The Catecholamines up (Catsup) Protein of *Drosophila melanogaster* Functions as a Negative Regulator of Tyrosine Hydroxylase Activity

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ABSTRACT

We report the genetic, phenotypic, and biochemical analyses of *Catecholamines up* (*Catsup*), a gene that encodes a negative regulator of tyrosine hydroxylase (TH) activity. Mutations within this locus are semidominant lethals of variable penetrance that result in three broad, overlapping effective lethal phases (ELPs), indicating that the *Catsup* gene product is essential throughout development. Mutants from each ELP exhibit either cuticle defects or catecholamine-related abnormalities, such as melanotic salivary glands or pseudotumors. Additionally, *Catsup* mutants have significantly elevated TH activity that may arise from a post-translational modification of the enzyme. The hyperactivation of TH in *Catsup* mutants results in abnormally high levels of catecholamines, which can account for the lethality, visible phenotypes, and female sterility observed in these mutants. We propose that Catsup is a component of a novel system that downregulates TH activity, making *Catsup* the fourth locus found within the *Dopa decarboxylase* (*Ddc*) gene cluster that functions in catecholamine metabolism.

THE biosynthesis of all catecholamines initiates with the hydroxylation of l-tyrosine into 3-4-dihydroxy-l-phenylalanine (DOPA), a reaction catalyzed by tyrosine 3-hydroxylase (TH; reviewed in Smeets and Reiner 1994). This first step is also the rate-limiting step and is considered the pivotal control point in the catecholamine biosynthetic pathway (reviewed in Kumer and Vrana 1996). This is in accordance with the complex array of regulatory mechanisms that direct both the synthesis of TH and its subsequent catalytic activity (reviewed in Kumer and Vrana 1996). The level and type of TH protein are controlled by transcriptional regulation, alternative splicing, transcript stability, and translational regulation. After synthesis, the enzymatic activity of TH is modulated by two forms of feedback inhibition, allosteric regulation, enzyme stability, and protein phosphorylation.

Much of the framework for the regulation of TH activity and catecholamine biosynthesis has been elaborated through biochemical, molecular, and pharmacological studies in mammalian cell culture systems (reviewed in Kumer and Vrana 1996). However, the genetic and molecular techniques available in the Drosophila model system offer an additional avenue of approach to elucidate and identify proteins that regulate TH activity and catecholamine biosynthesis (reviewed in Wright 1987; Restifo and White 1990). Encoded by the *pale* (*ple*) locus (Neckameyer and White 1993), Drosophila TH shares ~50% amino acid identity to its mammalian orthologs (Neckameyer and Quinn 1989). In addition, Drosophila TH is regulated by transcriptional and post-translational mechanisms that appear to be similar to those in mammals (Neckameyer and Quinn 1989; Birman *et al.* 1994), suggesting that the mechanisms directing this regulation may also be retained. These close similarities suggest that a genetic analysis of catecholamine regulation in Drosophila should be a fruitful avenue for uncovering further details of catecholamine regulatory mechanisms and functions that will have general applicability.

In addition to TH, >45 genes have been identified in Drosophila that appear to play a role in catecholamine metabolism (reviewed in Wright 1987). Of particular interest is a densely populated cluster of at least 21 genes surrounding the *Dopa decarboxylase* (*Ddc*) locus, the ortholog of mammalian AADC. The *Ddc* gene cluster seems to represent a large grouping of functionally related genes, because mutations in most loci result in similar morphogenetic defects of the cuticle or catecholamine-related abnormalities (Stathakis *et al.* 1995; Wright 1996). Seven of these genes are of particular interest because HPLC studies revealed that mutations in any of these loci result in mutants with aberrant catecholamine pool levels during prepupal and pupal development (T. Homyk, W. E. McIvor and T. R. F. Wright, unpublished data; cited in Wright 1996). One of these genes is *Ddc* itself (Wright *et al.* 1982), while a second is *diphenol oxidase A2* (*DOX-A2*), a protein

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that may regulate diphenol oxidase activity, another enzyme involved in catecholamine biosynthesis (Pentz *et al.* 1986; Pentz and Wright 1986; Kawamura *et al.* 1996). A third locus, *a-methyl DOPA* (*amd*), encodes a protein structurally related to DDC (Eveleth and Marsh 1986; Black *et al.* 1987) which may also function as a decarboxylase in catecholamine metabolism (Konrad *et al.* 1993; Wang and Marsh 1995). We report here the results of the genetic, phenotypic, and biochemical analyses of a fourth locus in this group, *Catecholamines up* (*Catsup*), so named because one of its mutant phenotypes is an elevation in catecholamine pools. We demonstrate that Catsup, the protein encoded by this gene, acts as a negative regulator of TH activity and thereby defines a new mode of regulation for catecholamine production.

MATERIALS AND METHODS

Strains: The locus *l(2)37Bc* (Stathakis *et al.* 1995) has been renamed *Catecholamines up* (*Catsup*). The *P*-element insertion strain P{lacW}*Catsup*^{k05402} was kindly provided by Paul Lasko. The *OvoD1* (B2121) and *T(2;3)ap*^{Xa} (B3234) strains were obtained from the Bloomington Stock Center, and the *odd* (1699) strain was obtained from the Mid-America Drosophila Stock Center. All other *Drosophila melanogaster* mutations, aberrations, and balancer chromosomes used were described previously (Lindsley and Zimm 1992; Stathakis *et al.* 1995).

Genetic testing for lethality: All crosses mated 12 males to 8 virgin females, and in most cases reciprocal crosses were set up. Hemizygous viability was tested over *Df(2L)TW130* and *Df(2L)OD15*, both of which delete the *Catsup* locus (Stathakis *et al.* 1995). Crosses were scored for the presence of non-*Curly O* progeny on days 14, 16, and 18. The severity of lethality was classified as follows: v, viable, 86% or more of expected progeny survive to eclose as adults; sv, subvital, 51–85% of expected progeny survive to eclose as adults; sl, semilethal, 6–50% of expected progeny survive to eclose as adults; l, lethal, 5% or less of expected progeny survive to eclose as adults.

Genetic testing for female sterility: Heteroallelic females were generated by mating 12 *Catsup*^{x}/*CyO* males to 8 *Catsup*^{y}/*CyO* virgin females and collecting the resulting *Catsup*^{x}/*Catsup*^{y} virgin females. Hemizygotes were generated in a similar fashion, except that *Catsup*^{x}/*CyO* males were mated to female virgins carrying either *Df(2L)TW130* or *Df(2L)OD15*. Female sterility was tested by mating 12 Oregon-R males to 10 *Catsup* heteroallelic heterozygous virgin females and scoring pupal stage progeny on day 7. To account for possible developmental delays, crosses were monitored for an additional 10 days. Fertility was classified as follows: ++, fully fertile (≥21 pupae), 0–5% reduction in female fertility; +, fertile (16–20 pupae), 6–25% reduction in female fertility; rf, reduced fertility (11–15 pupae), 26–50% reduction in female fertility; ss, semisterile (6–10 pupae), 51–75% reduction in female fertility; vs, virtually sterile (2–5 pupae), 76–95% reduction in female fertility; fs, female sterile (0–1 pupa), 96–100% reduction in female fertility.

***P*-element plasmid rescue:** Genomic DNA was purified from P{lacW}*Catsup*^{k05402} adult flies using Qiagen tips, and ~5.0 μg of genomic DNA was digested with *Sst*II (Life Technologies, Grand Island, NY). Digested DNA (~1.5 μg) was added to a 200-μl ligation reaction mix containing 2 units of T4 DNA ligase (Life Technologies), and the reaction was incubated at 14° overnight. Ligated DNA was precipitated in 0.3 m sodium acetate and 2 volumes absolute ethanol, microfuged for 15 min and resuspended in TE buffer. Resuspended DNA was transformed into DH5α *max efficiency* competent cells (Life Technologies), and plasmid-rescued colonies were selected on 100 μg/ml ampicillin Luria broth (LB) agar plates. Alkaline-lysis-purified plasmid DNA isolated from individual colonies was digested with *Sst*II, and P{lacW} transposon-positive clones were sequenced using the Thermo Sequenase Radiolabeled Terminator cycle sequencing kit (Amersham Life Sciences, Arlington Heights, IL).

***P*-element mutagenesis screen for imprecise excisions:** The PlacW transposon was remobilized by crossing P{lacW}*Catsup*^{k05402} virgin females *en masse* to males carrying the *P* transposase (Figure 1). Individual F_1 male progeny exhibiting somatic mosaicism in the compound eyes were isolated and mated to four virgin females from the *T(2;3)ap*^{Xa} balancer stock. Individual F_2 male progeny having lost the w^+ marker of the P{lacW} transposon were backcrossed individually to eight *Catsup*^{12} virgin females and scored for lethality. Crosses that did not produce viable heteroallelic heterozygous non-*Curly O* progeny in the next generation were considered potential *Catsup* mutations. A stock was established for all potential *Catsup* mutations, and each line was subjected to restriction fragment length polymorphism (RFLP) analysis to determine the extent of the imprecise excision event.

RFLP analysis: Genomic DNA from 30 adult flies was purified by a potassium acetate/ethanol precipitation procedure. Purified genomic DNA was digested with *Pst*I, electrophoresed, and transferred to a Zeta-probe nylon membrane (Bio-Rad, Richmond, CA) using an alkaline transfer method (Reed and Mann 1985) modified as follows: an acid pretreatment step was not used, and 0.4 N NaOH served as the transfer solution. *Catsup* gene-specific ^{32}P-labeled probes were synthesized from the 1.74-kb *Eco*RI-*Hin*dIII fragment from cDNA Bc541 (Stathakis *et al.* 1995) using the Prime-It II random primer labeling kit (Stratagene, La Jolla, CA). High stringency hybridizations and washes were carried out at 65° using the high SDS method (Church and Gilbert 1984). Membranes were exposed to Kodak XAR-5 film at −70° with intensifying screens for up to 24 hr before development.

Effective lethal phase analysis: The effective lethal phase of *Catsup* alleles was quantitatively measured by the direct method (Shearn 1978), using an egg-picking procedure (Sparrow and Wright 1974) with the following modifications. Cultures were established on egg-laying plates and transferred to fresh plates every 2 hr. Eggs were collected and transferred to new egg-laying plates in groups of ~50 and scored every day for viability until eclosion. Mortality at each stage was expressed as a percentage of the total number of fertile eggs. Embryos, larvae, pupa, and adults were also examined for any mutant phenotype. *or*^{49h}, a recessive visible mutant allele, was used to mark the *Catsup* mutant chromosome and phenotypically distinguish homozygous from heterozygous animals (Ashburner 1989).

Germline clone analysis: Germline clones of *Catsup*^{1} were generated using the dominant female sterile technique (Perrimon and Gans 1983; Perrimon *et al.* 1984). Balanced *Catsup*^{1} females were crossed *en masse* to males carrying two *P*-element insertions of the dominant female sterile mutation *OvoD1* {*P*[*OvoD1*](28A, 30D)}. Resulting *Catsup*^{1}/*OvoD1* virgin females were irradiated with 1200 rad of gamma radiation from a ^{137}Cs source. Irradiated females were mated to *Catsup*^{1}/*CyO* males *en masse* and placed on egg-laying plates. Plates were changed every 24 hr and scored for the presence of eggs.

Light and electron microscopy analysis: Dissected pupae and pharate adults were divided into head, thoracic, and abdominal regions and fixed for 24 hr in 100 mm sodium phosphate (pH 7.2) containing 2.5% glutaraldehyde and 4% para-

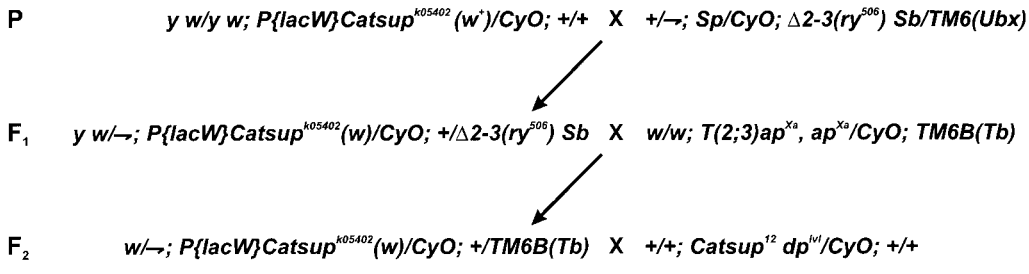

Figure 1.—Genetic screen to isolate imprecise excisions of P{lacW} transposon in P{lacW} *Catsup*^{k05402}. The genotype of P{lacW}*Catsup*^{k05402} is *y* $w^{67c23(2)}$/*y* $w^{67c23(2)}$, *Catsup*^{k05402} P{lacZ$^{P/T.W}$ w^{+mC} ampR ori}/*CyO*. Genotypes of all other fly strains used in this screen are indicated in the figure.

formaldehyde. Samples were washed twice and postfixed in 100 mm sodium phosphate containing 2% osmium tetroxide for 3 hr, dehydrated in acetone, and embedded in Epon 812. For light microscopy, both longitudinal and cross semithick sections (0.5 μm) were cut and stained with either 0.5% toluidine blue (5 min) or 2% acid fuchsin (10 min). For transmission electron microscopy, samples were trimmed and thin gold sections were collected on Formvar-coated copper slot grids, poststained with 5% uranyl acetate and 0.25% lead citrate, and examined in a JEOL 100CX electron microscope.

Phalloidin staining with confocal microscopy analysis: Adult females were dissected in Ringer's solution, and their ovarioles were fixed in Ringer's solution containing 4% formaldehyde for 60 min. Ovarioles were washed three times for 5 min each time in 1× PBS (pH 7.4) and incubated in blocking buffer [1× PBS (pH 7.4), 2% BSA, 0.2% NP-40, 0.02% sodium azide] for 30 min containing a 1:100 dilution of fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR) to visualize filamentous actin. Tissues were washed four times for 15 min each time in 1× PBS (pH 7.4) and mounted in Vectashield (Vector Laboratories, Burlingame, CA) to limit photobleaching. Tissues were visualized using an MRC 640 confocal microscope (Bio-Rad).

