

# Expression of proteasome subunit isoforms during spermatogenesis in *Drosophila melanogaster*

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## Abstract

**In this study, we sought to identify and characterize all the proteasome genes of *Drosophila melanogaster*. Earlier work led to the identification of two genes encoding  $\alpha$ 4-type 20S proteasome subunit isoforms that are expressed exclusively in the male germline. Here we extend these results and show that six of the 20S proteasome subunits, and four of the 19S regulatory cap subunits, have gene duplications encoding male-specific isoforms. More detailed analyses of two of these male-specific subunits (Pro $\alpha$ 3T and Pro $\alpha$ 6T), using GFP-tagged reporter transgenes, revealed that they are predominantly localized to the nucleus at later stages of spermatogenesis and are present there in mature, motile sperm. These results suggest a possible role of a 'spermatogenesis-specific' proteasome in sperm differentiation and/or function.**

**Keywords:** male-specific gene expression, gene duplication, sperm nucleus, regulated proteolysis

## Introduction

In eukaryotes, the regulated proteolysis of most cytosolic and nuclear proteins occurs via the ubiquitin/proteasome pathway (for review, see Hershko & Ciechanover, 1998). In this pathway, a protein is selected for destruction by the covalent attachment of a small, well-conserved polypeptide called ubiquitin (Wilkinson, 2000). Through a sequence of

reactions catalysed by enzymes called E1, E2 and E3, a polyubiquitin chain is added to the targeted protein, making it a substrate for a large, multisubunit protease called the 26S proteasome (Baumeister *et al.*, 1998). This remarkable 'proteolytic machine' is made up of two major components: a 20S core particle, and 19S regulatory 'caps' at each end. The structure of the 20S proteasome is known in great detail and is highly conserved across many kingdoms of life. Structural studies indicate that the 20S proteasome is a hollow, barrel-shaped cylinder made up of four stacked rings of seven subunits each (Gröll *et al.*, 1997). Each 20S proteasome comprises seven different  $\alpha$ -type and seven distinct  $\beta$ -type subunits, in an  $(\alpha 1-7)(\beta 1-7)(\beta 1-7)(\alpha 1-7)$  arrangement. The endopeptidase activities of the proteasome are conferred by three of the  $\beta$ -type subunits, with the  $\alpha$ -type subunits having a structural role, and possibly a regulatory function through their direct interactions with components of the 19S cap.

The 19S cap can be dissociated into two subcomplexes, the 'base' and the 'lid' (Glickman *et al.*, 1998). The base consists of a ring of six AAA-family ATPases and three other non-ATPase subunits. Among the likely functions of the base are: (1) opening the gated channel leading into the 20S core, (2) unfolding the substrate protein via a reverse chaperone activity, and (3) threading the unfolded polypeptide through the open channel into the core particle (Braun *et al.*, 1999; Glickman, 2000). The approximately eight subunits of the lid have not been well-defined in terms of their exact functions, but among them are one or more subunits that can bind polyubiquitin, and one or more deubiquitinating enzymes (Hözl *et al.*, 2000).

By controlling the rapid and irreversible turnover of key regulatory proteins, the ubiquitin/proteasome pathway has been shown to play important roles in a variety of processes, including cell cycle progression (Hoyt, 1997), transcriptional regulation (Desterro *et al.*, 2000), cell fate determination (Huang *et al.*, 1995; Schweisguth, 1999), long-term memory (Hegde *et al.*, 1997), circadian rhythms (Naidoo *et al.*, 1999), and programmed cell death (Grimm & Osborne, 1999). This pathway also carries out an important 'housekeeping' function, by ridding cells of potentially harmful abnormal proteins that arise as the result of

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mutation, translational errors, misfolding, or postsynthetic damage. In addition to its role as a protease that completely destroys proteins, the proteasome has also been shown to act in the proteolytic processing of a few regulatory proteins. For example, the NF $\kappa$ B transcription factor, a key regulator of the mammalian inflammatory response, is synthesized as an inactive 105 kDa precursor protein. Following its ubiquitination, it is partially degraded by the proteasome from its C-terminus to yield a 50 kDa active form (Palombella *et al.*, 1994).

One major question in proteasome research concerns whether there exist proteasome subtypes that carry special subunit isoforms that modify the functional properties of proteasomes in certain cells. In yeast, 20S proteasomes exist as a homogeneous population, each composed of the same 14 subunits (seven  $\alpha$ -type and seven  $\beta$ -type), and 14 corresponding genes have been identified in the yeast genome database. Similarly, database searches of the *Caenorhabditis elegans* genome also identify only 14 20S subunit genes, suggesting that in this species, 20S core particles are structurally uniform. In mammals there is at least one situation in which specialized 20S proteasomes containing alternative subunits occur: during the antiviral immune response,  $\gamma$ -interferon induces the synthesis of three new  $\beta$ -type subunits,  $\beta$ 1i,  $\beta$ 2i and  $\beta$ 5i, that replace the catalytic  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5 subunits, respectively (Rock & Goldberg, 1999). These reconfigured 'immunoproteasomes' have altered proteolytic properties that are more efficient at producing peptide antigens for MHC Class I-mediated antigen presentation.

In *Arabidopsis thaliana* there are at least 13  $\alpha$ -type and 10  $\beta$ -type 20S proteasome subunit genes (Fu *et al.*, 1998a), suggesting that there might be a structural heterogeneity of 20S proteasomes in that species. However, nothing is known about the functional differences, if any, between the different subunit isoforms, nor is it known if these genes are expressed in a cell type- or developmental stage-specific manner. At least five subunits of the 19S cap (Rpt1, Rpt2, Rpt4, Rpt5 and Rpt6) are each encoded by duplicate genes, although, again, it is not known if there are functional or cell-type specific differences between the two isoforms (Fu *et al.*, 1999).

To further investigate the extent and nature of proteasome structural heterogeneity, and to begin to examine its possible functional significance, we sought to identify and characterize all the proteasome genes in *Drosophila melanogaster*. Earlier studies led to the identification of two new  $\alpha$ 4-type subunit genes that are expressed exclusively in the male germline (Yuan *et al.*, 1996). In the present study, we extend these results and show that six of the 20S proteasome subunits, and four of the 19S regulatory cap subunits, likewise have gene duplications encoding male-specific isoforms. More detailed analyses of two of these male-specific isoforms, using GFP-tagged reporter transgenes,

reveal their expression and subcellular localization patterns, and suggest the possible role of a 'spermatogenesis-specific' proteasome in sperm differentiation and/or function.

