# The Dominant Temperature-Sensitive Lethal *DTS7* of *Drosophila melanogaster* Encodes an Altered 20S Proteasome β-Type Subunit

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ABSTRACT

Proteasomes are multicatalytic complexes that function as the major proteolytic machinery in regulated protein degradation. The eukaryotic 20S proteasome proteolytic core structure comprises 14 different subunits: 7 α-type and 7 β-type. *DTS7* is a dominant temperature-sensitive (DTS) lethal mutation at 29° that also acts as a recessive lethal at ambient temperatures. *DTS7* maps to cytological position 71AB. Molecular characterization of *DTS7* reveals that this is caused by a missense mutation in a β-type subunit gene, β2. A previously characterized DTS mutant, *l(3)73Ai*[1], results from a missense mutation in another β-type subunit gene, β6. These two mutants share a very similar phenotype, show a strong allele-specific genetic interaction, and are rescued by the same extragenic suppressor, *Su(DTS)-1*. We propose that these mutants might act as "poison subunits," disrupting proteasome function in a dosage-dependent manner, and suggest how they may interact on the basis of the structure of the yeast 20S proteasome.

REGULATED protein degradation is important for normal cell function, playing key roles in such diverse processes as growth control, metabolic regulation, embryonic development, cell cycle progression, and programmed cell death (see Ciechanover 1994; Hochstrasser 1995; Ciechanover and Schwartz 1998 for reviews). An essential component of intracellular proteolysis in eukaryotes is the 26S proteasome, a large, multisubunit complex that acts as the proteolytic machinery of the ubiquitin-dependent protein degradation pathway (Coux *et al.* 1996; Baumeister *et al.* 1998). The 26S proteasome has two major components: a 20S core particle and 19S regulatory complexes capping each end. The 20S proteasome is a hollow, barrel-shaped cylinder made up of four stacked rings of seven subunits each. In eukaryotes, this particle is comprised of seven different α-like and seven distinct β-like subunits, in an (α1-α7) (β1-β7) (β1-β7) (α1-α7) arrangement (Groll *et al.* 1997). It is within this hollow cylinder that hydrolysis of the polypeptide substrate takes place, catalyzed by the β1, β2, and β5 subunits. The composition and function of the 19S cap is less well understood, but components of this structure are thought to bind polyubiquitinated substrate proteins, unfold and translocate them into the 20S particle's inner degradative chamber, and remove their ubiquitin tags. An alternative regulatory complex, known as PA28, REG, or the 11S cap, has been identified in mammalian cells, where it is believed to play a role in activating the 20S proteasome to produce antigens for MHC class I presentation (Groettrup *et al.* 1995, 1996; Kuehn and Dahlmann 1997).

Although numerous studies have revealed much about the physical and biochemical properties of proteasomes, many aspects of their biological function remain obscure. We are interested in the roles of proteasome-mediated protein degradation during metazoan development and have initiated a molecular and genetic study of *Drosophila melanogaster* proteasomes as an approach to address this topic. One part of that study is to obtain proteasome subunit conditional mutants that can be used to manipulate the function of proteasomes *in vivo*, so that the role of the ubiquitin-proteasome pathway in specific processes can be assessed. The first such mutant to be identified in Drosophila was the *l(3)73Ai*[1] allele (Saville and Belote 1993). This mutant (previously known as *DTS5*) was discovered by Holden and Suzuki (1973) as part of a comprehensive study of temperature-sensitive mutations in *D. melanogaster* and was shown to have an unusual dominant temperature-sensitive (DTS) lethal phenotype, in which heterozygotes raised at the restrictive temperature die during the late pupal stage and show numerous developmental defects. Subsequent molecular genetic characterization of the *l(3)73Ai* locus revealed that it encodes the β6 subunit of the 20S proteasome (Saville and Belote 1993; see Zaiss and Belote 1997 for a list of previously used synonyms for the yeast, fly, and human 20S subunits). The dominant conditional nature of *l(3)73Ai*[1] makes it a potentially useful mutant for examining the *in vivo* role of proteasome-mediated protein degradation. We report the molecular cloning of a second domi-

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nant temperature-sensitive lethal mutant, *DTS7* [also isolated in Holden and Suzuki's (1973) original mutagenesis experiment] and show that it encodes another 20S proteasome subunit, β2. We also discuss a possible molecular basis for the dominant negative phenotype and the observed synthetic lethal interaction shown by these two DTS mutants.

## MATERIALS AND METHODS

**Fly culture:** Flies were cultured on standard Drosophila media at 25°, unless otherwise noted. Genetic variants of *D. melanogaster* are described in Lindsley and Zimm (1992), or the descriptions can be accessed using the Flybase (1997) database. The stocks used in this study were obtained from the Bloomington Stock Center and the Umeå Stock Center.

**Cytological localization of *DTS7*:** The *DTS7* mutant was mapped relative to the P{PZ}dev[00208] transposon inserted at 70D2 by crossing females of genotype *ru h DTS7 st tra in* $p^p$ *ry/P{ry + t7.2 = PZ}dev[00208]* $ry^{506}$ to $ry^{506}$ males and culturing the offspring at 29°. The rare rosy-eyed survivors (*i.e., DTS7*$^+$ *ry*) represent crossovers between the *DTS7* gene and the $ry^+$ gene associated with the transposon. The recombinants were then tested for the presence or absence of the *h* and *st* markers that flank *DTS7* and *P{ry + t7.2 = PZ}dev[00208]* by individually crossing the *ry* survivors to *ru h th st cu str* $e^s$ *Pri ca/TM6B, Hu Tb e ca* mates. In this experiment, of 811 survivors, 5 were rosy eyed. All 5 of these *DTS7*$^+$ *ry* recombinant chromosomes carried the *h* and $st^+$ flanking markers, indicating that the *DTS7* gene lies to the right of the P{PZ}dev[00208] transposon, ~0.6 map units away.

