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)73Ai1, 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 polyubiquitinylated 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)73Ai1 allele (Saville and Belote 1993). This mutant (previously known as DT5) 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)73Ai1 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)73Ai1 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-
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°C, unless otherwise noted. Genetic variants of D. melanogaster are described in Lindsay 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[ry506]dev[w] transposon inserted at 7D1 by crossing females of genotype ru h DTS7 st tra in ry P[ry + 17.2 = PZ]dev[w00208] ry02d to ry02e males and culturing the offspring at 29°C. 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 + 17.2 = PZ]dev[w00208] by individually crossing the ry survivors to ru h st cu str and Pri ca TM6B, HU T b e c a 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[ry]dev[w00208] 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/ + TM3, Sb + Ser were exposed to 5000 rad of γ-rays from a 60Co source or were fed 0.007 mM diepoxybutane and then mated to DTS7 + st tra in p/ + TM3, Sb + Ser females. After 2 days, the cultures were placed at 29°C and then cleared of parents at day seven. The rare survivors potentially represent pseudorevertants in which the DTS7-bearing chromosomal regions uncovered by the deficiency Df[3L]fz-M21.

In the second mutagenesis experiment, males of genotype DTS7 + st tra in p/ + TM3, Sb + Ser were irradiated with 3000 rad of X-rays and crossed to females of genotype I(3)73Ali/TM3, Sb + Ser and the progeny raised at 25°C. 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 I(3)73Ail. The mutagenized chromosomes were maintained over balancers and tested at 29°C 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 Axiosplan 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-l-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 Sau3A into the BamHI site of the λDASH vector (Stratagene). The ligation products were packaged using the Gigapack Gold III Bacteriophage 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 [35S]dATP and Sequenase (United States Bio-experiment, male adults of genotype

Polymorphic DNA in situ hybridization: DNA from D. melanogaster was digested with HindIII and labeled with [35S]dATP using the SuperScript III system from Life Technologies. The labeled DNA was hybridized to polytene chromosomes from late third instar larvae using standard methods. The hybridized DNA was visualized using autoradiography.
RESULTS

Genetic relationship between the $l(3)73Ai^1$ and DTS7 mutants: Individuals heterozygous for the $l(3)73Ai^2$ mutant allele develop normally when reared at $25^\circ$C but die during the late pupal stage when raised at $29^\circ$C (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 $\geq 25^\circ$, although at $18^\circ$C viable, but sterile, adults are observed (Saville and Belote 1993). The loss-of-function allele, $l(3)73Ai^{v10e}$, 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 $l(3)73Ai^2$, although certain aspects are more severe. Heterozygotes show no apparent phenotypic effects when raised at $25^\circ$, but at $29^\circ$, 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 ($18$–$29^\circ$). DTS7 hemizygotes usually die soon after hatching, although at $18^\circ$ some survive to the late larval or early pupal stage. Loss-of-function alleles of the DTS7 locus (e.g., DTS7-rv30, see below) are similar in phenotype to loss-of-function alleles of $l(3)73Ai^2$; they are recessive, early larval lethals.

While the similarity in mutant phenotypes is suggestive of a relationship between $l(3)73Ai^2$ and DTS7, more compelling is the strong synthetic lethal genetic interaction shown by the two mutants. Specifically, $l(3)73Ai^2$ 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 $l(3)73Ai^2$ 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 $l(3)73Ai^2$ and DTS7 mutants are functionally related. In a screen for extragenic dominant suppressors of the DTS lethal phenotype of $l(3)73Ai^2$, a mutant was found that rescues $l(3)73Ai^2$ heterozygotes from the 29°C 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 $l(3)73Ai^2$ or DTS7 are normally viable when reared at 29°C. This suppressor only partially rescues the synthetic lethal interaction of the two DTS mutants; i.e., a small fraction of triply heterozygous individuals (+ $l(3)73Ai^2$ Su(DTS)-1/DTS7 + +) can survive to adulthood when raised at 18°C.

The observations that (1) DTS7 and $l(3)73Ai^2$ have similar lethal phenotypes, (2) DTS7 and $l(3)73Ai^2$ exhibit a strong, allele-specific genetic interaction, and (3) the DTS lethal phenotypes of both DTS7 and $l(3)73Ai^2$ are rescued by the same extragenic suppressor mutant provide strong evidence that these genes are functionally related. Because $l(3)73Ai^2$ 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 $\sim$3-42.3. To better correlate this with a cytological map position within this interval, we mapped the DTS7 mutant relative to P$^+$Zdevel(00208), a genetically marked P-element transposon inserted in chromosome 7D02 (see materials and methods). The results indicated that the DTS7 locus lay $\sim$0.6 map units to the right of the ry$^+$ marker associated with this P element, which had been previously shown to be inserted at the daw
TABLE 1

<table>
<thead>
<tr>
<th>Rearrangement or mutant</th>
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<th>Source</th>
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<th>Discoverer</th>
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<td>Df(3L)D-1rv16</td>
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<td>X ray</td>
<td>+</td>
<td>A. Carpenter</td>
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<td>X ray</td>
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<td>This study</td>
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</tbody>
</table>

*These breakpoints are based on our determinations.

