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Temperature-Sensitive Mutations in *Drosophila melanogaster*, V. A Mutation Affecting Concentrations of Pteridines*

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Abstract. A temperature-sensitive *ras* allele has an effective lethal phase at 29°C about 12 hr after pupation and a temperature-sensitive period for lethality beginning midway through the third larval instar and ending around pupation. The mutation also alters the quantity of pteridines present in the eyes, testes, and Malpighian tubules at 29°C. The temperature-sensitive period for pigment production in the Malpighian tubules occurs in the egg and first instar larvae, and in eyes and testes after pupation. The demonstrated autonomy of the mutant in the eyes implies the tissue-specific functioning of the gene. We suggest that the different temperature-sensitive periods for a single mutation indicate tissue-specific activation of a gene at different times during development, although the possibility of activation of preformed polypeptides has not been eliminated.

A major question in developmental genetics is whether a single gene whose function is known to be required in different tissues is activated concomitantly in all these tissues in response to a single trigger or responds to tissue-specific stimuli at different developmental stages. Since many of the fluorescent pigments found in adult eyes and testes and in larval and adult Malpighian tubules of *Drosophila melanogaster* are regulated by the same set of genetic loci,[1] a single mutation affecting pigmentation in all three tissues might shed light on the problem. A recessive sex-linked mutation, *l (1)E6^{ts}*,[2] causes lethality at 29°C but results in a mutant eye color of viable adults at 22°C. Subsequent tests have indicated that, in addition to temperature-sensitive (ts) lethality, the presence and quantity of pteridines in the eyes, testes, and Malpighian tubules are also affected by temperature. The content of pigment in these organs was measured in flies grown at 29°C during different developmental intervals in order to delineate the temperature-sensitive period for the presence of pigment.

Materials and Methods. Genetic analysis: The mutation *l(1)E6^{ts}* (abbreviated as *E6*) was induced by ethyl methanesulfonate and found to be viable at 17°C and 22°C, but was lethal at 29°C. At 22°C, the eye color of *E6* males was obviously mutant, whereas homozygous females were visually indistinguishable from wild type. The lethal locus was initially localized genetically[2] close to *v* (33.0)[3] between *cv* (13.7) and *v* and subsequently positioned to the left of *v* within 0.7 map units by the markers *lz* (27.7) and *v*. A test of allelism of *E6* with *ras* (32.8) revealed that *E6/ras* females were viable at 29°C but had a mutant eye color. In order to determine whether ts lethality and the temperature-sensitive pigment phenotypes are caused by a single mutation, females heterozygous for *E6* and *m* (36.1) were mated at 29°C and the male offspring were scored for the pres-

ence of surviving crossover progeny with the mutant eye phenotype. This experiment showed that *E6* was 3.67 units to the left of *m* and that no separation of lethality from the pigment variant was found among 3,421 males scored. If, in fact, the lethal and eye phenotypes result from separate mutations, an upper limit of 0.09 map units[4] separates the two sites; this makes it probable that the two phenotypes result from a single mutation at the *ras* locus.

Developmental analysis: The "effective lethal phase",[5] that is, the time at which *E6* flies die when raised at 29°C, was established by maintaining eggs from the *E6* stocks collected within 2 hr of deposition at 29°C. The developmental stages reached were determined by inspecting the culture at 12-hr intervals. It was found that obvious autolysis and death occurred prior to the formation of any distinguishable adult structures, about 12 hr after puparium formation.

The actual developmental interval of sensitivity to the high temperature (temperature-sensitive period, TSP) during which growth at 29°C irrevocably commits the flies to death was established by reciprocal "shifts" at successive 12-hr intervals in which 29°C cultures were shifted to 22°C (shift-down) and vice versa (shift-up). The first shift-down which gave a reduction in survival was taken to indicate the beginning of the TSP and the first culture in a shift-up which yielded viable adults delimited the end of the TSP. By these criteria, the TSP was shown to begin midway through the third larval instar and to terminate just prior to pupation (Fig. 1).