Two different mutagenesis experiments were carried out to generate deficiencies around the 70D2 region. In the first experiment, male adults of genotype *DTS7 st tra in* $p^p$*/TM3, Sb Ser* were exposed to 5000 rad of γ-rays from a $^{60}$Co source or were fed 0.007 mm diepoxybutane and then mated to *DTS7 st tra in* $p^p$*/TM3, Sb Ser* females. After 2 days, the cultures were placed at 29° and then cleared of parents at day seven. The rare survivors potentially represent *DTS7*$^-$ "knockout" mutants that no longer exhibit the DTS lethal phenotype. The mutagenized chromosomes were balanced over *TM3, Sb Ser* and then the stocks were retested at 29° to confirm that they no longer carried a DTS lethal mutation. The mutagenized chromosomes were subsequently tested to see if they no longer exhibited a dominant synthetic lethal interaction with *l(3)73Ai*$^1$ and to determine if there was a recessive lethal mutation uncovered by the deficiency *Df(3L)fz-M21*.

In the second mutagenesis experiment, males of genotype *DTS7 st tra in* $p^p$*/TM3, Sb Ser* were irradiated with 3000 rad of X rays and crossed to females of genotype *l(3)73Ai*$^1$*/TM3, Sb Ser* and the progeny raised at 25°. Phenotypically wild-type (*i.e.*, non-Stubble, non-Serrate) survivors represented putative *DTS7*$^-$ "pseudorevertants" in which the *DTS7*-bearing chromosome no longer exhibited a dominant synthetic lethal interaction with *l(3)73Ai*$^1$. The mutagenized chromosomes were maintained over balancers and tested at 29° to confirm that they no longer acted as DTS lethals.

For each screen, the new mutants were examined for visible chromosome aberrations by crossing mutant-bearing males to wild-type females (Oregon R) and preparing salivary gland chromosome squashes from late third instar larvae. Orcein-stained chromosomes were examined under phase contrast optics with a Zeiss Axioplan microscope.

**Polytene chromosome *in situ* hybridization:** Biotinylated DNA *in situ* hybridization to larval salivary gland polytene chromosomes was performed as described in Ashburner (1989). Probes were prepared by random-primed labeling of recombinant phage DNA using a kit from Boehringer Mannheim (Indianapolis). The modified deoxynucleotide used was biotinylated dUTP from GIBCO-BRL (Gaithersburg, MD) and signal detection was done using a Detek-I-hrp Kit (ENZO).

**General molecular procedures:** All standard techniques (*e.g.*, DNA extraction, restriction digestion, plasmid and phage DNA isolation, Southern blots, Northern blots, etc.) were done as described in Sambrook *et al.* (1989). The *Escherichia coli* strains used for propagating phage and plasmids were LE392 [or XL1-Blue (Stratagene, La Jolla, CA)] and DH5α, respectively. The plasmid vector pGEM-4 blue (Promega, Madison, WI) was used for subcloning, unless otherwise noted.

**Recombinant P1 phage characterization:** Recombinant P1 bacteriophage were obtained from the Berkeley Drosophila Genome Project (BDGP). The Drosophila genomic DNA inserts of phage DS00138 and DS07775 were subcloned by ligating size-fractionated phage DNA that had been partially digested with *Sau*3A into the *Bam*HI site of the λDASH vector (Stratagene). The ligation products were packaged using the Gigapack Gold III Bacteriophage λ Packaging Kit (Stratagene) according to the supplier's protocol. The resulting recombinant λ phage clones were replica plated and filter lifts hybridized to random-primed labeled DS00138 and DS07775 DNA to determine which clones contained DNA from the region unique to DS00138, unique to DS07775, or common to both. Clones containing P1 phage vector sequences were identified by hybridization to unrelated P1 phage clones (DS06476 and DS03264 from region 70C). Representative λ phage were further characterized by restriction mapping, cross-hybridization, and *in situ* hybridization to deficiency chromosomes to generate a set of contiguous overlapping phage spanning the *DTS7* region.

**DNA sequencing:** The β2 cDNA and genomic clones were sequenced by the chain termination method of Sanger *et al.* (1977) using [$^{35}$S]dATP and Sequenase (United States Biochemicals, Cleveland). The primers used were either the T7, M13 Reverse, or SP6 promoter sequence primers, or gene-specific synthetic oligonucleotides obtained from GIBCO-BRL. In some cases, the sequencing was carried out by the Biotechnology Resource Center at Cornell University (Ithaca, NY). The sequences reported in this article have been deposited into the GenBank database under the accession numbers AF025791 and AF025792.

**Polymerase chain reaction:** The β2 genomic sequence was PCR amplified from a 5.5-kb *Eco*RI fragment of the λDm13 phage clone using the following primers: β2/5′ = 5′-ATG GATTTGGATAACGCACGCGAACTGCCC-3′ and β2/3′ = 5′-TAATAAATGTACGAAAAGGGTAGAAAGGGG-3′. The same primers were also used for the PCR amplification of β2 sequences from genomic DNA extracted from individual *DTS7/Df(3L)fz-M21* larvae or Samarkand adults using the method of Gloor and Engels (1992). PCR products were electrophoresed through a 1.5% agarose gel, excised and purified using the procedure of Boyle and Lew (1995), and cloned into pBlueScript II KS+ plasmid (Stratagene) that had been prepared for PCR product cloning according to the TA cloning method of Zhou *et al.* (1995).

***P*-element-mediated germline transformation:** Microinjection of Drosophila embryos was done using standard methods (Spradling 1986; Ashburner 1989). A 2.7-kb *Pst*I-*Eco*RI fragment of λDm13, containing the β2 transcription unit and no other identified gene, was inserted into the pW8 transformation vector (Klemenz *et al.* 1987) and plasmid DNA purified using the Wizard Plus Miniprep DNA Purification System (Promega). A mixture of pW8/2.7*Pst*I-*Eco*RI DNA (300 μg/ml) and helper plasmid pΔ2-3 wc (100 μg/ml) was injected into preblastoderm embryos of a *w* host strain. $G_0$ survivors were

singly crossed to *w; TM6B, Hu Tb/TM3, Sb Ser* mates and transformed progeny identified by their pigmented eyes. Transformants were individually crossed with *w; T(2;3)ap$^{Xa}$/CyO; TM3, Sb* mates and the appropriate offspring from subsequent generations were used to establish homozygous, or balanced heterozygous, transformed lines for testing. For each line, the chromosome containing the transgene was determined by linkage analysis of the $w^+$ gene associated with the pW8 transposon.

