GENETIC DISSECTION OF CLONALLY INHERITED GENOMES OF POECILIOPSIS: II. INVESTIGATION OF A SILENT CARBOXYLESTERASE ALLELE

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ABSTRACT

According to the ratchet mechanism hypothesis, deleterious mutations should accumulate in clonal genomes of unisexual fish of the genus Poeciliopsis. This study defines one such mutant, a silent carboxylesterase allele (Es-50) which is found in the heterozygous condition in a particular population of P. monach-occidentalis. An antiserum to purified Poeciliopsis carboxylesterase cross-reacts with the gene product of the Es-50 allele upon immunoelectrophoresis. This finding of cross-reacting material associated with the Es-50 allele provides a useful marker for the breeding of a carboxylesterase deficient strain.

STUDIES of clonal genomes from hybrid, all-female “species” of fish (Poeciliopsis, Poeciliidae) have revealed considerable genetic diversity. Unisexual Poeciliopsis populations often comprise multiple genetic clones characterized by electrophoretic, immunological, morphological, and ecological differences (VRIJENHOEK 1979). Most of the differences among clones arise from spontaneous recruitment of sexual genomes into the clonal mode of reproduction through interspecific hybridization. However, mutation also plays an important role in clonal evolution. The hidden genetic contents of these genomes can be revealed in the laboratory through crossing experiments that move the clonal genome from its hybrid genetic background into a homospecific genetic background (LESLIE and VRIJENHOEK 1978, 1980). These manipulations provided the first experimental evidence that asexual lineages are subject to genetic deterioration through accumulation of deleterious genes. This process, originally hypothesized by MULLER (1964), has subsequently become known as the “ratchet mechanism” (FELSENSTEIN 1974; MAYNARD SMITH 1978).

In addition to the recessive, developmental lethals observed in our earlier studies, we have uncovered mutant alleles that silence the enzymatic activity of loci encoding a carboxylesterase, an eye-tissue specific lactate dehydrogenase, and an alcohol dehydrogenase in various clonal genomes. Our present experiments have not identified the effects of these enzyme lesions on the fitness of their respective clones since these effects cannot easily be decoupled from those of closely linked deleterious genes.
The goal of the present study is to characterize the silent carboxylesterase mutant, Es-5\textsuperscript{0}, that occurs in clone Ia of \textit{P. monacha-occidentalis} from the Río de la Concepción of northern Sonora, Mexico (Vrijenhoek, Angus and Schultz 1977). \textit{P. monacha-occidentalis} is an all-female “species” that arose via natural, interspecific hybridizations between the sexual species \textit{P. monacha} and \textit{P. occidentalis}. During oogenesis these hybrids transmit only the haploid \textit{mónacha} genome (\textit{M}) to their ova; the \textit{occidentalis} genome (\textit{O}) is discarded. Cytogenetic, biochemical, and immunological studies all confirm that recombination between the \textit{M} and \textit{O} genomes does not occur (Cimino 1972; Vrijenhoek, Angus and Schultz 1977; Angus 1980). The hybrid (\textit{M-0}) genotype and phenotype are reestablished each generation by matings with males of \textit{P. occidentalis}. Thus the \textit{M} genome is transmitted clonally between generations, but the \textit{O} genome is substituted.

The \textit{Es-5} locus in \textit{Poeciliopsis} is expressed predominantly in liver tissue (Leslie and Pontier 1980). It is a highly polymorphic locus that lies within Linkage Group I (Leslie 1982):

\begin{center}
\begin{tabular}{cccc}
\textit{Ldh-1} & \textit{Es-5} & \textit{Ldh-2} & \textit{Idh-2} & \textit{Es-4} \\
21.1 & 6.4 & 10.0 & 20.3 \\
\end{tabular}
\end{center}

Several possible mutations could have given rise to the silent \textit{Es-5\textsuperscript{0}} phenotype. A mutation might have rendered the gene product enzymatically inactive without major alteration of the native protein. Alternatively, several classes of mutations could have eliminated the gene product or rendered it unidentifiable: e.g., a deletion, frame-shift, a chain terminating mutation, or alteration of a linked \textit{cis}-acting regulator. These latter possibilities are not easily distinguished from one another without detailed fine-structure analysis of this gene region. In order to test the enzymatic inactivation hypothesis we raised an antiserum to purified \textit{Poeciliopsis} carboxylesterase for use as a probe to detect cross-reacting material (CRM) associated with the \textit{Es-5\textsuperscript{0}} allele.