## Results

### Identification of proteasome subunit genes of *D. melanogaster*

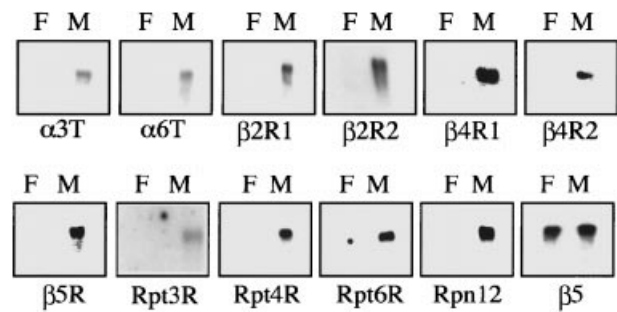
A major goal of this study was to identify and characterize all the proteasome subunit genes of *D. melanogaster*. Previous work, carried out by P. Kloetzel and colleagues (Humboldt University, Berlin) and by our group, focused on the 20S core particle genes, and involved a combination of approaches including screening embryonic cDNA expression libraries with antiproteasome antibodies (Haass *et al.*, 1989, 1990a,b; Frentzel *et al.*, 1992; Seelig *et al.*, 1993), PCR amplification of embryonic cDNAs using degenerate primers (Zaiss & Belote, 1997), and positional cloning of proteasome mutants (Saville & Belote, 1993; Smyth & Belote, 1999). These experiments resulted in the cloning and characterization of the *Prosa2* (also known as *Pros25*), *Prosa3* (*Pros29*), *Prosa4* (*Pros28.1*), *Prosa5* (*PSMA5*), *Prosa6* (*Pros35*), *Pros $\beta$ 2* (*DTS7*), and *Pros $\beta$ 6* (*DTS5*) genes. Later, with the partial sequencing of a large collection of *Drosophila* embryonic cDNAs by the Berkeley *Drosophila* Genome Project (i.e. the *Drosophila* EST database, <<http://www.fruitfly.org>>), sequences corresponding to the remaining 20S subunits were identified (*Pros1*, *Prosa7*, *Pros $\beta$ 1*, *Pros $\beta$ 3*, *Pros $\beta$ 4*, *Pros $\beta$ 5* and *Pros $\beta$ 7*). All 14 genes were mapped by *in situ* hybridization to widely scattered sites in the genome, and Northern blot analyses showed that they were all expressed at high levels at many stages of development (K.A. Smyth, J. Zeng, E. Katz and J.M. Belote, unpublished data). In the three cases where mutants were available (*Pros $\beta$ 2*, *Pros $\beta$ 5* and *Pros $\beta$ 6*) it was seen that these represent essential genes, with the lethal period of the null mutants occurring during the first larval instar (Saville & Belote, 1993; Smyth & Belote, 1999; J.M. Belote, unpublished data).

Previous studies of the *Prosa4* subunit gene, in which a reduced stringency hybridization screen of a genomic library was carried out, showed that there exist two additional genes, *Prosa4T1* (or *Pros28.1A*) and *Prosa4T2* (*Pros28.1B*), that encode related isoforms of this subunit and that are expressed in a testis-specific manner (Yuan *et al.*, 1996). These testis-specific genes are unlinked to the constitutively expressed *Prosa4* gene and their products share a 74% and 54% amino acid identity, respectively, with *Prosa4*. One major question concerns whether this situation of multiple proteasome subunit isoforms encoded by separate genes is unique, or whether other subunits also have developmentally regulated (i.e. testis-specific) isoforms. Since this class of proteasome genes would likely

have been missed by the experimental approaches described above, a series of reduced stringency hybridization screens of a recombinant  $\lambda$ -phage genomic library were begun using cDNA probes corresponding to each known proteasome gene, starting with the seven  $\alpha$ -type subunit genes. The conditions used were stringent enough to prevent spurious hybridization of the probes to unrelated sequences, but relaxed enough to allow the detection of related genes (see Experimental procedures for details). For *Prosa1*, *Prosa2*, *Prosa5* and *Prosa7*, no additional genes were discovered, since all of the clones that were isolated corresponded to the same gene that was used as the probe. In contrast, when *Prosa3* was used as a probe, two classes of phage clones were isolated. One type strongly hybridized to the probe, and was found to correspond to the *Prosa3* gene itself (by restriction mapping, *in situ* mapping and DNA sequencing). The other category of recombinant phage showed weak hybridization signals and mapped to a site unlinked to *Prosa3*. DNA sequencing revealed that this new gene, called *Prosa3T* (GENBANK accession no. AY147240), encoded a *Prosa3*-related subunit that shared a 58% amino acid identity with *Prosa3*. Similarly, when the *Prosa6* cDNA was used as a probe, two types of phage were isolated. One strongly hybridizing class of phage corresponded to the *Prosa6* gene itself (as determined by restriction mapping, *in situ* mapping and DNA sequencing), while the other more weakly hybridizing class of phage carried a *Prosa6*-related gene, called *Prosa6T* (GENBANK accession no. AY147241), that mapped to a different locus, and that shared a 64% amino acid identity with *Prosa6*.

During the course of these screens, the complete *Drosophila* genome sequence was released (Adams *et al.*, 2000; Rubin *et al.*, 2000; <<http://www.celera.com>>), obviating the need to carry out similar screens with the  $\beta$ -type subunit genes. A search of the *Drosophila* genome sequence database confirmed our experimental results with the  $\alpha$ -type subunit genes (i.e. that there are four  $\alpha$ -type subunit isoforms in addition to the seven previously identified constitutively expressed  $\alpha$ -type subunits), and they further revealed that there exist five additional  $\beta$ -type subunit genes: two new *Prosb2*-related genes, two new *Prosb4*-related genes, and one new *Prosb5*-related gene. The genomic sequence data also provided information about the map positions of these genes, and confirmed our experimentally deduced map positions of the proteasome genes mentioned above. Table 1 summarizes this information about the 23 *D. melanogaster* 20S proteasome genes.

The genomic sequence data also made it possible to identify additional genes encoding subunits of the proteasome's 19S regulatory particle. Direct experimental evidence had previously led to the identification of 18 subunits of the 19S cap structure (Haracska & Udvardy, 1995; Mounkes & Fuller, 1998; Cheng *et al.*, 1998; Hölzl *et al.*,



**Figure 1.** Northern blots of adult female (F) and male (M) RNA showing male-specific transcripts for the isoforms listed in Tables 1 and 2. Each blot was subsequently hybridized with a probe corresponding to a constitutive proteasome gene (e.g. *Prosb5*), or the ribosomal protein gene *rp49* (not shown), to confirm equal loading of male and female RNA.

2000). The existence of an additional closely related form of *Rpt6* (also known as *Dug* or *Prosa45*) was suspected, based on *in situ* hybridization results that showed two sites of hybridization with the *Rpt6* cDNA probe (Cheng *et al.*, 1998). A search of the *Drosophila* genome sequence database confirmed the existence of the second *Rpt6*-related gene, and revealed three other new genes that apparently encode isoforms of the Rpt3, Rpt4 and Rpn12 subunits. Table 2 lists these genes, with their corresponding map positions and degrees of amino acid similarity.