## RESULTS

**Genetic relationship between the *l(3)73Ai$^1$* and *DTS7* mutants:** Individuals heterozygous for the *l(3)73Ai$^1$* mutant allele develop normally when reared at 25° but die during the late pupal stage when raised at 29° (Holden and Suzuki 1973). The dead pupae exhibit multiple phenotypic abnormalities including reduced imaginal disc derivatives, frequent failure of head eversion, and absence of adult abdomen structures. The mutant behaves genetically as an antimorph, because a deletion of the locus has no phenotype in heterozygotes, and additional copies of the wild-type gene ameliorate the dominant lethal effect of the DTS allele (Saville and Belote 1993). Hemizygous or homozygous mutant individuals have a more severe lethal phenotype, dying during the early larval stages when cultured at temperatures ≥25°, although at 18° viable, but sterile, adults are observed (Saville and Belote 1993). The loss-of-function allele, *l(3)73Ai$^{rv10e}$*, which is a nonsense mutation that truncates the proteasome subunit open reading frame (ORF) at amino acid 78, is a recessive, nonconditional lethal (S. Brewer and J. Belote, unpublished results). Animals that are homozygous or hemizygous for this allele usually survive past hatching, but they are sluggish and die as first instar larvae.

The *DTS7* mutant phenotype is similar to that seen with *l(3)73Ai$^1$*, although certain aspects are more severe. Heterozygotes show no apparent phenotypic effects when raised at 25°, but at 29°, *DTS7*/+ larvae develop slowly, and at pupariation many of them fail to shorten, resulting in elongated, and sometimes curved, pupae. Metamorphosis is abnormal with grossly underdeveloped imaginal disc derivatives and a complete lack of adult abdomen structures. The recessive phenotype of *DTS7* is larval lethality at all temperatures tested (18–29°). *DTS7* hemizygotes usually die soon after hatching, although at 18° some survive to the late larval or early pupal stage. Loss-of-function alleles of the *DTS7* locus (*e.g.*, *DTS7-rv3D*, see below) are similar in phenotype to loss-of-function alleles of *l(3)73Ai*; they are recessive, early larval lethals.

While the similarity in mutant phenotypes is suggestive of a relationship between *l(3)73Ai$^1$* and *DTS7*, more compelling is the strong synthetic lethal genetic interaction shown by the two mutants. Specifically, *l(3)73Ai$^1$* and *DTS7*, when in *trans*-heterozygous condition, are lethal even at normally permissive temperatures (Holden and Suzuki 1973). We have confirmed this initial observation and have noted that the synthetic lethal period of these double mutants is much earlier in development than the DTS lethal period of either allele (early larval *vs.* late pupal stage) and resembles the homozygous phenotype of the loss-of-function alleles of either gene. The genetic interaction between *l(3)73Ai$^1$* and *DTS7* is allele-specific, as deletions or loss-of-function alleles of either locus do not show any observable dominant genetic interactions with the other DTS mutant. It should also be noted that no other DTS mutants isolated in the original screen were reported to show any genetic interactions with each other (Holden and Suzuki 1973).

One other genetic observation supports the notion that the *l(3)73Ai$^1$* and *DTS7* mutants are functionally related. In a screen for extragenic dominant suppressors of the DTS lethal phenotype of *l(3)73Ai$^1$*, a mutant was found that rescues *l(3)73Ai$^1$* heterozygotes from the 29° lethality (Saville 1992; Saville and Belote 1993). Curiously, this mutant, *Su(DTS)-1* (map position 3-48.5), also acts as a dominant suppressor of the DTS lethal effect of *DTS7* (J. Belote and J. Todd, unpublished results). Flies that are simultaneously heterozygous for both *Su(DTS)-1* and either *l(3)73Ai$^1$* or *DTS7* are normally viable when reared at 29°. This suppressor only partially rescues the synthetic lethal interaction of the two DTS mutants; *i.e.*, a small fraction of triply heterozygous individuals (+ *l(3)73Ai$^1$ Su(DTS)-1*/*DTS7* + +) can survive to adulthood when raised at 18°.

The observations that (1) *DTS7* and *l(3)73Ai$^1$* have similar lethal phenotypes, (2) *DTS7* and *l(3)73Ai$^1$* exhibit a strong, allele-specific genetic interaction, and (3) the DTS lethal phenotypes of both *DTS7* and *l(3)73Ai$^1$* are rescued by the same extragenic suppressor mutant provide strong evidence that these genes are functionally related. Because *l(3)73Ai* is known to be a proteasome subunit gene, it is reasonable to posit that the *DTS7* locus either encodes another proteasome subunit or has some role related to proteasome function. We therefore sought to clone the *DTS7* locus so that its relationship to proteasomes could be determined.

**Cytological mapping of the *DTS7* locus:** We established the cytological location of the *DTS7* gene as the first step toward its isolation. Meiotic recombination experiments by Holden and Suzuki (1973) placed this locus on the left arm of the third chromosome, between the *h* (3-26.5; 66D9-10) and *st* (3-44.0; 73A3-4) loci at ~3-42.3. To better correlate this with a cytological map position within this interval, we mapped the *DTS7* mutant relative to P{PZ}dev[00208], a genetically marked *P*-element transposon inserted in chromomere 70D2 (see materials and methods). The results indicated that the *DTS7* locus lay ~0.6 map units to the right of the $ry^+$ marker associated with this *P* element, which had been previously shown to be inserted at the *dev*

**TABLE 1**

**Chromosome rearrangements and mutants involving the DTS7 region**

| Rearrangement or mutant | Cytology[a] | Source | DTS7 | Discoverer |
|---|---|---|---|---|
| *Df(3L)D–1rv16* | 70C3-70D1 | X ray | + | A. Carpenter |
| *Df(3L)D–5rv6* | 70C7-70D6 | X ray | + | A. Carpenter |
| *Df(3L)fz–GS1a* | 70D2-70E5 | ? | + | ? |
| *Df(3L)fz–D21* | 70D2-70E8 | γ-Ray | + | P. Adler |
| *Df (3L)fz–M21* | 70D3-71E5 | γ-Ray | − | P. Adler |
| *Df(3L)DTS7–rv16X* | 71A1-71CD | X ray | − | This study |
| *Df(3L)DTS7–rv20X* | 70D3-71B2 | X ray | − | This study |
| *Df(3L)DTS7–rv24X* | 70D6-71B2 | X ray | − | This study |
| *DTS7–rv19x* | Normal | X ray | − | This study |
| *DTS7–rv3γ* | Normal | γ-Ray | − | This study |
| *DTS7–rv11γ* | Normal | γ-Ray | − | This study |
| *DTS7–rv12γ* | Normal | γ-Ray | − | This study |
| *DTS7–rv3D* | Normal | Diepoxybutane | − | This study |
| *T(2;3)DTS7–rv15X* | 21A; 28AB\|42A · 40; 28AB\|58D; 60F; 61A; 71A\|58EF; 42A\|71B, 80F · 81A; 100F | X ray | − | This study |

[a] These breakpoints are based on our determinations.

locus at 3-40.9. This places *DTS7* at position 3-41.5 and proximal to band 70D2.

