MOLECULAR AND CELLULAR BIOLOGY, June 2005, p. 4662–4675 Vol. 25, No. 11
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.11.4662–4675.2005

Identification and Characterization of a *Drosophila* Proteasome Regulatory Network†

Josefin Lundgren, Patrick Masson, Zahra Mirzaei, and Patrick Young*

Department of Molecular Biology and Functional Genomics, Stockholm University, S-10691 Stockholm, Sweden

Received 31 March 2004/Returned for modification 17 May 2004/Accepted 28 February 2005

Maintaining adequate proteasomal proteolytic activity is essential for eukaryotic cells. For metazoan cells, little is known about the composition of genes that are regulated in the proteasome network or the mechanisms that modulate the levels of proteasome genes. Previously, two distinct treatments have been observed to induce 26S proteasome levels in *Drosophila melanogaster* cell lines, RNA interference (RNAi)-mediated inhibition of the 26S proteasome subunit Rpn10/S5a and suppression of proteasome activity through treatment with active-site inhibitors. We have carried out genome array profiles from cells with decreased Rpn10/S5a levels using RNAi or from cells treated with proteasome inhibitor MG132 and have thereby identified candidate genes that are regulated as part of a metazoan proteasome network. The profiles reveal that the majority of genes that were identified to be under the control of the regulatory network consisted of 26S proteasome subunits. The 26S proteasome genes, including three new subunits, Ubp6p, Uch-L3, and Sem1p, were found to be up-regulated. A number of genes known to have proteasome-related functions, including Rad23, isopeptidase T, sequestosome, and the genes for the segregase complex TER94/VCP-Ufd1-Npl4 were also found to be up-regulated. RNAi-mediated inhibition against the segregase complex genes demonstrated pronounced stabilization of proteasome substrates throughout the *Drosophila* cell. Finally, transcriptional reporter assays and deletion mapping studies in *Drosophila* demonstrate that proteasome mRNA induction is dependent upon the 5′ untranslated regions (UTRs). Transfer of the 5′ UTR from the proteasome subunit Rpn1/S2 to a noninducible promoter was sufficient to confer transcriptional upregulation of the reporter mRNA after proteasome inhibition.

Proteasome-dependent degradation serves an essential role in the removal of a wide variety of key nuclear and cytosolic proteins (35, 42, 45, 52). This pathway also carries out an important housekeeping function by clearing cells from potentially harmful abnormal proteins that arise as the result of mutations, translational errors, misfolding, or postsynthetic damage and functions in the cytoplasm as a part of the protein quality control system for the endoplasmic reticulum (25).

Structurally, the 26S proteasome consists of a 20S catalytic core and a 19S regulatory complex that associates with the ends of the 20S proteasome in an ATP-dependent manner (3, 20, 46). The eukaryotic 20S proteasome is composed of 14 different subunits arranged in four stacked, seven-membered rings that form the barrel-shaped complex (18, 43). The 19S regulatory complex is itself composed of two distinct subcomplexes, the base and the lid (15). Six distinct ATPase subunits proposed to function in substrate unfolding and gating of the 20S pore have been localized to the base along with two additional subunits (7, 12, 16, 36). At least eight subunits form a lid subcomplex that is thought to be necessary for the processing of polyubiquitinated proteins and exhibit high similarities to the COP9/signalosome complex (15). An additional 19S subunit, Rpn10/S5a, has been observed to stabilize the interaction between the lid and the base subcomplexes. The Rpn10/S5a is a polyubiquitin binding protein (8, 44). Rpn10/S5a can be found both associated with the 26S proteasome and as a free form. For eukaryotic proteasomes, each subunit of the complex is coded by a unique gene, and the vast majority of these genes are essential for both proteasome activity and the survival of the organism (14, 17, 36, 37, 39).

Previously, we have observed that RNA interference (RNAi) depletion of the Rpn10/S5a subunit increases the level of 26S proteasome (28). This is in agreement with the observed overproduction of 26S proteasome observed in the Rpn10/S5a *Drosophila melanogaster* fly deletion which results in larval-pupal lethality (41). *Drosophila* cell lines lacking Rpn10/S5a show only minor increases in ubiquitin conjugate levels with no observable loss of in vivo proteasome activity (28). We sought to take advantage of this observation where knockdown of the Rpn10/S5a subunit apparently can induce proteasome levels without inducing substantial stress responses and thereby address the question of what genes are regulated components of the metazoan proteasome network. Transcriptional induction of the 26S proteasome mRNA levels has been recently observed for cells treated with proteasome inhibitors (32). To examine this in the *Drosophila* model system, we attempted to identify the pool of enriched mRNAs after two distinct treatments, MG132 inhibition of the 20S proteasome and knockdown of the Rpn10/S5a subunit by RNA interference. Treated *Drosophila* S2 cell lines were used to generate microarray probes to test for genome-wide expression pattern changes. The following set of RNAi-induced mRNAs was compared to the larger set of mRNAs that are induced by inhibition of the 20S proteasome. The combined results reveal a concise group

* Corresponding author. Mailing address: Department of Molecular Biology and Functional Genomics, Stockholm University, S-106 91 Stockholm, Sweden. Phone: 46-8-164135. Fax: 46-8-152350. E-mail: patrick.young@molbio.su.se.

† Supplemental material for this article may be found at http://mcb.asm.org/.

consisting of known proteasome genes from both the 20S and 19S complexes, proteasome related genes, and a small number of novel genes.

A proteasome negative feedback circuit has been described in *Saccharomyces cerevisiae* that functions in maintaining proteasome levels through the selected degradation of the Rpn4 protein, a transcription factor that regulates both 26S proteasome and other genes through binding to a 9-bp upstream activating sequence termed PACE (30, 50). Metazoan genomes apparently lack an Rpn4 homolog, and neither a transcription factor nor a DNA regulatory element that regulates proteasome levels has been identified in higher eukaryotes. From the presented genome profiles, a list of *Drosophila* genes can be grouped as potential members of a proteasome regulatory network. The gene sequences and promoter regions for proposed genes that were found to be transcriptionally upregulated were searched for regulatory elements. Promoter mapping and site-directed mutagenesis of a number of different *Drosophila* proteasome promoters indicate that an element within the 5′ untranslated region (UTR) allows the proteasome regulatory network to sense the cellular level of proteasomes.

MATERIALS AND METHODS

Chemical reagents and antibodies. Rabbit polyclonal antibodies against a sodium dodecyl sulfate-polyacrylamide gel electrophoresis purified recombinant *Drosophila* Rpn10/S5a subunit were raised in rabbit by Agrisera. Proteasome inhibitor MG132 and ubiquitin antibodies used in immunoblots, U5379 and U0508, were purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies and an ECL+ detection kit were from Amersham Biosciences. All primers were produced by Thermo Electron Corporation.

Cell culture. Schneider 2 (S2) cells were cultured in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin and L-glutamine (2 mM). Cells were maintained at 24°C and passed every 7 days at a 1:4 dilution. *Drosophila* S2 stable cell lines expressing the green fluorescent protein (GFP) for the in vivo assay of the 26S proteasome were described previously (28). For constitutive expression in *Drosophila* S2 cells, the genes for the short-lived green fluorescent proteins Ub^{G76V}GFP and Ub-R-GFP (6) were previously subcloned into the pAct vectors (Invitrogen).

dsRNA synthesis. For the RNAi experiments, double-stranded RNA (dsRNA) was synthesized with the aid of the MEGAscript kit (Ambion). Oligonucleotides were selected by searching the GadFly database. An approximately 700-bp fragment of exon sequence was amplified for GFP and Rpn10/S5a, respectively. The Rpn10/S5a T7-tailed DNA was produced with PCR amplification of a clone in a pET26b vector with the following primers: 5′-GAATTAATACGACTCACTATAGGGAGACGCGCTTAATGGCAAGGACG-3′ and 5′-GAATTAATACGACTCACTATAGGGAGAGCGAGTCCTTGCCGTCGCTC-3′. GFP T7-tailed DNA was produced with PCR amplification from recombinant GFP with the following primers: 5′-GAATTAATACGACTCACTATAGGGAGAGGTGAGCAAGGGCGAGGAGCTGTTC-3′ and 5′-GAATTAATACGACTCACTATAGGGAGAATGCCGAGAGTGATCCCGGCGGCGG-3′.

dsRNA synthesis to the new candidate targets was performed as described above with PCR amplification from genomic *Drosophila* DNA extracted from S2 cells with the QIAGEN DNeasy Tissue kit. The following primer sequences were used for the first PCR: for UMP1 CG9324, 5′-GTTGATTAGACGTGTTTTGCTTGAGGAATGTAATGCG-3′ and 5′-GCAACTTCATGGACGATGTCCTGACTGGCC-3′; for TER94 CG2331, 5′-GGGCAGCCTCAGAGCAGAGTGAAGCC-3′ and 5′-GCTGACCGACCCGGAGCCCTACTGC-3′; for Ufd-1 CG6233, 5′-GGTGGCTCCAGCGATTTCGCCGC-3′ and 5′-ATCCATAGGCACGGAGAGCCCAGCACAC-3′; for sequestosome, ref(2)p CG10360, 5′-GGAGTAGTTGGCTGAGTGGAACGTGGAGTCG-3′ and 5′-CAAGTGCGAGAGCAATATGCACG-3′; for CG5495, 5′-ATTCACGGGCACTCCACTCTCCAGCTCC-3′ and 5′-CCTATCACCATGTCCGTGCGCGTGATC-3′; for CG12321, 5′-CCGAATAGTAGGTTCCAGGACTTGGGCACGG-3′ and 5′-GGACAAGCGTACCACTCTGGACGTAGCAG-3′; for CG5039, 5′-GAATTAATACGACTCACTATAGGGAGACGTAGTTGTTGTGATTTCTTTGTACATCCCCTGAAAACG-3′ and 5′-GAATTAATACGACTCACTATAGGGAGAGCGTTATTCGCTCCCTGTGGAAGAAAAAGGG-3′; for CG2046, 5′-GAATTAATACGACTCACTATAGGGAGACCTCGTCGCGTGCCTTCTGGGACG-3′ and 5′-GAATTAATACGACTCACTATAGGGAGAGAGCTGCAGGCAACGCCCGCGGC-3′; for CG9828, 5′-GAATTAATACGACTCACTATAGGGAGACTGTCCGATCCCGAGAAGCGGCGC-3′ and 5′-GAATTAATACGACTCACTATAGGGAGAGGGTACGTGCGCAGACAGACGTTTCG-3′; for S6 CG16916, 5′-CCGTCCATCTGGTTGAGCAGCTCGAGC-3′ and 5′-GCAGGAGGAGTACATCAAGGACG-3′; for S6 homolog CG9475, 5′-CTGTCTGTGCATCAAAGCGCTTGGTGGC-3′ and 5′-GGAGCTGGAGCTCATCCAGGTGCAGGAG-3′; for S8 CG1489, 5′-GTGCACTCGGTGTGATGGGCAACGG-3′ and 5′-CGACATCTTGCATTGTGGTCCTTCAGTTCG-3′; for S8 homolog CG2241, 5′-CCAGTTCCAGGCGGTCCGTAGAGGAGC-3′ and 5′-CAATAGCTCGGGAAGTTGTTGAGCGCTATACAAGG-3′.