The DTS7 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 homozgyous 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-G51a, 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)73Ai1 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).

**Positional cloning of the DTS7 locus:** Having localized the DTS7 gene to the 71A2-71B4 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 DTS7 region.
DT57 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)DT57-rv16X and Df(3L)DT57-rv24X polytene chromosomes to determine their relationship to the defining breakpoints of the DT57 interval. Two clones, λDm10 and λDm13, cross the distal and proximal defining breakpoints, respectively. We expected the DT57 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 DT57 gene. The inserts from both λDm10 and λDm13 were digested with EcoRI, 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 EcoRI 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 DT57 mutant shows a strong genetic interaction with the l(3)73Ai1 mutant, and (3) the only identified transcription unit within the DT57 region encodes a β-type proteasome subunit, it seemed highly probable that DT57 and the β2 subunit gene correspond to the same locus.

Sequence analysis of the β2 gene from a DT57 mutant strain: The DT57 mutant l(3)73Ai1 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)73Ai1 and DT57, it is reasonable to suppose that the latter mutant is also a missense mutation involving a conserved amino acid.

To test the hypothesis that DT57 encodes an altered β2 proteasome subunit, we sequenced the gene encoding the β2 subunit from individuals hemizygous for the DT57 mutation. Dying non-Tubby larvae were selected from a cross of DT57/TM6B, Hua/Tb females and Df(3L)fz-M21/TM6B, Hua/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 DT57 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 DT57 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 archaeabacteria to humans, suggesting that it is functionally important and strengthening the view that this amino acid is absolutely conserved among β2 subunit genes from all species examined to date, from archaeabacteria to humans, suggesting that it is functionally important and strengthening the view that this amino acid

Figure 2.—Schematic diagram summarizing the positional cloning of DT57. (A) Schematic diagram of the 70C–71C region of the salivary gland chromosome map. For Df(3L)DT57-rv16X and Df(3L)DT57-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 EcoRI 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 DT57 (hatched bar), and the position and orientation of the β2 cDNA (arrowhead).
acids change in β2 corresponds to the DTS7 mutation (Figure 4).

Rescue of the DTS7 mutant phenotype by a β2 transgene: The DTS lethal phenotype of I(3)73Aii 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 EcoRI/PstI 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 reared at 29° and tested the transduced gene for its ability to rescue the DTS phenotype of DTS7, then all non-Stubble, non-Serrate (i.e., DTS7/+/ and DTS7/TM6B,
Hu Tb) survivors should be red eyed (that is, they must carry the transgene to survive at 29°C). 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., +/TM 3, Sb Ssr and TM 68, Hu Tb/TM 3, Sb Ssr) 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/TM 3, Sb were crossed to w/++; Df(3L)fz-M 21/TM 6, 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-M 21) 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 (Her skowitz 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 I(3)73A 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-
peasomes 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 DT57 and l(3)73Ai1, 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 DT57 and l(3)73Ai1, 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)73Ai1 and DT57 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)73Ai1 β6 subunit is in the loop between β-sheet2 and β-sheet3. The predicted position of the G170R mutation of the DT57 β2 subunit is between α-helix4 and β-sheet9. Thus, each mutant is altered near the active site. Structural analysis of the yeast proteasome enables us to locate the relative positions of the amino acid substitutions associated with both DTS mutations. The predicted position of the T18I mutation of the l(3)73Ai1 β6 subunit is in the loop between β-sheet2 and β-sheet3. The predicted position of the G170R mutation of the DT57 β2 subunit is between α-helix4 and β-sheet9. Thus, each mutant is altered near the active site. Structural analysis of the yeast proteasome enables us to locate the relative positions of the amino acid substitutions associated with both DTS mutations. The predicted position of the T18I mutation of the l(3)73Ai1 β6 subunit is in the loop between β-sheet2 and β-sheet3. The predicted position of the G170R mutation of the DT57 β2 subunit is between α-helix4 and β-sheet9. Thus, each mutant is altered near the active site.
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)73Ai1 and DTS7 heterozygotes could be used in similar temperature-shift experiments to examine these, or other, processes in Drosophila. The l(3)73Ai1 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)73Ai1 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 (Gilas 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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