**MATERIALS AND METHODS**

Purification of carboxylesterase: In a typical purification, wild-type liver carboxylesterase was extracted from the combined livers of more than 300 of these small, guppy-like fish. \textit{P. monacha-occidentalis} and \textit{P. occidentalis} collected in the Río Mayo near the town of El Tabelo, Sonora served as the source of the \textit{Es-5 a, d, and f} allozymes used in the purifications. Approximately two grams of liver tissue was crudely homogenized in 5 ml of cold phosphate buffered saline (PBS: 1M NaCl, 10 mM Na\textsubscript{2} HPO\textsubscript{4}, pH 7.0). The homogenate was diluted to 40 ml with PBS, agitated vigorously, and centrifuged at 2000 \texttimes g to remove cellular debris.

Carboxylesterase was purified by a two stage procedure involving group-specific affinity chromatography followed by preparative electrophoresis. Fish carboxylesterases are extensively glycosylated rendering them amenable to purification by affinity chromatography on Concanavalin-A coupled to an insoluble matrix (Leibel 1979). Commercially available Con-A coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was poured into a standard 5 ml glass syringe which served as a column. The column bed was equilibrated with PBS, and the liver extract was applied to the column through the buffer reservoir. This was followed by a wash with 50 ml of PBS to remove unbound proteins. Bound carboxylesterase was eluted from the column with 0.1 M \textit{a}-methyl mannoside in PBS with the column flow rate adjusted to about 2.5 ml/min (Lloyd 1976).
Five-ml fractions were collected and assayed for esterase activity using a modified Gomori assay (TRIPATHI and DIXON 1968). Positive fractions were pooled and concentrated to a final volume of about two ml by dialysis against a saturated solution of flake polyethylene glycol in water (Aquicide III, Calbiochem, Los Angeles, CA). The concentrated enzyme solution was assayed for protein concentration using the microbiuret assay of ITZHAKI and GILL (1974). Specific activity was increased from 0.0518 to 1.86 IU/mg over the crude homogenate.

The solution was then subjected to electrophoresis in a 5% acrylamide gel slab cast in the Borate buffer (III) of SHAW and PRASAD (1970). Electrophoresis proceeded for three hours at 350 V and 4°, whereupon the gel was lightly stained for esterase activity. The stained Es-5 bands were excised from the gel and ground in a small amount of 0.85% saline.

**Antiserum preparation:** Antiserum was raised in New Zealand White rabbits. The acrylamide-esterase slurry was mixed with an equal volume of complete Freund's Adjuvant (Difco, Detroit, MI) and homogenized by forceful passage through an 18 gauge hypodermic needle. The acrylamide itself also serves as a potent adjuvant (SPIELMAN, ERICKSON and EPSTEIN 1974). The entire mixture was injected into the thigh musculature of the rabbit on days 0, 7, and 14 of the immunization schedule. On day 21 the animal was given an intraperitoneal injection of the concentrated column eluant without Freund's adjuvant. The animal was bled by cardiac puncture on day 28 to obtain the serum.

The presence of precipitating serum antibodies was confirmed by the Ouchterloney double diffusion technique as described by GARVEY, CRAMER and SUSSDORF (1978). Antibody specificity was examined by the absorption technique of HOLMES and MARKERT (1969). A fresh liver extract was mixed with an equal volume of antiserum, and allowed to sit overnight at 4°. The mixture was then centrifuged at 7000 g and examined under our conventional starch gel electrophoretic procedures (LESLIE and VRIJENHOEK 1977). The antiserum-treated extract showed a complete removal of Poeciliopsis esterase activity. Controls which substituted normal rabbit serum revealed the typical Es-5 patterns.