High performance liquid chromatography analysis: Pool sizes of tyrosine and catecholamines were determined during late embryogenesis by reverse-phase high performance liquid chromatography (RP-HLPC) with electrochemical detection. Staged dechorionated embryos were sorted by phenotype using the *odd-skipped* allele *odd*^{IIID} as an embryonic marker (Nüsslein-Volhard *et al.* 1984). Sorted embryos were immediately homogenized in 100 mm perchloric acid, 4 mm EDTA (50 embryos in 100 μl buffer), chilled on ice for 15 min to precipitate proteins, and centrifuged for 10 min at 27,000 × *g* at 4° to remove tissue debris. Samples were immediately stored at −70° until needed.

Chromatographic separations were carried out on either a Beckman 334 liquid chromatography apparatus equipped with a 100A pump and 427 integrator or a Beckman 126 liquid chromatography apparatus equipped with the System Gold software package (Beckman, Fullerton, CA). Samples were loaded using a Spectrophysics SP8780 autoinjector fitted with a 10-μl injection loop. Separations were achieved using a 4.6 × 100-mm, 3-μm reverse-phase ODS particle column (Ranin Microsorb Short One) using an ESA Coulochem II EC detector fitted with a 5021 conditioning cell and a 5011 analytical cell. The conditioning cell was set at −0.04 V, while the two detector electrodes were set at +0.25 V to detect catechols and hydroxyindoles and +0.55 V to detect hydroxyphenyl compounds and indoles with substituted rings. Column temperature was held constant at 30°. The most commonly used mobile phase consisted of 75 mm sodium phosphate (pH 3.0), 1.7 mm octanesulfonic acid, 0.01% (v/v) triethylamine, 0.025 mm EDTA, and 11% (v/v) acetonitrile at a flow rate of 1.00 ml/min. Column retention times and linear detector response were calibrated using commercial standards, except for *N*-β-alanyl dopamine (NBAD), *N*-acetyl norepinephrine (NANE), and *N*-β-alanyl norepinephrine (NBANE), which were kindly provided by Karl J. Krammer. The limit of detection ranged from 10 fmol DOPA to 50 fmol tryptophan per injected sample. Typical standard elution times are as follows:

+0.25 V detector: DOPA 1.57 min, NANE 1.81 min, NBANE 2.13 min, *N*-acetyl dopamine (NADA) 2.73 min, normetanephrine 2.98 min, dopamine (DA) 3.65 min, 5-hydroxyindole acetic acid (5-HIAA) 4.2 min, acetyl serotonin 4.79 min, homovanillic acid (HVA) 6.11 min, and NBAD 7.90 min;

+0.55 V detector: l-tyrosine 2.00 min, *M*-tyrosine 2.44 min, normetanephrine 3.08 min, vanillic acid 5.04 min, and tryptophan 13.6 min.

Chromatograms of free catecholamine pool levels routinely resolved five major electroactive compounds in amounts of ≥1 pmol/embryo, of which three were identified as DOPA, DA, and NADA. Chromatograms of free tyrosine pool levels routinely resolved three electroactive compounds in amounts of ≥1 pmol/embryo, one of which was l-tyrosine.

Dopamine feeding experiments: Two different feeding experiments were conducted on media augmented with DA. In the first approach, 70–100 Oregon-R first instar larvae were harvested from 24-hr egg-laying plates and transferred to Petri dishes with filter paper containing a 25% yeast paste supplemented with either 1, 2, 3, 10, or 20 mg/ml DA. Cultures were monitored daily until eclosion, and animals were scored for delays in developmental rates and any visible larval or pupal phenotypes. In the second experimental approach, five Oregon-R virgin females were mated to five Oregon-R males and transferred to instant Drosophila medium (Carolina Biological) reconstituted with water supplemented with either 1, 2, 5, or 10 mg/ml DA. Adult flies were removed after 3 days, and the vials were subsequently monitored for the numbers of progeny that pupated and eclosed and for effects on developmental rates. Adult flies resulting from these crosses were transferred to vials containing unsupplemented media and were scored daily for fecundity and progeny viability.

Tyrosine hydroxylase assays: The l-tyrosine-3, 5-^{3}H microradioassay method (Nagatsu *et al.* 1964) was used with the following changes. Samples were homogenized in ice-cold 1× PBS (pH 7.0) containing 1 mm phenylmethylsulfonyl fluoride and 1 mm dithiothritol (25 larvae in 300-μl buffer). Debris was removed by centrifugation for 10 min at 4000 × *g*, and protein concentrations were determined. Two hundred microliters of fly extract, adjusted to 1 μg/μl protein, were added to a 300-μl reaction solution consisting of 200 μl 0.15 mm sodium phosphate (pH 6.7) containing 1950 units of catalase, 50 μl 1.0 mm ascorbic acid, 1.0 mm 6, 7-dimethyl 5, 6, 7, 8-tetrahydropterin ($DMPH_4$) in 1 mm ascorbic acid, and 50 μl 1.0 mm l-tyrosine with a ^{3}H-tyrosine tracer (100,000 cpm). The reaction components were incubated with shaking at 32° in uncapped, round-bottomed tubes for 25 min. The remainder of the procedure was essentially as described by Neckameyer and Quinn (1989). The enzyme reaction demonstrated

linear reaction kinetics with respect to increasing enzyme concentrations.

Western blot analysis: Total protein was extracted from third instar larvae, separated by 10% SDS-PAGE, and transferred to PVDF filters (Bio-Rad) using standard methods (Harlow and Lane 1988). Filters were blocked for 5 hr in 50 mm Tris-HCl (pH 7.6), 100 mm NaCl, 0.2% Tween-20, 5% nonfat dry milk, and incubated overnight in a 1:2500 dilution of anti-TH mouse monoclonal antibody (Pel-Freez), a 1:17,000 dilution of guinea pig polyclonal anti-Catsup antiserum, or a 1:15,000 dilution of anti-α-tubulin mouse monoclonal antibody (Sigma, St. Louis, MO). Blots were washed three times for 30 min each time in 50 mm Tris-HCl (pH 7.6), 100 mm NaCl, 0.2% Tween-20, incubated for 1 hr in a 1:2000 dilution of anti-mouse or a 1:2000 anti-guinea pig IgG-HRP-conjugated secondary antiserum (Jackson Immunological), and washed three times for 30 min in 50 mm Tris-HCl (pH 7.6), 100 mm NaCl, 0.2% Tween-20. Signals were visualized by the Renaissance chemiluminescence reagent (Dupont NEN) and demonstrated a linear response under the conditions used. Western blot films were scanned using a BioImage analyzer and Visage software. The signal in each band was quantitated as the integrated optical density of the band (OD × mm^2). Relative band densities were normalized to protein loads as determined by the band density of the control α-tubulin in each lane.

RESULTS

Genetic analysis of *Catsup*

Characterization of *Catsup* mutations: Twenty-nine mutant alleles of *Catsup* were recovered as part of a saturation mutagenesis for lethal and sterile mutations within the *Ddc* gene cluster (Stathakis *et al.* 1995). As hemizygotes, 25 *Catsup* alleles are nonconditional lethals and designated $Catsup^{1}$ to $Catsup^{25}$. $Catsup^{ts1}$ is a temperature-sensitive lethal with a restrictive temperature of 29° (Stathakis 1998). At permissive temperatures (25° or lower), homozygous $Catsup^{ts1}$ females are fully fertile. $Catsup^{cs1}$ and $Catsup^{cs2}$ are cold-sensitive lethals with restrictive temperatures of 22° or lower and permissive temperatures of 25° or higher (Stathakis 1998). Both alleles exhibit extremely reduced viability at 22° and complete lethality at 18°. Hemizygous escaper females for either allele are completely sterile. The last allele, $Catsup^{fs1}$, is a female sterile. $Catsup^{fs1}$ hemizygotes are completely viable but female sterile at all experimental temperatures tested (18°, 22°, 25°, and 29°).

***Catsup* mutations can be ordered into an allelic series:** The 25 nonconditional lethal *Catsup* mutations can be divided into five groups on the basis of the penetrance of lethality and female sterility (Table 1). Group I (GI) and group II (GII) lethal mutations have marginal effects on viability and fertility and are considered weak *Catsup* alleles. A single lethal allele, $Catsup^{11}$, is considered a GI lethal because it shows reduced viability as a hemizygote, behaving as a semilethal mutation with hemizygous female escapers being completely sterile (Table 2). This contrasts with GII lethals ($Catsup^{14}$, $Catsup^{17}$, and $Catsup^{19}$) that are hemizygous lethal. Homozygous mutants of either GI or GII lethal alleles exhibit incomplete penetrance for lethality, but differ in their effects on female fertility (Table 2). $Catsup^{11}$ homozygous adult female escapers are fertile, while $Catsup^{14}$, $Catsup^{17}$, and $Catsup^{19}$ homozygous female escapers exhibit drastically reduced fertility (Table 2). In addition, while GI/GII heteroallelic heterozygotes are completely fertile, GII/GII heteroallelic heterozygotes are female sterile (Table 2).

Group III (GIII) and group IV (GIV) lethal mutations have moderate effects on progeny viability and fertility. The 14 *Catsup* alleles comprising these two categories are lethal as hemi- and homozygotes (Table 2). However, these alleles can be separated into two categories on the basis of their interactions with GII lethal mutations and as heteroallelic heterozygotes with respect to each other. The seven alleles comprising GIII lethal mutations ($Catsup^{2}$, $Catsup^{6}$, $Catsup^{8}$, $Catsup^{9}$, $Catsup^{15}$, $Catsup^{18}$, and $Catsup^{23}$) produce progeny when heterozygous with any GII lethal allele (Table 2). This contrasts with GIV lethal mutations ($Catsup^{4}$, $Catsup^{5}$, $Catsup^{7}$, $Catsup^{10}$, $Catsup^{12}$, and $Catsup^{24}$), which are lethal when heterozygous with any GII lethal allele (Table 2). In addition, except for $Catsup^{15}$, GIII/GIII heteroallelic heterozygotes are viable and fertile, while all GIV/GIV heteroallelic heterozygotes are lethal (Table 2).

Allele-specific interactions are evident for GIII lethal mutations. Among GIII lethal mutations, $Catsup^{15}/Catsup^{6}$, $Catsup^{15}/Catsup^{8}$, and $Catsup^{15}/Catsup^{9}$ heterozygotes are viable, while $Catsup^{15}/Catsup^{2}$, $Catsup^{15}/Catsup^{18}$, and $Catsup^{15}/Catsup^{23}$ heterozygotes are lethal. This indicates that $Catsup^{15}$ is a strong negative complementer of the latter three alleles. Likewise, $Catsup^{8}$ negatively complements $Catsup^{2}$ and $Catsup^{6}$, as heterozygotes have dramatically reduced viability. This negative complementation suggests that GIII lethal mutations might affect two different functional aspects of the Catsup gene product. Allele-specific interactions also occur between GIII/GIV heterozygous combinations, suggesting that GIV lethal mutations are not complete loss-of-function mutations. These interactions are most evident with the group III lethal mutation $Catsup^{9}$. While other GIII lethals fail to complement GIV lethals, $Catsup^{9}$ complements all GIV lethal mutations, except for $Catsup^{12}$ (Table 2). The majority of GIII heteroallelic heterozygotes are female sterile (Table 2). However, heterozygotes between $Catsup^{9}$ and most GIII lethal mutations are fully fertile. Only $Catsup^{9}/Catsup^{15}$ heterozygotes are completely sterile (Table 2). GIV lethal mutations have a slightly more severe effect on female fertility because all GIV/GIV heteroallelic heterozygotes are completely sterile (Table 2). This contention is further supported by interallelic interactions of GIII and GIV lethal alleles with the female-sterile allele $Catsup^{fs1}$. $Catsup^{fs1}$/GIII heteroallelic heterozygotes exhibit a wide phenotypic range, with $Catsup^{fs1}/Catsup^{15}$ and $Catsup^{fs1}/Catsup^{23}$ female adults displaying full fertility (Table 2). In contrast, all $Catsup^{fs1}$/GIV heteroallelic heterozygotes show se-

TABLE 1

Lethal and female sterile effects of *Catsup* alleles

			Heteroallelic heterozygote					
Allele classification	Hemizygote	Homozygote	*Catsup*^{fs1}	GI lethals	GII lethals	GIII lethals	GIV lethals	GV lethals
Group I lethals (GI)								
Catsup^{11}	sl / fs	sv / ++	v / ++	—				
Group II lethals (GII)								
Catsup^{14}, *Catsup*^{17}, *Catsup*^{19}	l	sl-v / fs	v / ++-fs	v / ++	sl-v / fs			
Group III lethals (GIII)								
Catsup^{2}, *Catsup*^{6}, *Catsup*^{8}, *Catsup*^{9}, *Catsup*^{15}, *Catsup*^{18}, *Catsup*^{23}	l	l	v / ++-fs	v / ++	sl-v / ++-fs	sl-v / fs		
Group IV lethals (GIV)								
Catsup^{4}, *Catsup*^{5}, *Catsup*^{7}, *Catsup*^{10}, *Catsup*^{12}, *Catsup*^{24}	l	l	v / ss-fs	v-1 / fs	l	l	1	
Group V lethals (GV)								
Catsup^{1}, *Catsup*^{3}, *Catsup*^{13}, *Catsup*^{16}, *Catsup*^{21}, *Catsup*^{22}, *Catsup*^{25}	l	l	v / fs	sl / fs	l	l	l	l

Values are viability / fertility. When a range of phenotypes was seen in a given combination, the least and most severe traits observed are indicated. For viability: v, viable, on average ≥86% of expected progeny survive to eclose as adults; sv, subvital, on average 51–85% of expected progeny survive to eclose as adults; sl, semilethal, on average 6–50% of expected progeny survive to eclose as adults; l, lethal, on average ≤5% of expected progeny survive to eclose as adults. For fertility: ++, wild-type, fully fertile females; rf, reduced fertility, on average females show a 40% reduction of fertility; ss, semisterile, on average females show a 70% reduction of fertility; fs, female sterile, females are completely sterile; l, lethal, flies do not survive to eclose.