#### *Male-specific expression patterns of proteasome subunit isoform genes*

The results described above suggest that six subunits of the 20S proteasome (*Prosa3*, *Prosa4*, *Prosa6*, *Prosb2*, *Prosb4* and *Prosb5*) and four subunits of the 19S regulatory cap (*Rpt3*, *Rpt4*, *Rpt6* and *Rpn12*) are represented by two (or in some cases three) related isoforms, each encoded by a separate gene. Previous studies of the *Prosa4*-related isoforms showed that in that case, one form (i.e. the 'house-keeping' form) was expressed throughout development in multiple tissues, whereas the other two were expressed only in the testis (Yuan *et al.*, 1996). One obvious question is 'Does this property of testis-specific expression hold true for any of the additional proteasome subunit isoforms listed in Tables 1 and 2?' As a first step toward answering this, Northern blot analyses were done, using RNA extracted from sexed adult flies. In these experiments, the probes were synthesized from restriction fragments or PCR products that corresponded to poorly conserved 5' or 3' regions of the genes of interest, so that cross-hybridization to the related isoforms' transcripts was not a problem. As shown in Fig. 1, in every case where a proteasome subunit was represented by more than one gene, the additional gene(s) was expressed in a male-specific manner, similar to what had been seen for the *Prosa4* gene family. Prolonged exposure failed to detect any signal in the lanes corresponding to

**Table 1.** 20S proteasome subunit genes of *D. melanogaster*

Constitutive 'housekeeping' form	Synonym(s) CG no.*	Map position	Reference†	Additional isoform(s)	Synonym(s) CG no.	Map position	% amino acid identity‡	Expression pattern	Reference
<i>Prosa1</i>	CG18495	43E (2R)	1	–	–	–	–	–	–
<i>Prosa2</i>	<i>Pros25</i> , CG5266	87B (3R)	2	–	–	–	–	–	–
• <i>Prosa3</i>	<i>Pros29</i> , CG9327	57B (2R)	3	<i>Prosa3T</i>	CG1736	100B (3R)	58%	male-specific§	1
• <i>Prosa4</i>	<i>Pros28.1</i> , CG3422	14B (X)	4	<i>Prosa4T1</i> <i>Prosa4T2</i>	<i>Pros28.1A</i> , CG17268 <i>Pros28.1B</i> , CG4569	92F (3R) 60D (2R)	74% 54%	male-specific§ male-specific§	9 9
<i>Prosa5</i>	PSMA5, CG10938	54C (2R)	5	–	–	–	–	–	–
• <i>Prosa6</i>	<i>Pros35</i> , CG4904	31C (2L)	6	<i>Prosa6T</i>	CG5648	34B (2L)	64%	male-specific§	1
<i>Prosa7</i>	CG1519	46BC (2R)	1	–	–	–	–	–	–
<i>Prosβ1</i>	CG8392	52E (2R)	1	–	–	–	–	–	–
• <i>Prosβ2</i>	<i>DTS7</i> , CG3329	71A (3L)	7	<i>Prosβ2R1</i> <i>Prosβ2R2</i>	CG18341 CG12161	5B (X) 82F (3R)	62% 35%	male-specific¶ male-specific¶	1 1
<i>Prosβ3</i>	CG11981	85C (3R)	1	–	–	–	–	–	–
• <i>Prosβ4</i>	CG17331	36A (2L)	1	<i>Prosβ4R1</i> <i>Prosβ4R2</i>	CG17301 CG17302	23A (2L) 23A (2L)	43% 55%	male-specific¶ male-specific¶	1 1
• <i>Prosβ5</i>	CG12323	47B (2R)	1	<i>Prosβ5R</i>	CG9868	59D (2R)	53%	male-specific¶	1
<i>Prosβ6</i>	<i>DTS5</i> , l(3)73Ai	73AB (3L)	8	–	–	–	–	–	–
<i>Prosβ7</i>	CG12000	83A (3R)	1	–	–	–	–	–	–

• = Subunits with more than one isoform.

\*This is the gene name that can be found in the GADFLY Annotation Database <<http://hedgehog.lbl.gov:8001/cgi-bin/annot/query/>>.

†(1) This study, (2) Seelig *et al.* (1993), (3) Haass *et al.* (1990a), (4) Haass *et al.* (1990b), (5) Zais & Belote (1997), (6) Haass *et al.* (1989), (7) Smyth & Belote (1999), (8) Saville & Belote (1993), (9) Yuan *et al.* (1996).

‡Sequence comparison between the male-specific isoform and the corresponding 'housekeeping' form.

§Expressed in the testes during the mid- to late stages of spermatogenesis.

¶Tissue-specific expression pattern not determined.

**Table 2.** 19S regulatory cap genes of *D. melanogaster*

Constitutive 'housekeeping' form	Synonym(s) CG no.*	Map position	Reference†	Additional isoform	Synonym CG no.	Map position	% amino acid identity‡	Expression pattern	Reference
<i>Rpt1</i>	p48B, CG1341	43E (2R)	1	–	–	–	–	–	–
<i>Rpt2</i>	p56, S4, CG5289	95C (3R)	1	–	–	–	–	–	–
• <i>Rpt3</i>	p48A, CG16916	10B (X)	1	<i>Rpt3R</i>	CG9475	85E (3R)	77%	male-specific§	7
• <i>Rpt4</i>	p42D, CG3455	5E (X)	1	<i>Rpt4R</i>	CG7257	68E (3L)	82%	male-specific§	7
<i>Rpt5</i>	p50, TBP-1, CG10370	95B (3R)	1	–	–	–	–	–	–
• <i>Rpt6</i>	Pros45, Dug, p42C, CG1489	19F (X)	1, 2, 3	<i>Rpt6R</i>	CG2241	99F (3R)	88%	male-specific§	3, 7
<i>Rpn1</i>	p97, CG7762	76D (3L)	1	–	–	–	–	–	–
<i>Rpn2</i>	p110, CG11888	99A (3R)	1	–	–	–	–	–	–
<i>Rpn3</i>	p58, Dox-A2, CG10484	37B (2L)	1, 4	–	–	–	–	–	–
<i>Rpn5</i>	p55, CG1100	83C (3R)	1	–	–	–	–	–	–
<i>Rpn6</i>	p42B, CG10149	51C (2R)	1	–	–	–	–	–	–
<i>Rpn7</i>	p42A, CG5378	94B (3R)	1	–	–	–	–	–	–
<i>Rpn8</i>	p39B, Mov34, CG3416	83C (3R)	1, 5	–	–	–	–	–	–
<i>Rpn9</i>	p39A, CG10230	60C (2R)	1	–	–	–	–	–	–
<i>Rpn10</i>	p54, CG7619	78E (3L)	1, 6	–	–	–	–	–	–
<i>Rpn11</i>	37B, CG18174	25C (2L)	1	–	–	–	–	–	–
• <i>Rpn12</i>	p30, CG4157	73A (3L)	1	<i>Rpn12R</i>	CG11552	71A (3L)	49%	male-specific§	7

• = Subunits with more than one isoform.

\*This is the gene name that can be found in the GADFLY Annotation Database <<http://hedgehog.lbl.gov:8001/cgi-bin/annot/query/>>.

†(1) Hölzl *et al.* (2000), (2) Mounkes & Fuller (1998), (3) Cheng *et al.* (1998), (4) Pentz & Wright (1991), (5) Gridley *et al.* (1990), (6) Haracska & Udvardy (1995), (7) This study.