It was immediately noted that the eyes of flies hatching from a culture that was shifted up just after the TSP for lethality were dark brown. This suggested that defective pigment synthesis in testes and Malpighian tubules might also be affected by high tempera-

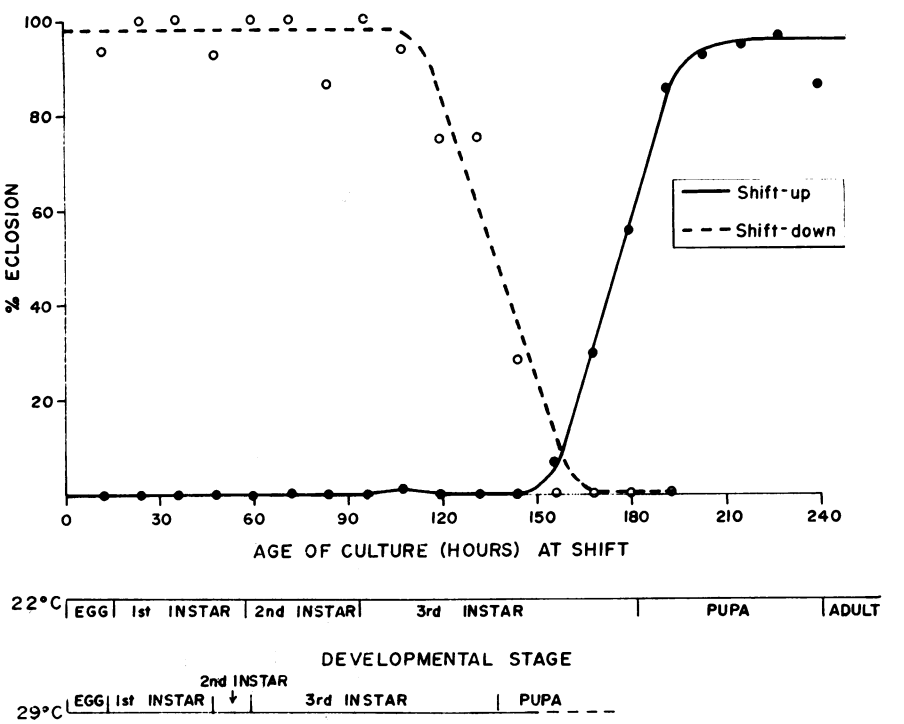

FIG. 1. Percentage of *E6* pupae which eclose after shift-ups and shift-downs at different times during development. The developmental stages present at each culture age at 22 and 29°C are shown at the bottom.

tures, and that TSPs for mutant pigment syntheses might be established. The shift studies carried out to determine the TSPs for pigment production will be described under *Results*.

Spectrofluorometric analysis of pigment production: The fluorescent pigments (FP) of the various tissues were separated by thin-layer chromatography, using MN 300 cellulose with $CaSO_4$ binder (Machery and Nagel) spread to a thickness of about 0.25 mm on glass plates. The plates were developed by ascending chromatography in the dark at 22°C for 3–4 hr in propanol–1% aqueous ammonia 2:1, or, on occasion, butanol–acetic acid–water 20:3:7. The latter solvent had the advantage of resolving FP-3 to FP-6 better than the propanol–ammonia, but it did not resolve (or only poorly resolved) FP-7 to FP-9. Consequently, the propanol–ammonia was used for most experiments. The plates were allowed to dry for 1 hr in a fume hood and were then scored visually under UV radiation. The quantity of each fluorescent pigment was determined spectrofluorometrically with an Aminco Bowman scanning fluoromicrophotometer using a Varicord recorder.

Eye pigments were obtained from intact heads of adult flies decapitated by a microscalpel. The heads were placed on the thin-layer cellulose plates and crushed with the end of a glass rod in order to release pigment directly onto the plate[6].