TABLE 2

Hemi-, homo-, and heteroallelic *Catsup* mutant relative viability and fertility (%)

		fs	GI	GII			GIII						
Classification	*Catsup*	fs1	11	14	17	19	2	6	8	9	15	18	23
fs	fs1	0	122[a]	104[b]	115	126[a]	106	90	103[c]	107[e]	97[a]	110	105[a]
GI	11	122[a]	79[a]	101[a]	103[a]	94[a]	71[a]	165[a]	97[a]	101[a]	62	82[a]	117[a]
GII	14	104[b]	101[a]	17	98	64	42	40	77	100[a]	76	104	118
GII	17	115	103[a]	98	109[e]	106	51	94	109[a]	93[a]	75	98	88
GII	19	126[a]	94[a]	64	106	71	33	55	108	89[a]	71	112	101
GIII	2	106	71[a]	42	51	33	0	46	12[e]	94[a]	0	87	76
GIII	6	90	165[a]	40	94	55	46	0	7	92[a]	89	102	105
GIII	8	103[c]	97[a]	77	109[a]	108	12[e]	7	0	110[a]	99	111[d]	102
GIII	9	107[e]	101[a]	100[a]	93[a]	89[a]	94[a]	92[a]	110[a]	0	69	106[a]	112[a]
GIII	15	97[a]	62	76	75	71	0	89	99	69	0	0	0
GIII	18	110	82[a]	104	98	112	87	102	111[d]	106[a]	0	0	115
GIII	23	105[a]	117[a]	118	88	101	76	105	102	112[a]	0	115	0
GIV	4	91[e]	0	0	0	0	0	0	0	97	0	59	0
GIV	5	104	85	0	0	0	0	0	0	38	0	105	5
GIV	7	95	3	0	0	0	0	0	0	102	0	66	0
GIV	10	104[d]	9	0	0	0	0	0	0	102	0	0	0
GIV	12	107	74	0	0	0	0	0	0	0	0	6	0
GIV	24	111	48[a]	0	0	0	0	0	0	13[a]	0	10	0
GV	1	92	43	0	0	1	0	0	0	1	0	2	0
GV	3	86	34	0	0	1	0	0	0	3	0	1	0
GV	13	106	51[e]	0	0	2	0	0	0	0	0	0	0
GV	16	104	52	0	0	0	0	0	0	0	0	0	0
GV	21	101	58	0	0	0	0	0	0	0	0	0	0
GV	22	105	57[e]	0	0	0	0	0	0	0	0	1	0
GV	25	110	51	0	0	0	0	0	0	0	0	4	0
Df	TW130[f]	91	49	0	0	0	0	0	0	0	0	0	0
Df	OD15[f]	88	30	0	0	0	0	0	0	0	0	0	0

The 155 crosses of *Catsup* alleles not shown did not produce viable homozygotes or complementing heteroallelic heterozygotes, with 350 or more progeny scored for each cross. All surviving heteroallelic heterozygous females are sterile (96–100% reduction in fertility), except as otherwise indicated.

[a] Fully fertile (0–5% reduction in fertility).
[b] Fertile (6–25% reduction in fertility).
[c] Reduced fertility (26–50% reduction in fertility).
[d] Semisterile (51–75% reduction in fertility).
[e] Virtually sterile (76–95% reduction in fertility).
[f] Deficiency is abbreviated without the Df(2L) designator.

verely reduced fertility, with the vast majority of these heterozygotes being completely female sterile (Table 2).

The seven remaining *Catsup* mutations (*Catsup*^{1}, *Catsup*^{3}, *Catsup*^{13}, *Catsup*^{16}, *Catsup*^{21}, *Catsup*^{22}, and *Catsup*^{25}) are considered group V (GV) lethal mutations as they have the most severe effects on viability and fertility. Heterozygotes between a GV lethal allele and any other *Catsup* allele, except for the two weakest mutations, *Catsup*^{fs1} and *Catsup*^{11}, are lethal (Table 2). Heterozygotes for *Catsup*^{11} or *Catsup*^{fs1} and a GV lethal allele are always female sterile. The results with GV lethal alleles are identical to those obtained from *Df(2L)TW130* and *Df(2L)OD15*, two different deficiencies that delete the *Catsup* locus (Table 2). This suggests that GV lethals are null mutations.

Taken together, this analysis indicates that the nonconditional lethal alleles of *Catsup* can be grouped in terms of increasing penetrance of lethality and female sterility. GI and GII lethals have weak effects, and GIII and GIV lethals display moderate effects. GV lethals have the most severe effects, which behave similarly to deletions of *Catsup*.

Generation of *Catsup* deletion mutation: The *Catsup* locus lies within the distal subcluster of the *Ddc* gene cluster (Stathakis *et al.* 1995). Both *Df(2L)TW130* and *Df(2L)OD15* remove many loci within the *Ddc* gene cluster, several of which also affect catecholamine pool levels. Therefore, there was concern that aspects of the *Catsup* null phenotype may have been altered or masked by the presence of only one copy of one or several of these other loci involved in catecholamine metabolism. To address this issue, we conducted a *P*-element mutagenesis screen to generate imprecise excision mutations to generate a deletion within the *Catsup* locus.

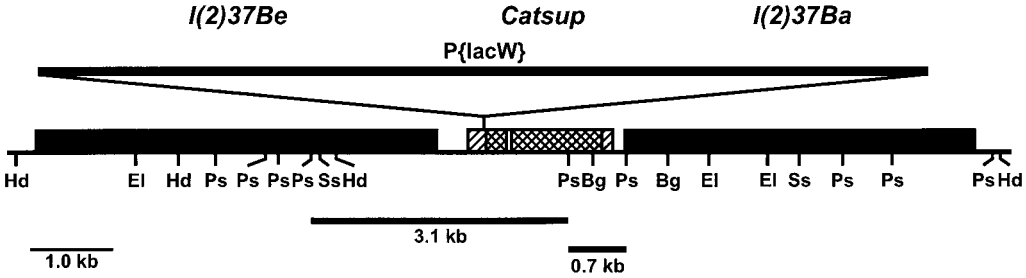

Figure 2.—RFLP analysis of *Catsup* imprecise excision mutations. The figure depicts the restriction endonuclease map of the genome region surrounding the *Catsup* locus. The genomic organization of the *Catsup* locus consists of two exons (Stathakis 1998) and is illustrated as follows: cross-hatched rectangles represent the protein coding regions of the exons, hatched rectangles represent the 5′ and 3′ UTRs of the exons, and the white rectangle represents the single intron. Also shown is the insertion site of the P{lacW} transposon within the 5′ UTR of *Catsup*. The location of the transcription units for *l(2)37Be*, *Catsup*, and *l(2)37Ba* are represented by black rectangles. The two *Pst*I DNA fragments recognized by the *Catsup* cDNA probe in the RFLP analysis are indicated below the restriction map. Restriction enzyme symbols are as follows: Bg, *Bgl*II; EI, *Eco*RI; Hd, *Hin*dIII; Ps, *Pst*I; Ss, *Sst*II. The diagram is drawn to scale.

We obtained a Berkley Drosophila Genome Project *P*-element insertion strain (Spradling *et al.* 1995), designated P{lacW}*Catsup*^{k05402}. P{lacW}*Catsup*^{k05402} behaves genetically as a severe GV lethal allele of *Catsup*. P{lacW}-*Catsup*^{k05402} mutations are lethal when heterozygous with the moderate GIV lethal allele *Catsup*^{12}, the severe GV lethal allele *Catsup*^{1}, or *Df(2L)TW130* and *Df(2L)OD15*. In addition, the penetrance of the weak GI lethal mutation *Catsup*^{11} is increased to similar levels when *in trans* to either P{lacW}*Catsup*^{k05402}, the severe GV lethal allele *Catsup*^{1}, or *Df(2L)TW130* and *Df(2L)OD15*. We cloned the genomic DNA region flanking the insertion site of the P{lacW} transposon in P{lacW}*Catsup*^{k05402} by plasmid rescue. Subsequent sequence analysis indicated that the P{lacW} transposon is inserted in the 5′ UTR of the *Catsup* transcription unit, 26 bp upstream from the AUG initiator methionine codon (Figure 2).

To generate deletion mutations in the *Catsup* locus, we screened for imprecise excisions of the P{lacW} transposon in P{lacW}*Catsup*^{k05402} by using the Δ2-3 transposase (Figure 1). Individual strains from 56 independent imprecise excision chromosomes were isolated, and males were backcrossed to *Catsup*^{12} females. Flies from 21 of these isolated lines proved lethal *in trans* to *Catsup*^{12}. These lines were considered new *Catsup* mutations and designated *Catsup*^{26}–*Catsup*^{46} (Table 3). Flies from each new allele were screened for disruption of the *Catsup* locus by RFLP analysis to determine the extent of the imprecise excision. A total of 16 lines exhibited visible polymorphisms relative to the 13.7-kb parental fragment that contains the 10.6-kb P{lacW} transposon of strain P{lacW}*Catsup*^{k05402}, while 5 lines showed no visible size reduction of the 13.7-kb parental fragment (Table 3). Of the 16 lines that did possess a visible polymorphism, the extent of the imprecise excision in 14 of these isolates ranges from 2.6 to 10.3 kb (Table 3). Because the P{lacW} transposon is 10.6 kb, these data suggest that these 14 lines still contain a remnant of the 10.6-kb transposon. Thus, we consider these 14 lines, in conjunction with the 5 isolates that show no visible polymorphism, as insertion mutations of *Catsup* (Table 3). The remaining two isolates (*Catsup*^{26} and *Catsup*^{27}) show a clear reduction in the 13.7-kb band to a size that is less than the 3.1-kb wild-type genomic DNA fragment. This suggests that in these two lines, regions of the *Catsup* locus in addition to the entire 10.6-kb P{lacW} transposon were removed. The first isolate, *Catsup*^{26}, exhibits an ~600-bp deletion of genomic DNA, while the second isolate, *Catsup*^{27}, shows a smaller deletion of ~300 bp. Because the excision event in both isolates does not affect the function of either locus flanking *Catsup* (see below), we deduce that *Catsup*^{26} and *Catsup*^{27} are deletions in the *Catsup* locus.

Two distal subcluster genes, *l(2)37Be* and *l(2)37Ba*, are located <500 bp on either side of the *Catsup* locus (Stathakis *et al.* 1995; see Figure 2). The close proximity of *l(2)37Be* and *l(2)37Ba* relative to *Catsup* raised concerns that an imprecise excision event of the P{lacW} transposon could also have removed essential regions of these two flanking transcription units. To determine whether potential *Catsup* mutations affected the function of either flanking gene, we conducted crosses with *l(2)37Be*^{4} and *l(2)37Ba*^{1}. Of the 22 *Catsup* mutations generated by this screen, three isolates (*Catsup*^{44}, *Catsup*^{45}, and *Catsup*^{46}) were also lethal for *l(2)37Be*^{4} (Table 3). None of the 22 *Catsup* mutations were lethal for *l(2)37Ba*^{1}.

Catsup^{26} was selected for further analysis because this mutation represents the largest deletion of the *Catsup* locus that did not affect the function of *l(2)37Be* and *l(2)37Ba*. With respect to genetic interactions, ELPs, fertility, and visible phenotypes, *Catsup*^{26} is indistinguishable from other GV lethal alleles. In addition, in every aspect examined, the lethal, sterile, and visible phenotypes of *Catsup* mutations were identical when tested with *Catsup*^{26}, *Df(2L)TW130*, and *Df(2L)OD15*. *Catsup*^{26} mutations are lethal when heterozygous with *Catsup*^{12} (GIV) or *Catsup*^{1} (GV) lethal alleles or deficiencies *Df(2L)TW130* and *Df(2L)OD15*. In addition, the penetrance of the weak lethal mutation *Catsup*^{11} (GI) is increased to similar levels when *in trans* to either *Catsup*^{26}, the GV lethal allele *Catsup*^{1}, or the deficiencies *Df(2L)TW130* and *Df(2L)OD15*. This analysis clearly indi-

TABLE 3

***Catsup* imprecise excision mutations**

Isolate line	Allele designation	Mutation class	RFLP analysis[a]		Genetic analysis[b]		
			RFLP fragment size (kb)	p{lacW} remnant size (kb)	*Catsup*	*l(2)3Be*	*l(2)3Ba*
M17	*Catsup*^{26}	Del	2.5	None	−	+	+
M19	*Catsup*^{27}	Del	2.8	None	−	+	+
M2	*Catsup*^{28}	Ins	None	10.6	−	+	+
M3	*Catsup*^{29}	Ins	None	10.6	−	+	+
M8	*Catsup*^{30}	Ins	None	10.6	−	+	+
M11	*Catsup*^{31}	Ins	None	10.6	−	+	+
M20	*Catsup*^{32}	Ins	None	10.6	−	+	+
M4	*Catsup*^{33}	Ins	8.0	5.6	−	+	+
M5	*Catsup*^{34}	Ins	7.3	6.3	−	+	+
M7	*Catsup*^{35}	Ins	7.3	6.3	−	+	+
M10	*Catsup*^{36}	Ins	7.0	6.6	−	+	+
M13	*Catsup*^{37}	Ins	4.0	9.6	−	+	+
M14	*Catsup*^{38}	Ins	3.4	10.2	−	+	+
M15	*Catsup*^{39}	Ins	3.8	9.8	−	+	+
M16	*Catsup*^{40}	Ins	6.8	6.8	−	+	+
M18	*Catsup*^{41}	Ins	4.7	8.9	−	+	+
M21	*Catsup*^{42}	Ins	6.8	6.8	−	+	+
M22	*Catsup*^{43}	Ins	8.0	5.6	−	+	+
M6	*Catsup*^{44}	Ins	7.1	6.5	−	−	+
M9	*Catsup*^{45}	Ins	4.5	9.1	−	−	+
M12	*Catsup*^{46}	Ins	5.0	8.6	−	−	+

[a] The expected parental *Pst*I fragment is an ~13.7-kb fragment containing the 3.1-kb *Pst*I genomic DNA fragment plus the 10.6-kb P{lacW} transposon.