In addition to its DTS lethal phenotype, the *DTS7* mutant also acts as a homozygous lethal at ambient temperatures (Holden and Suzuki 1973; see above). We therefore tested several deficiency chromosomes for their ability to complement this recessive lethal phenotype (Table 1, Figure 1). The results indicated that the recessive lethal effect of *DTS7* was complemented by the deficiencies *Df(3L)D-1rv16*, *Df(3L)D-5rv6*, *Df(3L)fz-GS1a*, and *Df(3L)fz-D21*, but not by *Df(3L)fz-M21*. This places the *DTS7* locus within chromosome interval 70F1-71C2. To better define the gene's position within this region and to generate chromosome breakpoints useful for its positional cloning, we carried out a mutagenesis experiment to isolate *DTS7*$^-$ "knockout" mutations associated with newly induced chromosome aberrations. We assumed that a mutation that deletes or inactivates the *DTS7* mutant would no longer behave as a dominant allele, but would be "reverted" to a recessive lethal. We treated males carrying a *DTS7*-bearing chromosome with mutagens that primarily induce chromosome breaks (X rays, γ-rays, or diepoxybutane) and looked at subsequent generations for the loss of the DTS lethal phenotype associated with *DTS7*. We also tested these "pseudorevertants" for the loss of the dominant synthetic lethal interaction with the *l(3)73Ai*$^1$ mutant. In these experiments 16 such mutants were recovered, and the *DTS7*-bearing chromosomes were examined for detectable aberrations (Table 1, Figure 1). Six of the pseudorevertant chromosomes were cytologically normal, 9 were deficiencies, and 1 was a translocation (also associated with a small deletion). Because all of the rearranged chromosomes were deleted for, or broken within, region 71A2-71B4, the *DTS7* gene must lie in this chromosomal interval. The most informative rearrangements obtained from the screen are *Df(3L)DTS7-rv16X* and *Df(3L)DTS7-rv24X*, which define the distal and proximal boundaries of the *DTS7* region (Figure 2).

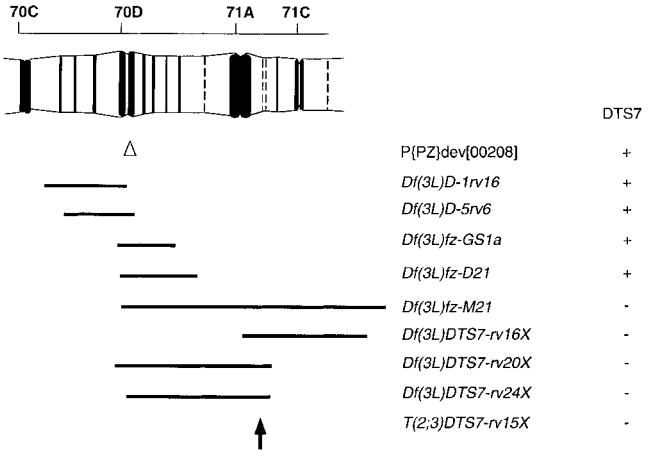


Figure 1.—Schematic diagram of the 70C-71C region of *D. melanogaster* polytene chromosomes, showing the deficiency breakpoints of a selection of the strains from Table 1. The triangle indicates the position of the transposon, P{PZ}dev [00208]. The arrow shows the chromosome breakpoint for *T(2;3)DTS7-rv15X*. The chromosome deficiencies (indicated by the solid bars) of the first four strains do not uncover *DTS7*. The next four deficiencies and *T(2;3)DTS7-rv15X* fail to complement *DTS7* and define *DTS7* to region 71AB.

**Positional cloning of the *DTS7* locus:** Having localized the *DTS7* gene to the 71A2-B4 interval, we next obtained a set of recombinant P1 phage containing large DNA inserts from this region (*i.e.*, DS00061, DS00138, DS04560, and DS07775) from the BDGP. These recombinant P1 phage were used as probes for *in situ* hybridization to salivary gland chromosomes of larvae heterozygous for *Df(3L)DTS7-rv16X* or *Df(3L)DTS7-rv24X* to identify those that span the defining breakpoints of the

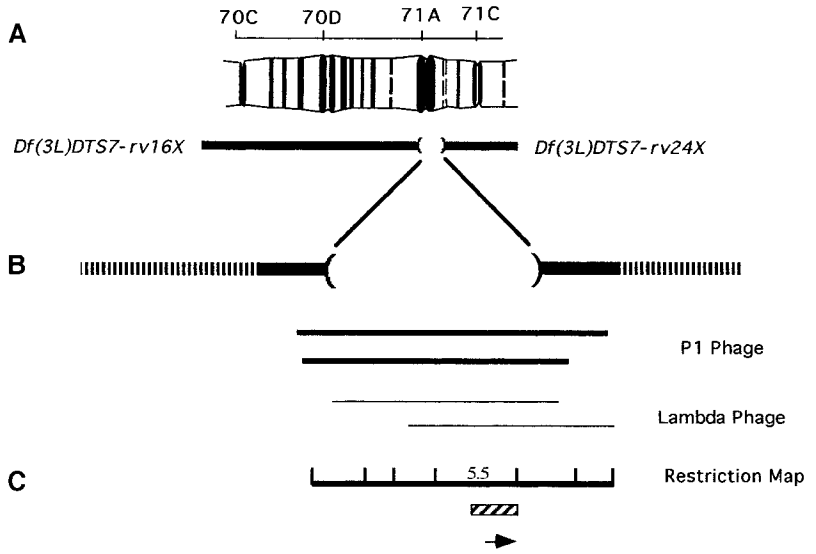


Figure 2.—Schematic diagram summarizing the positional cloning of *DTS7*. (A) Schematic diagram of the 70C-71C region of the salivary gland chromosome map. For *Df(3L)DTS7-rv16X* and *Df(3L)DTS7-rv24X*, the solid bars indicate the region not uncovered by the deficiencies, and the brackets indicate the deficiency breakpoints. (B) An expanded view of the deficiency region to show the positions of the P1 phage clones DS00138 and DS07775 (thick lines), and the λ phage clones λDm13 and λDm10 (thin lines). (C) The contiguous *Eco*RI restriction map of the λDm10 and λDm13 phage clones, indicating the location of the 5.5-kb fragment, the 2.7-kb fragment that rescues *DTS7* (hatched bar), and the position and orientation of the β2 cDNA (arrowhead).