‡Sequence comparison between the male-specific isoform and the corresponding 'housekeeping' form.

§Tissue-specific expression pattern not determined.

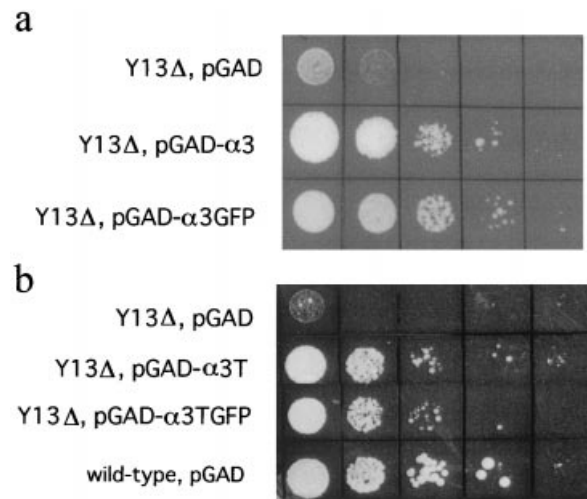
female RNA. The levels of expression of the male-specific genes, as deduced from the exposure times of the autoradiographs, were much lower than that of the non-sex-specific 'housekeeping' forms which are abundantly expressed.

*Construction of GFP-tagged Pro $\alpha$ 3 and Pro $\alpha$ 3T transgenes and complementation of Y13 $\Delta$ , a yeast  $\alpha$ 3 proteasome mutant*

Yuan *et al.* (1996) used *lacZ* reporter genes to investigate the tissue-specific expression patterns of the Pro $\alpha$ 4-related isoforms, Pro $\alpha$ 4T1 and Pro $\alpha$ 4T2. These experiments demonstrated that both of these male-specific isoforms are expressed in the male germline during the mid- to late stages of spermatogenesis. The Northern blot results described above, showing that all of the extra, non-constitutive proteasome genes exhibit male-specific transcription, suggest that the spermatogenic expression pattern of the Pro $\alpha$ 4-related subunits might be a general feature of proteasome subunit isoforms. To investigate whether any other male-specific proteasome genes are expressed during spermatogenesis, a more detailed examination of the Pro $\alpha$ 3-related isoforms was conducted.

In these experiments, both Pro $\alpha$ 3 and Pro $\alpha$ 3T were tagged at their C-termini with the green fluorescent protein (GFP) and constructs containing a few kb of upstream and downstream regulatory sequences were introduced into the *Drosophila* genome as transgenes. GFP-tagged reporters are superior to the *lacZ* reporters used for the Pro $\alpha$ 4 experiments in that the *lacZ* moiety ( $\beta$ -galactosidase) is a very large protein that most likely interferes with the ability of the fusion to become incorporated into the proteasome particle, and so its behaviour, stability and localization might not reflect that of the native protein. GFP is much smaller than  $\beta$ -galactosidase, and experiments in yeast indicate that GFP-tagged  $\alpha$ -type subunits can function well *in vivo* (Enenkel *et al.*, 1998).

Before using the GFP-tagged transgenes to examine the expression of Pro $\alpha$ 3 and Pro $\alpha$ 3T, it was important to know if the fusion proteins exhibited reasonably normal function *in vivo*. Ideally, this would involve testing the tagged genes for their ability to rescue the mutant phenotypes of Pro $\alpha$ 3 or Pro $\alpha$ 3T null mutants in *Drosophila*. Unfortunately, no such *Drosophila* mutants exist, so as a second-best approach a yeast complementation experiment was carried out. For this experiment, the *S. cerevisiae* strain Y13 $\Delta$  which carries a deletion of the  $\alpha$ 3 gene (Emori *et al.*, 1991), was used. This mutant is viable, but it is highly sensitive to the amino acid analogues canavanine and *p*-fluorophenylalanine (Emori *et al.*, 1991; Fu *et al.*, 1998a,b). For this experiment, the *Drosophila* coding regions, either normal or GFP-tagged, were cloned into the yeast expression vector pGAD and introduced into Y13 $\Delta$  cells by transformation. The plasmid carrying either Pro $\alpha$ 3 or



**Figure 2.** Complementation of the amino acid growth sensitivity of the yeast  $\alpha$ 3 proteasome mutant Y13 $\Delta$  by normal and GFP-tagged *Drosophila* orthologs. Serial dilutions of the Y13 $\Delta$  strain transformed with (a) pGAD vector only, pGAD/Pro $\alpha$ 3, or pGAD/Pro $\alpha$ 3-GFP; (b) pGAD vector only, pGAD/Pro $\alpha$ 3T or pGAD/Pro $\alpha$ 3T-GFP. The bottom dilution series is wild-type yeast transformed with pGAD. Cells were grown for 6–7 days at 30 °C in SD leucine<sup>-</sup> medium containing the amino acid analogues canavanine and *p*-fluorophenylalanine.

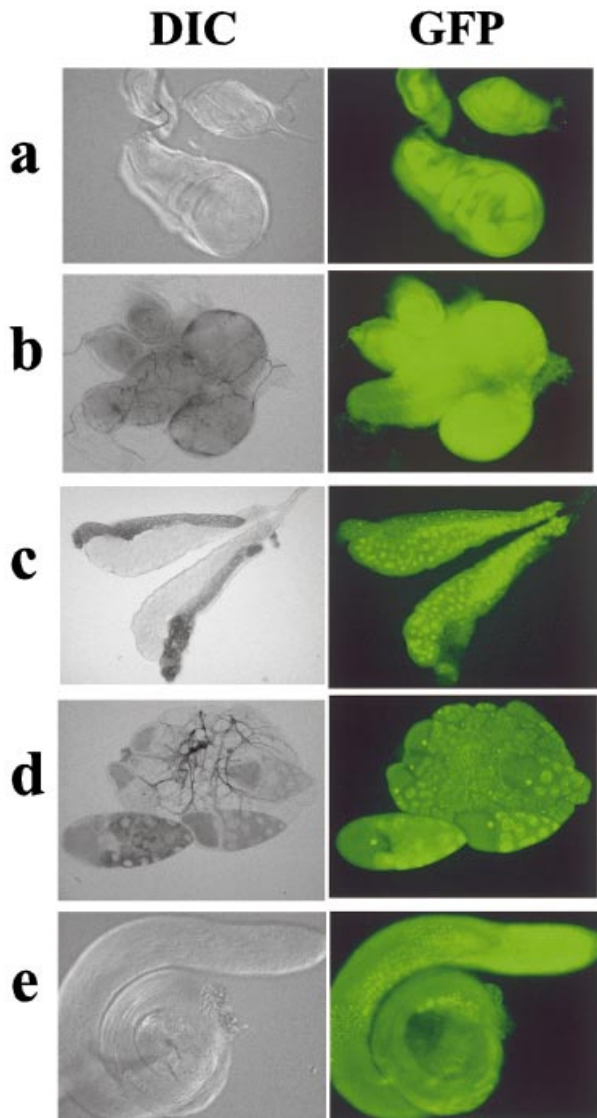
Pro $\alpha$ 3-GFP can rescue the amino acid analogue sensitivity of the Y13 $\Delta$  strain (Fig. 2a). Similar rescue is seen with plasmids carrying either the Pro $\alpha$ 3T or Pro $\alpha$ 3T-GFPORF (Fig. 2b). These results support the idea that both Pro $\alpha$ 3T-GFP and Pro $\alpha$ 3-GFP are capable of being incorporated into the proteasome complex and can function reasonably well as *bona fide* proteasome subunits.

