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ARTICLE *in* JOURNAL OF NEUROBIOLOGY · JANUARY 1998

Impact Factor: 3.84 · DOI: 10.1002/(SICI)1097-4695(199712)33:7<877::AID-NEU2>3.0.CO;2-5 · Source: PubMed

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Identification of a Phylogenetically Conserved Sug1 CAD Family Member that is Differentially Expressed in the Mouse Nervous System

Danhui Sun,[1,*] Jonathan C. Swaffield,[3,] Stephen Albert Johnston,[3] Carolanne E. Milligan,[2,***] R. Thomas Zoeller,[1,2] Lawrence M. Schwartz[1,2]**

[1] Molecular and Cellular Biology Program, University of Massachusetts, Amherst, Massachusetts 01003

[2] Department of Biology, University of Massachusetts, Amherst, Massachusetts 01003

[3] Department of Internal Medicine and Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75325

Received 14 March 1997; accepted 10 July 1997

ABSTRACT: **We have isolated a cDNA clone from mouse, m56, that encodes a member of the Conserved ATPase-containing Domain (CAD) protein family. Sequence analysis revealed that m56 is identical to mouse mSug1/FZA-B and shares high homology with human Trip1, moth 18–56, and yeast Sug1. When examined, Sug1-like CAD proteins appear to function in the regulation of the 26S proteasome, as well as associate with members of the steroid/thyroid receptor superfamily and other transcriptional activators. m56 can complement the lethal phenotype of loss of *SUG1* in yeast. We have examined the tissue distribution of m56 using Northern and Western blots, in addition to immunocytochemistry and *in situ* hybridization. While m56 was expressed in all tissues and cells examined, several classes of neurons, most notably in the hippocampus, olfactory bulb, and cerebellum, displayed elevated levels of m56 mRNA and protein. We also examined distribution of RNA polymerase II and 26S proteasome subunit 4 (S4) within the mouse brain by *in situ* hybridization. While all three genes had similar patterns of expression, there were significant differences among them. In moths, the expression of the Sug1 homolog 18–56 is dramatically up-regulated during programmed cell death. In addition, it has been previously demonstrated that the proteasome plays an essential role in the regulation of apoptosis in mammals. We examined the expression of m56 in mouse during natural and induced cell death in a variety of tissues and found no significant changes in expression. Taken together, the data presented here suggest that while m56 is a highly conserved gene that presumably plays essential but complex roles in basal and developmental processes, it may not represent a rate-limiting step in these processes.** © 1997 John Wiley & Sons, Inc. *J Neurobiol* 33: 877–890, 1997

Keywords: **CAD proteins; proteasome; ubiquitin; hippocampus; apoptosis; programmed cell death; *in situ* hybridization; RNA polymerase II; Sug1**

INTRODUCTION

The observation that some proteins are highly conserved throughout evolution suggests that they play essential roles in cellular development and homeostasis. One such group of evolutionarily conserved proteins has been designated the Conserved AT-

* *Present address:* Building 10, Rm. 1B43, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892

** *Present address:* Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614

*** *Present address:* Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157

Correspondence to: L. Schwartz

Contract grant sponsor: NIH

 CCC 0022-3034/97/070877-14

Pase-containing Domain (CAD) family (Dubiel et al., 1992; Peters et al., 1992; Ohana et al., 1993; Swaffield et al., 1995) or AAA family (Confalonieri and Duguet, 1995). These molecules appear to be essential for a variety of basic cellular functions such as secretion (Söllner et al., 1993), peroxisomal localization (Erdmann et al., 1991), protein degradation (Dubiel et al., 1993; Ghislain et al., 1993; Gordon et al., 1993) and gene transcription (Kim et al., 1994; Lee et al., 1995; Swaffield et al., 1995).

There has been controversy in the literature regarding the role(s) assumed by Sug1-like CAD family members. The best characterized function of these proteins is to serve as components of the 26S proteasome, a large ATP-dependent multisubunit proteinase that performs the bulk of targeted protein degradation in cells (Dubiel et al., 1993; DeMartino et al., 1994; Deveraux et al., 1994; Rock et al., 1994; Akiyama et al., 1995; Rubin et al., 1996; Swaffield et al., 1996; Russell et al., 1997). Several lines of data have also suggested that some members of the Sug1-like CAD family, most notably Sug1 from yeast, appear to participate in gene transcription (Kim et al., 1994; Lee et al., 1995; Swaffield et al., 1995). This view has been reappraised and recent reports provided convincing data that Sug1 in yeast localizes to the 26S proteasome and not the transcriptional complex as originally thought (Rubin et al., 1996; Swaffield et al., 1996). Consequently, the best characterized function for this class of CAD proteins is to function as 26S proteasomal subunits. The 26S proteasome is a symmetrical elongated complex that is involved in many cellular processes such as antigen presentation (Rock et al., 1994), degradation of oncoproteins and transcription regulators (Ciechanover, 1994), and cell cycle control (Glotzer et al., 1991).

Recently, homologs of Sug1 have been cloned from several taxa, including Trip1 from human (Lee et al., 1995), D56 from fruitfly (Cheng and Schwartz, unpublished) and 18–56 from moth (Sun et al., 1996). 18–56 was isolated using a cloning paradigm designed to identify genes that are upregulated during the programmed cell death (PCD) of muscle. PCD has been widely observed during embryonic and postembryonic development (reviewed in Oppenheim, 1991; Milligan and Schwartz, 1996), and serves a variety of essential functions, including eliminating deleterious cells, adjusting the size of interacting populations of cells, sculpting the body form, and removing obsolete tissues.

In this study, we report the isolation of the mouse Sug1/18–56/Trip1 homolog, m56, and show that it, too, is a structural and functional homolog of Sug1. Expression of m56 appears to be unchanged during PCD in mouse. Instead, we found that while m56 is present in all cells examined, it is preferentially expressed in cells that are metabolically active and display developmental plasticity, such as the hippocampus, olfactory bulb, and cerebellum. Interestingly, despite the fact that there is no apparent regulation of m56 during PCD in mouse, it has recently been demonstrated that the proteasome plays an essential regulatory role in cell death in mammalian cells (Grimm et al., 1996; Sadoul et al., 1996). Thus, while the proteasome is essential for cell death, it does not represent a rate-limiting factor in this process.

As this report was being prepared for publication, two groups reported the isolation of a mouse Sug1 homolog, *mSUG1* (Baur et al., 1996) and FZA-B (Wang et al., 1996), using yeast two-hybrid screens to identify proteins that can interact with nuclear receptors and c-*fos*.