T7 tails were added during a second PCR performed on purified products (PCR clean-up kit; QIAGEN). Primers for the second PCRs matched the 5′ half of the original PCRs with the addition of T7 tail sequences (5′-GAATTAATACGACTCACTATAGGGAGA-3′). For some genes, the T7 tail was already present in the primer in the first round of PCR from genomic DNA. The T7-tailed dsRNA was purified with the RNeasy kit (QIAGEN) followed by annealing in 0.1 M NaCl plus 20 mM Na-Citrate, pH 6.8, buffer for 30 min at 65°C followed by slow cooling to room temperature in a water bath. The amount and quality of the produced dsRNA were determined by spectrophotometric analysis and agarose gel electrophoresis.

Double-stranded RNAi. RNAi treatment of *Drosophila* S2 cells was performed as previously described (4, 28). Briefly, *Drosophila* S2 cell cultures were diluted to a concentration of 1×10^6 cells/ml in DES serum-free medium (Invitrogen). After cell attachment, the medium was replaced with 1 ml DES serum-free medium, and the cells were grown in the serum-free medium for 1 to 2 h. To initiate RNAi, 60 μg of dsRNA was directly added to the medium under constant agitation. After 5 h, 2 ml of Schneider's medium containing 10% fetal bovine serum was added, and the cells were cultured for 3 to 4 days prior to isolation of RNA, analysis by Western blot, reverse transcription (RT)-PCR, or 26S GFP in vivo assays.

MG132 treatment for proteasome induction studies. Approximately 12 million cells were treated with MG132 (affinity, dissolved in ethanol) at a final concentration of 10 μM for 5 h. In parallel, 12 million untreated cells were prepared and seeded under the same conditions. Total RNA was isolated as described below.

Isolation of total RNA and array probe synthesis. Total RNA was isolated using the RNeasy kit and QIAshredder (QIAGEN). Approximately 6 million RNAi-treated *Drosophila* S2 cells resulted in a total yield of 40 μg at a concentration of 2 μg/μl when eluted in 30 μl of RNase-free water. The quality of the RNA was analyzed on 1% agarose gel to visualize the two rRNA bands and by spectrophotometric determination of the value of the optical density at 260 nm/optical density at 280 nm. Ten micrograms of total RNA was used for synthesis of cDNA. Probe synthesis was carried out according to the Affymetrix Eukaryotic Target Preparation protocol. Enzymes, buffers, and additional reagents were purchased from Invitrogen. GeneChip T7-oligo(dT) 5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′ was ordered and purified from Thermo Hybaid. Double-stranded cDNA and biotin-labeled cRNA were purified using a GeneChip Sample Cleanup Module (Affymetrix). Purified cRNA and fragmented cRNA were analyzed on a 1% agarose gel.

Analysis of mRNA expression using oligonucleotide arrays. The *Drosophila* microarray chips were purchased from Affymetrix. The chips were hybridized using the Affymetrix GeneChip Fluidic Station 400 and analyzed with the Hewlett Packard Gene Array scanner. Triplicate RNAi experiments for both GFP and Rpn10/S5a and duplicate experiments for both MG132-treated cells and untreated control cells were performed and compared using the Microarray Suite program. The processed microarray data are in the supplementary material, and raw data files are available on request.

Flow cytometry. For cell cycle analysis, *Drosophila* S2 stable cell line fluorescence was measured using a FACScan flow cytometer (Becton Dickinson), while data collection and analysis were performed using the CellQuest software.

Transcription reporter constructs. The transcription reporter vector used to analyze the promoter region was pBSlac20, previously described by Engstrom et al. (10). PCR products corresponding to the upstream promoter regions were generated from genomic DNA from wild-type flies. For each PCR, the 5′ primer contained a SalI cleavage site, while the 3′ primer contained a HindIII cleavage site. The resulting fragment was cloned and ligated into pBSlac20 that was previously digested with SalI and HindIII. The following set of oligonucleotides was used for the transcriptional mapping as shown in Fig. 6: beta 2 5′ SalI, 5′-ACGCGTCGACGTTGACCACATGTGGCTCGGAAACATGG-3′; beta 2 3′ HindIII, 5′-CCCAAGCTTCAAGGAAAACTAAGGTTTTAATTCTGCCGAGCGC-3′; S2 5′ SalI, 5′-ACGCGTCGACGCCATGGATGGCCTTGCATCA

GGCC-3′; S2 3′ HindIII, 5′-CCCAAGCTTCTAGCTTGTCGGACTTAAGGG ATTTAATTGGCACTACG-3′; S2 UTR, 5′-CCCAAGCTTGCAGTGTGACC GCGCGGCGAACG-3′; S2 #1, 5′-ACGCGTCGACCCACAATACCTCGGAT CAAGAGG-3′; S2 #2, 5′-ACGCGTCGACGGAATTACAGCTAATACAACT GC-3′

Transfections of reporter constructs. A total of 6×10^6 *Drosophila* S2 cells were plated onto 5-cm dishes in 5 ml of Schneider's *Drosophila* medium (GIBCO). After 24 h of growth, the cells were transfected using calcium phosphate precipitation. For each transformation, a total of 1 μg of each specific expression plasmid (pBSlac20) was mixed with 100 ng of pAcCAT and 19 μg of carrier DNA. The cells were incubated with the DNA-calcium precipitate for 24 h and then washed three times and allowed to grow in 5 ml of medium. After an additional 24 h, half of the cells were treated with MG132 added directly to the medium at a final concentration of 10 μM. Cells were harvested by centrifugation after 5 h of MG132 treatment, and total RNA was isolated. Cells used for beta-galactosidase assays (10) were saved after 16 h of drug treatment when a total cell extract was generated (31).

Northern slot blots to determine mRNA levels. Total RNA was produced using an RNAeasy kit and QIAshredder (QIAGEN). Generally, different amounts of total RNA ranging from 5 to 1.25 μg were diluted in RNase-free water and blotted onto a nitrocellulose membrane, BrightStar-Plus (Ambion), using a Bio-Rad BioDot slot blot apparatus. After UV cross-linking, the membrane was labeled and washed using the buffers and instructions from the NorthernMax-Gly kit (Ambion). Specifically, the detection for each probe involved incubation overnight with a slot blot membrane in prehybridization buffer, ULTRAhyb (Ambion). For all experiments, the membranes were washed initially with a low-stringency buffer solution (Ambion) at room temperature for 10 min followed by two high-stringency washes (Ambion) at 68°C for 15 min. The slot blots were exposed and detected using a PhosphorImager. The radioactive RNA probes were produced using the Strip-EZ RNA StripAble RNA Probe Synthesis kit (Ambion). T7-tailed template DNA was produced with PCR from *Drosophila* genomic DNA using specific primers for each probe. [α-^{32}P]UTP was incorporated according to the manufacturer's protocol. The different probes were approximately 700 bp in length. Individual membranes were used for multiple probings, and the previous probe was removed using the protocol and solutions provided in the Strip-EZ RNA StripAble RNA Probe Removal kit (Ambion).

Semiquantitative RT-PCR to confirm RNA interference. Total RNA from dsRNA-treated cells was isolated using the RNAeasy kit from QIAGEN. Specific primers covering intron sequence or the same primers used for amplification from genomic DNA were used to amplify mRNA levels using the Promega Access RT-PCR system. PCR products were separated onto agarose gels and compared to molecular weight markers to confirm that the product matched the expected size for spliced mRNA product (see Fig. S1 in the supplemental material).

RESULTS

Concerted expression of proteasome genes through RNAi-mediated knockdown of Rpn10/S5a or MG132 treatment. We sought to confirm the apparent transcriptional up-regulation observed in the *Drosophila* Rpn10/S5a knockdown cells and attempted to determine the range and type of mRNAs that are induced when this proteasome subunit is partially removed. As a control to be able to screen out effects caused by the expected transcriptional changes induced by the RNAi itself, a triplicate set of control cells was treated with double-stranded RNA of the same length and concentration as that used to knock down Rpn10/S5a. The control double-stranded RNA corresponded to the GFP gene.

Out of a possible 14,010 probe sets from the *Drosophila* Affymetrix Gene Chip, the RNA targets identified 5,500 genes (40%) from the S2 *Drosophila* cell line that gave signal hybridization strengths that were significant. Initially, the three similar triplicate samples were evaluated, and these comparisons showed that no significant differences were present. A three-by-three matrix evaluation was then carried out; each Rpn10/S5a RNAi treatment was compared to the three GFP controls, resulting in nine total comparisons. After comparing the GFP and the Rpn10/S5a, the absent and unchanged genes were subtracted, and the average severalfold changes from the nine comparisons were determined. The candidate up-regulated genes were ranked based on this average severalfold increase (Fig. 1A). The top 80 out of 99 genes with mRNA levels increased in a majority of comparisons, at least five of the nine comparisons with a P value of <0.0025, are listed in Tables 1 and 2. The complete processed data and the gene expression matrix are presented in the supplemental material. Overall, the mRNA expression profiles at 3 days posttreatment to knock down the proteasome subunit Rpn10/S5a led to a modest increase in the expression of the mRNAs required to assemble a 26S proteasome (Fig. 1A). Strikingly, proteasome genes represented the majority of mRNAs that showed any detectable increases after RNAi treatment.