*DTS7* region. Two of these P1 phage (DS00138 and DS07775) were found to contain DNA crossing both breakpoints (Figure 2), because the probes showed detectable hybridization to both wild-type and deficiency chromosomes, with less signal associated with the deficiency chromosomes. To better define the relevant region within the recombinant phage, the inserts from both DS00138 and DS07775 were subcloned into the λDASH vector, which accommodates inserts of ~15–20 kb. The subclones were ordered into a contiguous set of overlapping clones by restriction mapping and cross-hybridization experiments. Representative phage were then tested by hybridizing them *in situ* to *Df(3L)DTS7-rv16X* and *Df(3L)DTS7-rv24X* polytene chromosomes to determine their relationship to the defining breakpoints of the *DTS7* interval. Two clones, λDm10 and λDm13, cross the distal and proximal defining breakpoints, respectively. We expected the *DTS7* locus to be contained on one or both of these recombinant phage, because these two phage overlap one another (Figure 2).

We looked for transcribed sequences within the cloned interval to identify the *DTS7* gene. The inserts from both λDm10 and λDm13 were digested with *Eco*RI, subcloned into plasmid vectors, and then used as probes against Northern blots of poly(A)$^+$ RNA isolated from all stages of development. A single transcript, ~1.0 kb in size, was detected with these probes, and it was present at all stages (data not shown). The 5.5-kb *Eco*RI fragment of λDm13 that hybridized to this RNA was then used as a probe to screen an embryonic cDNA library and two clones of similar structure were isolated and sequenced. A genomic clone of the corresponding region was also isolated and sequenced, revealing the presence of two introns and an ORF of 272 amino acids (Figure 3). A search of the available databases showed that this ORF encodes a protein with high-amino-acid identity to the yeast (54% identical) and human (72% identical) β2 proteasome subunits (also known as PUP1 and Z, respectively).

Given that (1) the *l(3)73Ai* gene encodes a β-type proteasome subunit, (2) the *DTS7* mutant shows a strong genetic interaction with the *l(3)73Ai*$^1$ mutant, and (3) the only identified transcription unit within the *DTS7* region encodes a β-type proteasome subunit, it seemed highly probable that *DTS7* and the β2 subunit gene correspond to the same locus.

**Sequence analysis of the β2 gene from a *DTS7* mutant strain:** The DTS mutant *l(3)73Ai*$^1$ is known to encode an aberrant β-type subunit in which there is a substitution of a highly conserved amino acid, T18I [this mutation is also referred to as T47I when the prosequence is included in the amino acid numbering system (see discussion)]. Given the similarities in the genetic properties of *l(3)73Ai*$^1$ and *DTS7*, it is reasonable to suppose that the latter mutant is also a missense mutation involving a conserved amino acid.

To test the hypothesis that *DTS7* encodes an altered β2 proteasome subunit, we sequenced the gene encoding the β2 subunit from individuals hemizygous for the *DTS7* mutation. Dying non-Tubby larvae were selected from a cross of *DTS7 / TM6B, Hu Tb* females and *Df(3l)fz-M21 / TM6B, Hu Tb* males, and the β2 coding region of these individuals was PCR amplified and sequenced. The β2 gene was also amplified and sequenced from flies of the Samarkand wild-type strain from which the *DTS7* mutant was derived. To confirm that any observed differences were not due to PCR or cloning artifacts, multiple independent PCR products were analyzed. Sequence analyses of these reveal that there is a single amino acid difference between the *DTS7* and wild-type β2 subunits (a G170R missense mutation; Figure 3). This glycine is absolutely conserved among β2 subunit genes from all species examined to date, from archaebacteria to humans, suggesting that it is functionally important and strengthening the view that this amino