MATERIALS AND METHODS

Cloning m56

Two degenerate primers, DEG1 5′-TA(C,T)GT(A,T,C,G)GG(A,T,C,G)GA(A,G)GT-3′ and DEG2 5′-(C,T)TT(A,T,G,C)C(T,G)(A,T,C,G)A(A,G)(A,G)TT(T,G,A)AT-3′, were designed based on the amino acid sequence ''YVGEV'' and ''LNLRK,'' which are conserved in both yeast Sug1 and moth 18–56 (Fig. 1). Total RNA isolated from phorbol ester (PMA 10 n*M*) treated T-cell hybrid DO11.10 cells (Haskins et al., 1983) and reverse-transcriptase polymerase chain reaction (RT/PCR) was performed using GeneAmp RNA PCR Kit (Perkin Elmer). The anticipated 0.8-kb fragment was subcloned into pBluescript (Stratagene) and sequenced with Sequenase (US Biochemicals) using the dideoxy method of Sanger et al. (1977) to verify that a true homolog had been identified. To obtain a full length clone of m56, we used the 0.8-kb fragment to screen a mouse cDNA library generated from RNA of dying thymocytes (Liu et al., 1994). Hybridizing recombinants were plaque purified and the cDNA clones recovered within pBluescript vector by *in vivo* excision. The full-length clone was sequenced as above.

Complementation of *SUG1* in Yeast

The yeast strain W303a_SUG1::URA3 carrying a single copy of the *SUG1* gene under the control of the Gal1/10 promoter on the plasmid pMTL was transformed with yeast plasmid pYEP112, pYEP112 expressing m56 gene under the control of yeast ADH1 promoter, pYEP112

expressing Sug1 under the control of the ADH promoter. Transformants were patched onto galactose and glucose plates.

Examining the Expression of m56 in Mouse Organs

RNA Isolation and Northern Blots. Adult Balb/C mice were decapitated and the brain, heart, kidney, lung, liver, thymus, testis, spleen, and skeletal muscle were rapidly dissected free of other tissues and frozen in liquid nitrogen. Total RNA was isolated using RNAzol B (Biotecx Laboratories). Northern blots were performed with 15 μg of total RNA denatured in formaldehyde and separated in a 1.5% agarose gel (Fourney et al., 1988). RNA was transferred to Zeta-Probe membrane (BioRad) and hybridized at high stringency to ^{32}P-labeled cDNA clones.

Protein Expression. We took advantage of the existing anti-*Manduca* 18–56 antisera (Sun et al., 1996) to affinity purify antibodies that recognize m56. The m56 cDNA was cloned into the pET-25b(+) bacterial protein expression vector (Novagen), expressed in *Escherichia coli* and the m56 protein partially purified by column chromatography according to the manufacturer's protocol. The bacterial expressed m56 was then fractionated in an 8% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) (Laemmli, 1970), transferred to Immobilon membrane (Millipore), and then incubated with anti–18–56 antiserum. The membrane was washed extensively and the antibody was dissociated from the bound protein by using a low pH buffer. Preimmune serum was used as a control.

Western Blots. Tissues from adult Balb/C mouse (brain, heart, kidney, lung, liver, thymus, spleen, and muscle) were homogenized in loading buffer and boiled for 5 min, and 10 μg of protein was fractionated in 12% SDS-PAGE. Proteins were transferred to Immobilon membrane (Millipore) in a semidry blotter using Towbin buffer (Towbin et al., 1979). Blots were reacted with a 1:200 dilution of the affinity purified rabbit anti-m56 antibody and detected with a horseradish peroxidase–labeled goat anti-rabbit antibody. The ECL Western blotting detection system (Amersham) and X-ray film (Konica Medical Corp.) were employed for signal detection. Preimmune serum was used as a control.

Immunocytochemistry. Adult Balb/C mice were perfused with FAA fixative (70% EtOH, 5% acetic acid, and 2% formaldehyde). The organs were dissected out and postfixed overnight in the same fixative. The tissues were incubated in 10% and then 20% sucrose in phosphate-buffered saline (PBS). These tissues were then frozen in embedding medium on dry ice, sectioned at 12 μm in a cryostat, and reacted with the affinity-purified anti-m56 antibody in a blocking solution (4% nonfat dry milk and 0.3% Triton X-100 in PBS). After being washed in PBS, the signal was detected by a Biostain Super ABC Kit (Biomeda), using diaminobenzidine as the chromogen. Tissue sections were then dehydrated and coverslipped with mounting media. Preimmune serum was used as a control.

In Situ *Hybridization.* Tissues were dissected from adult Balb/C mice flash-frozen on dry ice and then sectioned at 12 μm in a cryostat. Sections were thaw-mounted onto cold microscope slides previously treated with two coats of a gelatin/chromalum solution, then briefly dried on a warm plate at 37°C. Tissue sections were kept at −80°C until use. To prepare for hybridization, sections were warmed to room temperature and then loaded into stainless-steel racks that had been previously treated with diethylpyrocarbonate (DEPC) and autoclaved (Blumberg, 1987). The slides were immersed in 4% formaldehyde/PBS (0.15 *M* NaCl/1.0 m*M* KH_2PO_4/6.0 m*M* Na_2HPO_4) for 15 min, rinsed in PBS for 2 min, and then soaked in 0.25% acetic anhydride in TE × HCl (10 m*M* Tris × HCl/1 m*M* EDTA, pH 8.0) for 10 min. After rinsing in standard saline citrate (SSC) (150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.5), sections were dehydrated through a graded series of ethanol washes, delipidated in chloroform, rehydrated to 95% ethanol, then air-dried. Each section was incubated with 25 μL hybridization buffer: 50% formamide; 2× SSC; transfer RNA (250 μg/mL); sheared, single-stranded salmon sperm DNA (500 μg/mL); Denhardt's solution [0.02% each of bovine serum albumin (BSA), Ficoll, and polyvinylpyrrolidone]; 10% (w/v) dextran sulfate mol wt = 500,000; 0.2 m*M* dithiothreitol (DTT); and 4.0–5.0 × 10^5 cpm probe. Each slide was covered with a glass coverslip and incubated at 55°C for 16 h in humid chambers. After hybridization, the coverslips were floated off in 1× SSC buffer and washed two times in 1× SCC for 10 min each. After washing in 2× SSC/50% formamide at 52°C twice for 10 min each, the slides were rinsed in 2× SSC twice for 1 min each, and then incubated with RNase A 100 μg/mL in 2× SSC/1 m*M* EDTA at 37°C for 30 min. Following rinsing in 2× SSC twice for 1 min each, the slides were incubated in 2× SSC/50% formamide twice for 10 min each at 52°C. Sections were then dehydrated through an ethanol series, air-dried, and dipped in Kodak NTB3 nuclear track emulsion to visualize the radioactive probe.