To confirm candidates found in the Rpn10/S5a RNAi experiments, duplicates of samples were treated with the proteasome inhibitor MG132 at a concentration of 10 μM for 5 h. Previously, it was determined that 5 h of posttreatment with proteasome inhibitor gave the maximum induction of mRNA levels for *Drosophila* proteasome genes in S2 cells as determined by Northern slot blots of samples at various time points (Fig. 4A). Total RNA from cells treated for 5 h with MG132 was compared to that of cells that had no drug treatment in expression analysis on microarrays. Compared to the RNAi treatment, exposure of *Drosophila* cell lines with the proteasome inhibitor MG132 resulted in larger severalfold inductions of proteasome mRNA levels (Fig. 1B). However, as expected, the inhibition of proteasome activity on cells influenced a wide variety of pathways, and the number of genes that were induced by proteasome inhibition was quite large (Fig. 1B). Yet the MG132 expression results were still found to contain all the reported proteasome family genes as found in the Rpn10/S5a RNAi treatments. In addition, a number of the novel genes discovered in the RNAi experiments were also found to be up-regulated after proteasome inhibitor treatment (Table 2). The majority of 26S proteasome genes showed similar increases for both profiles, with an increase typically approaching twofold for the Rpn10/S5a RNAi-treated cells and three- to fourfold for the MG132 treatments (Fig. 1 and Table 1). While the *Drosophila* genome contains nine additional isoforms of 20S proteasome genes (29, 51), only 14 20S mRNAs, 7 alpha and 7 beta, were found to be up-regulated. Previous studies have shown that the additional isoforms are expressed in a male-specific pattern (29). All of these male-specific 20S proteasome genes were present on the genome microarray but showed no upregulation. For the 19S regulatory complex, 18 mRNAs that matched this complex were found to be up-regulated. For these 18 mRNAs, 16 of the genes match the identical subunits that have been previously identified in a purification and characterization study of the *Drosophila* 19S regulatory complex (19). Unlike the 20S complex that shows up-regulation for only the minimum set required for formation of the proteasome, the 19S regulatory complex had two additional ATPase subunit homologs strongly overexpressed, CG9475 and CG2241. The corresponding protein sequences from CG9475 and CG2241 show high similarity to 19S ATPase base subunits Rpt3/S6 (77% similarity) and Rpt6/S8 (88% similarity), respectively, and have previously been characterized by sequence comparison as homologs to the two base subunits

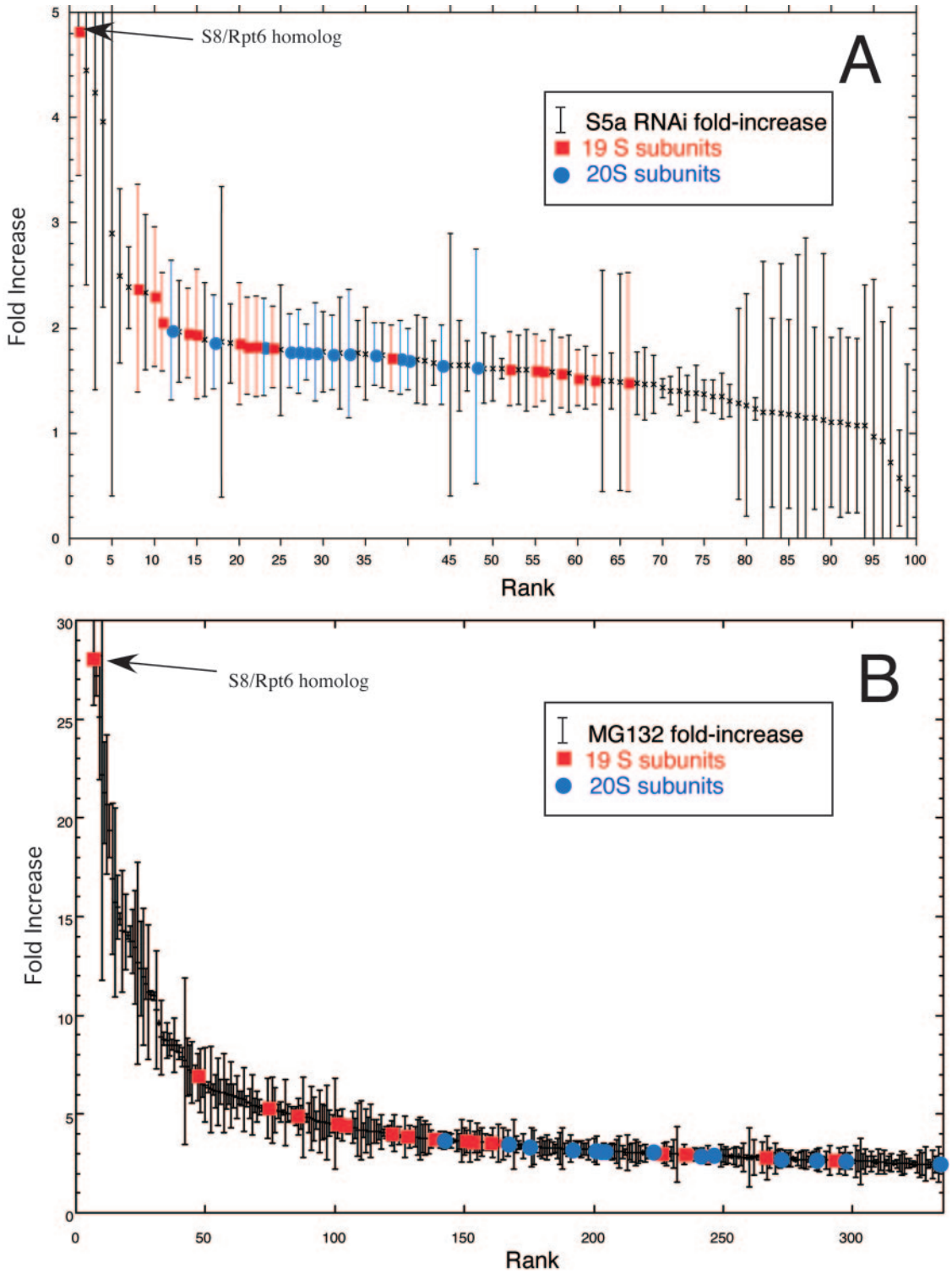

FIG. 1. Transcriptional up-regulation of genes after RNAi-mediated knockdown of proteasome subunit Rpn10/S5a or MG132 treatment. Triplicate S5a RNAi samples were compared to profiles obtained from three independent controls that were treated with dsRNA of GFP. Each S5a RNAi-treated microarray profile was compared to each of the three GFP samples. (A) For each gene, a total of nine comparisons were averaged and used for the ranking shown on the *x* axis. The standard deviations for the nine comparisons are drawn as *y*-axis error bars. Genes that were not up-regulated in a majority of comparisons were excluded from the ranking list. Up-regulated 20S proteasome genes are shown with a blue filled circle, while 19S regulatory complex subunits are marked with a red filled square. (B) Microarray profiles of duplicate samples of MG132-treated cells were compared to those of nontreated cells and graphed according to the same principles described above. The genes included in the graph are all induced with a fold increase higher than that of the lowest 26S subunit.

TABLE 1. mRNA changes for 26S proteasome genes[a]

CG no.	Name or description	RNAi depletion of Rpn10/S5a subunit		MG132 treatment	
		Rank	Increase (fold)	Rank	Increase (fold)
20S Subunits					
CG18495	Alpha-1	26	1.8	223	3.0
CG5266	Alpha-2 (Pros25)	48	1.6	242	2.9
CG9327	Alpha-3 (Pros29)	36	1.8	175	3.4
CG3422	Alpha-4 (Pros28.1)	28	1.8	286	2.7
CG10938	Alpha-5 (ProsMA5)	12	2.0	167	3.5
CG4904	Alpha-6 (Pros35)	23	1.8	142	3.7
CG1519	Alpha-7	33	1.8	333	2.5
CG8392	Beta-1 (1(2)05070)	17	1.9	202	3.2
CG3329	Beta-2 (Prosbeta2)	31	1.8	246	2.9
CG11981	Beta-3 (Prosbeta3)	29	1.8	272	2.7
CG17331	Beta-4	39	1.7	192	3.2
CG12323	Beta-5 (Prosbeta5)	44	1.7	200	3.2
CG4097	Beta-6 (Pros26)	40	1.7	297	2.6
CG12000	Beta-7	27	1.8	205	3.2
19S base subunits					
CG11888	S1, Rpn2 (p110)	38	1.7	86	4.9
CG7762	S2, Rpn1 (p97)	22	1.8	128	3.9
CG5289	S4, Rpt2 (Pros26.4)	56	1.6	151	3.6
CG10370	S6′, Rpt5 (Tbp-1)	62	1.5	160	3.6
CG16916	S6, Rpt3 (p48A)	52	1.6	165	3.5
CG1341	S7, Rpt1 (p48B)	21	1.8	240	3.0
CG1489	S8, Rpt6 (Pros45)	55	1.6	225	3.0
CG3455	S10b, Rpt4 (p42D)	20	1.9	236	3.0
CG7619	S5a Rpn10 (pros54)		−1.8	138	3.8
19S lid subunits					
CG10484	S3, Rpn3 (Dox-A2)	15	1.9	122	4.0
CG1100	p55, Rpn5 (p55)	59	1.6	267	2.8
CG5289	S9, Rpn6 (Pros26.4)	57	1.6	293	2.6
CG5378	S10a, Rpn7 (p42A)	66	1.5	245	2.9
CG10230	S11, Rpn9 (p39A)	11	2.1	75	5.3
CG3416	S12, Rpn8 (Mov34)	14	2.0	102	4.5
CG18174	S13, Rpn11 (37B)	24	1.8	162	3.5
CG4157	S14, Rpn12 (p30)	8	2.4	47	6.9
Recently proposed subunits					
CG5384	Similar to Ubp6p	16	1.9	154	3.6
CG3431	p37A, Uch-L3	60	1.5	139	3.7
CG13779	Similar to Sem1p	46	1.6		<2

[a] RNAi screens were carried out on underlined genes.

and have been proposed to be male-specific expressed genes (11, 29). The Rpt6/S8 homolog had the highest mRNA increase of all genes measured on the Rpn10/S5a RNAi microarray while induced 28-fold after 5 h of proteasome inhibitor treatment (Fig. 1 and Table 2).

As expected, Rpn10/S5a subunit mRNA levels were found to decrease in the three RNAi experiments. The gene was one of three genes that showed decreased levels in all nine comparisons. The other two genes found to be down-regulated in all nine comparisons were the Cyp4ac2 cytochrome P450 subunit gene and the neutral endopeptidase gene. The list for down-regulated genes is presented in the supplemental material.

Up-regulation of proteasome-associated genes and novel candidates. For the Rpn10/S5a RNAi-treated cells, proteasome genes rank from 1 for the proposed Rpt6/S8 homolog to 66 for the Rpn7/S10a subunit. Within this range of 66 genes, a number of genes with similarity to proteasome-associated proteins are present (Table 2). An additional three genes that have recently been proposed to be additional subunits of the 19S complex, Uch-L3, p37A, and Sem1p, were also up-regulated (13, 19).