The GFP-tagged proteasome subunit genes were introduced into the fly genome using *P*-element transformation, and fluorescence microscopy used to examine their expression and subcellular localization patterns during development.

*Expression pattern of GFP-tagged Pro $\alpha$ 3*

As expected, the Pro $\alpha$ 3-GFP subunit was detected in all tissues examined, including imaginal discs, brain, salivary glands, ovary and testis (Fig. 3). Consistent with other studies of both yeast and vertebrate proteasomes (Reits *et al.*, 1997; Enenkel *et al.*, 1998; Wilkinson *et al.*, 1998), the signal was seen in both the cytoplasm and the nucleus.

To date, there have been no reports about when the maternal contribution of a proteasome subunit is depleted and when the zygotic proteasome gene becomes expressed. The Pro $\alpha$ 3-GFP transgenic flies provide an opportunity to look at this. To examine the timing of the depletion of the maternal Pro $\alpha$ 3 contribution, virgin females carrying one copy of the Pro $\alpha$ 3-GFP gene, heterozygous over a balancer chromosome, were crossed to males carrying no transgene. Offspring not carrying the transgene showed a significant level of Pro $\alpha$ 3-GFP



**Figure 3.** Expression patterns of the *Proα3-GFP* transgene in different tissues. (a) imaginal discs, (b) brain and attached leg discs, (c) salivary glands, (d) ovary, (e) testis. DIC, differential interference contrast; GFP, green fluorescent protein fluorescence.

fluorescence throughout the embryonic stages and into the first instar larval stage. By the second instar larval stage, however, this maternally contributed *Proα3-GFP* signal was essentially gone. To examine the timing of the zygotic activation of the *Proα3* gene, males homozygous for the *Proα3-GFP* transgene were crossed to females lacking the transgene, and the resulting embryos examined for GFP fluorescence. In this cross, any appearance of the GFP signal would have to be derived from zygotic expression of the paternally contributed *Proα3-GFP* transgene. Here, it was observed that there was little, if any, GFP fluorescence apparent in embryos or first instar larvae, but during the second instar larval stage the signal became

prominently expressed in many tissues, in individuals carrying the transgene.

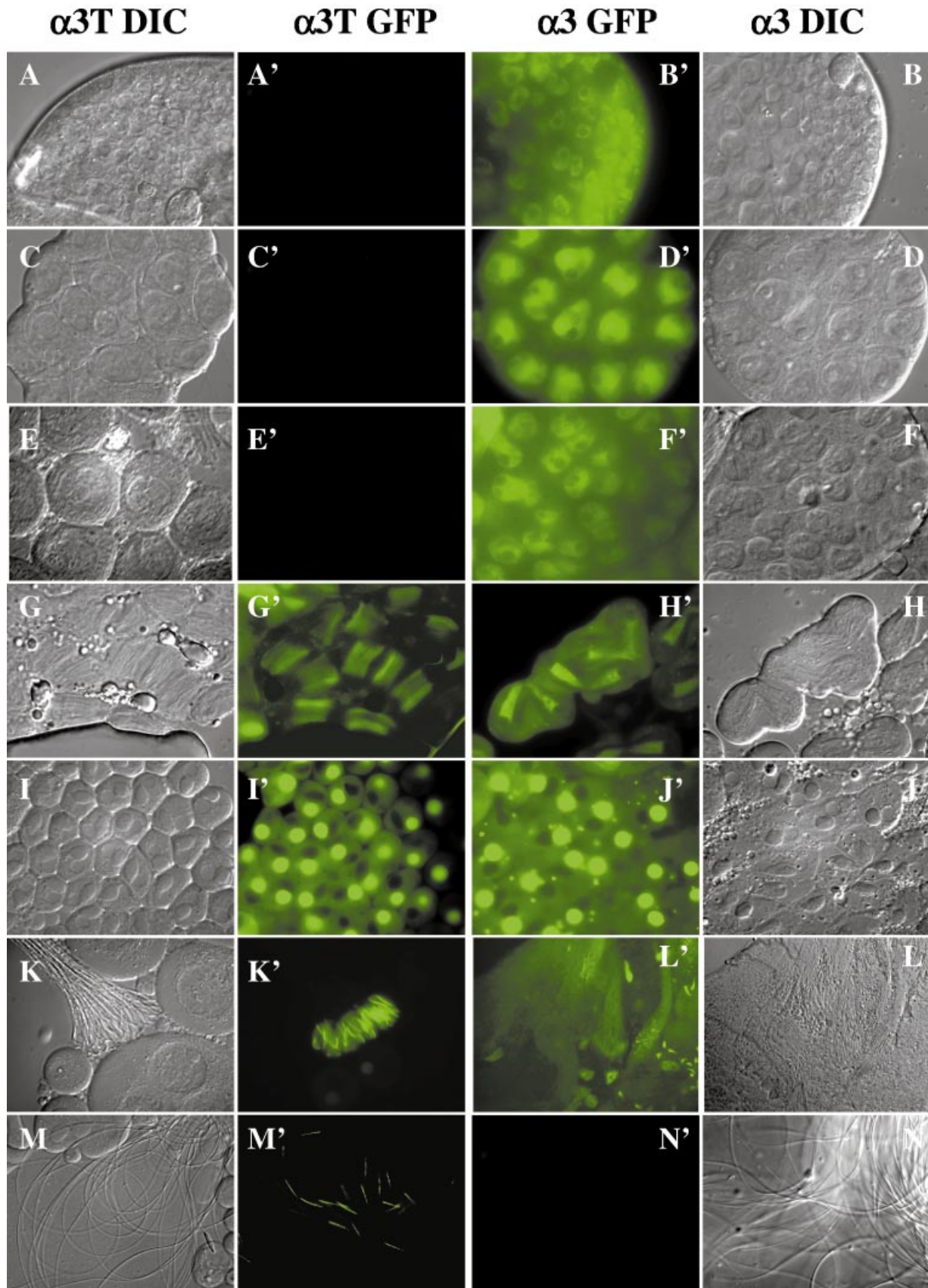
#### *Spermatogenic expression pattern of GFP-tagged Proα3T*

For *Proα3T-GFP*, there was no signal detected in any tissue except the testis. Moreover, the expression pattern of *Proα3T-GFP* in the testis was strikingly different from that of *Proα3-GFP* (Fig. 4). *Proα3-GFP* expression is clearly seen in the early spermatogonial stages, the primary spermatocytes, meiotic cells, secondary spermatocytes, 64 haploid spermatids, and elongating spermatids (Fig. 4). The green fluorescence was detected in both the cytoplasm and the nucleus before meiosis (Fig. 4B', D' and F'). After meiosis, at the 64-cell cyst stage, there was a strong nuclear signal and prominent punctate spots in the more weakly fluorescing cytoplasm (Fig. 4J'). The nuclear signal then faded as the spermatid nuclei elongate and condense (Fig. 4L'). In mature sperm, the green signal was only seen along the sperm tail as a faint fluorescence (not visible in Fig. 4N').