Probe Preparation. The last 350 nucleotides of m56 cDNA containing the 3′ untranslated region and 18 poly A residues were subcloned into pBluescript and linerized with *EcoRI* or *XhoI* for the generation of antisense and sense probe, respectively. The linerized DNA was transcribed *in vitro* in the presence of 0.5 m*M* of each ATP, GTP, and CTP, 6 μM UTP, and 6 μM ^{33}P-UTP (DuPont NEN) for antisense and sense probe.

Examination of m56 Expression in PCD Models

Cell Culture. The T-cell hybrid DO11.10 was grown to a density of 0.5–1 × 10^6 cells/mL at 37°C in 5% CO_2

```
        0                                                            49
  m56       MALDGP EQMELEEGKA GSGLRQYYLS KIEELQLIVN DKSQNLRRLQ
Trip1       MALDGP EQMELEEGKA GSGLRQYYLS KIEELQLIVN DKSQNLRRLQ
18-56       MTL... TKMEVDSTK. GEGFRPYYIT KIEELQLIVA EKSQNLRRLQ
 sug1   MTAAVTS... SNIVLE.TH. ESGIKPYFEQ KIQETELKIR SKTENGRRLE

        50                                                   >       99
  m56   AQRNELNAKV RLLREELQLL QEQGSYVGEV VRAMDKKKVL VKVHPEGKFV
Trip1   AQRNELNAKV RLLREELQLL QEQGSYVGEV VRAMDKKKVL VKVHPEGKFV
18-56   AQRNELNAKV RMLREELQLL QEQGSYVGEV VKPMDKKKVL VKVHPEGKFV
 sug1   AQRNALNDKV RFIKDELRLL QEPGSYVGEV IKIVSDKKVL VKVQPEGKYI

        100                                                          149
  m56   VDVDKNIDIN DVTPNCRVAL RNDSYTLHKI LPNKVDPLVS LMMVEKVPDS
Trip1   VDVDKNIDIN DVTPNCRVAL RNDSYTLHKI LPNKVDPLVS LMMVEKVPDS
18-56   VDLDKNVDIN DVTANCRVAL RNESYTLHKI LPNKVDPLVS LMMVEKVPDS
 sug1   VDVAKDINVK DLKASQRVCL RSDSYMLHKV LENKADPLVS LMMVEKVPDS

        150                                                          199
  m56   TYEMIGGLDK QIKEIKEVIE LPVKHPELFE ALGIAQPKGV LLYGPPGTGK
Trip1   TYEMIGGLDK QIKEIKEVIE LPVKHPELFE ALGIAQPKGV LLYGPPGTGK
18-56   TYEMVGGLDK QIKEIKEVIE LPVKHPELFD ALGIAQPKGV LLYGPPGTGK
 sug1   TYDMVGGLTK QIKEIKEVIE LPVKHPELFE SLGIAQPKGV ILYGPPGTGK

        200                                                          249
  m56   TLLARAVAHH TDCTFIRVSG SELVQKFIGE GARMVRELFV MAREHAPSII
Trip1   TLLARAVAHH TDCTFIRVSG SELVQKFIGE GARMVRELFV MAREHAPSII
18-56   TLLARAVAHH TECTFIRVSG SELVQKFIGE GSRMVRELFV MAREHAPSII
 sug1   TLLARAVAHH TDCKFIRVSG AELVQKYIGE GSRMVRELFV MAREHAPSII

        250                                                          299
  m56   FMDEIDSIGS SRLEGGSGGD SEVQRTMLEL LNQLDGFEAT KNIKVIMATN
Trip1   FMDEIDSIGS SRLEGGSGGS SEVQRQMLEL LNQLDGFEAT KNIKVIMATN
18-56   FMDEIDSIGS SRIESGSGGD SEVQRTMLEL LNQLDGFEAT KNIKVIMATN
 sug1   FMDEIDSIGS TRVEGSGGGD SEVQRTMLEL LNQLDGFETS KNIKIIMATN

        300                                                  <   349
  m56   RIDILDSALL RPGRIDRKIE FPPPNEEARL DILKIHSRKM NLTRGINLRK
Trip1   RIDMLDSALL RPGRIDRKIE FPPPNEEARL DILKIHSRKM NLTRGINLRK
18-56   RIDILDPALL RPGRIDRKIE FPPPNEEARL DILKIHSRKM NLTRGINLRK
 sug1   RLDILDPALL RPGRIDRKIE FPPPSVAARA EILRIHSRKM NLTRGINLRK

        350                                                          399
  m56   IAELMPGASG AEVKGVCTEA GMYALRERRV HVTQEDFEMA VAKVMQKDSE
Trip1   IAELMPGASG AEVKGVCTEA GMYALRERRV HVTQEDFEMA VAKVMQKDSE
18-56   IAELMPGASG AEVKGVCTEA GMYALRERRV HVTQEDFEMA VAKVMQKDSE
 sug1   VAEKMNGCSG ADVKGVCTEA GMYALRERRI HVTQEDFELA VGKVMNKNQE

        400    409
  m56   KNMSIKKLWK
Trip1   KNMSIKKLWK
18-56   KNMSIKKLWK
 sug1   TAISVAKLFK
```

Figure 1 (A)

percent identity	Trip1	18-56	SUG1	MSS1	26sIV	TBP-1
overall sequence	99.3	93.0	74.6	47.7	45.6	42.5
CAD domain	98.7	96.9	87.2	61.7	61.3	60.4

Figure 1 (B)

in complete RDG medium supplemented with 10% horse serum. Cells were stimulated with the phorbol ester PMA (10 n*M*), dexamethasome (12.5 μM), or the anti−T-cell receptor (anti-TCR) antibody F23.1. To stimulate cells with anti-TCR, flasks were coated with sheep anti-mouse immunoglobulin (0.8 $\mu g/cm^2$) for 1 h, washed two times with PBS, and then coated with culture supernatant of the monoclonal antibody F23.1 for 1 h.