Pigments from the testes and Malpighian tubules were more difficult to obtain. Testes were removed from adult flies by microdissection in *Drosophila* Ringer's solution[7] and transferred to the thin-layer plates with a minimum of accompanying Ringer's solution. One pair of testes from a single fly was used per run. Malpighian tubules of either 3rd instar larvae or adult flies were dissected and chromatographed in the same manner. Although the fluorescent pigment of Malpighian tubules from single individuals (four strands) was sufficient for quantitation, Malpighian tubules from two identically-treated individuals were usually pooled to increase the amount of pigment per run and the accuracy of measurement. Crushing of the testes and Malpighian tubules was not necessary for good recovery of fluorescent pigment.

Results. Temperature effects on eye and testis pigmentation. The TSP for eye and testis pigmentation was determined as follows. 15–20 prepupae from a 22°C culture were collected within a 15-min interval and shifted up to 29°C. Starting 12 hr after this shift-up, different vials of pupae were shifted down again at successive 1-hr intervals until 70 hr after prepupal formation. Adult flies from each vial were counted and their eyes and testes were analyzed for pigment production. The per cent eclosion (number of adults/number of pupae) for each vial was calculated, to show that the TSPs for lethality and eye–testes pigmentation are definitely separable.

The heads of a total of 362 *E6* and 173 Oregon-R flies raised at different temperatures (17, 22, and 29°C) were chromatographed and compared. It was found that 7–11 fluorescent pigments could be separated from both the mutant and the + strain, depending on the preparation and the quality of the thin-layer plate. Generally, eight pigments were resolved easily in propanol–ammonia. These pigments were designated as FP-1 (fluorescent pigment with lowest R_f) to FP-8 (highest R_f). We did not attempt to characterize the pigments by their absorption or fluorescent spectra, but by comparing our plates with the data and descriptions of other workers,[8,9] we could tentatively identify some pteridine pigments. The two eye pigments consistently affected in *E6* by growth at high temperature were FP-1 and FP-8; FP-1 corresponds to drosopterin. The drosopterin concentration in the mutant raised at 29°C was about 70% that of Oregon-R (Fig. 2*A*).