In contrast, the signal from *Proα3T-GFP* was undetectable during the early stages of spermatogenesis, and only became prominent after meiosis II (Fig. 4). The expression of *Proα3T-GFP* first appeared in late phases of the second meiotic division and was expressed at a relatively low level (note that in Fig. 4G' the exposure time for this stage was fivefold longer than for other stages). After meiosis, *Proα3T-GFP* was predominantly localized to the haploid spermatid nuclei of the 64-cell cysts (Fig. 4I'), and the nuclear signal remained during the elongation and condensation of the spermatid nuclei (Fig. 4K'). In mature, motile sperm, the green fluorescence of *Proα3T-GFP* was seen exclusively in the sperm head (Fig. 4M').

#### *Nuclear co-localization of a 19S cap proteasome subunit with Proα3T-GFP*

Because previous studies on mammalian sperm have shown that they contain an abundance of proteasome particles (Tipler *et al.*, 1997; Wojcik *et al.*, 2000), it seems likely that the *Proα3T* subunits present in *Drosophila* sperm nuclei are incorporated into proteasomes. If so, one would expect to find other known components of proteasomes in sperm nuclei. To investigate this, immunofluorescence staining was used to examine the expression and subcellular localization of the proteasome subunit Rpt3, a highly conserved component of the 19S cap structure of the 26S proteasome. For this experiment, a commercially available rabbit polyclonal antiserum that reacts with the *Drosophila* Rpt3 proteasome subunit (anti-TBP7 antiserum) was used to immunostain dissected testes. The results showed that this proteasome subunit was found throughout the testes, and most importantly, it was seen to be present in the nuclei of elongating and mature spermatids. The coincident



nuclear localization of this 19S cap subunit and Pro $\alpha$ 3T-GFP suggested that there are 26S proteasomes in the developing sperm nuclei, and that these proteasomes contain the Pro $\alpha$ 3T subunit.

#### Spermatogenic expression of GFP-tagged Pro $\alpha$ 6T

A second male-specific proteasome  $\alpha$ -type subunit gene, Pro $\alpha$ 6T, was also tagged at the end of the coding region with GFP and introduced into the genome as a transgene. As was seen for Pro $\alpha$ 3T-GFP, the expression of Pro $\alpha$ 6T-GFP was limited to the testes. Examination of dissected testes showed that the expression pattern was very similar to that of Pro $\alpha$ 3T-GFP. That is, GFP fluorescence was not detected in premeiotic cells but became prominent in the cytosol and nuclei of the 64-cell spermatid cysts. As the spermatids elongated, the GFP signal remained in the condensing sperm nuclei and was present there in mature, motile sperm. In this case there was also detectable fluorescence distributed along the sperm tail bundles.

#### Discussion

Using a combination of genomic library screens and computer database searches, we have identified the genes encoding subunits of the *D. melanogaster* 26S proteasome. An important finding was that many of the subunits appear to be represented by more than one gene, with one corresponding to a constitutively expressed 'housekeeping' form, and the other(s), in every case, being expressed in a male-specific manner. Of the 14 20S proteasome subunits, six (Pro $\alpha$ 3, Pro $\alpha$ 4, Pro $\alpha$ 6, Pro $\beta$ 2, Pro $\beta$ 4 and Pro $\beta$ 5) have male-specific isoforms encoded by separate genes. Most of these show a degree of similarity with their paralogous subunit of between 50 and 60% amino acid identity, and the degrees of divergence are highest at the C-terminal portions.

As for the 19S regulatory cap subunits, four of the known 17 subunits (Rpt3, Rpt4, Rpt6 and Rpn12) are represented by duplicate genes. Again, in every case, one of the two isoforms is expressed in a male-limited manner. The Rpt3, Rpt4 and Rpt6 subunits are members of the AAA-family of ATPases that make up the base of the 19S cap, while the Rpn12 subunit is a part of the lid. The male-specific forms of the base subunits are highly similar to their constitutive counterparts, showing 75–90% amino acid identity, while the Rpn12 isoforms are less similar (49% identical).

The expression patterns of the male-specific Pro $\alpha$ 3T and Pro $\alpha$ 6T subunits are very similar to what was seen for the Pro $\alpha$ 4T1 and Pro $\alpha$ 4T2 isoforms described previously

(Yuan *et al.*, 1996). That is, these subunits are expressed exclusively during spermatogenesis, with the subunits first appearing during meiosis and persisting throughout spermiogenesis. Both Pro $\alpha$ 3T and Pro $\alpha$ 6T localize to the elongating spermatid nuclei, and are present in the nuclei of mature, motile sperm. This is in contrast to the expression pattern of the 'housekeeping' isoform Pro $\alpha$ 3, which is expressed throughout spermatogenesis, but which fades from the spermatid nuclei as elongation and nuclear condensation occur. While the other nine male-specific proteasome subunit isoforms have not yet been examined in detail with respect to their expression patterns, it is likely that they are also expressed during spermatogenesis. Whether they show a similar nuclear localization during spermatid elongation must await further experimentation.

Given the dynamic changes in expression patterns of Pro $\alpha$ 3, Pro $\alpha$ 3T and Pro $\alpha$ 6T during spermatogenesis, we hypothesize that as sperm elongation and nuclear condensation occurs, there is a replacement of the 'housekeeping subunits' by their male-specific isoforms, resulting in a 'sperm-specific' proteasome. Although there is not yet direct evidence that these male-specific isoforms are incorporated into proteasome particles in the developing sperm, the results with Pro $\alpha$ 3T, showing that it can completely complement a deletion mutant of the orthologous subunit in yeast, support this idea. There is also genetic evidence that suggests that the spermatogenesis-specific Pro $\alpha$ 3T subunit and the housekeeping form, Pro $\alpha$ 3, are not functionally identical. That is, when the Pro $\alpha$ 3T subunit is inappropriately expressed in the soma [using the UAS/GAL4 system (Brand & Perrimon, 1993)] it causes late pupal lethality, whereas similar constitutive expression of Pro $\alpha$ 3 has no effect (J. Ma, unpublished data). The physiological basis for this is not known, but it does demonstrate that the Pro $\alpha$ 3T and Pro $\alpha$ 3 subunits do not have totally redundant activities.