A *Drosophila* candidate with 22% similarity to the yeast 20S maturation factor UMP1p and 40% similarity to the human UMP1 homolog was identified (34, 47). The valosin-containing protein (VCP) ATPase (TER94, cdc48) and three gene products known to associate with this ATPase, Ufd1, Npl4, and p47 (2), were also found to be up-regulated, and this complex, sometimes termed the segregase, has been implicated in the recognition and presentation of ubiquitinated proteins to the proteasome (1). Recently, the ubiquitin-binding protein sequestosome 1 (SQSTM1/p62) was reported to serve a central role in polyubiquitin chain binding and proteasomal degradation in mammalian cells and *Saccharomyces* (38, 40). No homolog to this gene has been described in *Drosophila*. Our results show induction of a *Drosophila* gene that has similarity

TABLE 2. Genes up-regulated by Rpn10/S5a depletion and comparison with MG132 treatment[a]

CG no.	Name or description	RNAi depletion of Rpn10/S5a subunit		MG132 treatment	
		Rank	Increase (fold)	Rank	Increase (fold)
CG2241	S8, homolog	1	4.8	7	28
CG12493	Double-stranded RNA binding	2	4.4		<2
CG12477	*Similar to makorin 4 ring finger*	3	4.2	109	4.2
CG10630	*Double-stranded RNA binding domain*	4	4.0	24	12.6
CG14027	*TotM*	5	2.9	25	12.3
CG10810	Drosomycin, antifungal peptide	6	2.5	49	6.5
CG14628	Novel, RNA-binding region RNP-1	7	2.4		<2
CG12321	Carcinoma susceptibility protein	9	2.3	48	6.7
CG9475	S6, homolog	10	2.3	104	4.4
CG10360	Similar to sequestosome 1, ref(2)P	13	2.0	16	15.5
CG4463	Hsp23	18	1.9	2	162
CG9324	POMP, 20S maturase	19	1.8	285	2.7
CG18372	*Attacin, anti-microbial*	25	1.8	230	3.0
CG7415	Dipeptidyl-peptidase III	30	1.8	184	3.3
CG7340	Leucyl-aminopeptidase	32	1.8	108	4.3
CG6697	Ub-like domain *ctd*-like phosphatases	34	1.8	185	3.3
CG6776	*glutathione transferase*	35	1.8	11	21.3
CG5495	Tx1 thioredoxin-like	37	1.7	174	3.4
CG1836	Rad23	41	1.7	173	3.4
CG12795	Arsenite induced protein O9JII7 mouse	42	1.7	152	3.6
CG4572	Similar to CPVL carboxypeptidase	43	1.7	460	2.1
CG1667	Novel	45	1.7		<2
CG8209	Novel; Zn-finger, UBA, UBX	47	1.6		<2
CG13914	Novel	49	1.6		<2
CG2046	Novel	50	1.6	239	3.0
CG9828	DnaJ-H proposed cochaperone HSC70	51	1.6	87	4.8
CG13184	Novel BRCT domain	53	1.6		<2
CG11885	Novel	54	1.6	342	2.4
CG2331	TER94, VCP ATPase (p97, cdc48)	58	1.6	368	2.3
CG10245	Cyp6a20 p450	61	1.5	3	62.3
CG5039	Novel	63	1.5	140	3.7
CG4673	NPL4	64	1.5	131	3.8
CG4466	Hsp27	65	1.5	4	62.0
CG13349	Glycoprotein, N terminal, similar to Rpn13	67	1.5	207	3.1
CG3962	*Keap1 molecular sensor for inducers*	68	1.5		<2
CG10602	Leukotriene-A4 hydrolase like	69	1.5		<2
CG11139	p47 cofactor TER94	70	1.4		<2
CG15141	Novel PHD finger, N-recognin Zn finger	71	1.4	322	2.5
CG13122	1D-*myo*-Inositol-trisphosphate-3-kinase	72	1.4		<2
CG12082	Isopeptidase T	73	1.4	157	3.6
CG3356	Similar to HECT E3/Hul5	74	1.4		<2
CG12628	Microsomal glutathione *S*-transferase like	75	1.4		<2
CG4265	Uch-D ubiquitin C-terminal hydrolase	76	1.4		<2
CG5009	Similarity to palmitoyl-CoA oxidase	77	1.4	264	2.8
CG7176	Isocitrate dehydrogenase ($NADP^+$)	78	1.3		<2
CG6233	Ubiquitin fusion degradation, UfD-1 like	79	1.3	385	2.3
CG4454	Novel	80	1.3	403	2.2

[a] The italicized genes were also found to be induced by nonspecific double-stranded RNA to GFP compared to nontreated cells. RNAi was carried out on underlined genes. CoA, coenzyme A; ctd, carboxyl-terminal domain.

(27%) to the sequestosome protein sequence CG10360. This gene contains the conserved ubiquitin-associated (UBA) domains and a specific zinc-binding domain found in the mammalian form. In general, heat shock proteins were not identified as up-regulated genes after Rpn10/S5a RNAi. *Drosophila* HSP23 and HSP27 were exceptions, with significant increases in mRNA levels for six of nine comparisons. Recently, HSP27 has been identified to bind ubiquitin and interact directly with the 26S proteasome (33). A number of genes with RNA binding domains were identified as having increased mRNA levels and ranked high on the overall comparisons (Table 2). Approximately a dozen proteins within the top 80 up-regulated genes have no described function and were classified as novel. The majority of the novel *Drosophila* genes identified show high similarity to an uncharacterized mammalian gene.

RNAi-mediated knockdown of proteasome pathway candidates. To test if the genes that showed concerted up-regulation with the known proteasome genes had essential proteasome functions, RNAi was carried out on a set of these candidates. A total of 15 *Drosophila* genes were selected for RNAi experiments (Tables 1 and 2). To confirm that RNAi of the targeted gene had occurred, semiquantitative RT-PCRs were carried out on the treated cells, and the data are presented in the supplemental material. The majority of genes tested showed a

clear reduction of the targeted mRNA. However, repeated attempts for RNAi against CG4673 (NPL4) and CG6697, a gene containing a Ub-like motif and phosphatase domain, failed to decrease their mRNA levels. The RNAi treatments were specific for decreasing the target mRNA while maintaining the levels of other proteasome genes.

To test the potential functions of the candidate up-regulated genes, immunoblots to examine overall ubiquitin conjugate levels and in vivo proteasome assays using stable cell lines expressing short-lived GFP proteasome substrates were carried out on the RNAi-treated cells (Fig. 2 and 3). Immunoblots from whole-cell lysates were carried out from treated and control cells and probed using ubiquitin antibodies. The in vivo 26S proteasome assay measures the ability of the cells to degrade GFP that has been engineered to act as an N-end rule or ubiquitin fusion degradation (UFD) substrate and was originally tested and described in mammalian cell lines (6). We have recently used the GFP substrates in *Drosophila* stable S2 cell lines to measure in vivo proteasome activity (28). The short-lived GFP reporters used in those studies lack endoplasmic reticulum signal sequences and were observed to be localized to the cytoplasm and nucleus when stabilized through proteasome inhibitor treatment.

The RNAi-mediated inhibition of proteasome subunit Rpn11/S13 and the *Drosophila* candidates POMP, TER94, and UFD1 resulted in the accumulation of high-molecular-weight ubiquitin-conjugated proteins and stabilization of GFP reporters (Fig. 2). As expected, loss of the Rpt6/S8 or Rpt3/S6 19S ATPase subunit led to the appearance of stabilized GFP throughout the cell (Fig. 3). A large fraction of the treated cells depleted of Rpt3/S6 or Rpt6/S8 were found as doublet GFP-positive cells. However, the RNAi knockdown of the Rpt3/S6 and Rpt6/S8 homologs gave no apparent increases in ubiquitin conjugates or GFP levels (data not shown).

TER94, the *Drosophila* VCP ATPase, and CG6233, a Ufd1 gene, showed strong GFP stabilization for both the UFD and N-end rule substrates (Fig. 2C). The knockdown of TER94 yielded the highest percentage of GFP-stabilized cells and the highest levels of fluorescence per cell, even higher than at 7 h of 50 μM MG132 treatment. Cells lacking TER94 were generally found to have GFP stabilized throughout the cell with the greatest accumulations in the nucleus and were also found to contain cytoplasmic vacuoles containing stabilized GFP (Fig. 3).

RNAi against CG10360, which has similarity to sequestosome 1 binding protein, showed no increase in ubiquitin chain levels or GFP stabilization. The other tested candidates (underlined in Table 2) also showed no significant increases in ubiquitin conjugate pools or GFP stabilization after RNAi knockdown (data not shown).

Mapping of *Drosophila* proteasome transcription response elements. The availability of complete sequenced genomes from different *Drosophila* species allowed us to search for conserved regions in proteasome promoters among the different fly species. No obvious conserved motifs were found upstream of the proteasome genes, but almost all genes were found to contain 5′ conserved UTRs. These sites are well conserved within individual proteasome genes for different species. However, only limited conservation exists between different proteasome genes. The conserved areas typically resided directly