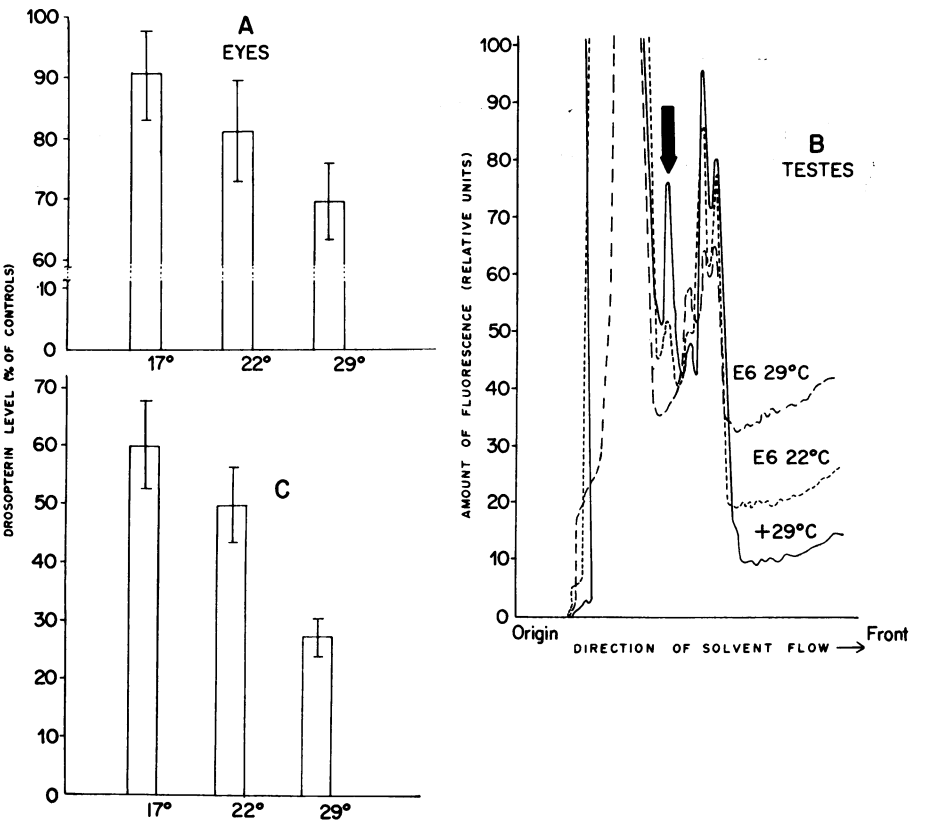

FIG. 2. (*A*) Drosopterin (FP-1) levels (with confidence limits) in the eyes of *E6* females relative to the concentration in Oregon-R females raised at 29°C. (*B*) Distribution of fluorescent pigments in thin-layer chromatograms of testes of *E6* and Oregon-R males raised at different temperatures. The arrow indicates the pigment (FP-2) measured to determine the TSP. (*C*) Drosopterin (FP-1) levels (with confidence limits) in the Malpighian tubules of *E6* flies relative to the level in Oregon-R flies raised at 29°C.

At 22°C, *E6* female eyes were visually wild type whereas males were distinguishably mutant; at 17°C, the eyes of both females and males were indistinguishable from wild type. Drosopterin concentration in flies raised at 17°C were 80% (males) and 90% (females) of those in comparable Oregon-R flies. (Interestingly, the drosopterin concentrations in females raised at 29°C and 22°C were approximately equal to those in males raised at 22°C and 17°C, respectively.) From shift studies, the TSP for eye pigment formation was found to begin 52–56 hr after pupation at 29°C. The end of the TSP was not determined, but the period was assumed to continue until eclosion.[9]

The pigment content of the testis differed somewhat from that of the eye and Malpighian tubules. Only five fluorescent pigments were consistently separable from the testes of either *E6* mutant or Oregon-R males. The drosopterin pigment was either missing, or masked by the bright blue isoxanthopterin, which constituted FP-1 for the testes. Although FP-2 was present in *E6* males grown

at 22°C, or 17°C, it was absent from the *E6* males shifted to 29°C at the onset of pupation (Fig. 2*B*). The concentration of FP-2 in males raised at 22°C or 17°C never reached that of the wild type controls.

The shift studies showed that the TSP for pigment synthesis in testes at 29°C began 56–60 hr after pupation, which is similar to the TSP for eye pigment. The end of the TSP was not determined. The per cent eclosion in the various vials varied from 70 to 90% of the rate of survival determined for *E6* at 22°C. Therefore, the TSP for lethality was definitely earlier than the TSPs for eye and testis pigmentation.

Temperature effects on Malpighian tubule pigmentation. The effect of temperature on pigment production in the Malpighian tubules was determined by growing cultures of *E6* at the restrictive temperature until 3rd instar and then shifting them down to 17°C. Pigments in Malpighian tubules from either 3rd instar larvae or adult *E6* flies were chromatographed after the shift-down and compared with those of *E6* larvae or adults raised entirely at 17°C and with those of Oregon-R flies from high or low temperatures. The beginning of the TSP for pigment synthesis in the Malpighian tubules was determined by collecting eggs laid within 1 hr at 29°C and shifting individual cultures down to 17°C at successive 4-hr intervals until the 36th hr. The Malpighian tubules of 3rd instar larvae were scored for pigment as for eyes and testes. The end of the TSP for Malpighian tubule pigment synthesis was delineated by sequential shift-ups at successive 12-hr intervals of cultures established from 2-hr egg lays. Shift-ups were performed from the time of egg deposition to late third larval instar stages and Malpighian tubules removed from late third instar larvae were assayed for pigmentation.