[b] (−) Lethal, ≤5% of expected progeny survive to eclose; (+) viable, ≥86% of expected progeny survive to eclose.

cates that GV lethal alleles are genetic nulls, and that all the observed lethal, sterile, and visible phenotypes can be solely attributed to mutations in the *Catsup* locus.

***Catsup* nonconditional mutations are semidominant lethals that exhibit three broad ELPs:** To elucidate when the *Catsup* gene product is required during development, we determined the ELP for each of the five nonconditional lethal categories of *Catsup* mutants. In conjunction with our phenotypic examination, this analysis indicates that the onset of lethality can be divided into three broad periods and that a distinctive phenotype is associated with each ELP (Table 4).

Early larval lethals: The first ELP occurs between the late embryonic period and the second larval instar. Approximately 65–75% of the total lethality observed in *Catsup* mutants hemi- or homozygous for the moderate lethal alleles *Catsup*^{23} (GIII) and *Catsup*^{12} (GIV) and the severe lethal allele *Catsup*^{1} (GV) takes place in this ELP (Table 5). However, while the overall level of total lethality is comparable among these three lethal groups, the relative distribution of lethal onset between each of the three developmental periods is different. The lethality seen in larvae hemi- or homozygous for the *Catsup*^{1} (GV) allele is more evenly distributed, with total observed lethality occurring in ~22% of the embryos, 30% of the first instars, and 23% of the second instars (Table 5). In larvae hemi- or homozygous for *Catsup*^{12} (GIV), lethality occurs primarily in the first instar, with total observed lethality occurring in ~20% of the embryos, 44% of the first instars, and 10% of the second instars (Table 4). The distribution of lethality arises even later in hemi- or homozygous *Catsup*^{23} (GIII) mutants, with total lethality occurring in ~8% of embryos, 28% of first instars, and 30% of second instars (Table 5).

The most common phenotype associated with early larval lethality is arrested development. Hatched *Catsup* mutant first instar larvae may survive up to 4 days, but do not grow or molt. In addition, these stunted larvae are akinetic, crawling slowly across the food or moving only when prodded, and may also exhibit patches of internal melanization. Likewise, the majority of dying embryos undergo internal melanization several hours before hatching. However, this late embryonic lethality cannot be attributed solely to their inability to hatch because the majority of manually dissected, living larvae are akinetic. Larvae that reach second instar are of normal size, but they exhibit the same akinetic behavior and internal patches of melanization seen in *Catsup* mutant first instar larvae. These second instar larvae may survive up to 2 days, but do not grow or molt.

Late larval lethals: The second ELP occurs during the third instar, although some lethality is seen earlier in

TABLE 4

Phenotypes of *Catsup* mutants

Allele classification	Effective lethal phase[a]			Arrested larval development[b]	Melanotic salivary glands[c]	Melanotic body tumors[d]	Comments
	Hemizygote	Homozygote	Heterozygote				
Female sterile	None	None	None	No	No	Yes	A small percentage (<5%) of balanced heterozygous third instar larvae develop melanotic tumors.
GI lethals	Third instar through pupa	Third instar through pupa	Third instar through pupa	No	Yes	No	Approximately 10–20% of homozygous and balanced heterozygous third instar larvae develop melanotic salivary glands.
GII lethals	Third instar through pupa	Third instar through pupa	Third instar through pupa	No	Yes	Yes	Approximately 10–20% of homozygous and balanced heterozygous third instar larvae develop melanotic salivary glands.
GIII lethals	First instar through second instar	First instar through second instar	Third instar through pupa	Yes	Yes	Yes	Approximately 10–20% of balanced heterozygous third instar larvae develop melanotic salivary glands.
GIV lethals	Embryonic through first instar	Embryonic through first instar	Second instar through pupa	Yes	Yes	Yes	Approximately 10–20% of balanced heterozygous third instar larvae develop melanotic salivary glands, except for *Catsup*^{15} and *Catsup*^{24} heterozygotes.
GV lethals	Embryonic through first instar	Embryonic through first instar	Second insta through pupa	Yes	Yes	Yes	Approximately 10–20% of balanced heterozygous third instar larvae develop melanotic salivary glands, except for *Catsup*^{1} and *Catsup*^{3} heterozygotes.

[a]Larval lethality occurs throughout larval development in the indicated instar; pupal lethality occurs throughout prepupal and pupal development. While balanced heterozygotes were examined for each *Catsup* allele, a representative allele from each lethal category was also examined as a + / *Catsup* heterozygote (see Table 4).

[b]These larvae remain in the first instar stage for up to 4 days before dying; in weaker alleles, these mutants may undergo one molt and then die as second instar larvae.

[c]Melanotic salivary glands occur primarily in third instar larvae, and there is a high correlation of this phenotype with lethality. A low number (<5%) of second instar larvae can develop this phenotype.

[d]Melanotic body tumors do not cause lethality and occur in second and third larval instars and adults, developing throughout the body.

TABLE 5

Lethal stages of *Catsup* mutants

Cross			Number of fertile eggs	% mortality					
♀♀	×	♂♂		Embryo	First instar	Second instar	Third instar	Pupa	Total
+ / +		+ / +	323	0.6	0	0.5	1.9	1.1	4.1
Df(2L)TW130, or^{49h} / CyO		+ / +	478	0.8	0	1.1	2.7	1.8	6.4
Df(2L)OD15, or^{49h} / CyO		+ / +	618	0.8	0	2.4	6.9	7.2	17.3
Df(2L)OD15, or^{49h} / CyO		*or^{49h}/or^{49th}*	224	0	0	2.7	6.5	7.5	16.7
Df(2L)OD15, or^{49h} / CyO		*Catsup^{11} or^{49h}* / +	492	0.8	0	0	10.4	14.0	25.2
Df(2L)OD15, or^{49h} / CyO		*Catsup^{11} or^{49h}/CyO*	336	26.2	0	0	9.8	14.6	50.6
Df(2L)OD15, or^{49h} / CyO		*Catsup^{19}* / +	358	0.5	0	0.1	10.8	19.1	30.5
Df(2L)OD15, or^{49h} / CyO		*Catsup^{23} or^{49h}* / +	409	3.0	10.8	11.5	6.6	6.8	38.7
Df(2L)OD15, or^{49h} / CyO		*Catsup^{12} or^{49h}* / +	566	9.0	22.3	5.3	4.2	7.6	48.4
Catsup^{12} or^{49h} / CyO		*Catsup^{12} or^{49h}* / +	507	10.1	19.6	4.0	8.0	6.2	47.9
Df(2L)OD15, or^{49h} / CyO		*Catsup^{25} or^{49h}* / +	428	11.2	12.2	10.0	5.4	7.5	46.3
Catsup^{25} or^{49h} / CyO		*Catsup^{25} or^{49h}* / +	276	10.1	16.7	11.2	6.1	5.2	49.3

second instars. Approximately 35–40% of the total lethality seen in hemi-, homo- and heterozygotes for the weak lethal alleles *Catsup^{11}* (GI) and *Catsup^{19}* (GII) occurs in this ELP. In addition, this ELP accounts for ~12–17% of the total lethality seen in *Catsup* mutants heterozygous for the moderate lethal alleles *Catsup^{23}* (GIII) and *Catsup^{12}* (GIV), and the severe lethal allele *Catsup^{1}* (GV; Table 5). The death of ~10–20% of either balanced or nonbalanced third instar heterozygotes indicates that *Catsup* mutations are semidominant lethals (Table 5). Surprisingly, this lethality occurs in heterozygotes from any of the five lethal groups.

Regardless of the allele, dying third instar larvae manifest a very distinctive "melanotic salivary gland" phenotype (Figure 3). In these larvae, individual cells of the salivary glands begin to melanize until, in the most common form, the entire gland is a completely black, mulberry-shaped organ. In the more severe examples, a secondary site of melanization occurs in the mid-dorsal region, probably in the lymph glands, which then spreads throughout the hemolymph. This phenotype can be expressed at any time during third instar.

Pupal lethals: The third ELP exhibits the most variable time of onset, occurring at any time during pupariation (Table 4). Total lethality occurs in ~45–55% of pupae hemi-, homo-, and heterozygotes for the weak lethal alleles *Catsup^{11}* (GI) or *Catsup^{19}* (GII), and ~15–20% of pupae heterozygotes for the moderate lethal alleles *Catsup^{23}* (GIII) and *Catsup^{12}* (GIV) and the severe lethal allele *Catsup^{1}* (GV, Table 5). As observed in the second ELP, lethality occurs in ~10–20% of heterozygotes from any of the five lethal groups.

The *Catsup* mutant phenotype during prepupal development is characterized by large regions of internal melanization in prepupae and pupal cases that are darker than normal, especially after aging (Figure 4A).

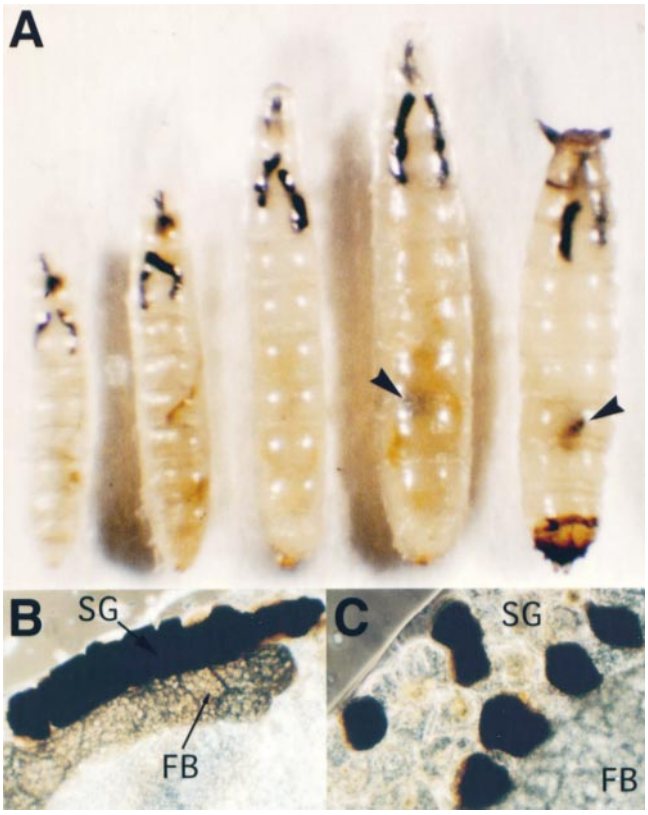

Figure 3.—Late larval lethal phenotypes associated with *Catsup* mutations. (A) Onset of melanotic salivary gland phenotype. Homozygous *Catsup^{11}* larvae from left to right: second instar, early third instar, mid-third instar, late third instar and white prepupa. There is abnormal melanization centered around the lymph area (arrows) in the posterior third of the last two larvae. (B and C) Phase-contrast light microscopy of dissected salivary glands from homozygous *Catsup^{11}* third instar larvae.

