Ontogenic Expression of Translocated Purple and Vermilion Genes in *Drosophila Melanogaster*

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개체발생에 따른 초파리의 Purple과 Vermilion Gene 발현에 관한 연구

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(Received April 25, 1981)

요   약

초파리의 유전자 purple과 여기서 만들어내는 효소 sepiapterin synthase를 사용하여 염색체상의 위치에 따른 유전자의 ontogenic expression과의 관계에 대해 연구하였다. 전위된 purple mutant $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$의 성체는 wild type과 pr^1에 비하여 이 효소의 activity가 현격한 감소를 나타내었으나 이 activity의 감소는 larval stage에서 발견되지 않았다. 이것으로 미루어 보아 Y 염색체상의 purple 유전자가 늦은 larval stage나 이른 pupal stage에 불활성화 된 것으로 사료된다. Tryptophan pyrrolase는 eye pigment 생합성에 관여하는 유전자인 vermilion에서 만들어지는데, 이 효소의 활성도 역시 초파리의 발생 단계에 따라 측정하였다. 전위된 vermilion gene을 가지고 있는 초파리의 한 mutant는 발생과정중 늦은 larval stage에서 activity peak을 보여주는데 반하여 Oregon-R은 이 시기에서 가장 낮은 activity를 나타내었다.

INTRODUCTION

Progress in understanding the regulation of gene activity in microbial systems has generated increased interest in analogous processes in higher forms. The genetic and ontogenic complexity of the Metazoa, however, requires levels of functional integration not found in bacteria. It has been well established that although the characteristics of cells, tissues, and organs within an individual may differ drastically, the genetic complements are equivalent and their totipotency is maintained (Davidson, 1976). One is left, therefore, with the questions of how genetically identical cells become morphologically and functionally distinct, how differential functions of tissues and organs are controlled,

and how separate metabolic activities are integrated into a viable functional unit, the metazoan organism. One possible mechanism of regulation of gene activity is the differential enzyme activity observed during development of an organism. Gene-specific changes in enzyme activity during development must result, at least in part, from the turning on and off of structural genes for that enzyme.

It has been established that the phenotypic expression of a gene may depend on its position in the genome. This phenomenon, termed "variegated position effect", has been the subject of several reviews (Baker, 1968; Spofford, 1976). Because relocation does not change the gene *per se*, an alteration in the control processes of that gene is indicated. In order to investigate the interrelationship between the position effect and the eukaryotic control processes, ontogenic profiles of sepiapterin synthase (Fan *et al.*, 1975), and tryptophan pyrrolase (Tobler, 1975) activity in relocated and normally positioned genotypes were determined. The results of this study and its implications are the subject of this report.

MATERIALS AND METHODS

1. Drosophila Stocks

All stocks examined in these studies were maintained on the standard cornmeal-agar medium at 25°C. $T(Y:2)pr^{c5}$ is a translocation from the second to the Y chromosome involving the pr^+ gene induced in an Oregon-R fly. pr^{c4}/pr^{c4} is inviable. Both pr^{c5} and pr^{c4} were induced by ethyl methanesulfonate (Yim *et al.*, 1977). $T(Y:2)pr^{c5}$ was balanced against pr^{c4}, a mutant which is homozygous lethal. Cinnabar (*cn*, 2:57.5) contained the pr^+ allele from Oregon-R and was introduced into the genotype to eliminate ommochrome deposition in the eye, thereby providing a white background to observe pr^+ variegation. All other stocks were obtained from Dr. E.H. Grell and are described in Lindsley and Grell (1968).

2. Culturing and Collection Procedure

Appropriate genotypes were placed on freshly yeasted bottle (a layer of moist bakers' yeast on 1.5% agar with propionic acid added as a mold inhibitor) and were allowed to lay eggs for up to 6 hrs. The flies were then removed and the eggs were incubated at 25°C. At 12-and 24 hrs intervals after hatching, the larvae were harvested by washing onto a fine-mesh screen to remove the yeast cells. After several washes with distilled water, the larvae were assayed for enzyme activities. Pupal stages were synchronized and collected by using a floatation procedure based on the phenomenon of head eversion occurring at the larva to pupa transition. The adults used in the ontogenic study were collected at 24-hr intervals after emergence, aged the desired period of time, and then assayed for enzyme activity. In all cases freshly harvested organisms were the source of extract.