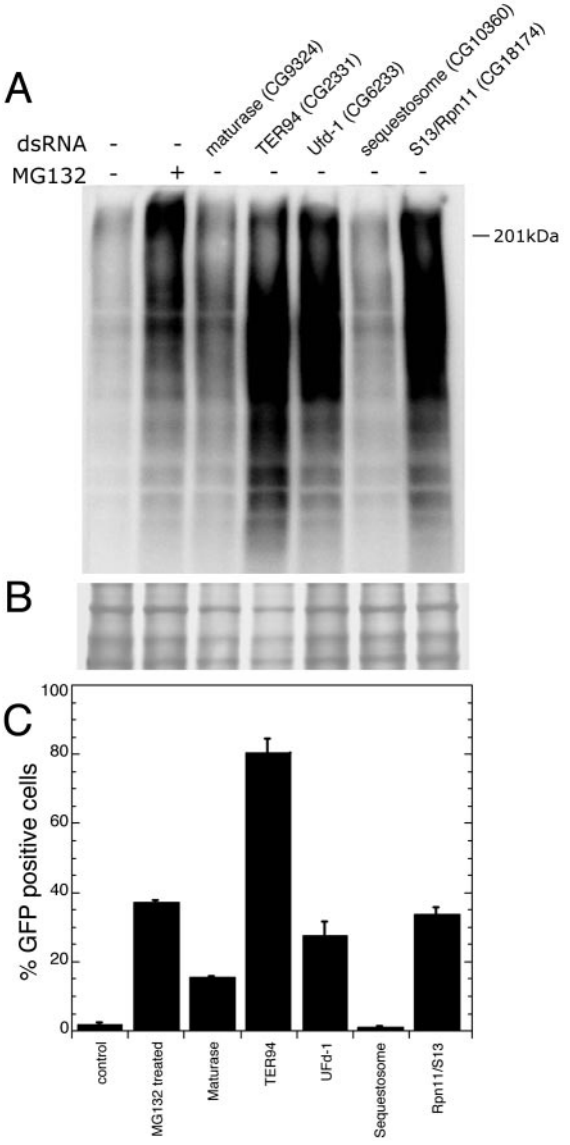

FIG. 2. Ubiquitin conjugate levels and effects on in vivo 26S proteasome substrates after RNAi-mediated knockdown of candidate genes. (A) Western blots of total cell extracts from S2 cells exposed to dsRNA against proposed coregulated genes. The immunoblot was probed with monoclonal ubiquitin antibodies. (B) To confirm that equivalent amounts of total protein were present in each lane, the blot from panel A was Coomassie stained. (C) In vivo 26S activity assays were performed in stable S2 cell lines constitutively expressing short-lived GFPs. Similar overall GFP stabilization profiles were found for both the N-end rule (Fig. 3) and the UFD (Ub-G76V-GFP) stable cell lines. Four days posttreatment, flow cytometric analysis was performed to detect cells positive for GFP. Values are averages of duplicate experiments. As a comparison, cells treated with 50 μM MG132 for 7 h are shown for both assays.

downstream of the proposed start site of transcriptional initiation. Through the use of plasmid-based transcriptional reporter assays, we have examined the importance of these conserved regions. Due to the difficulty in carrying out transcriptional reporter assays on RNAi-treated cells, we chose to focus on the mechanism of proteasome mRNA induction after MG132 treatment. Initially, treatment of *Drosophila* cells

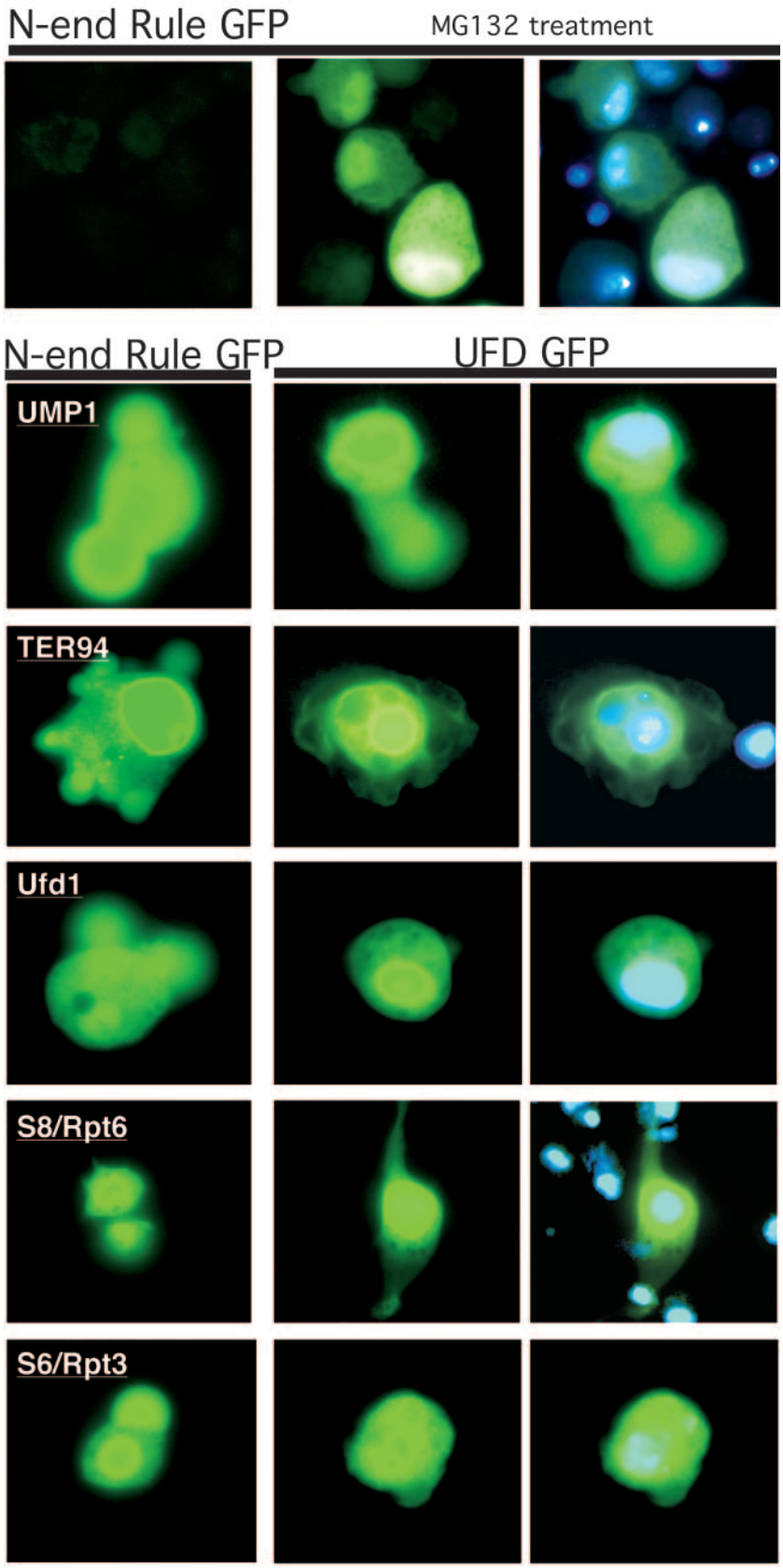

FIG. 3. RNAi-mediated knockdown of candidate genes and cellular localization of in vivo 26S proteasome substrates. Stabilization of the N-end rule (Ub-R-GFP) and UFD (Ub-G76V-GFP) substrates is shown. Three days after RNAi treatment, the locations of stabilized substrates were obtained by live-cell fluorescent imaging. To identify the location of the nucleus, UFD (Ub-G76V-GFP) cells were DNA stained with bisbenzimide H 33342.

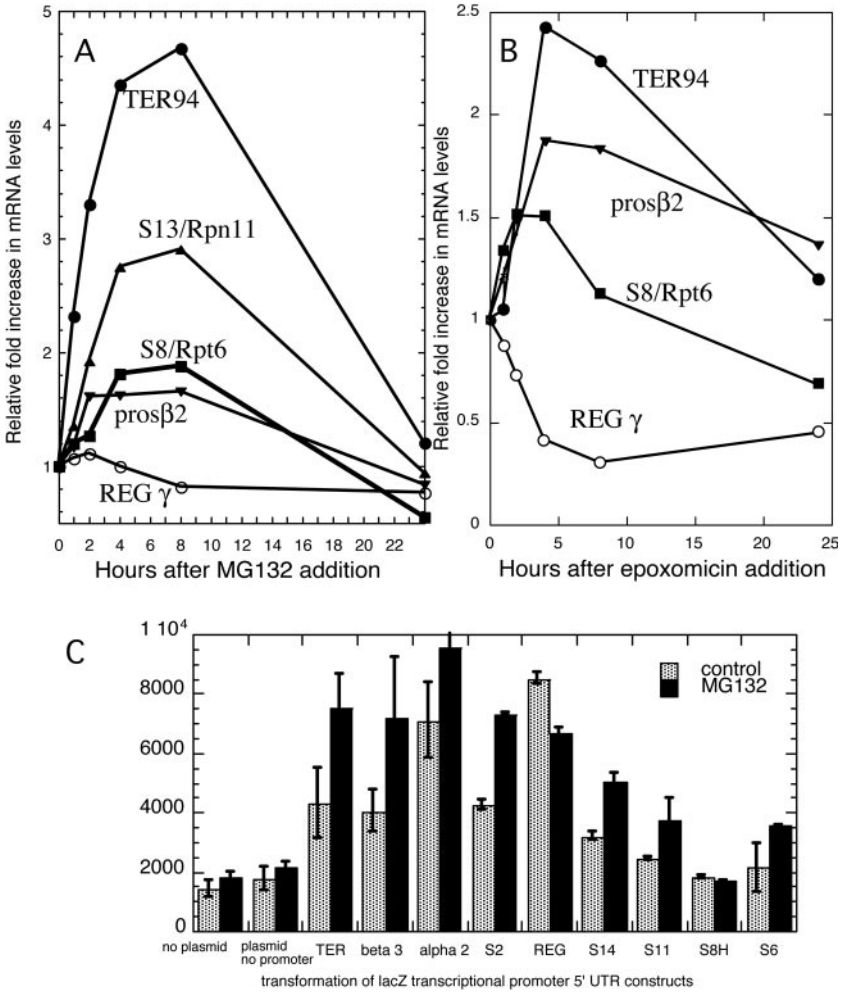

FIG. 4. (A, B) Induction of VCP ATPase (TER94) mRNA after proteasome inhibition follows a similar response curve to that seen with proteasome mRNAs. Total RNA was purified from cells treated with 10 μM MG132 or 5 μM epoxomicin at various time points and blotted onto nylon membrane. Membranes were hybridized with ^{32}P antisense RNA probes specific for each mRNA. The levels of mRNA for different concentrations of total RNA are graphed against the time of treatment. REG proteasome activator was not found to be induced in the genome-wide microarray survey and was therefore used as a comparison. (C) To understand the mechanism of transcriptional induction of proteasome genes, the promoter regions containing 5′ UTRs of 19S and 20S *Drosophila* proteasome genes and REG were cloned into *lacZ* transcriptional promoter constructs and transfected into S2 cells. LacZ mRNA levels were measured using Northern slot blot experiments after 3 days posttransfection in combination with 5 h of treatment with 10 μM proteasome inhibitor MG132.

with 10 μM MG132 or 5 μM epoxomicin revealed increased levels of mRNA for proteasome genes and TER94, with a maximum increase at 5 h (Fig. 4A and B), and these conditions were used for the transcriptional assays.