These results, suggesting that there is a dramatic reorganization of the proteasome in spermatogenic cells, are exciting, as they represent the most striking example of cell-type specific proteasome gene expression in any system described to date. What role(s) might the hypothetical sperm-specific proteasome play in sperm differentiation or function? The observed localization patterns of both Pro $\alpha$ 3T and Pro $\alpha$ 6T in elongating spermatids and mature spermatozoa point to a role in the nucleus. Studies in vertebrates have suggested that during the late stages of spermiogenesis, the ubiquitin/proteasome pathway plays a key role in chromatin remodelling, in which the histones are replaced first by testis-specific transition proteins and

**Figure 4.** The expression patterns of Pro $\alpha$ 3T-GFP and Pro $\alpha$ 3-GFP during spermatogenesis. DIC images (A through M), and GFP fluorescence images (A' through M'), of dissected testes from transgenic flies carrying Pro $\alpha$ 3T-GFP (A, C, E, G, I, K, M) or Pro $\alpha$ 3-GFP (B, D, F, H, J, L, N), showing different stages of spermatogenesis. (A and B) early spermatogonial stages. (C and D) 16-cell cysts of primary spermatocytes. (E and F) secondary spermatocytes, following first meiotic division. (G and H) late phases of the second meiotic division. (I and J) 64-cell cysts of spermatids. (K and L) elongating spermatid nuclei bundle. (M and N) mature, motile sperm. DIC, differential interference contrast; GFP, green fluorescent protein fluorescence. Magnification 400 $\times$ .

then by protamines (Sassone-Corsi, 2002). For example, histones H3 and H2A become ubiquitinated in elongating spermatids, just before the histones are replaced (Agell & Mezquita, 1988; Chen *et al.*, 1998). Moreover, a mouse knockout mutant of the Ub-conjugating enzyme HR6B causes male sterility, with the spermatogenic defects suggesting a failure in nuclear condensation (Roest *et al.*, 1996). While sperm chromatin proteins have not been well studied in *Drosophila*, database searches do identify two male-specific protamine-related genes, *Mst35Ba* and *Mst35Bb*, suggesting that a similar transition in sperm histones might also occur in flies. If so, one possible role for the sperm-specific proteasomes might be in mediating the degradation of histones as the spermatid nuclei condense.

Another possibility is that these sperm-specific proteasomes act post-fertilization. Given that we observe the Pro $\alpha$ 3T-GFP nuclear signal in mature, motile sperm that are transferred to the female sperm storage organ (J. Ma, unpublished data), it is likely that paternal proteasomes are brought into the egg during fertilization. There, they might play a role in the decondensation of the male pronucleus that is necessary for successful zygotic development to occur (Fitch & Wakimoto, 1998). This is presumably accompanied by the degradation of sperm-specific chromosomal proteins and their replacement by conventional histones. Perhaps paternally contributed proteasomes are responsible for this. Of course, there are other possibilities, and it is hoped that a detailed examination of sperm development and function in flies with impaired proteasomes will provide clues as to what role(s) these sperm-specific proteasomes play. Whatever the functional significance of these testis-specific subunits might be, this question can be best addressed by genetic studies. A future goal is to obtain mutations in one or more of these testis-specific proteasome genes. It would be of interest to see whether these mutants are male steriles, whether the phenotypic effects on spermatogenesis are informative, and whether such mutants cause changes in the 2D-gel profile of testis-specific proteins that might identify putative substrates for proteasome-mediated proteolysis during spermatogenesis.

## Experimental procedures

### Fly culture

*D. melanogaster* strains were cultured at 25 °C on standard media. Stocks were obtained from the Bloomington Stock Center, and the descriptions are available on the FLYBASE server <<http://flybase.bio.indiana.edu/>>. *P*-element transformation was done using standard procedures (Spradling, 1986), with *w*<sup>1118</sup> as the host strain.

### General molecular procedures

All standard molecular techniques (e.g. genomic DNA extractions, restriction digestions, Southern and Northern blots, phage pro-

pagation and DNA isolation, PCR, plasmid subcloning, etc.) were done essentially as described in Sambrook *et al.* (1989). DNA sequencing was performed by the Biotechnology Resource Center at Cornell University (Ithaca, NY).

### Genomic library screening

A *D. melanogaster* genomic library, constructed in  $\lambda$ EMBL3 using DNA from a *th st tra cp in ri p<sup>o</sup>/TM3, Sb e p<sup>o</sup>* strain (J. M. Belote, unpublished data), was screened using <sup>32</sup>P-labelled probes prepared from PCR products or restriction fragments using the Random Primed Labeling Kit (Roche, Indianapolis, IN). Hybridizations were carried out overnight at 42 °C using the following hybridization solution: 37% formamide, 5 $\times$  SSPE, 1 $\times$  Denhardt's solution, 0.1% SDS, and 0.2 mg/ml salmon sperm DNA. Filters were washed for 1 h at 50 °C in 0.5 $\times$  SSPE, 0.1% SDS.

### Northern blot analyses

Total RNA was extracted from sexed adult flies (about 200 flies/preparation) using the hot phenol method. Poly(A)<sup>+</sup> RNA was purified from about 1 mg total RNA using the PolyA Tract mRNA Isolation System III Kit (Promega Corporation, Madison, WI), following the manufacturers' instructions. The entire sample was then run on a 1.2% agarose denaturing formaldehyde gel and blotted on to a Gene Screen Plus® nylon membrane (NEN Life Sciences Products, Boston, MA). The RNA blot was hybridized and autoradiographed as described in Sambrook *et al.* (1989).

Probes specific for the *Pro $\alpha$ 3*, *Pro $\alpha$ 3T*, *Pro $\alpha$ 6* and *Pro $\alpha$ 6T* genes were made using restriction fragments corresponding to their poorly conserved C-terminal portions of the ORFs and the 3'-UTRs. Gene specific probes for the other male-specific proteasome subunit isoforms were made using DNA fragments produced by PCR amplification of genomic DNA. The following oligonucleotide primers were used to generate gene specific probes for the Pro $\beta$ 2-related (CG18341 and CG12161), Pro $\beta$ 4-related (CG17301 and CG17302), Pro $\beta$ 5-related (CG9868), Pro $\beta$ 6-related (CG9475), Pro $\beta$ 7-related (CG7257), Pro $\beta$ 8-related (CG2241) and Pro $\beta$ 9-related (CG11552) genes: CG18341(5') = (ATCACCGCAAAGGGAGCGGTTTAC) and CG18341(3') = (AAGCCATGTGGCTATTTAACGGT); CG12161(5') = (GCGGGCATGAAGAACGACCTGT) and CG12161(3') = (GGATACCCAAGCCGGGTCCTA); CG17301(5') = (CCATCTCGAGGAGTTCTACAAACCG) and CG17301(3') = (ATATCCTTCGCTTGGGACCAGCTAAG); CG17302(5') = (TATGGCGCTGCTCAGTCAAT) and CG17302(3') = (TTC-CGCTTTACCAACTCAGG); CG9868(5') = (CTATCGGAAACGCATGACCGTG) and CG9868(3') = (TGCACGACAGAAGTTTTACTTCC); CG9475(5') = (CTGGT-TACGCTCATGTATTGAAGC) and CG9475(3') = (AGTCCATCAT-TCTCTCCAGCGAC); CG7257(5') = (GAACCGTTTCATCGAAGTGCTC) and CG7257(3') = (TCAC-GACCTTGACTTGCTCG); CG2241(5') = (TAGCTCG-GGAAGTTGTTGAGCGCT) and CG2241(3') = (TTGGCGTTTCACAGTGAAGT); CG11552(5') = (TCTAT-GCCCTCGGAGATCTATTCTGA) and CG11552(3') = (CGAGT-GAAGCCATTGAGATGTGTGA).