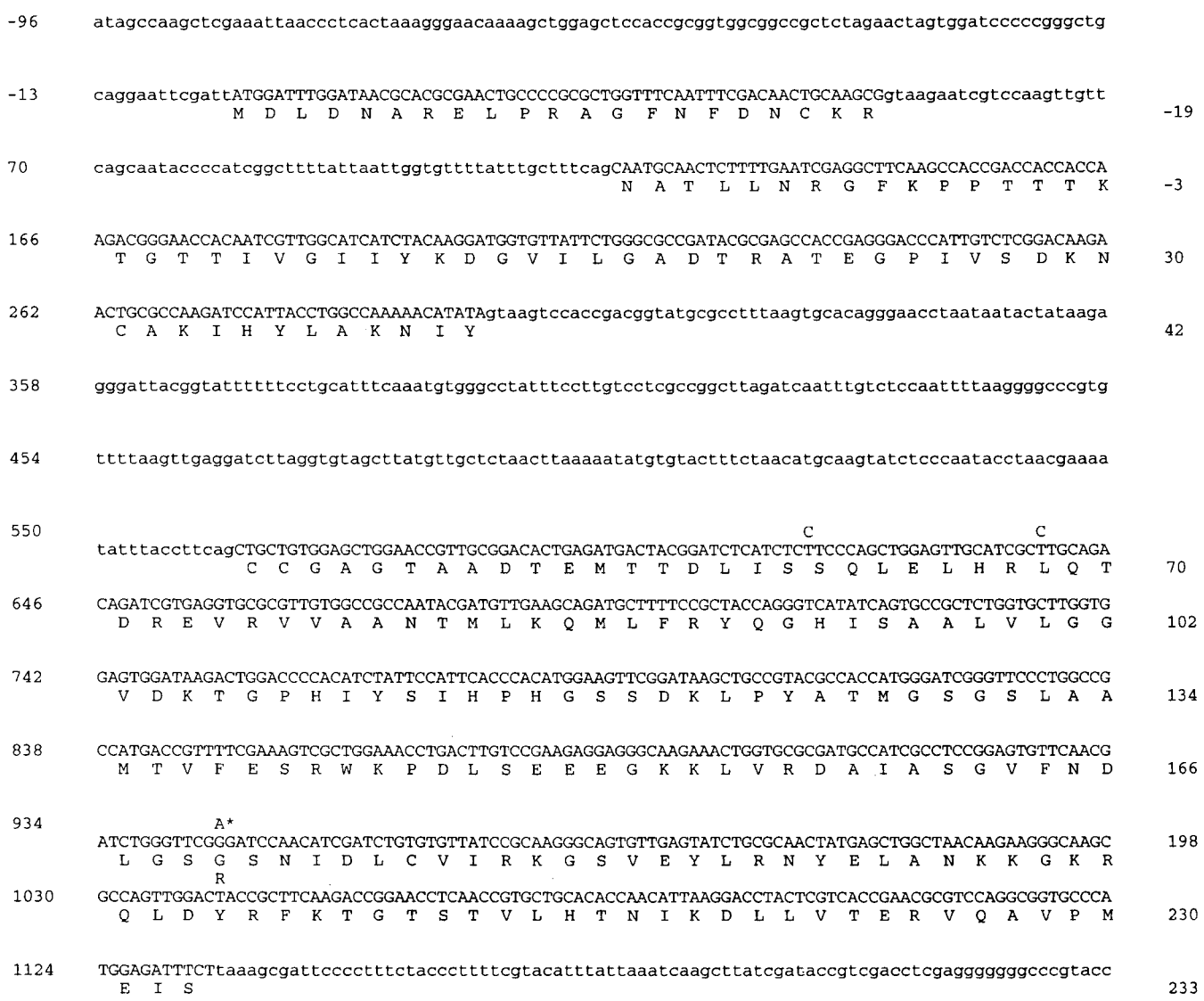


Figure 3.—Genomic DNA and predicted amino acid sequence of the β2 gene. The numbers on the left represent the nucleotide position relative to the beginning of the coding sequence. The numbers on the right represent the amino acid position relative to the N-terminal active site (Thr 1). The nucleotide differences between the wild-type (Samarkand) and the mutant (*DTS7*) β2 genes are indicated above the sequence. The asterisk denotes the G→A transition resulting in the G170R substitution.

acid change in β2 corresponds to the *DTS7* mutation (Figure 4).

**Rescue of the *DTS7* mutant phenotype by a β*2* transgene:** The DTS lethal phenotype of *l(3)73Ai*[1] is rescued by an extra copy of the wild-type gene, supplied as either a transgene or as a conventional chromosome region duplication (Saville and Belote 1993). Similarly, we expected that an extra copy of the wild-type β2 gene might ameliorate the DTS lethal effect associated with *DTS7* if the β2 subunit gene does correspond to the *DTS7* locus. To examine this, we used *P*-element-mediated germline transformation methods to introduce a wild-type copy of the β2 gene into the genome and tested the transduced gene for its ability to rescue the 29° lethality associated with *DTS7*. A 2.7-kb *Eco*RI/*Pst*I fragment containing the wild-type β2 gene along with ~0.9 kb of 5′ upstream sequences and 0.5 kb of 3′ downstream sequences was subcloned into the pW8 transformation vector and used to generate transgenic lines. This fragment contains no other identified gene, as defined by Northern blot analysis and DNA sequencing of the fragment. Four independent lines carrying the transgene were obtained. Individuals from one line, in which the transgene was linked to chromosome 2, were crossed to the appropriate mates to generate flies of the genotype *w; P[w*$^+$*,* β*2]/+; +/TM6B, Hu Tb.* These flies were crossed to *w;+/; DTS7/TM3, Sb Ser* and the progeny reared at 29°. If the *P[w*$^+$*,* β*2]* transgene is able to rescue the DTS phenotype of *DTS7*, then all non-Stubble, non-Serrate (*i.e.*, *DTS7*/+ and *DTS7*/*TM6B,*

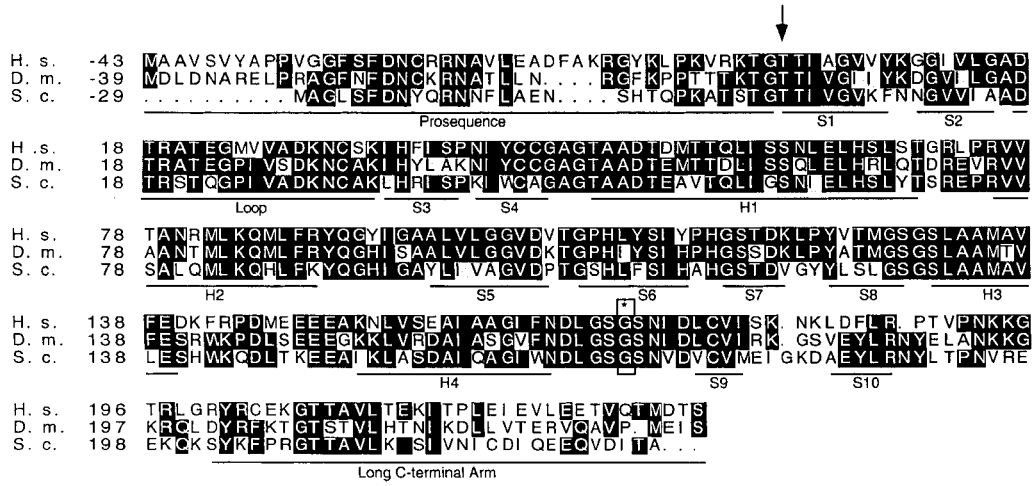


Figure 4.—The amino acid sequence alignment of the β2 subunits from human (H. s.), *Drosophila melanogaster* (D. m.), and *Saccharomyces cerevisiae* (S. c.). The darkly shaded areas indicate identical amino acids and the lightly shaded areas denote conservative amino acid changes. The arrow indicates the position of the N-terminal Thr 1. The asterisk within the box indicates the site of the G170R substitution in *DTS7*. The extent of the yeast secondary structures is indicated by the lines below the alignment. The α-helices are labeled by H and the β-sheets by S.