The fluorescent pigments found in the Malpighian tubules were the same as those in the eyes. FP-1 (drosopterin) was barely preceptible in Malpighian tubules in third instar *E6* larvae raised at 29°C, while it was clearly detectable in *E6* larvae raised at 22°C and increased to approximately 60% of that found in Oregon-R larvae at 17°C (Fig. 2*C*). FP-8 was not present in sufficient quantity in either Oregon-R or *E6* Malpighian tubules to permit quantitative comparisons.

Shift studies indicated that the TSP for pigment synthesis was from about 12 hr after egg deposition at 29°C to about 120 hr at 17°C. The end of the TSP for pigment synthesis (delineated at 17°C) corresponded to the end of the 2nd larval instar.

To summarize, for flies growing at 22°C, the TSP for Malpighian tubule pigmentation was 12–90 hr, for lethality 153–187 hr, for eye pigmentation 224 hr to eclosion, and for testis pigmentation 228 hr to eclosion (Fig. 3).

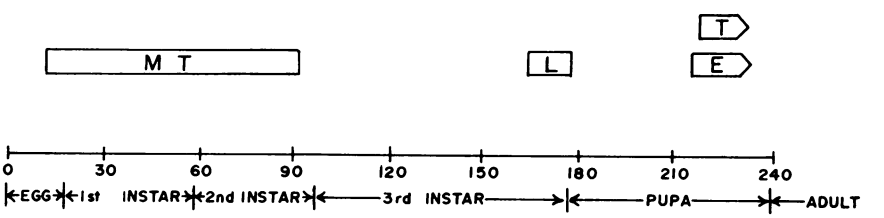

FIG. 3. The temperature-sensitive periods in *E6* for lethality and pigment concentrations in the eyes, testes, and Malpighian tubules (in hours, on a 22°C time scale).

Autonomy of pigment formation in different organs and pigment turnover. Since the TSPs for pigment synthesis varied from organ to organ, it is tempting to conclude that the time of gene activity varies from organ to organ. Indeed, Stern and Tokunaga[10] demonstrated the autonomous pleiotropy of the *spl* mutation in eyes and mesonota. However, in our experiments, the presence of pigment in a tissue does not preclude transport of the molecules from other tissues; the presence of pigment in a tissue cannot, therefore, be assumed to reflect its synthesis in that tissue. A demonstration of tissue autonomy for pigment production would strengthen the suggestion that pigments are synthesized at the site of deposition.

The first experiment consisted of a series of shift studies. Cultures collected within a 2-hr period at 29°C were shifted down to 22°C at 80 hr (mid-3rd instar) just prior to the TSP for lethality. Half of these flies were left at 22°C until eclosion (Group A) while the other half was shifted back up 12 hr after pupation (Group B). Thus, flies in Group B were kept at 29°C except during the TSP lethality. Concomitantly, eggs were collected at 22°C and half were shifted up 12 hr after pupation (Group C) and the other half left at 22°C until eclosion (Group D). The adult flies that emerged from these shift studies were scored for eye, testis, and Malpighian tubule pigmentation.

Table 1 shows that the mutant Malpighian tubule phenotype was determined

TABLE 1. *Phenotypes of Malpighian tubules and eyes after each kind of shift (see Fig. 3).*

Temperature shifts	Type of shift	Phenotype		
		Malpighian tubules†	Eyes*	Testes†
A	Shift-down before TSP‡	Mutant	Wild type	Wild type
B	Shift-down before and up after TSP	Mutant	Mutant	Mutant
C	Shift-up after TSP	Wild type-like	Mutant	Mutant
D	22°C control	Wild type-like	Wild type	Wild type

* Determined visually.
† Based on chromatographic profile.
‡ Temperature-sensitive period.

irreversibly by early exposure to 29°C whereas growth at low temperatures up to pupation did not affect eye or testis pigmentation after a shift-up. These experiments ruled out significant transport of pigment from the Malpighian tubules to the eyes and testes and vice versa, a result supporting the autonomy of each tissue.