### GFP reporter constructs

Translational fusions of the green fluorescent protein (GFP) to Pro $\alpha$ 3, Pro $\alpha$ 3T and Pro $\alpha$ 6T were made by inserting the entire

coding region of EGFP (Clontech, Palo Alto, CA) into a newly created *NotI* site at the end of each proteasome gene's coding region. The *NotI* sites were generated using the Quik Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following primers: *Prosa3-Not(5')* = (GCGAAGCAGCCG-ACCGCGGCCGCAAGTAATCCCAAGGA) and *Prosa3-Not(3')* = (TCCTTGGGATTACTTGCGGCCGCGGTGCGCTGCTTCGC); *Prosa3T-Not(5')* = (AAGAGGCGCGTGGGGCGGCCGCTCG-TAAAGAGAAAAT) and *Prosa3T-Not(3')* = (ATTTTCTTTTAC-GAGCGGCCGCCCGACGCGCCTCTT); *Prosa6T-Not(5')* = (GCAGTGACGCACATGCGGCCGCTGATCTAATCTGTGATT) and *Prosa6T-Not(3')* = (AATCACAGATTAGATCAGCGGCCGCGATGTGCGT-CACTGC). A 738 bp EGFP *NotI* cassette was made by PCR amplification of pEGFP (GENBANK accession no. U76561) using the following primers: EGFP-5'-Not = (GCGCGGCCGCCA-TGGTGAGCAAGGGCGAGGAGCTGTTCAC) and EGFP-3'-JM = (AAGGGCCCGTACGGCCGACTAGTAGGCCTA). This product was treated with *NotI* and ligated into pBlueScript KS to create pBS/EGFP-Not that served as the source of the EGFP coding region for subsequent reporter gene constructs.

For construction of the *Prosa3-GFP* reporter gene an 8.3 kb *HinDIII* genomic (phage clone fragment, containing the *Prosa3* gene, was subcloned into pGEM3 and site-directed mutagenesis used to create a *NotI* site at the end of the *Prosa3* ORF. The EGFP *NotI* cassette (see above) was then ligated into the *NotI* site to create an in-frame fusion of *Prosa3* and EGFP. A 5.4 kb *SphI* fragment containing the *Prosa3-EGFP* reporter gene, with 2.2 kb of upstream sequences and 2.2 kb of downstream sequences, was cut out and inserted into the *SphI* site of the *P*-element transformation vector pW8 (Klemenz *et al.*, 1987) to give pW8/*Prosa3-GFP*. The *Prosa3T-GFP* reporter gene was constructed by creating a *NotI* site at the end of the *Prosa3T* coding region in the genomic clone pGEM3/9R1, which contains about 3.8 kb of sequence upstream, and 1.6 kb of sequence downstream, of the *Prosa3T* ORF. The EGFP *NotI* cassette was then ligated into the newly created *NotI* site to yield pGEM3/9R1-EGFP. The 6.2 kb *EcoRI* fragment of pGEM3/9R1-EGFP was then subcloned into pW8 to give pW8/*Prosa3T-GFP*. The *Prosa6T-GFP* reporter was made using a similar strategy, starting with the genomic clone pGEM4/*Prosa6T-2.8E* that consists of a 2.8 kb *EcoRI* fragment containing the *Prosa6T* coding region flanked by 0.8 kb of upstream sequences and 1.1 kb of downstream sequences. The *NotI* site was inserted just before the stop codon and the EGFP *NotI* cassette ligated into that site to create a *Prosa6T-EGFP* translational fusion. The 3.6 kb *EcoRI* fragment was then cut out and ligated into pBlueScript KS to give pBS/*Prosa6T-EGFP*. This insert was then removed by cutting with *XhoI* and *XbaI*, and ligated to pW8 that had been cut with *XhoI* and *XbaI* to yield pW8/*Prosa6T-GFP*.

#### Yeast complementation experiments

The *Prosa3* cDNA was PCR amplified from the EST clone LD29759 (Berkeley *Drosophila* Genome Project) using the following primers containing *HinDIII* sites: *Prosa3-H(5')* = (GCG-CAAGCTTGCAACCCTACACTGAATGACAAGTAAATTC) and *Prosa3-H(3')* = (GGCCAAGCTTCAATAAATGCATCCTTGGG-ATTACTTGG). The PCR product was treated with *HinDIII*, gel purified and ligated into the *HinDIII* site of the yeast expression vector pGAD (Chien *et al.*, 1991), to give pGAD/*Prosa3*. The pGAD/*Prosa3-GFP* construct was made by replacing a C-terminal *BamHI-SphI* restriction fragment of *Prosa3* in pGAD/*Prosa3* with

a corresponding fragment from pW8/*Prosa3-GFP*. The following primers were used to PCR amplify the *Prosa3T* or the *Prosa3T-GFP* coding regions, using genomic DNA or pW8/*Prosa3T-GFP* plasmid, respectively, as templates, for subcloning into the *HinDIII* site of pGAD: *Prosa3T-H(5')* = GCGCAAGCTTTCAATCCATAT-TCCAAGTAATCTGCTATAT and *Prosa3T-H(3')* = GGCCAAGCTT-TATATAAACATTATAAAAGTAACTAATGAC.

These plasmids were introduced into the *Saccharomyces cerevisiae Y13Δ* mutant strain (kindly provided by W. Heinemeyer, University of Stuttgart) using the yeast transformation method of Chen *et al.* (1992). Complementation of the amino acid analogue sensitivity of *Y13Δ* by the transformed plasmids was performed using the method of Fu *et al.* (1998a,b).

#### Microscopy and immunofluorescence

Tissues were dissected from larvae or adult flies in PBS or *Drosophila* Ringer's buffers. For the fluorescence microscopy of spermatogenic cells, live squashed testis preparations were performed as described in Kempfues *et al.* (1980). For immunofluorescence staining, testis preparations were made as described in Lin *et al.* (1996). Testes were fixed in 4% formaldehyde PBS, washed three times in PBS with 0.1% Triton X-100 (PBTx), and incubated for 16 h at 4 °C in a 1 : 6000 dilution of rabbit anti-TBP7 polyclonal antibodies (Affiniti Research Products Ltd, Exeter, UK). They were then washed three times (20 min each) in PBTx at room temperature, and incubated with a 1 : 800 dilution of Cy3-conjugated goat anti-rabbit IgG (H + I) secondary antibody (Jackson Immuno-Research Laboratories Inc., West Grove, PA) for 1–3 h at room temperature. After washing three times in PBTx at room temperature and counterstaining with 1 μg/ml DAPI in PBS, the testes were mounted in 80% glycerol : 20% PBS.

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