As an initial step to understand the mechanism of transcriptional induction of proteasome genes, the promoter regions of 19S and 20S *Drosophila* proteasome genes were cloned into *lacZ* transcriptional promoter construct pBSlac20 (10) and transfected into S2 cells. Depending upon the distance between the proteasome gene and the preceding upstream gene, the transcriptional promoter constructs contained from 200 to 1,000 bp of upstream sequence and included the 5′ UTRs of each gene tested. Previously, 5′ rapid amplification of cDNA ends and Northern blots showed that proteasome inhibition did not change the transcriptional start points or overall messenger sizes for proteasome mRNA after inhibitor treatment (data not shown). As a control, the promoter region of the *Drosophila* proteasome activator REG was used. The *Drosophila* PA28 proteasome activator is under the transcriptional control of the DNA replication elements and is not transcriptionally up-regulated in the current microarray experiments. LacZ mRNA was measured using Northern slot blot experiments after 3 days posttransfection in combination with 5 h of treatment with proteasome inhibitor MG132. The promoter constructs contained 5′ UTRs, and all proteasome subunits revealed a transcriptional increase in *lacZ* mRNA levels, while the REG promoter showed no change or a minor decrease (Fig. 4C). These inductions also correspond to increases in protein levels as determined by beta-galactosidase activity (data not shown).

Deletion mapping of upstream promoter regions alpha-2 and Rpn1/S2 was unable to identify MG132 response elements. All deletions in the upstream promoter regions that had remaining basal transcriptional activity were also re-

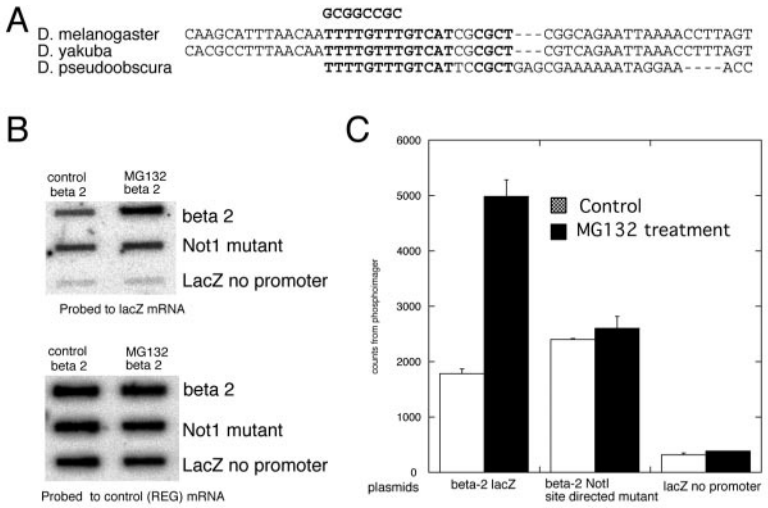

FIG. 5. A conserved sequence in the 5′ UTR of the 20S subunit beta-2 is required for transcriptional upregulation after proteasome inhibition. Plasmids containing the beta-2 proteasome gene promoter region along with the entire 5′ UTR upstream of the *lacZ* open reading frame were transfected and contained either the wild-type 5′ UTR or a NotI site-directed mutant in a conserved sequence region present in three different *Drosophila* species (A). To prevent differences due to various transfection efficiencies, the control and MG132-treated samples originated from the same calcium phosphate transfection for each reporter plasmid tested. After 2 days of recovery, the cells were treated for 5 h with 10 μM MG132 before harvesting and *lacZ* mRNA quantification. Equivalent amounts of total RNA were blotted onto a nylon membrane and probed with a specific ^{32}P probe. (B) Example of Northern slot blots probed for plasmids expressing *lacZ* reporter mRNA or endogenous REG mRNA as a loading control. (C) Quantification of slot blot experiments.

sponsive to MG132 treatment as long as the 5′ UTRs were intact (data not shown). However, site-directed mutations in the conserved region of the 5′ UTR of a beta-2 20S proteasome reporter construct revealed that the conserved region is required for the induction of mRNA (Fig. 5). Placement of a NotI site within the conserved region did not abolish basal transcription levels but did prevent the induction after proteasome inhibition. Mutations in the conserved 5′ UTRs for beta-3 and S14 also inhibited induction of reporter mRNAs (data not shown).

To confirm the importance of the 5′ UTR of the Rpn1/S2, the 5′ UTR was swapped into the control REG promoter construct that previously did not respond to proteasome inhibition (Fig. 6A). The REG promoter with the Rpn1/S2 5′ UTR was found to be responsive for inhibition of the proteasome after MG132 treatment (Fig. 6B).

To confirm these results, the Rpn1/S2 5′ UTR was mapped by the insertion of HindIII restriction sites throughout the 5′ UTR (Fig. 7A). Transcriptional assays were initially carried out on the original HindIII site-directed mutants (Fig. 7B). The reporter constructs were then digested with HindIII enzyme and religated, giving a series of deletion constructs. These deletions were also assayed for their ability to respond to proteasome inhibition by MG132 (Fig. 7C). A 67-nucleotide region of the S2 5′ UTR was sufficient to respond to proteasome inhibitor treatment. This area corresponds to a highly conserved region directly downstream of the site of transcriptional initiation.

DISCUSSION

Little is known about the regulation of the metazoan proteasome pathway. Under conditions where additional proteasome proteolytic activity is required, a regulatory mechanism that provides both adequate and balanced levels of proteasome subunits and ancillary proteins must exit. A number of proteins that interact with the 26S proteasome have recently been identified (27) but were originally overlooked due to disassociation from the protease during purification. In this study, we have tried to identify genes that are part of a metazoan proteasome regulatory network to find proteins that are functionally important for the proteasome pathway.

Previously, we carried out RNAi against two *Drosophila* 19S proteasome subunits, Rpn10/S5a and Rpn11/S13 (28). Loss of either subunit results in the induction of 20S proteasomes. Other groups have reported similar findings with the loss of other 19S subunits and demonstrated that the loss of a specific 19S subunit increased the mRNA levels for fellow subunits (48). While the Rpn10/S5a subunit has recently been shown to be essential for the development of *Drosophila* (41), our work on *Drosophila* cells lacking Rpn10/S5a revealed almost no defects except for a modest increase in ubiquitin conjugate chains. In contrast, knockdown of essential proteasome subunits markedly affects proteasome activity and leads to a wide range of cellular responses.

The loss of the proteasome Rpn10/S5a subunit results in increased levels of both 20S and 19S subunits. The observed increased levels were modest, with less than a twofold increase for the majority of proteasome genes. Yet the increased levels are significant and result in a list of induced genes that is abundant in proteasome subunit genes. As a comparison, a previous study that examined proteasome inhibitor induction of subunit concentrations revealed a two- to threefold increase at the protein level (32). The induction levels observed likely underestimate the actual severalfold increase of mRNA induction possible after the complete loss of the Rpn10/S5a subunit. Unlike MG132 treatments, the RNAi effect on *Drosophila* cells

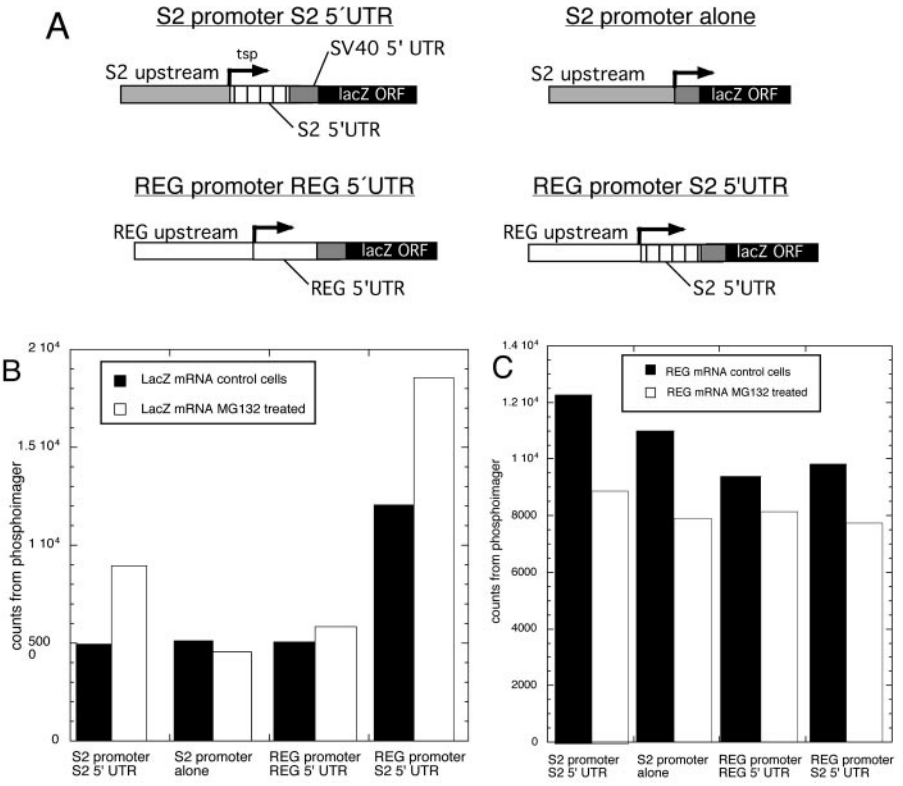

FIG. 6. The S2 5′ UTR region is essential and sufficient for mRNA induction after proteasome inhibition. For each transfection, the calcium DNA solution was divided equally between two identical cell culture plates. One plate served as a control while the other plate was treated with MG132. (A) Schematic of the promoter-5′ UTR constructs generated and tested for transcriptional up-regulation. All constructs contained a short simian virus 40 5′ UTR directly upstream of the *lacZ* open reading frame. *lacZ* mRNA of plasmid-expressed *lacZ* mRNA (B) and endogenous REG mRNA as a control (C) are quantified.

occurs over a long time period, 3 days, and therefore, it is likely that a proportion of the Rpn10/S5a RNAi-treated cells had proteasome induction occur prior to the harvesting of mRNAs. We consider that the very high density of known proteasome genes present on the microarray ranking list (Fig. 1A) is the best evidence that knockdown of Rpn10/S5a provides valuable data to identify new members. The obtained low increases do make it likely that a number of genes that are in fact coregulated with the proteasome will be missed in this survey due to the lack of sensitivity in detecting up-regulation. As an example, the Ecm29 gene has been characterized to have a role in proteasome-dependent degradation (22). However, we were unable to find any change in Ecm29 mRNA levels after S5a RNAi treatment even though up-regulation (3.6-fold) was observed after proteasome inhibition with MG132.