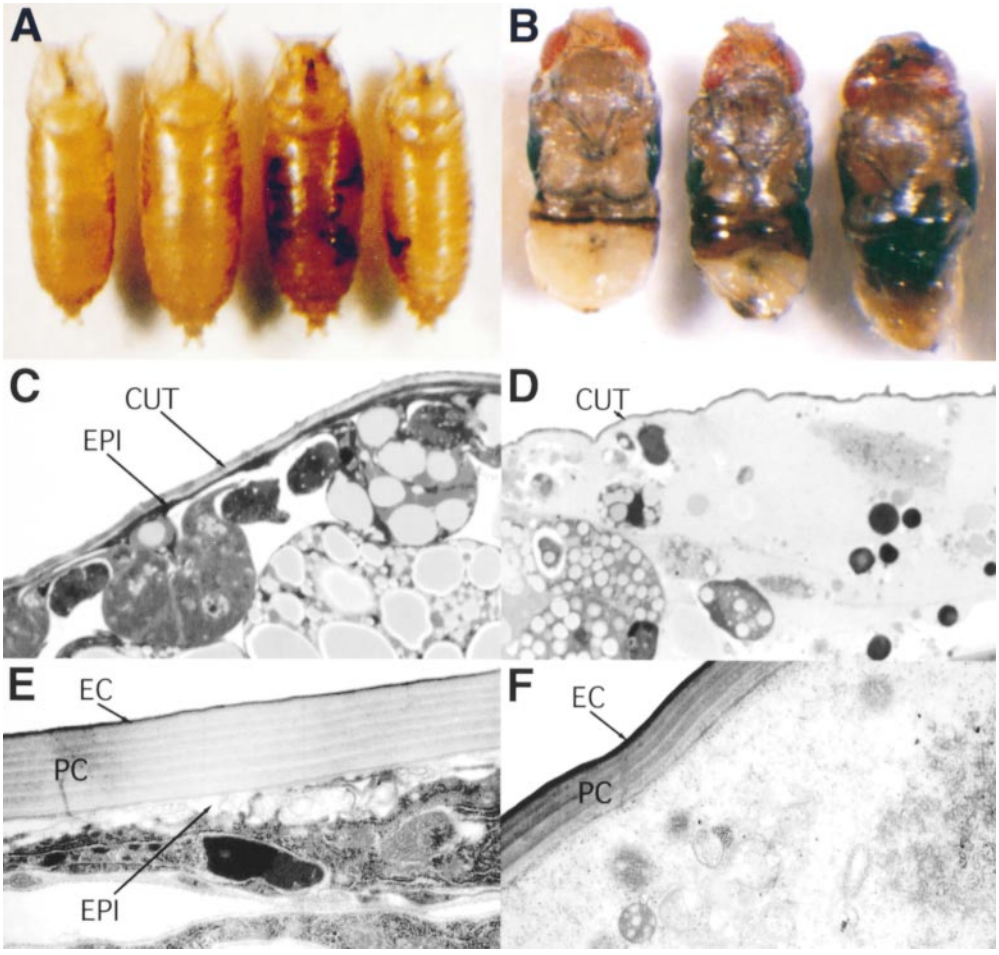

Figure 4.—Pupal lethal phenotypes associated with *Catsup* mutations. (A) Comparison of wild-type (left two) and *Catsup*^{11}/*Df(2L)TW130* mutant (right two) pupae. There are internal regions with intense melanization within *Catsup* mutant pupae. (B) Dissected *Catsup*^{11}/*Df(2L)TW130* late pharate adult. There is an absence of tergite and bristle formation in the posterior abdominal region. Melanization is apparent through the undamaged pupa case at the time of imaginal disc eversion and darkens as development proceeds. (C and D) Micrographs of pharate adult abdominal cuticle. Light micrographs (×2000) of serial sections stained with toluidine blue in a control (C) and *Catsup*^{11} *or*^{49h} homozygote (D). Controls show well-defined epidermis (EPI) and cuticle (CUT). There is a complete absence of a dark-staining epidermal layer in *Catsup*^{11} mutants, but a CUT is present. The lack of internal organs or tissues in these mutants also suggests that the disappearance of the epidermis may result from necrosis. All sections are ×2000, oil immersion. (E and F) Transmission electron micrographs (×16,000) of cross-sections of a control (E) and a *Catsup*^{11} *or*^{49h} homozygote (F). Controls show a well-formed, simple cuboidal epithelium (EPI) and the two subdivisions of the cuticle, an electron-dense epicuticle (EC), and a distinctly lamellar procuticle (PC). While *Catsup*^{11} mutants exhibit no epidermis, cuticle structure appears normal, albeit thinner relative to controls, because both well-formed epicuticle and procuticle layers are present.

In addition, most prepupae that die start autolysis but cannot undergo metamorphosis, producing empty or partially empty pupal cases. Both *Catsup* mutant pupae and pharate adults undergo severe, abnormal melanization and display varying degrees of incomplete cuticle formation. The cuticle is relatively normal on the head and thorax, but is incompletely formed over much of the abdomen (Figure 4B). No bristles are formed in the abdomen, and the abdomen shows no indications of either segmentation or normal patterns of tanning. Furthermore, the abdomens of these pharate adults are usually extensively melanized in a blotchy pattern. While the head, thoracic, and leg cuticles appear to be formed normally, the thoracic cuticle in particular is not completely hardened. Initial inspection of the gross morphology by light microscopy reveals that *Catsup* pharate adults appear to have normal cuticles, but they lack an epidermis in regions exhibiting incomplete cuticles. Further examination by electron microscopy indicates that while thinner relative to control pharate adults, the cuticles of *Catsup* mutants have both well-defined epicuticles and procuticles with normal appearance (Figure 4, E and F).

***Catsup* mutant adult escaper phenotypes:** Incomplete cuticle formation, melanotic pseudotumors, and female sterility are associated with *Catsup* mutant adult escapers. Approximately 30–50% of the adult escapers produced by crosses between balanced GI and GII lethal mutations, as well as various heteroallelic combinations of GIII, GIV, and GV lethal mutations, exhibit minor malformations of the cuticle, primarily on the abdominal tergites (Figure 5, A–D). Another phenotype associated with *Catsup* adult escapers is melanotic pseudotumors. These acellular tumors occur in ~5–10% of *Catsup*^{3}, *Catsup*^{5}, *Catsup*^{6}, *Catsup*^{8}, *Catsup*^{11}, *Catsup*^{13}, *Catsup*^{22}, *Catsup*^{fs1}, *Catsup*^{cs1}, and *Catsup*^{cs2} balanced heterozygotes. Although present in only a subset of *Catsup* alleles, there is no association with mutation severity because

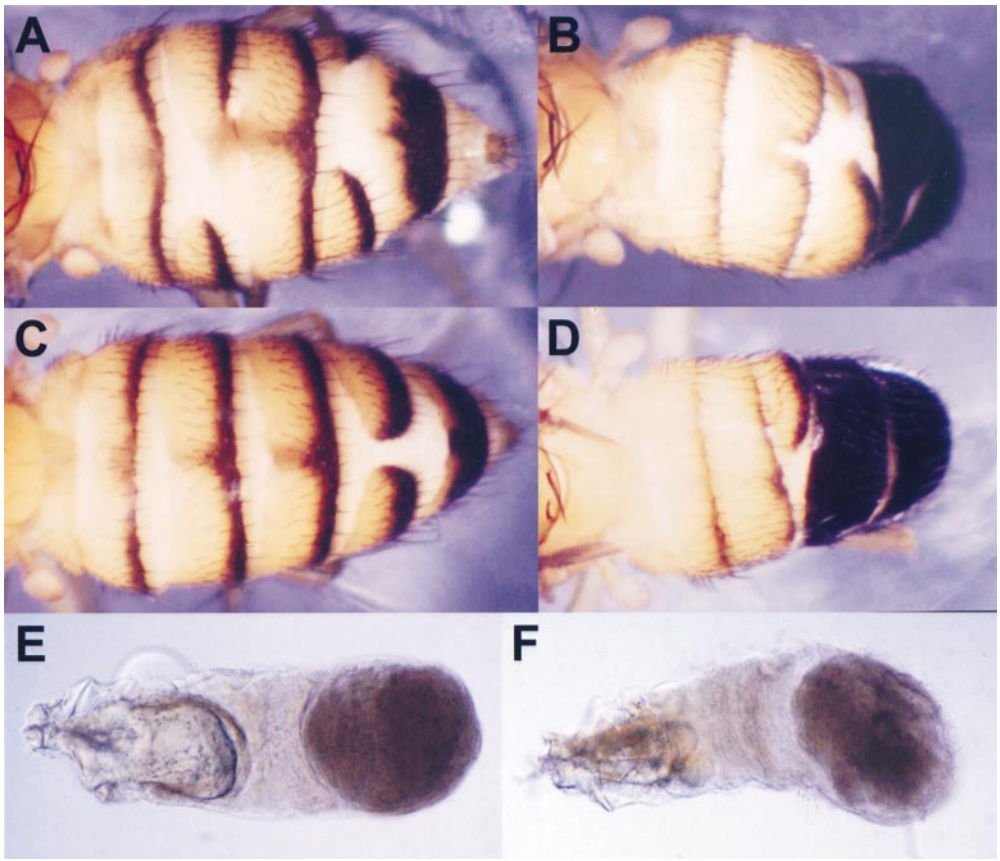

Figure 5.—Adult visible phenotypes associated with *Catsup* mutants. (A and B) Incomplete formation of abdominal tergites in $Catsup^{11}$ homozygous female (A) and male (B) adults. (C and D) Incomplete formation of abdominal tergites in $Catsup^{12}/Catsup^{fs1}$ heterozygous female (C) and male (D) adults. (E and F) Phase-contrast light microscopy of eggs produced by adult females whose germline is homozygous for the $Catsup^{1}$ mutation.

these 10 alleles represent weak, moderate, and severe lethal mutations, as well as the female-sterile and both cold-sensitive mutations. In addition, while melanotic pseudotumors may occur in mutants as early as the second larval instar, their formation does not affect viability because larvae will survive to become fertile adults.

The most common adult phenotype is female sterility. Female sterility is seen in all adults homozygous for $Catsup^{14}$, $Catsup^{19}$, or either cold-sensitive allele raised at permissive temperatures. In addition, ~66% of the crosses that produce heteroallelic heterozygotes are female sterile, with an additional 10% exhibiting some loss of fertility (Table 2). Dissected spermatheca of *Catsup* female-sterile mutants contain large numbers of spermatozoa after mating with wild-type males, indicating that these mutants are inseminated. In addition, female-sterile mutants contain bloated ovarioles containing large numbers of apparently mature, yolk-filled eggs. However, the fecundity of these mutants is dramatically reduced with females depositing only a few small flaccid eggs.

Closer examination of hemizygous $Catsup^{fs1}$ females by DIC and confocal microscopy indicates an abnormal retention of eggs, with every ovariole of a 4-day-old adult containing two to five normal-sized oocytes (Figure 6). Approximately 50% of oocytes from these *Catsup* female-sterile mutants lack dorsal appendages. However, rudimentary filaments do form in the remainder of these oocytes, suggesting that oogenesis can proceed until stage 13. A chorion membrane is always present. The formation of micropyles in addition to the presence of rudimentary dorsal appendages suggests that oocytes from these *Catsup* female-sterile mutants undergo normal pattern formation. Mature, stage 14 oocytes are never present in ovarioles from these *Castup* female-sterile mutants. Likewise, ovarioles contain <5% stage 8–11 egg chambers. A similar egg retention phenotype is seen in $Catsup^{cs1}$ and $Catsup^{cs2}$ hemizygous or $Catsup^{fs1}/Catsup^{1}$ and $Catsup^{fs1}/Catsup^{12}$ $Catsup^{cs1}/Catsup^{cs2}$ heterozygous escaper females.

Germline clone analysis of the GV lethal allele $Catsup^{1}$ confirms a critical role for the *Catsup* gene product during oogenesis. Females with homozygous $Catsup^{1}$ mutant germlines deposit few, morphologically defective eggs that appear to have been arrested at mid- to late oogenesis (Figure 5, E and F). The eggs produced are flaccid when laid or collapse during egg deposition, suggesting the chorion is weak or absent, and the egg is only surrounded by the vitelline membrane. Intact eggs have rudimentary dorsal appendages that are fused to the eggshell, or they lack such appendages all together. Large cuboidal cells, reminiscent of nurse cells, comprise the anteriocentral region of the follicle, with a yolk-filled oocyte occupying the posterior end, a cellular organization similar to that of the "dumpless" phenotype (Perrimon *et al.* 1989). This indicates that females with homozygous $Catsup^{1}$ mutant germlines produce egg chambers with more severe abnormalities relative to hemizygous $Catsup^{fs1}$ females.

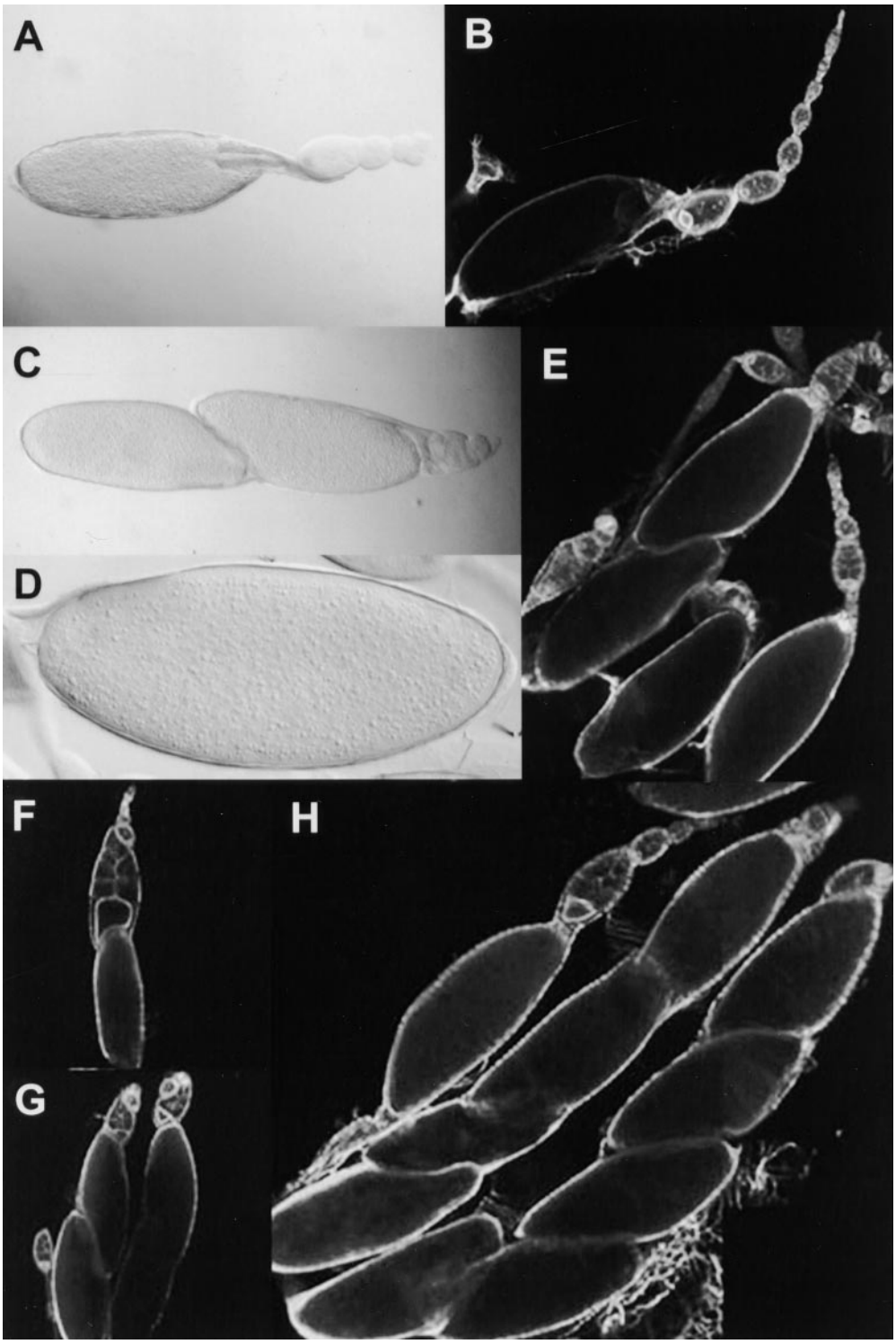

Figure 6.—Visible phenotypes associated with oogenesis in *Catsup* mutants. (A, C, and D) DIC microscopy of ovarioles dissected from a 2- to 4-day-old Oregon-R adult female (A) and from 2- to 4-day-old $Catsup^{fs1}$ hemizygous adult females (C and D). Although arresting at stage 13 of oogenesis, *Catsup* mutant ovarioles appear morphologically normal, possessing a micropyle, chorion membrane, and dorsal appendages. (B and E–G) Confocal microscopy of ovarioles stained with fluorescein-conjugated phalloidin to visualize filamentous actin. Ovarioles dissected from 2- to 4-day-old Oregon-R adult female (B) and from 2- to 4-day-old $Catsup^{fs1}$ hemizygous adult females (E–G). Similar phenotypes are observed in 2- to 4-day-old $Catsup^{cs1}$ and $Catsup^{cs2}$ hemizygous adult females and 2- to 4-day-old $Catsup^{fs1}/Catsup^{1}$ and $Catsup^{fs1}/Catsup^{12}$ and $Catsup^{cs1}/Catsup^{cs2}$ heterozygous adult females.