Thymuses from adult mice were removed and thymocytes isolated from the thymic matrix by gently grinding the cells between glass slides in the presence of PBS. Cells were pelleted and then resuspended in RDG medium. Thymocytes were then incubated in either media alone (controls) or in the presence of 1 μM dexamethasone to induce apoptosis.

PC12 cells were grown in flasks coated with calf skin collagen (Sigma) in a medium containing 85% RPMI-1640, 10% horse serum, 5% fetal calf serum (FCS), and penicillin/streptomycin at 100 U/mL medium. Cells were induced to undergo differentiation when the medium was supplemented with 2.5S nerve growth factor (NGF) (Boehringer Mannheim Biochemicals) at 100 ng/mL. After 7 days, cells were triturated and then grown in F12/Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with 10% FCS, antibiotics, and NGF as above for an additional 7 days. Cells were then washed in F12/DMEM medium and half of the cells were plated in collagen-coated plates in F12/DMEM medium supplemented with 10% FCS and antibiotics as above; the other half was grown in the same medium with NGF as a control.

Total RNA was isolated from the T-cell hybrid DO11.10, the adrenal chromaffin cell line PC12 cells, immature thymocytes, and mammary glands using RNAzol B as above, and used to generate Northern blots.

Seizure Induction in Rats. Adult male rats (200−250 g) were injected intraperitoneally with kainic acid (10 mg/kg body weight) dissolved in saline, and then decapitated 2, 6, and 24 h following the onset of kainate-induced seizures. Control animals were injected with normal saline. The brains were dissected out and *in situ* hybridization was performed as described above. The last 350 nucleotides of m56 cDNA were transcribed *in vitro* in the presence of 0.5 m*M* each of ATP, GTP, and CTP, 6 μM UTP, and 6 μM ^{35}S-UTP (Dupont NEN) for antisense and sense probe. c-*fos* cDNA was transcribed *in vitro* in sense and antisense orientation in the presence of ^{35}S-UTP as above.

Examination of RNA Pol II Expression in Adult Mouse Brain by *In Situ* Hybridization

A 235-bp exon from mouse RNA polymerase II (RNA Pol II) genomic DNA clone was amplified by PCR using the following primers: 5′-GACATTCTTTGCCGC TTGG-3′ hybridizing to nucleotides 9554−9572 and 5′-TTCTCAGCAATCTGTTCCATG-3′ recognizing nucleotides 9789−9769 of the genomic DNA (Ahearn et al., 1987). The PCR product was cloned into pBluescript and transcribed *in vitro* in both anti-sense and sense orientations in the presence of ^{33}P-UTP as above. Sequential tissue sections were hybridized to labeled m56 and RNA Pol II probes. Sense strands were used as controls.

Examination of Subunit 4 of the Mouse 26S Proteasome in Adult Brain by *In Situ* Hybridization

To examine the expression pattern of the S4 subunit of the 26S proteasome in the mouse brain, we chose a fragment from the 5′ region of S4 that shared low homology to other CAD family members and designed oligonucleotide probes. The antisense oligo, m26SIV3′, was 5′-ATGGGGGTCCCCCTGAGATCATCCACTTTTG ATCTTTCCTCCTCTTGC-3′, and the sense oligo m26-S4 sense was 5′-GCAAGAGGAGGAAAGATCAAA AGTGGATGATCTCAGGGGGACCCCCAT-3′. Both oligonucleotides were synthesized and OPC-purified by the Great American Gene Company. The oligos were labeled with a ^{33}P-dATP by using Terminal Transferase (Boehringer Mannheim), and then purified by phenol/chloroform and chloroform/isoamyl alcohol extraction in the presence of 1 μL of yeast tRNA (25 mg/mL). The probes were then precipitated twice and dissolved in 1× SSC.

Tissue sections were prepared as described above. Each section was incubated with 25 μL hybridization buffer as described above, except using 4× SSC instead of 2× SSC, and 1.4×10^5 cpm probe was used. Slides were coverslipped and incubated at 37°C for 16 h in humid chambers. After hybridization, the coverslips were floated off in 1× SSC buffer and washed twice in 1× SSC for 15 min each. After washing in 2× SSC/50% formamide at 40°C four times for 15 min each, the slides

Figure 1 (A) Sequence comparison of m56 with other CAD family members. Sequence alignment of m56, human Trip1, *Manduca* 18−56, and yeast Sug1. Identical amino acids are shaded. (B) Comparison of m56 and CAD members at the protein level.

were washed twice at room temperature in 1× SSC for 30 min each. Following a quick rinse in distilled water, the slides were washed in 70% ethanol for 5 min and then air-dried. Sections were initially exposed to X-ray film and then dipped in emulsion as described above.

RESULTS

Isolating m56 and DNA Sequencing

The observation that Sug1/18-56 is a phylogenetically conserved protein that may play a role in the programmed death of the intersegmental muscles of the moth *Manduca sexta* motivated us to clone the mouse homolog of this gene for study. The profound sequence conservation between Sug1 and 18–56 (74% at the protein level) allowed us to design two degenerate primers that could be used to clone the conserved regions between Sug1 and *Manduca* 18–56 (Fig. 1). We selected this region for amplification because it shares low identity to the corresponding region of other non–Sug1-like CAD family members. Using RT/PCR, we cloned the anticipated 0.8-kb fragment from PMA-treated DO11.10 hybridoma cells. When sequenced, it displayed high identity to both clone 18–56 and *SUG1* at the nucleotide level.

To isolate a full length of this recombinant, termed m56, this 0.8-kb fragment was labeled with ^{32}P and used as a probe to screen a mouse cDNA library generated from the mRNA of dying thymocytes (Liu et al., 1994). Of 400,000 recombinants screened, 28 clones were obtained. Two were selected for further study owing to differences in size of their restrictive inserts. Upon DNA sequencing, it was found that the smaller clone was a truncated version of the larger one. The large clone contained 1308 nucleotides plus a long poly A tail and encoded a predicted protein of 406 amino acids. The deduced amino acid sequence of m56 cDNA shares 98%, 93%, and 75% identity with human Trip1, moth 18–56, and yeast Sug1, respectively (Fig. 1), and is identical with the recently reported mouse mSug1/FZA-B (Baur et al., 1996; Wang et al., 1996). The amino acid sequence of m56 also shares about 45% identity with those of the CAD proteins previously shown to be associated with the proteasome.

m56 Functionally Substitutes for *SUG1* in Yeast

The high sequence identity between m56, yeast Sug1, *Manduca* 18–56, and human Trip1 suggested that m56 might be the mouse homolog of Sug1/18–56/Trip1. To determine if m56 shared functional homology with Sug1, an m56 expression construct was introduced into a yeast strain deleted for the endogenous *SUG1* but viable because of a plasmid borne *SUG1* under *GAL1* promoter (galactose-inducible) control. Therefore, this strain can grow only in the presence of galactose. When a plasmid-bearing m56 under the control of the yeast *ADH1* promoter was introduced into this strain, it could grow in the absence of galactose (Fig. 2). This indicates that m56 can functionally substitute for yeast Sug1. MSS1, a related CAD 26S proteasome subunit, could not substitute for Sug1 in this assay.