*Hu Tb*) survivors should be red eyed (that is, they *must* carry the transgene to survive at 29°). This expectation was borne out: among the 90 surviving progeny scored from this cross, all 43 non-Stubble, non-Serrate flies were red eyed, while the 37 Stubble, Serrate (*i.e.*, *+/TM3, Sb Ser* and *TM6B, Hu Tb/TM3, Sb Ser*) progeny were of mixed eye color (25 red and 12 white). Similar results were obtained for the other three transformed lines.

To test whether the *P[w$^+$, β2]* transgene is also able to rescue the recessive lethal phenotype characteristic of *DTS7$^-$* loss-of-function alleles, males of genotype *w/Y; P[w$^+$, β2]/+ ; DTS7-rv3D/TM3, Sb* were crossed to *w; +/+; Df(3L)fz-M21/TM6, Ubx* females. If the *P[w$^+$, β2]* transgene is able to rescue the lethality of *DTS7-rv3D* hemizygotes, then some non-Stubble, non-Ubx flies (*i.e.*, *DTS7-rv3D/Df(3L)fz-M21*) should survive, and they should all have pigmented eyes (*i.e.*, they should carry the transgene). Of the 624 progeny scored, 115 were non-Stubble, non-Ubx, and all of these were red eyed. Similar results were seen with three other loss-of-function alleles, all cytologically normal revertants isolated in the above screen (*DTS7-rv3γ*, *DTS7-rv11γ*, and *DTS7-rv12γ*). The transgene was not able to rescue the recessive lethal phenotype of *DTS7-rv19X*, suggesting that this strain may carry a small deletion or other mutation in the 71A-B region that we were unable to visualize.

## DISCUSSION

The identification of *DTS7* as a gene encoding the 20S proteasome β2 subunit is supported by three lines of evidence: (1) the only transcription unit detected within the *DTS7* region, as defined by overlapping deficiencies, is the β2 gene; (2) sequencing of the β2 genes of the *DTS7* mutant strain, and the Samarkand wild-type strain from which it was derived, shows that the *DTS7* β2 gene encodes an altered 20S subunit, involving the substitution of a highly conserved residue; and (3) a transgenic DNA fragment containing the β2 transcription unit and no other identified gene is able to rescue both the dominant temperature-sensitive lethal phenotype of *DTS7* and the recessive lethal phenotype of *DTS7$^-$* mutant alleles. Taken together, these results provide compelling evidence that the *DTS7* gene and the β2 gene are one and the same.

The *DTS7* mutant behaves genetically as an antimorphic, or dominant negative, mutation. Such an allele is characterized by the property that its dominant mutant phenotype is ameliorated by increasing, and exacerbated by decreasing, the dosage of the wild-type gene (Muller 1932). It has been hypothesized that if a protein normally acts as part of a multimeric complex, then a mutant variant that is capable of becoming integrated into the complex might behave as a "poison subunit" and disrupt the function of the entire structure (Herskowitz 1987). Under this model, the altered form of the β2 subunit encoded by the *DTS7* allele would disrupt the function or stability of 20S proteasomes that contain either one or two mutant β2 subunits. Because each 20S proteasome contains two β2 subunits, this would mean that only one-fourth of the 20S proteasomes of such a heterozygote would be functionally normal (*i.e.*, would have only wild-type β2 subunits). So, while individuals with half the number of functional proteasomes (for example, those heterozygotes for a loss-of-function *DTS7$^-$* allele) might be normal, those with only one-fourth the number would be severely compromised, and this may explain the dominant effect. This model could also explain the synthetic lethal interaction between *DTS7* and *l(3)73Ai$^1$*. If both mutant subunits were expressed and could act as poison subunits, then in a double heterozygote only one-sixteenth of the 20S pro-

teasomes would be completely normal (*i.e.*, have two wild-type β2 subunits and two wild-type β6 subunits), thus resulting in the severe phenotype of the double mutant. That proteasome subunit mutations can act in such a dominant negative fashion has experimental support in other systems. In yeast, a missense mutation in the 19S cap gene DOA4, encoding a deubiquitinating enzyme, acts as a dominant negative by inhibiting ubiquitin-dependent proteolysis (Papa and Hochstrasser 1993). Similarly, missense mutations in subunits of the PA28 (or REG) regulatory cap of the proteasome can inhibit proteasome activation in an *E. coli* expression system assay, even when other normal subunits are present in the complex (Zhang *et al.* 1998).

Given our results with *DTS7* and *l(3)73Ai*[1], it is reasonable to expect that certain mutations in other β-type proteasome subunit genes might also exhibit dominant temperature-sensitive phenotypes. However, none of the other DTS mutants characterized by Holden and Suzuki (1973) exhibits phenotypes or genetic interactions similar to those shown by *DTS7* and *l(3)73Ai*[1], nor do any of the other cloned β-type subunit genes map to the sites of any well-characterized DTS mutants (our unpublished results). Thus, it does not appear that any of the other extant DTS mutants are likely to represent β-type subunit genes.

The structure of the 20S proteasome may help explain how the *l(3)73Ai*[1] and *DTS7* mutant subunits act as poison subunits. Although the exact structure of the fly 20S proteasome is not known, the yeast particle has been solved by X-ray crystallography (Groll *et al.* 1997). The yeast structure shows that β2 interacts with β1 and β3 in the same ring and with β6 and β7 in the adjacent β ring. Conversely, β6 interacts with β5 and β7 in the same ring and with β2 and β3 in the adjacent ring. Moreover, the yeast structure suggests that the specific interactions between β2 and β6 are extensive and intimate, involving the β2 loop 162–167 and the long C-terminal arm (Figure 5). Extrapolating from the yeast structure enables us to locate the relative positions of the amino acid substitutions associated with both DTS mutants. The predicted position of the T18I mutation of the *l(3)73Ai*[1] β6 subunit is in the loop between β-sheet2 and β-sheet3. The predicted position of the G170R mutation of the *DTS7* β2 subunit is between α-helix4 and β-sheet9. Thus, each mutant is altered near the β2-β6 interfacing regions (Figure 5). It is possible that the physical proximity of the two mutational lesions plays a role in the strong genetic interaction seen in the double-mutant heterozygote.