Biochemical analysis of *Catsup*

***Catsup* mutants exhibit elevated catecholamine pool levels during embryogenesis:** The aberrant melanization observed in *Catsup* mutants could be indicative of abnormal regulation of catecholamine pool levels. In Drosophila, the four major catecholamines produced are DOPA, DA, NADA, and NBAD (Figure 7). DOPA serves primarily as a precursor compound in the catecholamine biosynthetic pathway. DA functions as a neurotransmitter (reviewed in Restifo and White 1990) and is the precursor compound for all subsequent catecholamines used in the melanin and sclerotin biosynthetic pathways (reviewed in Hopkins and Kramer 1992). NADA and NBAD are the two major cross-linking agents in sclerotization. NADA and its derivatives (dehydro-NADA, NANE) are found primarily in colorless (clear) sclerotin, while NBAD and its derivatives (dehy-

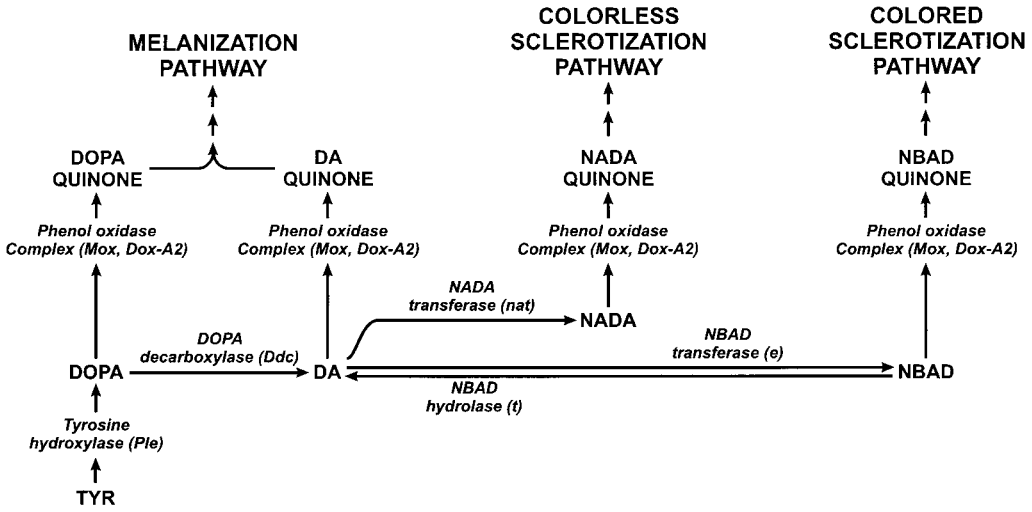

Figure 7.—Catecholamine biosynthesis pathway in *D. melanogaster*. The figure depicts the initial enzymatic steps required to synthesize the four major catecholamines necessary for neurotransmitter production, melanization, and sclerotization (reviewed in Wright 1987). The hydroxylation of tyrosine (TYR) to form DOPA is catalyzed by TH. The decarboxylation of DOPA to form DA is catalyzed by DOPA decarboxylase (DDC). The N-acylation of DA with acetate to form NADA is catalyzed by NADA transferase (NAT), while the N-acylation of DA with β-alanine to form NBAD is catalyzed by NBAD transferase (NBAT). The conversion of all catecholamines to their respective quinones is catalyzed by the phenol oxidase enzyme complex. Loci encoding the enzyme(s) responsible for each reaction are in parentheses, with references for gene assignments as follows: *ple*, Neckameyer and White (1993); *Ddc*, Wright *et al.* (1976); *Nat*, Wright (1987); *t* and *e*, Black (1988); *Mox*, Fujimoto *et al.* (1995); *Dox-A2*, Pentz *et al.* (1986).

dro-NBAD, NBANE) are present principally in colored (brown) sclerotin (Anderson 1989a,b; Hopkins and Kramer 1992; Sugumaran *et al.* 1992). Therefore, measuring the levels of these four catecholamines should determine whether misregulation of catecholamine biosynthesis is occurring in *Catsup* mutants.

We determined catecholamine pool levels in embryos homozygous for the GIV lethal allele $Catsup^{12}$. Because we estimate that ~70% of $Catsup^{12}$ homozygous mutants die during the first larval instar, catecholamine pool levels were determined in 22-hr-old embryos, a time point representing peak catecholamine synthesis during embryogenesis (Wright 1987; D. G. Stathakis, W. E. McIvor and T. R. F. Wright, unpublished data). However, because ~30% of $Catsup^{12}$ homozygous mutants die during embryogenesis, great care was taken to select embryos that were still alive and did not exhibit patches of internal melanization. $Catsup^{12}$ mutant embryos exhibit pronounced increases in DA and NADA pools at 22 hr of embryonic development, indicating that catecholamine biosynthesis is misregulated during embryogenesis (Table 6). Relative to controls, DA pools increase more than fourfold in homozygous $Catsup^{12}$ mutants. Similarly, NADA pools increase more than fivefold in $Catsup^{12}$ mutant embryos. Interestingly, heterozygous $Catsup^{12}$ mutants also exhibit significantly higher levels of DA and NADA relative to controls (Table 6). This increase is approximately midrange between homozygous $Catsup^{12}$ mutants and controls, indicating that one normal allele provides a single functional dose of the *Catsup* gene product.

In contrast to the effects observed for DA and subsequent catecholamines, DOPA pool levels in $Catsup^{12}$ mutant embryos are not significantly elevated relative to wild-type levels. Interestingly, both homo- and heterozygous *Catsup* mutant embryos exhibit significantly reduced levels of free tyrosine relative to controls (Table 6). Tyrosine pools decrease ~3-fold in homozygous $Catsup^{12}$ mutants relative to controls. A similar decrease (2.6-fold) of tyrosine pool levels was also observed in heterozygous $Catsup^{12}$ mutant embryos (Table 6).

Our findings are consistent with HPLC analysis of $Catsup^{11}$ (GI) hemizygotes, which showed dramatic increases in catecholamine pool levels at four distinct times during the first 72 hr of pupariation (T. Homyk, W. E. McIvor and T. R. F. Wright, unpublished data, cited in Wright 1996). During this period, mutants from this GI lethal allele exhibit ~7.5-fold higher levels of NADA, 4-fold higher levels of NBAD and NBANE, and 3-fold higher levels of DA relative to controls. DOPA levels were unaffected.

Exogenous dopamine phenocopies *Catsup* mutations: To test directly whether any of the phenotypes associated with *Catsup* mutations are caused by elevated DA levels, we transferred wild-type first instar larvae to food supplemented with 1, 2, 3, 10, and 20 mg/ml of DA. Many of the phenotypes associated with mutations in *Catsup* are phenocopied under these conditions and are positively correlated with increasing DA concentrations with respect to severity of the observed phenotypes. At higher DA concentrations, these larvae exhibit several early larval lethal phenotypes associated with *Catsup* mutants. Larval lethality is evident on media supplemented with 10 or 20 mg/ml DA, with only 45% of the larvae reaching pupation at 20 mg/ml DA (Table 7). In addition, although not completely akinetic, there is a dramatic reduction in larval movement on media supplemented with 20 mg/ml DA. On lower DA concentrations, *Catsup* mutant phenotypes associated with late larval and pupal ELPs are induced (see Figure 4). Devel-

TABLE 6

HPLC analysis of catecholamine pool levels in *Catsup*^{12} mutant embryos

Genotype[a]	*n*	Mean compound detected (pmol/embryo)[b]			
		Tyrosine	DOPA	DA	NADA
+/+	5	19.86 ± 1.29	0.99 ± 0.57	4.23 ± 0.32	6.58 ± 0.25
+/*Catsup*^{12}	5	7.46 ± 1.42*	1.57 ± 0.54	9.28 ± 0.76*	20.52 ± 2.11*
Catsup^{12}/*Catsup*^{12}	5	6.67 ± 0.42*	2.21 ± 0.80	17.60 ± 1.30*	34.11 ± 1.61*

*The amount of a compound in a mutant combination that is significantly different from the control ($P < 0.0005$). Significant differences were determined by calculating the *F* value for a single-factor analysis of variance and then determining which compounds were significantly different from the others using the Tukey multiple comparison test.

[a]Control and homozygous *Catsup* embryos are homozygous for the *odd*^{IIID} and *or*^{49h} alleles. Heterozygous *Catsup* embryos are heterozygous for the *odd*^{IIID} and *or*^{49h} alleles.

[b]NANE, NBAD, and NBANE were not detected. The numbers given are the amount of compound detected (mean ± standard error) and are expressed in terms of pmol/embryo.

opment of larvae is slowed at concentrations of 3 mg/ml DA or greater, with a delay in pupation of ~24–36 hr at 3 mg/ml DA. Larvae that do undergo pupation are undersized, with the pupal cases tanning much more rapidly and significantly darker relative to the controls. In addition, most pupae that die start autolysis but cannot undergo metamorphosis, producing empty or partially empty pupal cases. Some unhatched pupae exhibit melanotic patches, although this effect is not severe. However, not all phenotypes were induced by exogenously supplemented DA. DA-supplemented, wild-type larvae did not exhibit melanotic salivary glands or pseudotumors at any concentration tested.

We also tested the effects of DA by transferring adults to media supplemented with 1, 2, 5, and 10 mg/ml of DA. The DA supplementation did not have any effect on adult viability and fertility. However, as observed in the first series of experiments, progeny raised in DA-supplemented media resulted in similar effects on larval viability, developmental rates, and late larval and pupal phenotypes. In fact, the effects of DA supplementation were enhanced in this second series of experiments. For example, only 66% of larval progeny from the second series of experiments pupated when raised in media containing 2 mg/ml DA, as compared to 86% of larvae pupating from the first series of experiments. In addition, a low incidence of melanotic tumors were observed in larval progeny of DA-fed adults, with the proportions exhibiting tumors increasing with increasing DA concentrations. The tumors varied in size from very tiny to very large, ultimately filling as much as one half of the pharate adult bodies in the pupal cases. Those with very large tumors did not eclose.

To examine the effects of DA on fertility, adult progeny reared on media supplemented with DA were transferred to unsupplemented media and tested for decreased fecundity and progeny viability. Eggs laid per female during the first 48 hr after eclosion were reduced from 33 to 67% relative to controls. However, these differences largely disappeared by the third day. Thus, effects on egg deposition were seen in adults newly removed from DA, but these deficiencies were not permanent. Furthermore, embryo viability and hatching rates of these eggs were identical to those of control females.

We conclude from these studies that most of the phenotypes observed in *Catsup* mutants can be directly attributed to increases in catecholamine levels, as each

TABLE 7

Viability effects on larvae of DA feeding

DA concentration (mg/ml)	Survival[a]		
	Day 3 (%)	Day 6 (%)	Day 11 (%)
0	65/70 (93)	61/70 (87)	61/70 (87)
1	64/70 (91)	57/70 (81)	55/70 (79)
2	70/70 (100)	60/70 (86)	53/70 (76)
3	65/70 (93)	64/70 (91)	62/70 (89)
10	94/100 (94)	85/100 (85)	59/100 (59)
20	75/100 (75)	45/100 (45)	31/100 (31)

[a]Day 3 corresponds to third instar larvae, day 6 to pharate adults, and day 11 to 4-day-old adults.

TABLE 8

Specific activity of tyrosine hydroxylase in *Catsup* mutant prepupae

Genotype[a]	*n*	Specific activity range[b]	Mean specific activity	Relative activity
+ / +	4	0.42–0.62	0.66 ± 0.09	1×
Catsup^{11} / *CyO*	3	3.21–4.35	3.68 ± 0.19*	5.6×
Catsup^{11} / *Catsup*^{11}	3	1.35–2.89	2.18 ± 0.29*	3.3×
Catsup^{12} / *CyO*	3	4.61–5.16	4.89 ± 0.17*	7.4×
Catsup^{1} / *CyO*	3	2.19–3.06	2.74 ± 0.24*	4.1×
Catsup^{26} / *CyO*	6	1.68–3.19	2.63 ± 0.09*	4.0×

*The specific activity of a mutant combination that is significantly different from the control ($P < 0.0005$). Significant differences were determined by calculating the *F* value for a randomized block single factor analysis of variance and then determining which compounds were significantly different from the others using the Tukey multiple comparison test.