3. Preparation of H_2-Neopterin Triphosphate

GTP was incubated at a concentration of 670 uM with 100 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10 mM EDTA (pH 7.5) and pure GTP cyclohydrolase. GTP cyclohydrolase was prepared from *E. coli* B by conventional purification procedures and the chromatography on a ATP-coupled Sepharose (Yim and Brown, 1976). The amount of H_2-neopterin triphosphate produced was determined from measurements of the release of ^{14}C-formate by filtration of reaction mixtures through charcoal (Burg and Brown, 1968). Conversion was consistantly greater than 95%. An aliquot of the GTP cyclohydrolase reaction was used as the source of H_2-neopterin triphosphate for sepiapterin synthase assay discussed below.

4. Assay of Sepiapterin Synthase by High Pressure Liquid Chromatography

Drosophila extract was prepared with 50 mM PIPES buffer (pH 7.0) in a glass tube and pestle homogenizer at a ratio of 1 ml buffer per 0.2 g flies. The homogenate was centrifuged twice, first at 7,000 g for 10 min and then at $100,000 \times g$ for 60 min. The protein precipitating between 40 and 60% saturated $(NH_4)_2SO_4$ (4°C) was collected at 15,000 g for 30 min, redissolved in 50 mM PIPES, pH 7.0, and filtered through Sephadex G25 (medium). Sepiapterin synthase reactions were run with a total reaction volume of 70 ul with the following components; 15 ul of the enzyme described above, 40 ul of H_2-neopterin triphosphate, 5 ul of 35 mM NADPH, 5 ul of 0.1 M $MgCl_2$, and 5 ul of 0.7 M PIPES (pH 7.5). The reactions were carried out in the dark at 42°C for 30 min and heated at 100°C for 5 min. After centrifugation, 40 ul of the resulting supernatant was subjected to high pressure liquid chromatography. A Waters Liquid Chromatograph with a 6.3 mm × 30 cm μ Bondapak C-18 reverse phase column was used to separate sepiapterin from the reaction mixture. The column was eluted at 2 ml/min with 7% methanol at room temperature. The enzymatically produced sepiapterin was quantitated using a UV monitor (Waters at 260 nm (Dorsett *et al.*, 1980, for details). Calibration of the peaks was accomplished using $[U-^{14}C]H_2$-neopterin-$(P)_3$ as a substrate (specific activity, 22.5 uCi/umol).

5. Tryptophan Pyrrolase Assay

The tryptophan pyrrolase assay, a modification of the method of Kaufman (1962) and Marzluf (1965), consisted of homogenizing 0.5–1 g of flies in cold 0.14 M KCl, 2.5 mM NaOH. The ratio of flies to solution was 1:5. Following centrifugation ($25,000 \times g$, 30 min), the supernatant was rapidly filtered through Pasteur pipettes stuffed with glass wool. Two-tenths milliliter of the filtered supernatant was added to 1.8 umol L-tryptophan, 50 umol potassium phosphate, 0.275 umol 2-mercaptoethanol, and 1 umol 1-phenyl-2-thiourea contained in 0.3 ml at pH 7.4. This mixture was incubated in a shaking water bath at 37°C for 2 hrs (a period during which the increase in kynurenine is linear). At the end of the incubation period, 1.5 ml of 6.7% trichloroacetic acid was added to stop the reaction, and the sample was filtered through Whatman No. 1 filter paper. Two-tenths milliliter of 0.4% sodium nitrate, 0.2 ml of 1.0% ammonium sulfamide, and 0.2 ml of 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride were added in that order at 5 min

intervals to 1.0 ml of cold TCA filtrate. This mixture was stored in the dark at 4°C for at least 18 hr, after which it was centrifuged. The optical densities of the supernatants were determined at 560 nm. Assays in which the enzyme preparations were replaced with known amounts of kynurenine and samples to which TCA was added prior to incubation provided the necessary controls.