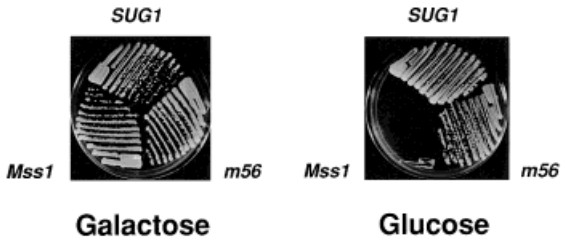

Figure 2 m56 can functionally substitute for SUG1 *in vivo*. W303a_SUG1::URA3 pMTL Gal1/10-*SUG1* (a strain in which the lethal deletion of the chromosomal copy of *SUG1* is covered by the expression of Sug1 from the controllable yeast Gal1/10 promoter) was transformed with plasmids expressing Sug1, m56, or Mss1 (from the yeast ADH I promoter). Transformants were selected on galactose plates and streaked onto glucose plates.

Expression of m56 mRNA and Protein in Adult Mouse Organs

To examine the expression of m56 at the RNA level, we probed a Northern blot of RNA isolated from a variety of adult mouse organs. A single abundant transcript was detected in every tissue examined, with the exception of brain and testis, where expression was somewhat higher, and spleen where it was lower (Fig. 3). In separate studies, we found that m56 was expressed in all tissue culture cells examined, including T-cell hybrids (DO11.10), PC12 cells, and Rat-1 fibroblasts (data not shown).

To analyze the expression of m56 at the protein level, the m56 cDNA was expressed in bacteria and the resulting protein was used to affinity purify an antisera generated against *Manduca* 18–56 (Sun et al., 1996), which in turn was used to probe Western blots generated from various adult mouse organs. In

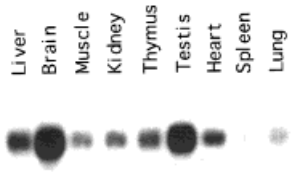

Figure 3 Expression of mouse 56 mRNA in various organs of the adult mouse. Total RNA from different organs of adult mice was isolated, fractionated under denaturing conditions, and blotted onto a membrane. The blot was probed with ^{32}P-labeled m56 cDNA.

agreement with the predicted size of m56, a single protein of approximately 45 kD was detected in almost all tissues and cells examined, including liver, brain, kidney, thymus, spleen, lung, thymocytes, DO11.10 cells, and PC12 cells (Fig. 4) (data not shown). The lower-molecular-weight bands may have resulted from either degraded protein within the sample or cross-reaction of this polyclonal antibody to other CAD family members. Surprisingly, very little protein expression was seen in heart and no signal was detected in skeletal muscle (Fig. 4). This observation may be an artifact due to the low abundance of nuclei and cytoplasm in these cells. Control blots reacted with preimmune serum produced no signals above background (data not shown).

Expression of m56 Is Not Altered during Cell Death in Mouse

As previously mentioned, we cloned m56 in part because its moth homolog is dramatically up-regulated coincident with the commitment of the intersegmental muscles to die (Sun et al., 1996). To determine if the expression of m56 was also associated with vertebrate cell death, we examined a variety of mammalian cell death models for alterations in m56 expression.

DO11.10 T-cell hybridoma cells were induced to undergo apoptosis in response to either anti-TCR antibody or glucocorticoid treatment, or, as a control, induced to proliferate in response to the phorbol ester PMA. While m56 RNA and protein could be detected in all samples, no change in the steady-state levels of these molecules was detected in response to apoptotic or mitotic stimuli (Table 1) (data not shown). The level of m56 expression was also unaffected when immature thymic T cells were induced to undergo apoptosis in response to dexamethasone treatment (Table 1). Furthermore, m56 did not show enhanced expression in PC12 cells when they were induced either to differentiate in response to NGF or to undergo apoptosis following its removal (Fig. 5 and Table 1). In fact, there were reductions in the level of m56 protein in older cultures which were exacerbated when NGF was absent from the media. Another classic model for programmed cell death in mammals is the postlactation regression of the mammary gland (Strange et al., 1992; Feng et al., 1995). Again, while m56 mRNA could be detected at every developmental stage, there was no enhanced expression with either differentiation or regression (Table 1) (Sun, Jerry, and Schwartz, unpublished).

The excitotoxic death of neurons in the hippocampus, amygdala, cortex, and thalamus following kainic acid seizures occurs with an apoptotic morphology (Sakhi et al., 1994). The expression of c-*fos* is dramatically elevated up to 16 h after kainic acid administration and can be used as a marker for the induction of seizures (Schreiber et al., 1993). We induced seizures in rats with kianic acid and monitored m56 expression via *in situ* hybridization at 2, 6, and 24 h post-treatment. Although c-*fos* expression was dramatically up-regulated, no change in m56 expression was observed (data not shown). Therefore, it does not appear that changes in the steady-state level of m56 participate in mammalian apoptosis.

Cellular Distribution of m56 mRNA and Protein in Adult Mouse Central Nervous System (CNS) and Other Tissues

We further examined expression of the m56 message at the cellular level by *in situ* hybridization.

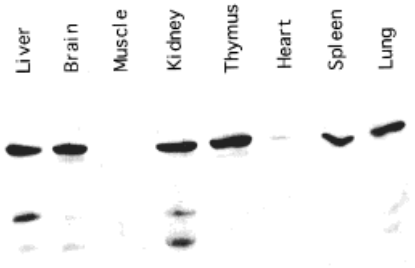

Figure 4 Expression of mouse 56 protein in various organs of the adult mouse. Protein from different organs of adult mice was extracted and fractionated by SDS-PAGE. A Western blot was prepared and reacted with an affinity-purified anti-m56 antibody. The signal was detected with a horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody.