[a]Control larvae are Oregon-R. Homozygous and balanced heterozygous *Catsup* larvae are homozygous for the *or*^{49h} allele.

[b]Specific activity (mean ± standard error) is expressed in terms of cpm / mg protein × 10^5.

phenotype was observed to some degree in wild-type individuals reared in DA-supplemented medium.

***Catsup* mutants exhibit elevated tyrosine hydroxylase activity:** The depletion of free tyrosine and the concomitant increase in catecholamine levels in *Catsup* embryos suggest that mutations may result in the hyperactivation of TH. To examine this possibility, we measured TH activity in *Catsup* mutants. We chose for this analysis *Catsup*^{11} (GI) and *Catsup*^{12} (GIV) mutants, both of which were subjected to the analysis of catecholamine pools described above, and *Catsup*^{1} (GV) and *Catsup*^{26} (GV), presumptive null mutations. Assays were performed at the late third larval instar white prepupal stage. Here, TH activity is at sufficiently high levels in wild-type strains to allow for reliable and consistent measurements.

These assays demonstrate that *Catsup* mutations are indeed associated with significant increases in TH activity (Table 8). Because it is a weak allele, ~75% of *Catsup*^{11} homozygous flies survive, allowing us to assay TH activity in these mutants. Mean TH activity in homozygous *Catsup*^{11} mutant larvae is fourfold higher than wild-type levels. Consistent with other dominant characteristics of *Catsup* mutations, *Catsup*^{11} balanced heterozygotes also demonstrate significantly higher TH activity, showing more than a fivefold increase relative to controls. Surprisingly, TH activity does not display a dose-response relative to the dose of the *Catsup*^{11} allele, indicating that the effect of *Catsup*^{11} on TH activity is a fully dominant trait. TH activity in *Catsup*^{1}, *Catsup*^{12}, and *Catsup*^{26} balanced heterozygotes is also significantly elevated. *Catsup*^{1} / *CyO* larvae exhibit an approximately fourfold increase and *Catsup*^{12} / *CyO* larvae more than a sevenfold increase in TH activity. To verify the effects of the presumptive null allele *Catsup*^{1} on TH activity, we assayed TH activity in a newly generated null mutation, *Catsup*^{26}. Balanced heterozygotes of *Catsup*^{26} exhibit a fourfold increase in TH activity (Table 8), a level similar to the increases detected in *Catsup*^{1} mutants. These data demonstrate that both weak and severe mutations of *Catsup* dramatically increase TH activity, and they suggest that GV lethal alleles behave genetically and biochemically as null mutations.

TH-hyperactivating *Catsup* alleles are loss-of-function mutations: Genetic complementation, phenotypic analysis, and TH assays indicate that *Catsup* alleles are loss-of-function mutations and that GV lethal alleles are null mutations. To verify these findings, we conducted Western blot analysis with anti-Catsup antibody to determine whether *Catsup* mutants show reduced protein levels. The anti-Catsup antibody detects only a single band of ~50 kD, a size consistent with the predicted molecular mass of Catsup (Stathakis 1998).

Third instar larvae protein extracts of *Catsup*^{11} (GI), *Catsup*^{1} (GV), *Catsup*^{12} (GIV), and *Catsup*^{26} (GV) balanced heterozygotes all exhibit a clear reduction in the level of Catsup protein (Figure 8). This finding is consistent with our genetic analysis that indicates all three of these alleles are either severe or null mutations (Table 1). Surprisingly, the amount of protein detected by anti-Catsup antiserum in *Catsup*^{1} (GV), *Catsup*^{12} (GIV), and *Catsup*^{26} (GV) mutants is ~16–22% of the level relative to wild-type controls (Figure 8). Because the *Catsup* mutant larvae assayed were all balanced heterozygotes, protein nulls would be expected to express ≥50% of the wild-type Catsup protein. The apparent >50% reduction in protein levels suggests that the dominant characteristics of these mutations might be explained by the destabilization of wild-type Catsup protein in these mutants or possibly the perturbation of an autoregulating mechanism normally involved in Catsup function. On the other hand, protein extracts from mutants for the weak allele *Catsup*^{11} (GI) do not exhibit a visible reduction in Catsup protein levels. Interestingly, the band of immunoreactivity observed in the *Catsup*^{11} lane is broader relative to all other lanes and may reflect the presence of a second smaller band (Figure 8). This observation

is consistent with sequence analysis of *Catsup*^{11} mutants, which identified a frameshift mutation that prematurely truncates the Catsup protein at its carboxyl end (Stathakis 1998). This analysis indicates that at least some *Catsup* mutations, including two severe GV lethals, are associated with a reduction in the amount of *Catsup* gene product and confirms that these alleles are loss-of-function mutations. In turn, this analysis suggests that the hyperactivation of TH appears to be caused by a reduction in the function of the *Catsup* gene product.

Hyperactivation of TH enzyme activity is not associated with an increase in TH protein: TH activity is controlled by a complex array of mechanisms that act at the level of gene expression, mRNA translation, and protein modification. As a first step in assessing how *Catsup* might regulate TH activity, we determined whether TH protein levels were altered in *Catsup* mutants. Western blots of late third instar larval proteins were probed with an anti-TH antibody. The expected 58-kD TH protein is detected in the mutant strains *Catsup*^{1}, *Catsup*^{11}, *Catsup*^{12}, and *Catsup*^{26} (Figure 8). In none of these was the TH protein level elevated; hence, the increase in TH activity in *Catsup* mutants cannot be attributed to increased production or stabilization of the TH protein. In fact, for *Catsup*^{1} (GV), *Catsup*^{11} (GI), and *Catsup*^{26} (GV) mutants, the opposite effect is observed because the amount of TH protein detected is only 35–74% of the level found in wild-type controls (Figure 8). In the course of our experiments, we found that the activity of TH in *Catsup* mutants is much less stable than in wild-type strains. It would appear that the less stable activity is accompanied by a loss of the protein itself.

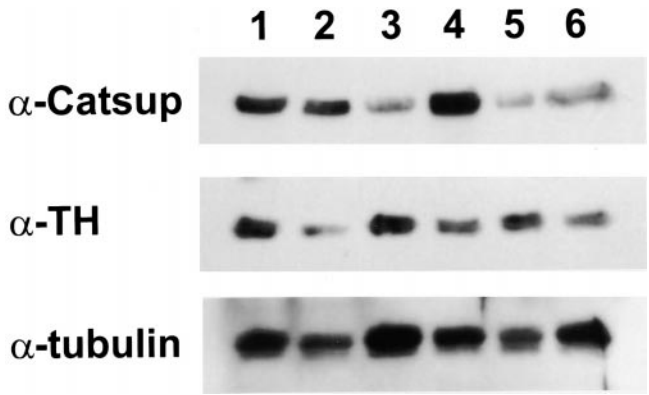

Figure 8.—Western blot analysis of Catsup and TH expression in *Catsup* mutants. Autoradiographs of a Western blot probed sequentially with an anti-Catsup antiserum, an anti-TH antibody, and an anti-α-tubulin antibody. Except where noted, each lane contains 5.0 μg of total protein extracted from third instar larvae of the following strains: Oregon-R (lane 1), 1/2 Oregon-R, 2.5 μg total protein loaded (lane 2), *Catsup*^{1}/*CyO*, a GV lethal (lane 3), *Catsup*^{11}/*CyO*, a GI lethal (lane 4), *Catsup*^{12}/*CyO*, a GIV lethal (lane 5), and *Catsup*^{26}/*CyO*, a GV lethal (lane 6). Top, anti-Catsup guinea pig polyclonal antiserum detects a single band of ~50 kD with relative band ODs as follows: Oregon-R, 1.00; 1/2 Oregon-R, 0.90; *Catsup*^{1}/*CyO*, 0.22; *Catsup*^{11}/*CyO*, 1.83; *Catsup*^{12}/*CyO*, 0.20; *Catsup*^{26}/*CyO*, 0.16. This band is significantly reduced in *Catsup*^{1}, *Catsup*^{12}, and *Catsup*^{26} mutants relative to the Oregon-R controls. Middle, an anti-TH rabbit polyclonal antibody detects a single band of ~58 kD with relative band ODs as follows: Oregon-R, 1.00; 1/2 Oregon-R, 0.33; *Catsup*^{1}/*CyO*, 0.74; *Catsup*^{11}/*CyO*, 0.61; *Catsup*^{12}/*CyO*, 1.39; *Catsup*^{26}/*CyO*, 0.35. The amount of TH protein detected in each of the *Catsup* mutants is equivalent to or less than that observed in Oregon-R larvae. Bottom, an anti-α-tubulin antibody detects a band of ~35 kD. This protein serves as a control for protein loads and to establish that general degradation of the proteins was not occurring.

To verify these findings, we repeated the Western blot experiments but varied the amount of protein extract loaded by one-half to twofold. While immunoreactive signal intensity increased or decreased as we increased or decreased the amount of protein extract loaded, we never detected increased levels of TH protein in *Catsup* mutants. In all cases, TH protein seems to be reduced below wild-type levels. Similar results were obtained when we repeated the Western blot experiments but varied the concentration of anti-TH mouse monoclonal primary antibody (1:250 to 1:2500) or anti-mouse IgG-HRP-conjugated secondary antiserum (1:1000 to 1:5000). These data indicate that our detection method demonstrates a linear response under the conditions used. Taken together, this analysis indicates that *Catsup* mutations do not affect TH protein levels and suggests that the elevated TH activity observed in *Catsup* mutants is most likely attributed to a post-translational mechanism that alters TH catalytic activity.

DISCUSSION

***Catsup* mutations hyperactivate TH activity by a post-translational mechanism:** Our data demonstrate that *Catsup* mutations result in a significant elevation in TH activity that corresponds to increased catecholamine pool levels. *Catsup*^{1} (GV), *Catsup*^{11} (GI), *Catsup*^{12} (GIV), and *Catsup*^{26} (GV) mutant third instar larvae show a four- to sevenfold increase in TH activity (Table 8). Because *Catsup* mutants exhibit three broad overlapping ELPs with associated cuticle defects or catecholamine-related abnormalities (Table 4), we infer that TH activity is hyperactivated throughout development. The significantly elevated catecholamine pool levels measured in *Catsup* mutant embryos (Table 6) and pupae (Wright 1996) are consistent with elevated TH activity at these developmental time points.

Our data also suggest that the regulation of TH activity by the *Catsup* gene product is achieved through a posttranslational mechanism. Western blot analysis of protein extracts from *Catsup* mutant third instar larvae shows TH protein levels that are at or below wild-type levels, indicating that TH synthesis is not elevated in

these mutants (Figure 8). A wide range of anti-TH antibody dilutions and protein concentrations were tested extensively to verify that our detection of TH protein was in a linear range. We are confident that a four- to sevenfold increase in the amount of TH protein corresponding to the elevated degree of TH activity would have been detected. We therefore conclude that the wild-type *Catsup* gene product acts posttranslationally to modify the catalytic activity of TH, and that mutations in this gene result in significant hyperactivation of TH activity.

Catsup functions as a negative regulator of tyrosine hydroxylase activity: Two alternative genetic mechanisms could explain the hyperactivation of TH activity and catecholamine biosynthesis in *Catsup* mutants. *Catsup* alleles might be gain-of-function mutations in a gene that is normally required to activate TH activity. Such mutations could either be neomorphic, causing inappropriately timed or mislocalized TH activity, or hypermorphic mutations resulting in the hyperactivation of TH. Alternatively, these *Catsup* alleles could represent loss-of-function or hypomorphic mutations in a gene that normally functions as a negative regulator of TH activity. Several lines of evidence support the conclusion that the *Catsup* alleles described in this report are loss-of-function mutations, and that the most severe class, the GV lethals, are null mutations. First, with the exception of GV lethal alleles, *Catsup* hemizygotes produce stronger phenotypes than homozygotes, as would be expected for hypomorphic alleles (Table 1). Second, the penetrance of weaker alleles is increased to similar levels when *in trans* to either GV lethal alleles of *Catsup* or to deficiencies that delete the *Catsup* locus, as would be expected if the GV lethal alleles were null mutations. A clear illustration of this point is presented in Table 2, where it is shown that placing the weak $Catsup^{11}$ (GI) lethal allele *in trans* to either *Catsup* deficiencies or any GV lethal allele reduces viability to an equivalent extent. Third, all GV lethal alleles fail to complement each other for any of the mutant phenotypes and, therefore, behave as expected if no functional gene product was expressed (Table 1). Fourth, the GV lethal allele $Catsup^{1}$ behaves identically with respect to TH activity as $Catsup^{26}$, a GV lethal allele that deletes part of the *Catsup* locus (Table 8). Fifth, four *Catsup* mutants representing GI, GII, and GV lethal alleles exhibit either reduced levels or aberrant migration of the Catsup protein on Western blots, including $Catsup^{26}$, a mutation that deletes part of the *Catsup* coding region (Figure 8). Taken together, our analysis indicates that *Catsup* mutations reduce wild-type function; therefore, we conclude that *Catsup* is a gene that normally functions as a negative regulator of TH activity. In addition, as nonconditional lethal mutations in *Catsup* are semidominant zygotic lethals, *Catsup* is a haploinsufficient locus with respect to lethality, morphological phenotypes, and the regulation of TH activity.

