclear proteasome preparation; (*ii*) that the immobilized antibody retained much of the proteasomal protein reacting with anti-26S antiserum (as well as proteasome-associated c-Fos); and (*iii*) that mSug1 is a nuclear protein and is present in nuclear proteasomes, but not in cytoplasmic proteasomes of HeLa cells (or present to a much lesser extent relative to hydrolytic activity). Our findings thus extend the recent report that SUG1 in yeast is in proteasomes (6).

c-Fos and several other transcription factors have been shown to be degraded through the proteasomal pathway (30–35). The interaction of c-Fos and mSug1 described in this report and interactions of mSug1 with other transcription factors reported by others (4, 18, 27) suggest that these transcription factors might be recruited by Sug1 to the 26S proteasome for degradation. Thus, Sug1 may play a role in the regulation of specific transcription by controlling the rate of degradation of transcription factors, perhaps coupled to the initiation of transcription. It is also possible that Sug1 has two independent functions: degradation of transcription factors via nuclear proteasomes and regulation of their activities via a mediator complex.

Finally, we note that Sug1 is a member of a family of ATPases present in proteasomes (13, 21, 36–39). The functional differences between the proteasomal ATPases are not understood. However, at least in the case of Sug1, our results indicate that this ATPase is present in all or nearly all nuclear proteasomes, excluding the possibility that a given proteasome has only one member of the ATPase family.

We thank M. Rechsteiner for anti-proteasome serum, J. C. Swaffield for *S. cerevisiae* strains, and Laura Sanders for technical assistance. This research was supported in part by the National Cancer Institute Grant PO1 CA16519. P.M.C. was a trainee of the Medical Scientist Training Program (GM-07309).

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