Table 1 Relative Levels of m56 mRNA Determined from Northern Blots of Total RNA Isolated from Cells Before or After Initiation of Cell Death

A				
m56 expression	Untreated	Anti-TCR	PMA	Dex
DO11.10 T cell hybrid	+	+	+	+
Thymocytes	+	ND	ND	+
B				
m56 expression	Untreated	+NGF		−NGF
PC12 cells	+	+		+
C				
m56 expression	Virgin	Pregnancy	Lactation	Weaning
Mammary glands	+	+	+	+

(A) DO11.10 T cell hydrid and immature thymocytes. Phorbol ester (PMA) acts as a mitogen for T-cell hybrids, while treatment with anti-TCR antibody or glucocorticoid (dexamethasone) results in massive apoptosis. (B) Treatment of PC12 cells with nerve growth factor (NGF) induces differentiation, while its removal results in neuronal death. (C) Pregnancy and lactation involve massive proliferation of cells in the mammary gland, while weaning results in massive apoptosis. + = low level of expression; ND = not determined.

Various organs were removed from adult mice, sectioned, and hybridized to ^{33}P-labeled probes generated from an m56 fragment. In the brain, m56 mRNA was found to be very broadly distributed with several notable features [Fig. 6(A,B)]. There was prominent expression in the olfactory bulb and cerebellum, throughout the hippocampus including dentate gyrus and all subfields of Ammon's horn, and in specific forebrain structures such as zona incerta, ventromedial hypothalamus, and preoptic area. In addition, there was strong expression in entorhinal cortex, brain stem, and cerebral cortex, with little evidence of selective expression in specific layers. There was no detectable expression in areas of white matter such as corpus callosum, fornix, and cerebellar leaflet. Nonneural tissues such as the liver and kidney displayed a uniform and low-level signal (data not shown).

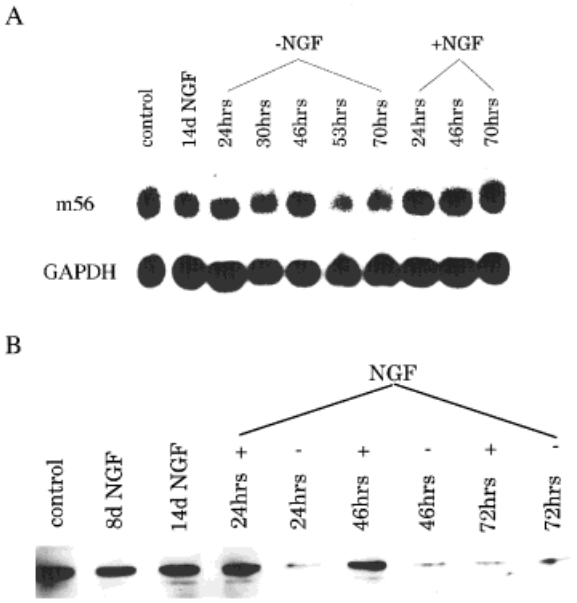

Figure 5 Lack of induction of mouse 56 mRNA or protein in PC12 cells following differentiation or initiation of apoptosis. PC12 cells were grown in culture and induced to differentiate for 8 or 14 days in the presence of NGF. In some cultures, cells were subsequently incubated in media lacking NGF to induce apoptosis. At the times indicated, cells were analyzed for their expression of mouse 56 RNA (A) and protein (B) by Northern and Western blots, respectively. Following film autoradiography, the Northern blot was stripped and reprobed with the constitutively expressed GAPDH gene to monitor RNA loading.

To characterize the expression of m56 at the protein level, we used immunohistochemistry and an affinity purified anti-m56 antisera to examine the distribution of this protein in the mouse CNS. In agreement with the *in situ* hybridization data above, staining was not uniform in all regions of the brain. Cells of the hippocampus [Fig. 7(A)] and Purkinje cells in the cerebellum [Fig. 7(B)] displayed elevated levels of staining relative to the rest of the CNS. As has been observed in yeast and moths, m56 staining was predominantly restricted to the nucleus. No signal above background was observed when preimmune serum was used as a control (data not shown).

Expression of m56 Parallels that of RNA Polymerase II and Subunit 4 of the 26S Proteasome

Previous work by Kim et al. (1994) suggested that Sug1 is a subunit of the transcriptional mediator complex that links specific transcription factors to