Catsup may regulate TH activity by controlling the phosphorylation status of this enzyme: Catsup appears to be the first identified component of a regulatory system that negatively controls TH activity. In mammals, three known post-translational mechanisms act to increase TH catalytic activity: by relieving end-product feedback inhibition of catecholamines, by increasing the synthesis of the ordinarily rate-limiting tetrahydropterin (BH_4) cofactor, and by increasing the affinity of TH for the BH_4 cofactor (reviewed in Zigmond *et al.* 1989; Fillenz 1993; Kumer and Vrana 1996). The critical process that appears to control all three post-translational mechanisms is the regulation of phosphorylation. The phosphorylation of four specific sites in the regulatory domain of TH simultaneously relieves the feedback inhibition of catecholamines and increases the affinity of TH for the BH_4 cofactor, resulting in an increased catalytic activity of TH (reviewed in Zigmond *et al.* 1989; Kumer and Vrana 1996). The four phosphorylation sites shown to be critical for modulating the activity of mammalian TH are conserved in Drosophila TH (Neckameyer and Quinn 1989; Birman *et al.* 1994). Similarly, increased pteridine biosynthesis can be regulated post-translationally and appears to be linked to cyclic AMP-dependent-and-independent-mediated phosphorylation mechanisms (Abou-Donia *et al.* 1986). The most likely target in the pteridine pathway is GTP cyclohydrolase, the first and rate-limiting step in the biosynthesis of BH_4 from GTP (Blau and Niederwieser 1985; O'Donnell *et al.* 1989). Recent studies have revealed that phosphorylation increases the activity of GTP cyclohydrolase and results in increased BH_4 levels (Hesslinger *et al.* 1998; Lapize *et al.* 1998). Similar to TH, there are at least six phosphorylation sites conserved among mammalian and fly GTP cyclohydrolase orthologs (McLean *et al.* 1993). This suggests that the activity of both Drosophila TH and GTP cyclohydrolase may be regulated by similar phosphorylation events. Therefore, we hypothesize that the function of the *Catsup* gene product is to control the phosphorylation state of TH and GTP cyclohydrolase.

Under our hypothesis, two possible mechanisms would account for the negative regulation of TH activity by wild-type Catsup function (Figure 9). First, Catsup may activate the signals necessary to dephosphorylate TH, thereby downregulating its activity. This suggests that Catsup might function in the activation of a phosphatase. Alternatively, Catsup may inactivate a protein kinase, thereby keeping TH in a less active form. Disruption of Catsup function by either of these mechanisms would result in a constitutively active phosphorylated form of TH. Consistent with this model is the inability of high levels of catecholamines to negatively regulate TH activity in *Catsup* mutants. Our model also proposes a similar regulatory mechanism for the suspected coordinated downregulation of GTP cyclohydrolase in the pteridine biosynthesis pathway (Figure 9).

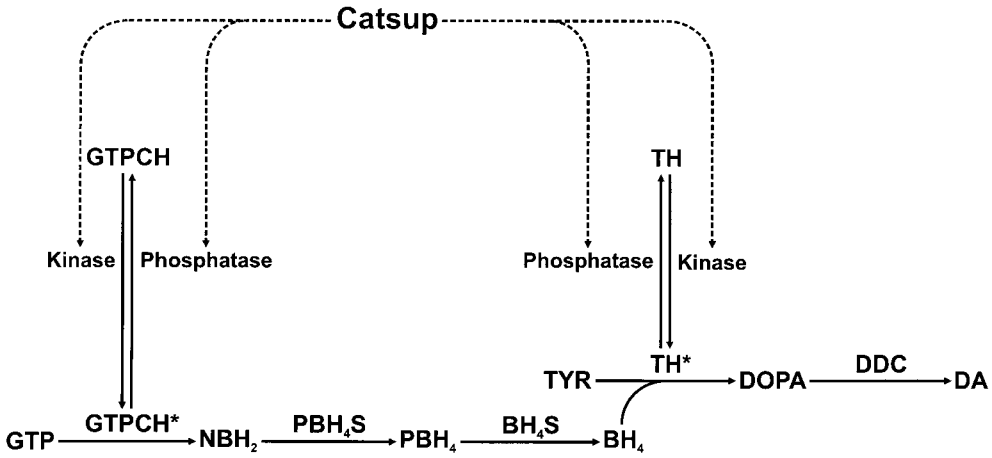

Figure 9.—Model for Catsup function in regulating TH activity. The figure depicts the initial two enzymatic steps of catecholamine biosynthesis necessary to produce DA and the three steps required to synthesize BH_4. Both TH and GTP cyclohydrolase (GTPCH) exist in two forms, a dephosphorylated inactive form (TH and GTPCH) and a phosphorylated active form (TH* and GTPCH*). With respect to regulating the activity of either enzyme, Catsup could act by one of two potential pathways. Catsup could regulate a pathway that increases the phosphatase activity necessary to dephosphorylate TH or GTPCH. Alternatively, Catsup could act via a pathway that decreases the kinase activity necessary to phosphorylate TH or GTPCH. In *Catsup* mutants, loss of either controlling mechanism would allow TH and GTPCH to exist primarily in the phosphorylated form. This would result in a constitutive hyperactivation of these enzymes and subsequent elevation of catecholamine pool levels. Abbreviations are as defined in Figure 7 or as follows: NBH_2, dihydroneopterin; PBH_4S, 6-pyrovoyl tetrahydropterin synthase; PBH_4, 6-pyrovoyl tetrahydropterin; BH_4S, sepiapterin synthase; BH_4, tetrahydropterin.

The experiments described here primarily address effects on TH activity. The large increases in several catecholamine pool levels detected in *Catsup* mutants might also suggest that, in addition to TH activity, other enzymes within this biosynthetic pathway may also be hyperactivated in a coordinate fashion. However, evaluation of DDC activity in *Catsup* mutants indicates no difference relative to controls (Stathakis 1998). In addition, while diphenol oxidase activity is marginally increased by 1.5-fold in *Catsup* mutants (Stathakis 1998), this effect could be a consequence of the 4- to 7-fold increase in TH activity, the rate-limiting step in the pathway. This argues that *Catsup* mutations result primarily in the hyperactivation of TH activity, not a systemic misregulation of the catecholamine biosynthetic pathway.

***Catsup* mutations may result in increased instability of TH catalytic activity:** There are certain discrepancies between the specific TH activity assayed in *Catsup* mutants in relation to their genetic, phenotypic, and catecholamine pool level characteristics. *Catsup*^{11} (GI), *Catsup*^{12} (GIV), *Catsup*^{1}(GV), and *Catsup*^{26} (GV) heterozygous mutants all exhibit increases in TH activity of at least fourfold relative to wild-type levels. With respect to *Catsup*^{11}, TH activities are clearly elevated in both homozygous and heterozygous mutants, indicating that TH hyperactivation is a completely dominant trait. Furthermore, relative to the null alleles *Catsup*^{1} and *Catsup*^{26}, heterozygous mutants for the less severe alleles *Catsup*^{11} and *Catsup*^{12} are associated with more extreme stimulation of TH activity; 5.6- and 7-fold increase in activity, respectively, relative to the null alleles *Catsup*^{1} and *Catsup*^{26} (Table 8). However, all *Catsup* mutants exhibit a semidominant mode of inheritance with respect to the effects of *Catsup*-associated lethality, morphological phenotypes, and catecholamine pool levels. With respect to these phenotypes, *Catsup*^{11} mutants exhibit the least severe phenotypes, *Catsup*^{12} shows moderate effects and *Catsup*^{1} and *Catsup*^{26} show the most severe phenotypes. Thus, the complete dominance of TH activity exhibited by *Catsup* mutants is somewhat at variance with the semidominant mode of inheritance of *Catsup* lethality and morphological phenotypes.

While the basis for this difference is not clear, an intriguing possible explanation is that *Catsup* mutations cause TH to be less stable, which would affect *in vitro* activity assays. Under our hypothesis for Catsup function, a reduction in TH stability would be predicted. In mammals, phosphorylation of TH converts the enzyme to a more active form that simultaneously decreases its stability (Lazar *et al.* 1981; Gahn and Roskoski 1995). If *Catsup* mutations do indeed cause hyperphosphorylation of TH and do so to different degrees, they may cause differential instability of the resulting catalytic hyperactivation. Therefore, it seems reasonable to suspect that endogenous TH activity is, in fact, further elevated in more severe *Catsup* mutants, but that increased instability of the phosphorylated form of TH counters its detection. Consistent with this hypothesis is the observation that catecholamine pool levels are higher in *Catsup*^{11} homozygotes relative to heterozygotes (T. Homyk, W. E. McIvor and T. R. F. Wright, unpublished data, cited in Wright 1996), suggesting that TH activity may indeed be higher in homozygous *Catsup* mutants *in vivo*.

***Catsup* lethality and associated phenotypes are a consequence of elevated catecholamine pool levels:** Because catecholamines are toxic (Hopkins and Kramer 1992; Kumer and Vrana 1996), the systemic increase of these compounds is most likely responsible for the

lethality observed in *Catsup* mutants. This assertion is supported by our DA feeding experiments, where an increase in DA concentration is correlated with increased lethality. The penetrance of lethality in *Catsup* mutants is highly variable and leads to the semidominant nature of *Catsup* mutations, the polyphasic character of ELPs, and the randomness of lethal onset, indicating that *Catsup* mutants may possess a functional threshold effect. Homozygous or heterozygous $Catsup^{11}$ mutants can die either as third instars, prepupae, pupae, or pharate adults, or they survive as fertile adults (Table 4). This suggests that Catsup activity in mutants may be sufficient to overcome a threshold requirement for normal function. However, when increased demands for Catsup function occur, its activity might fall below this functional threshold, resulting in lethality. Activation thresholds appear to be intrinsic to enzymatic cascades in which the enzymes are subject to inhibitory control (Beltrami and Jesty 1995) and are consistent with the function of Catsup as a negative regulator of TH activity. While a similar threshold effect may also be occurring in early larvae carrying GIV and GV lethal mutations for *Catsup*, the broad ELP seen during early larval development may also be evidence for the presence of a maternal product (Table 5). These observations are supported by RNA *in situ* hybridization experiments, which clearly demonstrate the presence of a maternal *Catsup* transcript (Stathakis 1998). We also suspect that the cuticular defects observed in *Catsup* mutant pharate adults (Figure 4) are a consequence of lethality and that the *Catsup* gene product may not have a direct role in cuticle synthesis. During adult cuticle synthesis, the epicuticle is initially deposited in the abdomen ~40–45 hr after puparium formation (Roter *et al.* 1985). Subsequently, the lamellar procuticle is deposited underneath the epicuticle in successive layers between 55 and 80 hr after puparium formation (Roter *et al.* 1985). The presence of both a well-formed epicuticle and procuticle in *Catsup* mutants suggests that the actual process of cuticle synthesis was proceeding normally and that this process appears to have been interrupted rather than disrupted. In addition, the lack of an epidermal layer or any internal tissue organization and the presence of a thinner cuticle in *Catsup* mutants can be explained by necrosis subsequent to death.

Our analysis indicates that all larval and pupal visible phenotypes associated with *Catsup* mutations are also a consequence of the abnormally high accumulation of free catecholamine pool levels, principally DA. DA is critically important in Drosophila, because it is the only catecholamine that functions as a neurotransmitter (reviewed in Restifo and White 1990), it is the principal catechol used in melanin synthesis, and it is the precursor compound for all catecholamines involved in sclerotization (reviewed in Hopkins and Kramer 1992). While the akinesis seen in early *Catsup* mutant larvae could result from toxic catechol levels, the high accumulation of DA in late embryos homozygous for $Catsup^{12}$ also suggests that normal neurotransmission at the synapse could be disrupted in these first instar larvae. The promiscuous melanization seen in body tissues of *Catsup* early larval and pupal mutants, or in the formation of melanotic salivary glands and pseudotumors in late larval mutants, also implicates excessive levels of free DA at these stages.

It is not clear whether the female sterility observed in *Catsup* mutants is a direct result of abnormally high catecholamine levels. Both phenotypic examination of *Catsup* mutations that produce female sterile escapers and germline clone analysis of $Catsup^{1}$ mutants clearly indicate that Catsup function is critical for oogenesis. Abnormal catecholamine pool levels could result in an abnormal egg-laying behavior in these female-sterile mutants (Wright 1996). However, *Catsup* female-sterile mutations arrest egg development during stage 13 oogenesis, arguing against a defect in egg-laying behavior. Recently, it was shown that pharmacological depletion of catecholamines in larvae leads to defects in ovarian development and female sterility, indicating a developmental role for catecholamines in oogenesis (Neckameyer 1996). Our DA supplementation experiments support these findings and indicate that elevated DA levels also reduce fecundity. Interestingly, octopamine has also been implicated in female fertility in Drosophila. Tyramine β-hydroxylase (TBH) catalyzes the hydroxylation of tyramine into octopamine, and null mutations in the *Tβh* locus result in significantly reduced octopamine pool levels and produce female-sterile adults with an egg-retention phenotype (Monastirioti *et al.* 1996). While this trait appears remarkably similar to that of *Catsup* female-sterile adults, it is not yet known whether *Catsup* mutations either upregulate or downregulate monoamine biosynthesis.

***Catsup* is the fourth locus involved in catecholamine metabolism found within the *Ddc* gene cluster:** The *Ddc* gene cluster contains at least 21 genes (Stathakis *et al.* 1995), and extensive genetic analysis suggests that as many as 15 of these may play a role in catecholamine metabolism (Wright 1996). In addition to *Catsup*, molecular analysis of the protein products encoded by *Ddc*, *amd*, and *Dox-A2* indicate their involvement in catecholamine metabolism (Wright 1996). Furthermore, mutations in 11 other loci within the *Ddc* gene cluster also result in similar morphogenetic defects of the cuticle or in catecholamine-related abnormalities (Wright 1996). HPLC studies on three of these mutants revealed that all exhibited aberrant catecholamine pool levels during prepupal and pupal development, suggesting that the corresponding genes may encode proteins involved in or regulating catecholamine metabolism (T. Homyk, W. E. McIvor and T. R. F. Wright, unpublished data). This interpretation is consistent with the HPLC analysis of *Catsup*, *Ddc*, *amd*, and *Dox-A2* mutants, all of which show abnormal catecholamine profiles

(Wright 1996). Taken together, this evidence strongly argues that the *Ddc* gene cluster represents one of the largest groupings of functionally related genes involved in an enzymatic pathway found in higher eukaryotes.

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