RESULTS

The two major eye pigments of *Drosophila* consist of drosopterins and ommochromes. The biosynthesis of the red eye pigments drosopterins involves the conversion of GTP to dihydroneopterin triphosphate as the first step in the pteridine biosynthesis (Dorsett *et al.*, 1979). Sepiapterin synthase then converts the dihydroneopterin triphosphate to sepiapterin in the presumptive second step. The subsequent production of drosopterin is accomplished by a series of enzymatic steps that are yet to be defined. Yim *et al.* (1977) have shown that purple (*pr+*) is probably the structural gene for sepiapterin synthase. This finding provides a system for examining the expression of a variegating gene as a function of its associated enzyme activity. On the other hand, tryptophan pyrrolase which catalyzes the production of formyl kynurenine from tryptophan is at the first step in the biosynthesis of ommochromes. It has been suggested that tryptophan pyrrolase is the enzyme product of vermilion (*v+*) gene (Baglioni, 1960). Dependence of sepiapterin synthase reaction on the incubation time and on the concentration of the enzyme extract is shown in Figure 1. The production of sepiapterin is linear with time up to 60 min and that the rate is proportional to concentration of the enzyme. Figure 2 illustrates that the time and
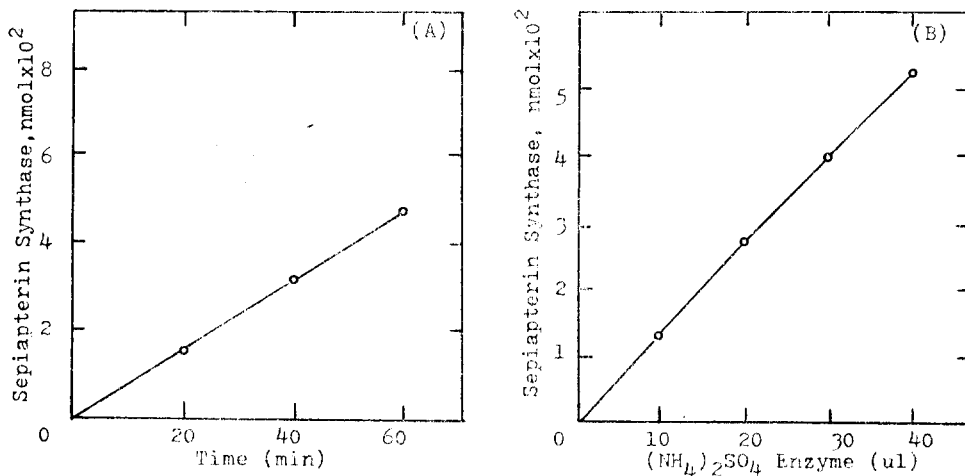

Fig. 1. Dependence of the enzymatic formation of sepiapterin on the incubation time (A) and on the concentratration of enzyme (B). The $(NH_4)_2SO_4$ enzyme was prepared and assayed as described in Materials and Methods.
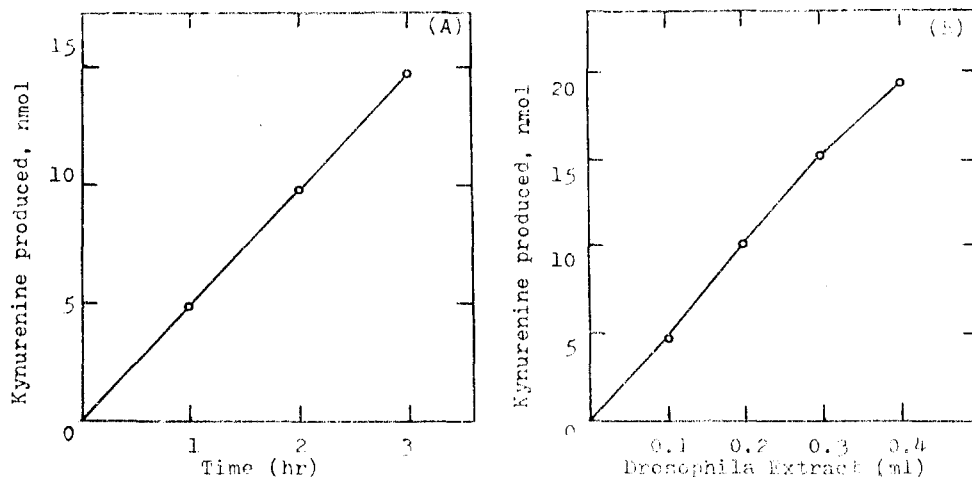
Fig. 2. Tryptophan pyrrolase activity in Oregon-R extract, showing time (A) and enzyme (B) dependency. Preparation of the *Drosophila* extract and the assay conditions were as described in Materials and Methods.

enzyme dependency of tryptophan pyrrolase activity.