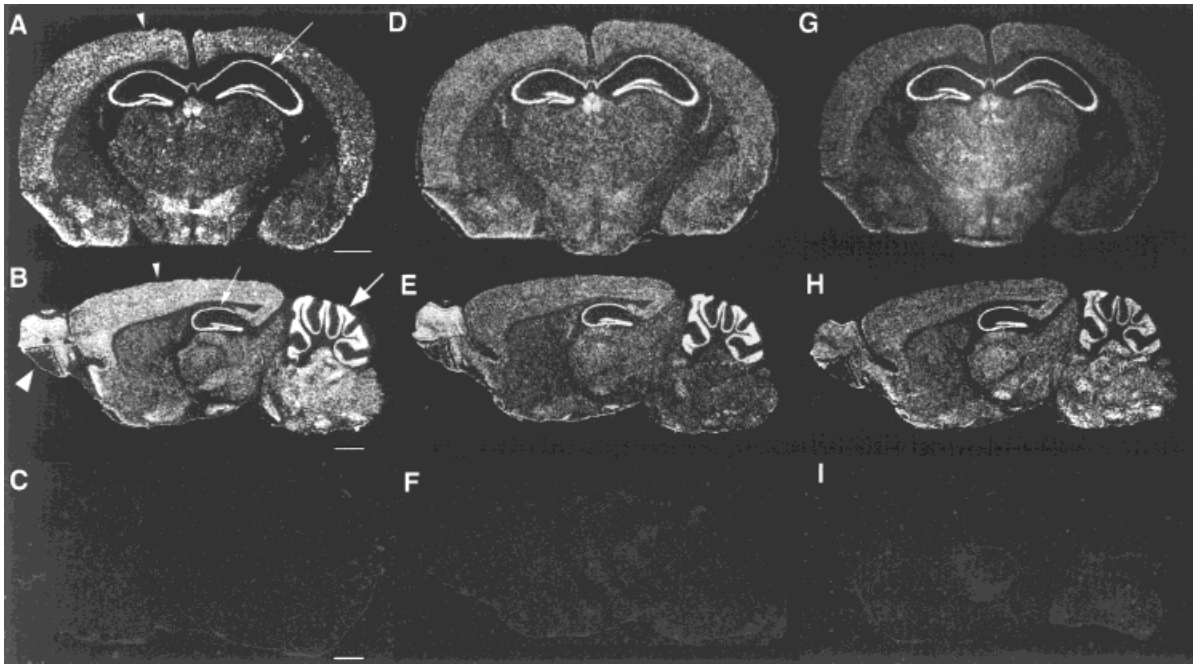

Figure 6 Distribution of mouse 56, RNA Pol II, and S4 mRNA in the adult mouse brain. Frozen sections of mouse brains were made in coronal (A,D,G) or sagittal (B,C,E,F,H,I) plane and hybridized to ^{33}P-labeled probes: m56 antisense (A,B), or sense (C); Pol II antisense (D,E) or sense (F); and ^{33}P-labeled S4 antisense oligomer (G,H) or sense oligomer (I). Each panel represents a dark-field micrograph of sections following exposure of hybridized sections to liquid emulsion. Small arrow = hippocampus; small arrowhead = cerebral cortex; large arrow = cerebellum; large arrowhead = olfactory bulb. Bar = 1 mm.

the basal transcription apparatus. Upon association with the mediator complex, RNA Pol II assumes a greater specific activity in promoter-dependent transcription. Recently, both Rubin et al. (1996) and Swaffield et al. (1996) presented convincing evidence that Sug1 is instead a subunit of the 26S proteasome. To determine if m56 expression parallels that of RNA Pol II or the 26S proteasome, we used a riboprobe generated from a portion of RNA Pol II gene and oligonucleotide probe derived from subunit 4 of the 26S proteasome (S4) for *in situ* hybridization. Expression in brain for both Pol II and S4 mRNA were similar in several respects to that of m56 (Fig. 6); both mRNAs appeared to be broadly distributed with notable expression in olfactory bulb, hippocampus, and the cerebellum relative to other brain areas. In addition, S4 mRNA exhibited specific features that were more similar to that of m56, including higher relative expression in brain-stem nuclei, habenulum, and forebrain structures [Fig. 6(G,H)]. Interestingly, based on exposure times, it appears that the steady-state levels of RNA Pol II mRNA was lower than that of m56.

DISCUSSION

In this report, we describe the isolation and initial characterization of clone m56, the mouse homolog of the yeast *SUG1* gene. Sequence analysis demonstrates that m56 is a member of the conserved ATPase-containing CAD protein superfamily that is identical with the recently described mSug1/FZA-B (Baur et al., 1996; Wang et al., 1996). The nucleotide-binding site is a common feature shared by all CAD family members, and in some cases CAD proteins have been shown to possess ATPase activity (Peters et al., 1992). Several members of the CAD family including, MSS1, S4, p45, Sug1, and mSug1/FZA-B have been shown to be subunits of the 26S proteasome (Dubiel et al., 1992, 1993; Akiyama et al., 1995; Rubin et al., 1996; Wang et al., 1996).

There has been some controversy in the literature regarding whether Sug1 is a component of both the transcriptional complex and the 26S proteasome or is specifically proteasome associated. Recent reports have provided convincing data that Sug1, expressed

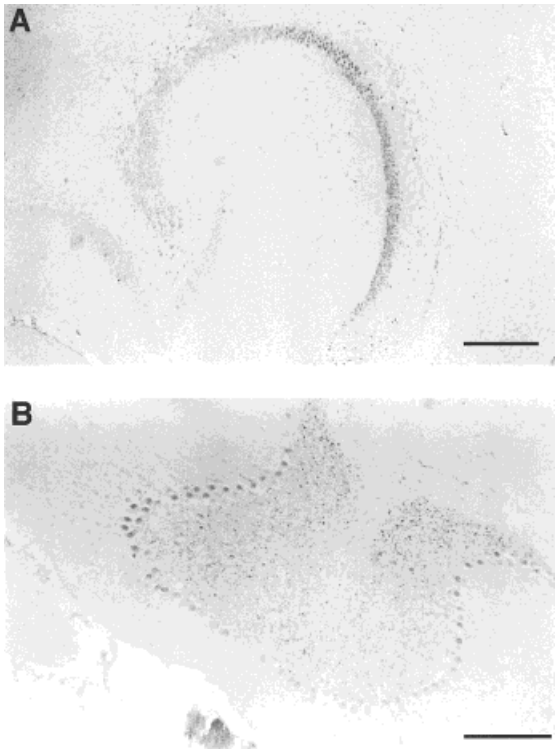

Figure 7 Immunocytochemical detection of mouse 56 protein in the adult mouse brain. Sagittal sections from the adult mouse brain were fixed in FAA and reacted with an affinity-purified anti-m56 antibody. A horseradish peroxidase (HRP)-labeled goat anti-rabbit polyclonal antibody was used for detection. The cells in the hippocampus (A) and the Purkinje's cells in the cerebellum (B) were intensely stained. The antigen is localized in the nucleus. Bar = 0.2 mm.

at normal levels in yeast, stably associates with the 26S proteasome in cell extracts (Rubin et al., 1996; Swaffield et al., 1996). Only when overexpressed does Sug1 associate with the transcriptional complex as originally observed (Swaffield et al., 1996). A possible transcriptional role for Sug1 has been suggested by several lines of evidence. The *sug1-1* mutation of *SUG1* was identified as a second site suppresser of a mutation in the activation domain of yeast transcription activator Gal4 (Swaffield et al., 1992). Two additional alleles of *SUG1* (*SUG1-20* and *SUG1-26*) have also been isolated in a similar fashion to *SUG1-1,* owing to their ability to function as second site suppressers of a transcriptionally compromised cdc68 in yeast (Xu et al., 1995). Sug1 has also been implicated in the transcription mediator complex (Kim et al., 1994) and has been shown to be capable of direct interaction with activation domains and the TATA-binding protein (TBP) in yeast (Swaffield et al., 1995). Finally, Trip1 has been shown to interact with thyroid hormone receptor in a hormone-dependent fashion (Lee et al., 1995), and mSug/FZA-B interacts with a number of transcription factors, including retinoic acid receptor (Baur et al., 1996) and c-*fos* (Wang et al., 1996).