During the preparation of the manuscript, a number of coregulated genes (Table 2) have shown relevance for the proteasome pathway in other model systems. The *Drosophila* gene CG13779 has 35% identity to the small protein Sem1p, a recently proposed 26S proteasome subunit from *Saccharomyces cerevisiae* (40), and has 60% identity to the proposed human protein homolog DSS1. Surprisingly, MG132-treated cells showed no observed increases in the *Drosophila* Sem1p mRNA. Another candidate, CG10360, shows similarity to a human protein that has recently been found to bind polyubiquitin and contribute to proteasome degradation, the sequestosome 1/p62 (38). While lower in overall identity (27%), both proteins share positionally conserved dystrophin-like zinc-binding domains and UBA domains. A recent study using RT-PCR quantification of proteasome inhibitor-treated cells revealed that the mammalian version of the 20S proteasome maturase was transcriptionally induced after inhibitor treatment (32). Our results confirm a metazoan coregulation between proteasome genes and the 20S maturase (CG9324). In addition, the concerted up-regulation of p37A/Uch-L3 (CG3431) and a gene with high sequence similarity to Ubp6p (CG5384) lends support to the idea that these two conserved proteins are actual proteasome subunits that easily dissociate from the complex during purification (27).

Recent interest has focused on proteins that assist in the transfer of ubiquitinated proteins to the 26S proteasome (1). Our results support a central role for the segregase complex (24) in proteasome-dependent degradation. These results indicate conserved regulation between metazoan and *Saccharomyces cerevisiae*. The yeast homolog of VCP, cdc48, is transcriptionally coregulated with proteasome subunits and has a PACE element that interacts with the yeast Rpn4 transcription factor (23). Past studies have implicated VCP in an extremely wide range of cellular functions, from fusion of the endoplasmic reticulum to degradation of IκBα (5, 26). For the current microarray data, the VCP ATPase (CG2331), Npl4 (CG4673), and Ufd-1 (CG6233) genes of the segregase complex (2) were found to be consistently up-regulated. RNA interference was successful against two of the genes, the VCP ATPase and

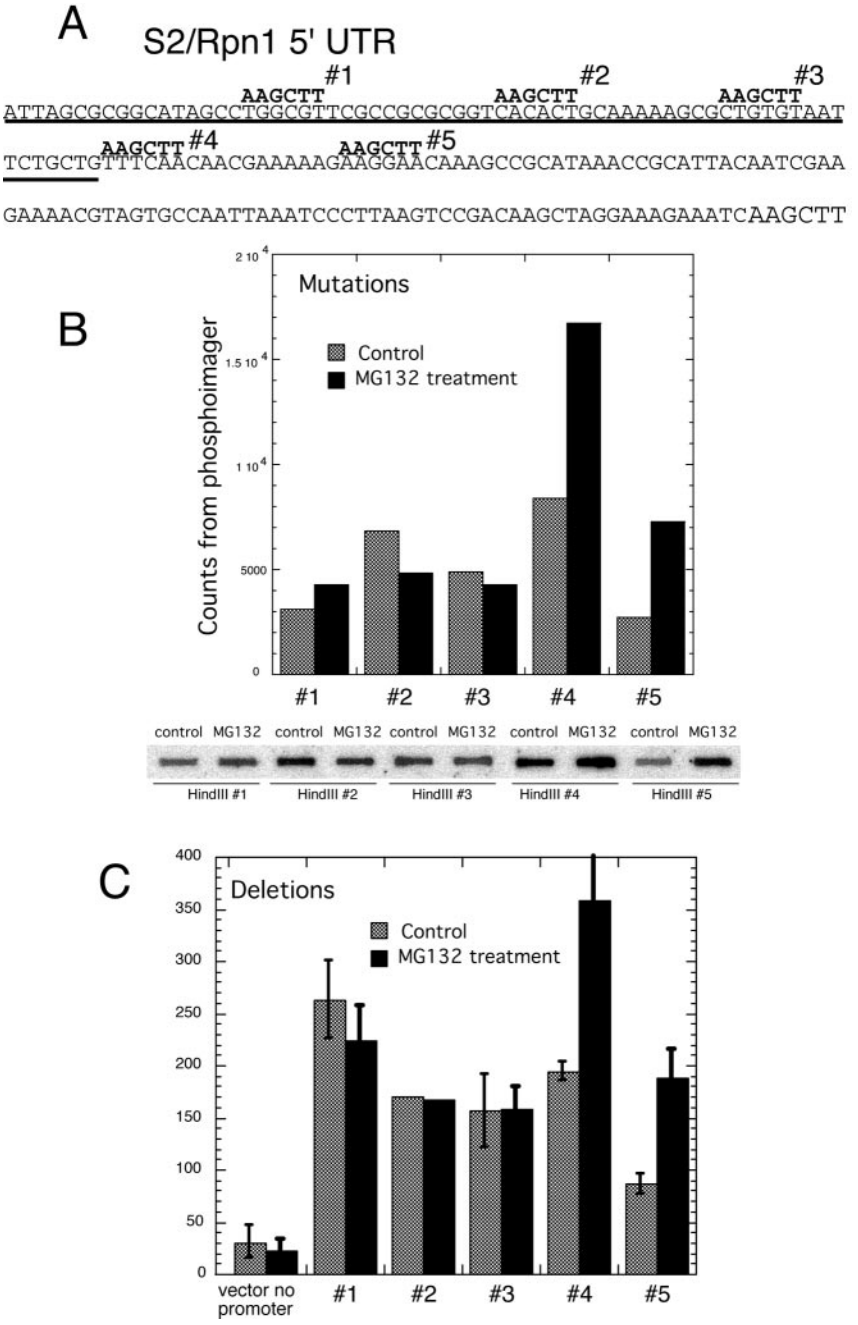

FIG. 7. (A) Mutational and deletional mapping of the Rpn1/S2 5′ UTR. Transfections were performed as described in the legend of Fig. 5. (A) Boldface type shows the position of HindIII. #1 to #5, site-directed mutations within the conserved region of the S2/Rpn1 5′ UTR. For all constructs, a HindIII site was also present between the 5′ UTR and the *lacZ* open reading frame. (B) Inducibility of HindIII site-directed mutant reporter plasmids after proteasome treatment. Northern slot blots of total RNA purified from control and MG132-induced transformations are shown. The slot blot was probed with an antisense RNA probe of the *lacZ* open reading frame. 5′ UTR deletions were generated by digestion and religation between the corresponding HindIII site-directed mutation and the HindIII site present between the 5′ UTR and the *lacZ* ORF and used for transfection studies.

Ufd-1, resulting in stabilization of overall ubiquitin conjugates and in vivo GFP substrates. This confirms recent results showing that RNAi knockdown of the VCP ATPase stabilized ubiquitin conjugates in both *Drosophila* and HeLa cell lines (49). RNAi against the *Drosophila* VCP and Ufd-1 shows surprisingly strong stabilizations of both UFD and N-end rule substrates throughout the cell. This is in contrast to the yeast Ufd1 that is required only for UFD substrate degradation and was found to be nonessential for N-end rule substrate proteolysis (21).

The microarray profiling revealed that two homologs of ATPase subunits, Rpt6/S8 and Rpt3/S6, were strongly up-regulated. Both ATPase homologs have been previously proposed to be male-specific expressed homologs; however, little is

known about their potential function (29). Previous 19S *Drosophila* proteasome purification studies did not identify these homologs as significant components of the 19S regulatory complex (19). In this study, RNAi treatment against the homologs showed no increase in overall ubiquitin chain levels or loss of proteasome activity (data not shown). In comparison, RNAi knockdown of either bona fide ATPase subunit resulted in increased ubiquitin conjugates and stabilization of 26 proteasome subunits in vivo. Currently, it is unclear how the ATPase homologs participate and function within the proteasomal pathway, but it is clear that they are coregulated with other proteasome genes and expressed in *Drosophila* cell lines.

Little is known about potential downstream members of the proteasome pathway. The 26S proteasome degrades proteins preferentially to small peptides (9), and additional proteases are required for the complete hydrolysis of substrates to individual amino acids. A number of peptidases were found to be up-regulated in our microarray analysis. A proposed carboxypeptidase (CG4572), a leucyl-aminopeptidase (CG7340), and dipeptidyl-peptidase III (CG7415) were all found to be up-regulated and may indicate a functional interaction with the proteasome in the downstream processing of short peptides to amino acids.

To start addressing the mechanism of proteasome mRNA induction after a loss of proteasome activity, we have tested promoter reporter plasmids using sequences directly upstream of 20S and 19S proteasome open reading frames. As expected, induction of the reporters was observed after proteasome inhibition. Alignments of upstream promoter regions have so far failed to reveal conserved DNA sequence motifs. However, when individual proteasome genes are compared across different *Drosophila* species, conserved regions are present in the vast majority of genes just downstream of the site of transcriptional initiation in the 5′ UTRs. Removal of 5′ UTRs from promoter constructs eliminates the previously observed induction after proteasome inhibition. Site-directed mutagenesis mapping of the Rpn1/S2 5′ UTR confirms that the conserved region in the 5′ UTR is essential for up-regulation of *Drosophila* proteasome genes. Finally, transfer of the proteasome subunit Rpn1/S2 5′ UTR to a noninducible control promoter was sufficient to confer inducibility after proteasome inhibition of the reporter plasmid. These initial results indicate that an element(s) within the 5′ UTR of *Drosophila* proteasome genes is essential for the cell to regulate proteasome levels. Sequence comparisons of the 5′ UTRs show no obvious consensus sequence for all regulated genes. This suggests that either a number of distinct factors interact with a variety of conserved sites or the sites reflect a folded element on the 5′ end of the newly synthesized mRNA. Future studies to determine what factor(s) is interacting with the 5′ UTRs at either the DNA or RNA level should greatly aid in understanding metazoan proteasome regulation. Our results demonstrate that the concerted expression of proteasome genes includes both 26S proteasome subunit genes and functionally related genes. Future use of bioinformatic tools and promoter mapping studies should allow further characterization of the system that globally regulates proteasome levels in higher eukaryotes.

ACKNOWLEDGMENTS

We thank Nico Dantuma and Maria Masucci for the original short-lived GFP vectors and Jenni Reimari for generating site-directed mutants of S2 promoter constructs.

This work was supported by grants from the Swedish Research Council and the Carl Tryggers Stiftelse.