It has been demonstrated that the translocated purple mutant, $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$, had approximately 5% of the Oregon-R drosopterin content and approximately 6% of the Oregon-R sepiapterin synthase activity. The original purple mutant (pr^{1}) had approximately one-third the drosopterin content and enzyme activity of Oregon-R flies. Since the decrease in the amount of drosopterin and the enzyme activity correlate rather closely, they both apparently reflect the extent of gene activity in these flies (Tobler *et al.*, 1979). In order to determine if the purple gene in the translocated genotype was low throughout development, the sepiapterin synthase activity was measured at several developmental stages. The larval stages for all three genotypes were assayed as a group; similarly, the pupal and adult stages were each assayed as a group. This study was repeated on four different cultures and utilized both sexes. Figure 3 shows the results of this experiment. Throughout development, Oregon-R had more activity than either pr^{1} or $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$ genotype and showed two peaks of activity one in adults and one in early larvae. Purple had a similar profile during development but with considerable less (approximately 20-30%) activity in any one stage. The profile of activity of $T(Y:2)pr^{c5}$, cn/pr^{c4}, however, was different than those of the other two genotypes. The translocated genotype showed the larval peak of activity, having about one-half Oregon-R levels and approximately twice pr^{1} levels. The adult peak, however, was missing or at least drastically reduced in size as compared with the other genotypes. Sepiapterin synthase activity in 1-day-old adult purple flies was as much as 3 times the activity in translocated genotypes. Wild-type flies has as much as 10 times the $T(Y:2)pr^{c5}$, cn/pr^{c4} adult levels of sepiapterin synthase activity. It seems clear then that the flies bearing the translocation

Fig. 3. Developmental profile of sepia-
pterin synthase in Oregon-R
(○), pr^1(□), and $Y(T:2)pr^{c5}$,
$cn/pr^{c4}cn$(△). The enzyme
activity was assayed under stan-
dard conditions described in
Material and Methods.

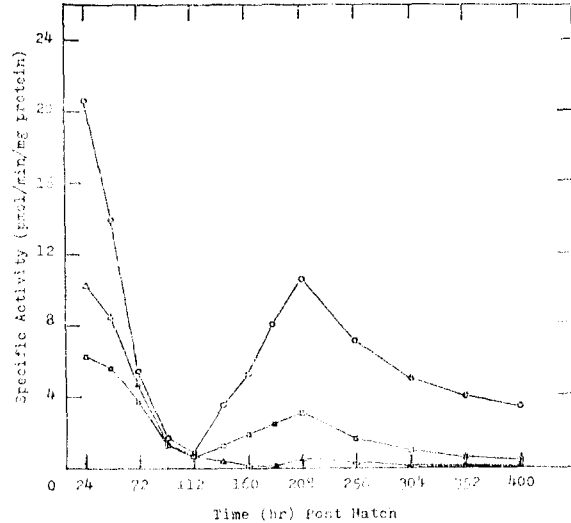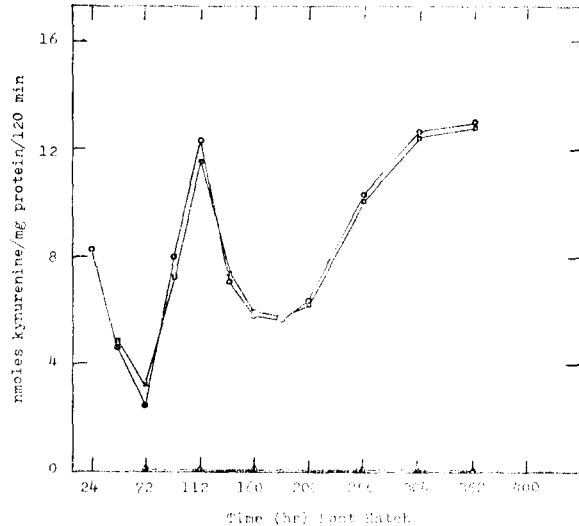Fig. 4. Ontogenic profiles of tryptophan
pyrrolase in Oregon-R (○), bw
(□), and $v; bw$ (△). The activi-
ties are the result of at least
three independent determina-
tions.

have a different profile of activity which is in all probability caused by an inactivation of
the relocated pr^+ gene sometime prior to eclosion. The next experiment was designed to
compare v^+ gene activity in *vermilion, vermilion;brown,* and wild type flies during onto-
geny. Synchronously developing progeny from eggs laid in bakers' yeast during 6-hr
periods were harvested at the times stated above and examined for tryptophan pyrrolase
activity. The results (Fig. 4) show the tryptophan pyrrolase activity profile of Oregon-R
along with *bw*, and *v;bw* flies. Wild-type stocks displayed high levels of activity in early
pupae and adults, with lower levels in 72-and 160 hph (hrs. post hatch) larvae and pupae.
The same pattern of activity was observed for *bw* flies. *v;bw* showed essentially no
activity as expected.