The 26S proteasome is responsible for selective proteolysis, such as the presentation of major histocompatibility complex (MHC) class I-restricted antigens, as well as the bulk of protein turnover in cells (Rock et al., 1994). It is possible that Sug1 and Sug1-like molecules exert their purported effect on transcription by selective degradation of transcription factors. Alternatively, the ATPase domain-containing Sug1 and Sug1-like proteins may be involved in modulating protein–protein interactions within assembled or assembling transcriptional complexes, thus altering transcriptional regulation.

The moth homolog of m56, 18–56, was isolated as a gene whose expression was dramatically increased coincident with the initiation of PCD (Sun et al., 1996). Despite extensive examination, no correlation could be detected between PCD in the mouse and enhanced m56 expression. It is worth noting, however, that while all of the mammalian cell deaths examined display the morphology of apoptosis, the death of the intersegmental muscles of *Manduca* does not (Schwartz et al., 1993). The molecular mechanism that mediates the death of the ISMs may therefore be distinct from that of apoptosis. When the ISMs become committed to die, there are dramatic increases in the levels of: polyubiquitin mRNA, ubiquitin protein, ubiquitin–protein conjugates, proteasomal subunits, and proteasomal enzymatic activity (Schwartz et al., 1990; Haas et al., 1995; Dawson et al., 1995; Jones et al., 1995; Takayanagi et al., 1996; Sun et al., 1996). In contrast to apoptotic cells which are phagocytosed, the dying ISMs are not removed by other cells (Jones, Fahrbach, and Schwartz, in preparation). Therefore, all of the machinery required to dismantle the corpse must reside within the condemned cell itself. Nevertheless, while there does not seem to be induction of proteasome subunits in apoptotic cells, this moiety does serve an essential regulatory role for in apoptosis. Transient inhibition of the 20S proteasome with peptide aldehyde inhibitors can block the apoptotic death of immature thymocytes (Grimm et al., 1996), sympathetic neurons (Sadoul et al., 1996), and rat-1 fibroblasts (Hu and Schwartz, unpublished). Chronic inhibition of the proteasome however is lethal in cells (Imajoh-Ohmi et al., 1995; Shinohara et al., 1996). Interestingly,

mild derangement of 26S proteasome function is not lethal to rat-1 fibroblasts but does sensitize them to apoptotic stimuli (Yu, Wang, Woo, Sun, Goldberg, and Schwartz, in preparation). Therefore, basal levels of the proteasome are sufficient to mediate apoptosis and other developmental changes in these cells.

While the pattern of expression and relative abundance of m56 in mouse brain does not immediately reveal its role in CNS function, several features of its expression suggest possibilities. First, its broad distribution of expression throughout the brain and in nonneural tissues indicates that it plays a basic role in cellular processes. However, because its abundance is much greater in olfactory bulb, hippocampus, cerebellum, and several brain stem and forebrain nuclei, the specific role(s) it plays may be related to cellular processes particularly important to neurons in these brain areas. Moreover, because m56 was not detected in areas of white matter (e.g., corpus callosum), oligodendrocytes do not appear to require significant levels of m56 expression. These data at the RNA level agree well with the recent immunohistochemical description of 20S proteasome expression in the mouse brain (Mengual et al., 1996).

One cellular feature common to neurons of the olfactory bulb, hippocampus, and cerebellum is that they display dramatic developmental and functional plasticity. For example, the hippocampus and cerebellum display long-term potentiation, processes that are dependent on both *de novo* gene expression (Montarolo et al., 1986; Frey et al., 1988; Nguyen et al., 1994) and proteasome activity (Hegde et al., 1997). It has recently been demonstrated in *Aplysia* that long-term facilitation requires the activation of the proteasome (Hedge et al., 1997). In addition, cell proliferation, differentiation, and death are constitutive processes within the olfactory bulb (Kaplan et al., 1985; Corotto et al., 1993), processes that are dependent on proteasomal activity.

We compared the pattern of m56 expression to that of RNA Pol II and the S4 26S proteasome subunit, as both yeast Sug1 and its mouse homolog have also been implicated in the association with RNA Pol II (Kim et al., 1994; Wang et al., 1996). While the hippocampus and cerebellum exhibited high relative levels of both RNA Pol II and 26S mRNA, several features of S4 expression appeared to be more similar to that of m56, including elevated levels of expression in specific brain-stem nuclei, nuclei of the forebrain, and the entorhinal nucleus. Similar findings were seen at the protein level using antisera directed against the 20S proteasome (Mengual et al., 1996). Although these findings do not unequivocally confirm an association of m56 and S4, they do indicate that these genes covary in their relative levels of expression and may parallel the distribution of the 20S proteasome.

The data presented here represent the first characterization of the cellular distribution of RNA Pol II in the mouse CNS. While expression of RNA Pol II might reasonably be expected to be uniform in all cells, the selective elevation of its steady-state level in specific neurons indicates that perhaps some neurons are more transcriptionally active than others.

Finally, the differential abundance and distribution of expression of m56, RNA Pol II, and S4 in brain are not likely to be related to cell size or cell density alone. Our reasoning is that several populations of classic magnocellular neurons did not appear to be highly labeled by these probes. For example, notable levels of expression were not found in the magnocellular division of the hypothalamic paraventricular nucleus, the substantia nigra, or the striatum. In addition, cell density is not uniform throughout Ammon's horn, cerebral cortex, or within the olfactory bulb; yet m56 expression appeared to be relatively uniform throughout these particular structures.

While the elevated basal levels of m56 may merely reflect a higher metabolic rate, it may also function to allow cells to respond to developmental signals either through selective protein degradation or by altered transcriptional regulation. We are currently engaged in direct tests of these hypotheses.

NOTE ADDED IN PROOF

Further support for a role by *sug-1* family members in transcriptional regulation was provided by the recent observation that yeast SUG1 has 3′-5′ DNA helicase activity and that mammalian SUG1 is present in multiple complexes within the cell [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].

The authors thank Dr. B. Osborne, Dr. J. Nambu, and Dr. J. Jerry for helpful discussions; Yanhui Hu for help with Northern blots; Dr. J. Jerry for the Northern blot of mammary gland mRNA; Dr. Jeffery Corden for providing the mouse RNA polymerase II DNA; Dr. Edward Westhead for providing PC12 cells; Dr. Wolfgang Bielke for

help with the PC12 experiments, Dr. Sallie Smith for help with T cells and T cell hybrids; and Dr. Eric Martz and Dr. Bruce MacDonald for providing mice. This study was supported by NIH grants to LMS, RTZ and SAJ.

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