Tissue autonomy of pigment synthesis was also tested in mosaic females using the unstable ring X chromosome, $In(1)w^{vC}$.[11] $In(1)w^{vC}/l(1)E6^{ts}$ females were raised continuously at 29°C and the adults were scored for the presence of mutant eye tissue. Since the somatic loss of $In(1)w^{vC}$ produced cells hemizygous for *E6*, the observation that mutant patches of eye tissue were readily detected in a wild type background constituted the unequivocal genetic proof of autonomy of *E6*. Several bilateral mosaics for external genitalia were also observed. Both approaches suggest that the *E6* gene is acting autonomously in all three organs.

Discussion. It has been shown[10] that a mutation which results in a mutant phenotype in different tissues acts autonomously in each tissue. Our experiments have corroborated this and have demonstrated that the amounts of those

fluorescent pigments affected by *E6* are sensitive to temperature in different organs at different times during development (Fig. 3). Since the genetic mapping studies indicated that a single mutation was responsible for both lethality and pteridine synthesis or deposition, at least three separable TSPs have been delineated during development. We assume that the lethal effect of *E6* occurred in tissues other than the organs scored chromatographically, since mutant patches of eye and external genital tissue were observed in mosaic flies raised at 29°C.

The significance of these results is dependent upon the interpretation of the TSP in molecular terms. In microorganisms, temperature-sensitive lethality has been shown[12] to be a consequence of missense mutation, which results in the thermolability of the polypeptide gene product. The genetic properties of such lethal mutations in Drosophila strongly suggest a basis for temperature sensitivity similar to that in microorganisms (ref. 2 and manuscript in preparation).

The genetic proof that expression of the *E6* phenotype in each organ was autonomous suggests that the *E6* locus does indeed function in the organs in which pigment was measured. However, it should be stressed that the concentrations of the pigments measured were quite probably an indirect result of the activity of the *E6* locus, since many different pteridine and ommachrome eye mutants affect the amount of drosopterin produced.[6] Furthermore, the complete viability of white-eyed mutants (which are totally lacking in pigments) shows that the fluorescent compounds themselves are not necessary for viability. Hence, it could be suggested that a single period of genetic activation (transcription) occurs early in development in all tissues; the resultant gene product could be a long-lived masked messenger RNA[13] or an inactive polypeptide[14] with a temperature-sensitive translational or activation mechanism, respectively. Although these possibilities cannot be eliminated, their contrivance and the special assumptions required render them unattractive. Waddington's experiments[15] showed that pigment synthesis in optic discs was suppressible by actinomycin D treatment in early pupation, a result strongly supporting the hypothesis that the syntheses of RNA and proteins involved in pigment formation occur close to the time of pigment production in the eye itself. We therefore suggest that the *E6* locus is activated in different organs in response to tissue-specific stimuli at different developmental stages.

The occurrence of the TSP for a mutant phenotype after pupation suggests a number of experiments. The effects of ecdysone on macromolecular synthesis in imaginal discs may be tested on the induction of pigmentation in explanted and transplanted imaginal discs. The discs in which *E6* manifests its lethality may also be indirectly detected using somatic elimination of the unstable ring X chromosome. For example, the recovery at 29°C of viable mosaics in which one eye was wild type while the other was mutant in color, or which carried male genitalia, indicates that lethality due to *E6* does not occur in tissues derived from eye and genital imaginal discs. Nolte[16] has shown by light and electron microscopy that ras^s, a temperature-insensitive allele of *E6* which also manifests depressed levels of drosopterin, has a disorientation of the secondary pigment cells of the eye and some irregularly shaped pigment granules. Of immediate

interest to us is whether temperature affects the formation of secondary pigment cells and pigment granules, or transport and deposition of pigment, in the eyes of the *E6* mutant.

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