DISCUSSION

There has been considerable effort in the past to determine the time of the variegating process. Research has, however, largely been restricted to the study of mosaic phenotype patterns, which requires interpretation of events that have occurred much earlier in the development. The basic criterion one is forced to use in such studies is the number and the size of the mosaic spots in the tissue; this information is then extrapolated to the number of primordial cells present during the variegating process. By using available information concerning cell number and lineage maps, the developmental stage at the time of the variegating event can be estimated. In general, phenotypes with many small patches of mutant and wild-type cells are thought to represent events that took place later in development than those with a smaller number of large mosaic patches. Estimates for the time of the variegation process have varied from blastoderm to pupal stages, depending on the system examined (Spofford, 1976). Although valuable information has been obtained with these types of systems, one is limited in an analysis because (1) mosaic patterns could be altered by migrating primordial cells, (2) the variegating gene may have tissue-specific position effects without a visible phenotype, and (3) the "variegating" process may be reversible. The system developed with pr^+ and its associated sepiapterin synthase activity may be free of these complications. Migration of cells and tissue-specificity problems would not interfere with the interpretation because all cell types are included in the assay. The reversibility of "inactivation" is monitored by enzyme activity. To my knowledge, this is the first system providing such an advantage.

If one assume that enzyme activity reflects gene activity, this system seems to provide a reliable means for determining the timing of the purple gene variegation. Inactivation of the translocated pr^+ gene probably occurs sometime after the larval stage (Fig. 3). Since pr^{c4}/pr^{c4} is inviable, it seems that pr^{c4} has no gene product. $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$, therefore, is a heterozygote having only one active pr^+ gene that has been translocated to the Y chromosome. If that gene acting normally in $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$, one could expect approximately one-half the enzyme activity of wild-type flies. As shown in Fig. 3, this seems to be true throughout the larval stages. In pupal and adult stages, however, the relative amount of enzyme activity in the translocated genotype is drastically reduced, indicating a loss of activity of the translocated gene. The cn mutant cannot be the cause of the lack of sepiapterin synthase in the adult stage of $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$; activity in cn was even higher than in Oregon-R (data not shown). Since the larvae have relatively higher activity than adults, this indicates that the maternal enzyme is not carried over in the egg. This leaves us with hypothesis that low activity in the adult $T(Y:2)$ pr^{c5}, $cn/pr^{c4}cn$ is the result of an inactivated gene. The inactivation probably occurs sometime during late larval stages. The relatively late inactivation of $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$

is similar to that observed with $T(1:4)w^{m258-18}$ and $In(1)y^{3p}$ systems (Spofford, 1976). This similarity is of interest since the system involve different chromosomes and genes. Furthermore, $T(Y:5)pe^{ml}$, an eye-color gene, seems to show early inactivation when translocated from an autosome to the Y chromosome (Baker, 1967).

It has been previously demonstrated that relocation of *vermilion* gene can affect the ontogenic profile of its associated enzyme (Tobler, 1971). $T(13)ras^v$ flies have v^+ flies have v^+ translocated into the heterochromatin of the third chromosome. Genotypes carrying this translocated v^+ show a burst of tryptophan pyrrolase activity in late larval stages, a time in which wild type activity is at its lowest ebb (Fig. 4). Although the reason for this activity remains unknown, speculation is made that it may reflect another control function operating at the gene level. The interrelationship between this observation and the finding with $T(Y:2)pr^{c5}/pr^{c4}$ awaits further experimentation.

SUMMARY

The purple (pr^+) gene of *Drosophila* and its associated enzyme, sepiapterin synthase, were employed in a study of the relationship between ontogenic expression and the location of the gene in the genome. Enzyme assays performed at different developmental stages indicate the $T(Y:2)pr^{c5}$, $cn/pr^{c4}cn$ flies (files in which pr^+ has been translocated and which exhibit variegation) have a reduced amount of enzyme activity as compared with both wild-type and pr^1 flies. This reduction in activity was not found in larval stages, which suggests that the inactivation process probably occurs in late larval or early pupal stages. Tryptophan pyrrolase, the enzyme system associated with vermilion (v^+), was also examined for activity in different developmental stages of the fly. Genotype carrying a translocated v^+ shows a peak of tryptophan pyrrolase activity in late larval stages, whereas, Oregon-R exhibits the lowest activity at this period.

ACKNOWLEDGEMENTS

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