REFERENCES

1. **Bays, N. W., and R. Y. Hampton.** 2002. Cdc48-Ufd1-Npl4: stuck in the middle with Ub. Curr. Biol. **12:**R366–R371.
2. **Braun, S., K. Matuschewski, M. Rape, S. Thoms, and S. Jentsch.** 2002. Role of the ubiquitin-selective CDC48$^{UFD1/NPL4}$ chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J. **21:**615–621.
3. **Chu-Ping, M., J. H. Vu, R. J. Proske, C. A. Slaughter, and G. N. DeMartino.** 1994. Identification, purification, and characterization of a high molecular weight, ATP-dependent activator (PA700) of the 20 S proteasome. J. Biol. Chem. **269:**3539–3547.
4. **Clemens, J. C., C. A. Worby, N. Simonson-Leff, M. Muda, T. Maehama, B. A. Hemmings, and J. E. Dixon.** 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA **97:**6499–6503.
5. **Dai, R. M., E. Chen, D. L. Longo, C. M. Gorbea, and C. C. Li.** 1998. Involvement of valosin-containing protein, an ATPase co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. J. Biol. Chem. **273:**3562–3573.
6. **Dantuma, N. P., K. Lindsten, R. Glas, M. Jellne, and M. G. Masucci.** 2000. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat. Biotechnol. **18:**538–543.
7. **DeMartino, G. N., C. R. Moomaw, O. P. Zagnitko, R. J. Proske, M. Chu-Ping, S. J. Afendis, J. C. Swaffield, and C. A. Slaughter.** 1994. PA700, an ATP-dependent activator of the 20 S proteasome, is an ATPase containing multiple members of a nucleotide-binding protein family. J. Biol. Chem. **269:**20878–20884.
8. **Deveraux, Q., V. Ustrell, C. Pickart, and M. Rechsteiner.** 1994. A 26 S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. **269:**7059–7061.
9. **Emmerich, N. P., A. K. Nussbaum, S. Stevanovic, M. Priemer, R. E. Toes, H. G. Rammensee, and H. Schild.** 2000. The human 26 S and 20 S proteasomes generate overlapping but different sets of peptide fragments from a model protein substrate. J. Biol. Chem. **275:**21140–21148.
10. **Engstrom, Y., L. Kadalayil, S. C. Sun, C. Samakovlis, D. Hultmark, and I. Faye.** 1993. kappa B-like motifs regulate the induction of immune genes in Drosophila. J. Mol. Biol. **232:**327–333.
11. **Frohlich, K. U.** 2001. An AAA family tree. J. Cell Sci. **114:**1601–1602.
12. **Fu, H., N. Reis, Y. Lee, M. H. Glickman, and R. D. Vierstra.** 2001. Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. **20:**7096–7107.
13. **Funakoshi, M., X. Li, I. Velichutina, M. Hochstrasser, and H. Kobayashi.** 2004. Sem1, the yeast ortholog of a human BRCA2-binding protein, is a component of the proteasome regulatory particle that enhances proteasome stability. J. Cell Sci. **117:**6447–6454.
14. **Ghislain, M., A. Udvardy, and C. Mann.** 1993. S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature **366:**358–362.
15. **Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley.** 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell **94:**615–623.
16. **Glickman, M. H., D. M. Rubin, H. Fu, C. N. Larsen, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, R. Vierstra, W. Baumeister, V. Fried, and D. Finley.** 1999. Functional analysis of the proteasome regulatory particle. Mol. Biol. Rep. **26:**21–28.
17. **Gordon, C., G. McGurk, P. Dillon, C. Rosen, and N. D. Hastie.** 1993. Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. Nature **366:**355–357.
18. **Groll, M., L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H. D. Bartunik, and R. Huber.** 1997. Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature **386:**463–471.
19. **Holzl, H., B. Kapelari, J. Kellermann, E. Seemuller, M. Sumegi, A. Udvardy, O. Medalia, J. Sperling, S. A. Muller, A. Engel, and W. Baumeister.** 2000. The regulatory complex of Drosophila melanogaster 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme. J. Cell Biol. **150:**119–130.
20. **Hough, R., G. Pratt, and M. Rechsteiner.** 1986. Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. J. Biol. Chem. **261:**2400–2408.
21. **Johnson, E. S., P. C. Ma, I. M. Ota, and A. Varshavsky.** 1995. A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. **270:**17442–17456.
22. **Kajava, A. V., C. Gorbea, J. Ortega, M. Rechsteiner, and A. C. Steven.** 2004.

New HEAT-like repeat motifs in proteins regulating proteasome structure and function. J. Struct. Biol. **146:**425–430.
23. **Kapranov, A. B., M. V. Kuriatova, O. V. Preobrazhenskaia, V. V. Tiutiaeva, R. Shtuka, H. Feldmann, and V. L. Karpov.** 2001. [Isolation and identification of PACE-binding protein rpn4—a new transcription activator, participating in regulation of 26S proteosome and other genes.] Mol. Biol. (Moscow) **35:**420–431. (In Russian.)
24. **Koller, K. J., and M. J. Brownstein.** 1987. Use of a cDNA clone to identify a supposed precursor protein containing valosin. Nature **325:**542–545.
25. **Kostova, Z., and D. H. Wolf.** 2003. For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. EMBO J. **22:**2309–2317.
26. **Latterich, M., K. U. Frohlich, and R. Schekman.** 1995. Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. Cell **82:**885–893.
27. **Leggett, D. S., J. Hanna, A. Borodovsky, B. Crosas, M. Schmidt, R. T. Baker, T. Walz, H. Ploegh, and D. Finley.** 2002. Multiple associated proteins regulate proteasome structure and function. Mol. Cell **10:**495–507.
28. **Lundgren, J., P. Masson, C. Realini, and P. Young.** 2003. The use of RNA interference and complementation to study the function of the *Drosophila* and human 26S proteasome subunit S13. Mol. Cell. Biol. **23:**5320–5330.
29. **Ma, J., E. Katz, and J. M. Belote.** 2002. Expression of proteasome subunit isoforms during spermatogenesis in Drosophila melanogaster. Insect Mol. Biol. **11:**627–639.
30. **Mannhaupt, G., R. Schnall, V. Karpov, I. Vetter, and H. Feldmann.** 1999. Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett. **450:**27–34.
31. **Masson, P., J. Lundgren, and P. Young.** 2003. Drosophila proteasome regulator REGgamma: transcriptional activation by DNA replication-related factor DREF and evidence for a role in cell cycle progression. J. Mol. Biol. **327:**1001–1012.
32. **Meiners, S., D. Heyken, A. Weller, A. Ludwig, K. Stangl, P. M. Kloetzel, and E. Kruger.** 2003. Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of mammalian proteasomes. J. Biol. Chem. **278:**21517–21525.
33. **Parcellier, A., E. Schmitt, S. Gurbuxani, D. Seigneurin-Berny, A. Pance, A. Chantome, S. Plenchette, S. Khochbin, E. Solary, and C. Garrido.** 2003. HSP27 is a ubiquitin-binding protein involved in I-κBα proteasomal degradation. Mol. Cell. Biol. **23:**5790–5802.
34. **Ramos, P. C., J. Hockendorff, E. S. Johnson, A. Varshavsky, and R. J. Dohmen.** 1998. Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. Cell **92:**489–499.
35. **Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg.** 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78:**761–771.
36. **Rubin, D. M., M. H. Glickman, C. N. Larsen, S. Dhruvakumar, and D. Finley.** 1998. Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. EMBO J. **17:**4909–4919.
37. **Saville, K. J., and J. M. Belote.** 1993. Identification of an essential gene, l(3)73Ai, with a dominant temperature-sensitive lethal allele, encoding a Drosophila proteasome subunit. Proc. Natl. Acad. Sci. USA **90:**8842–8846.
38. **Seibenhener, M. L., J. R. Babu, T. Geetha, H. C. Wong, N. R. Krishna, and M. W. Wooten.** 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol. Cell. Biol. **24:**8055–8068.
39. **Smyth, K. A., and J. M. Belote.** 1999. The dominant temperature-sensitive lethal DTS7 of Drosophila melanogaster encodes an altered 20S proteasome beta-type subunit. Genetics **151:**211–220.
40. **Sone, T., Y. Saeki, A. Toh-e, and H. Yokosawa.** 2004. Sem1p is a novel subunit of the 26 S proteasome from Saccharomyces cerevisiae. J. Biol. Chem. **279:**28807–28816.
41. **Szlanka, T., L. Haracska, I. Kiss, P. Deak, E. Kurucz, I. Ando, E. Viragh, and A. Udvardy.** 2003. Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in Drosophila melanogaster. J. Cell Sci. **116:**1023–1033.
42. **Ulrich, H. D.** 2002. Natural substrates of the proteasome and their recognition by the ubiquitin system. Curr. Top. Microbiol. Immunol. **268:**137–174.
43. **Unno, M., T. Mizushima, Y. Morimoto, Y. Tomisugi, K. Tanaka, N. Yasuoka, and T. Tsukihara.** 2002. The structure of the mammalian 20S proteasome at 2.75 Å resolution. Structure **10:**609–618.
44. **van Nocker, S., S. Sadis, D. M. Rubin, M. Glickman, H. Fu, O. Coux, I. Wefes, D. Finley, and R. D. Vierstra.** 1996. The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. **16:**6020–6028.
45. **von Arnim, A. G.** 2001. A hitchhiker's guide to the proteasome. Sci. STKE **2001:**PE2.
46. **Walz, J., A. Erdmann, M. Kania, D. Typke, A. J. Koster, and W. Baumeister.** 1998. 26S proteasome structure revealed by three-dimensional electron microscopy. J. Struct. Biol. **121:**19–29.
47. **Witt, E., D. Zantopf, M. Schmidt, R. Kraft, P. M. Kloetzel, and E. Kruger.** 2000. Characterisation of the newly identified human Ump1 homologue POMP and analysis of LMP7(beta 5i) incorporation into 20 S proteasomes. J. Mol. Biol. **301:**1–9.
48. **Wojcik, C., and G. N. DeMartino.** 2002. Analysis of Drosophila 26 S proteasome using RNA interference. J. Biol. Chem. **277:**6188–6197.
49. **Wojcik, C., M. Yano, and G. N. DeMartino.** 2004. RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis. J. Cell Sci. **117:**281–292.
50. **Xie, Y., and A. Varshavsky.** 2001. RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc. Natl. Acad. Sci. USA **98:**3056–3061.
51. **Yuan, X., M. Miller, and J. M. Belote.** 1996. Duplicated proteasome subunit genes in Drosophila melanogaster encoding testes-specific isoforms. Genetics **144:**147–157.
52. **Zwickl, P., E. Seemuller, B. Kapelari, and W. Baumeister.** 2001. The proteasome: a supramolecular assembly designed for controlled proteolysis. Adv. Protein Chem. **59:**187–222.