These insights into proteasome structure shed light on the possible ways in which the β2 mutation affects proteasome function. For example, in the *DTS7* mutation G170R, the substitution of an arginine, a large basic amino acid, for a glycine, small and uncharged, may interrupt the folding of the β2 protein between α-helix4 and β-sheet9 and structurally alter the proteasome or cause it to fail to assemble correctly at the elevated temperature. Proteasome assembly is a two-step process where an α-ring is initially bound to a β-ring and two β-rings are subsequently joined to form the four-ringed 20S proteasome (Schmidtke *et al.* 1996). During the last step, the β subunit prosequences are autocatalytically cleaved, creating the active particle. The G170R substitution of *DTS7* might affect how the β-rings align or interfere with the cleavage of the prosequences. Alternatively, the G170R mutation could affect the catalytic function of the β2 subunit by altering the structure of the active site. Structural analysis of the yeast proteasome shows that Ser 169 (immediately adjacent to the

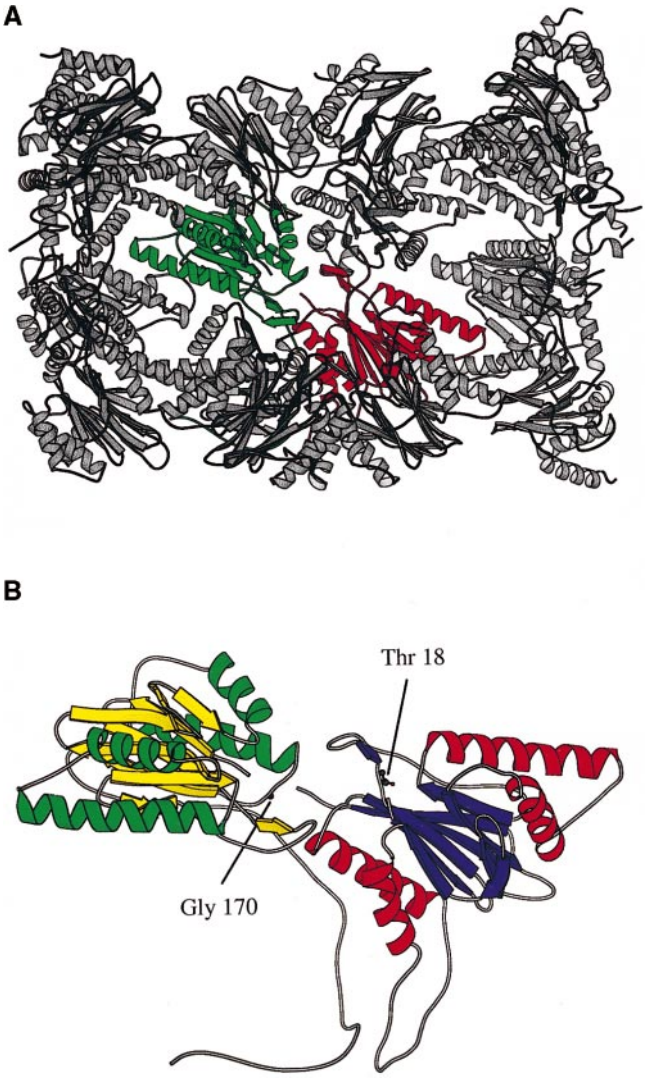


Figure 5.—(A) Ribbon drawing of the yeast 20S proteasome. The β2 subunit is shown in green and the β6 in red, in adjacent β-rings. (B) Ribbon drawing of the yeast β2 and β6 subunits in adjacent β-rings. α-helices are in green (β2) or red (β6) and β-sheets in yellow (β2) or blue (β6). The position of the Gly 170 of β2, between α-helix4 and β-sheet9, is shown in purple. The position of the Thr 18 of β6, between β-sheet2 and β-sheet3, is also in purple.

Gly 170 substituted in *DTS7*) is close to the active site Thr 1, and, along with Ser 129 and Asp 166, it has a role in maintaining the structure of the active site (Groll *et al.* 1997). Additionally, Gly 170 is hydrogen bonded to Asp 17, another residue that plays an important role in formation of the active site. The G170R substitution may severely alter the active site structure and thus affect the proteolytic function of mutant proteasomes.

The availability of two separate conditional lethal proteasome mutants should provide a useful tool for assessing the role of the ubiquitin-proteasome pathway in Drosophila development. For example, temperature-sensitive mutants of two cyclosome components, cdc23 and cdc27, have been used to study the roles of the ubiquitin-proteasome pathway in the regulation of cyclin proteolysis and cell cycle control in yeast (Prinz *et al.* 1998). Mutant *l(3)73Ai*$^{1}$ and *DTS7* heterozygotes could be used in similar temperature-shift experiments to examine these, or other, processes in Drosophila. The *l(3)73Ai*$^{1}$ mutation has already been used to help elucidate the role of the Fat facets protein, a Drosophila deubiquitinating enzyme, as a regulator of cell fate decisions (Huang *et al.* 1995). A variation of these types of experiments would make use of the UAS/GAL4 binary system of Brand and Perrimon (1993) to target the expression of these antimorphic mutant subunits to specific cells or developmental stages to see what effects proteasome malfunction has on particular processes of interest. Such experiments would provide a complementary approach to the use of proteasome inhibitors to investigate the myriad roles that proteasome-related protein degradation plays in the life of a complex organism. This type of genetic approach would have advantages over the use of exogenous proteasome inhibitors, whose specific delivery to the cells of interest could be very problematic.

Finally, one question of future investigation concerns the molecular identity of the extragenic suppressor mutant, *Su(DTS)-1*, which is able to rescue both β subunit DTS alleles, *l(3)73Ai*$^{1}$ and *DTS7*. One obvious possibility is that the suppressor might encode a variant of one of the other β-type subunits that is able to interact directly with both β2 and β6 and somehow compensate for the changes in the DTS mutant subunits. Another explanation is suggested by recent work in mammalian cells, where it was found that under the continuous presence of potent proteasome inhibitors, another proteolytic system is apparently able to compensate for the loss of proteasome function in some cells and allow their proliferation (Glas *et al.* 1998). Perhaps the *Su(DTS)-1* mutant results in the upregulation of expression of such a compensatory system in the fly. In any case, the future molecular characterization of *Su(DTS)-1* will be necessary to understand its relationship to proteasomes and clarify how it acts to ameliorate the effects of these DTS mutants.

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