**Detection of CRM:** Since the Es-5 silent allele exists only in the heterozygous condition (e.g., Es-5O/4) in *P. monacha-occidentalis*, it was necessary to distinguish antibody response to the functional allozyme from that directed against CRM associated with the silent allele. Two-dimensional crossed immunoelectrophoresis was employed for this purpose. One percent agarose gels were cast in barbital buffer (Stock buffer: 0.05M Na Barbital, 0.01M Barbituric acid, 0.5 g/liter calcium lactate; after LAURELL 1965). Stock buffer was diluted 1:4 for the gels and 1.2 for the buffer chambers. Gels were cast on 80 x 100 mm glass plates. Four equidistant sample slots were cut into the hardened gel about 15 mm from the narrow end of the plate and filled with liver extracts. Replicate samples were run in adjacent pairs of slots 1–2 and 3–4. Electrophoresis in this first dimension proceeded for one hour at 100 V.

Following first dimensional electrophoresis, 25 mm wide tracks from slots two and four were excised from the gel and stained for esterase activity. The relative mobility of the bands was measured for later comparison with precipitin arcs formed during the second dimension. Electrophoresis in the second dimension involved running the remaining sample lots (1 and 3) perpendicularly into newly cast tracks (2 and 4) that incorporated the anti-esterase serum in the gel matrix. This was prepared by allowing 10 ml of 1% agarose in barbital buffer to cool to 50° in a water bath. One ml of undiluted antiserum was then added, and the mixture was cast in the vacant tracks. Electrophoresis proceeded for 12 hr at 30 V, followed by a 12 hr incubation at 37° to insure completion of the immune reaction. Precipitin arcs were stained with Coomassie Brilliant Blue R (WEEK 1973).

**RESULTS**

Poeciliopsis *Es-5* genotypes are readily assessed by conventional starch gel electrophoresis as described previously (LESLIE and VRIJENHOEK 1977). Figure 1 depicts the electromorph patterns of relevant genotypes. Unisexual *P. monacha-occidentalis* (e.g., strain IIa, Rio Sonora) are typically heterozygous at the *Es-5*
locus, and express the two-banded phenotype seen in slots 1 and 6. A fast migrating $a$ allozyme is contributed by occidentalis (slot 2), while the slower migrating $d$ or $f$, allozyme is encoded by the clonal monacha genome. Strain Ia of *P. monacha-occidentalis* from the Rio de la Concepción however (slot 3), expresses a single-banded phenotype corresponding to the *a* electromorph pattern of *P. occidentalis* in this river (slot 2). Laboratory crosses of five females of strain Ia *P. monacha-occidentalis* with males of a highly inbred strain of *P. lucida* (slot 5) produced *P. monacha-lucida* offspring which express only the $Es-5^d$ phenotype which characterizes this strain of *P. lucida* (slot 4).

The clonal *M* genome of strain Ia contributes nothing to the $Es-5$ phenotypes of these *M-O* or *M-L* hybrids (slots 4 and 5); it carries a silent allele. The $Es-5^a/o$ and $Es-5^d/o$ heterozygotes express only the $Es-5^a$ or $Es-5^d$ allozymes contributed by the substitutable paternal alleles.

Liver extracts of fish with four different $Es-5$ genotypes were subjected to crossed immunoelectrophoresis as described above. The precipitin patterns are illustrated in Figure 2. Dotted lines reflect the mobility of known $Es-5$ allozymes as determined by staining duplicate samples for esterase activity. Track 1 depicts the immunoelectrophoretic phenotype of *P. occidentalis* from the Rio de la Concepción (*Es-5^a/a*). A single sharp precipitin arc overlies the position of the fast migrating $a$ allozyme (dotted line). A series of smaller arcs (black arrows) which overlie no esterase allozymes is seen in all tracks. These arcs do not fuse with the esterase immunoprecipitin lines (i.e., share no common antigenic de-
track 1

**ES-5 genotype**

- **a/a**
- **a/d**
- **a/o**
- **d/o**

**FIGURE 2.**—Precipitin patterns associated with *Es-5* genotypes of *P. monacha-occidentalis* on crossed immunoelectrophoresis. Dotted lines correspond to mobilities of active allozymes.

Terminants) and probably represent antibody response to glycoprotein contaminants which remained after esterase purification.

Track 2 illustrates the phenotype of an *Es-5a/d* heterozygous *P. monacha-occidentalis* from the Rio Mayo (clone IIIa). It has the sharp peak corresponding with the *a* allozyme and a prominent shoulder on the right side which overlies the position of the *d* allozyme. Track 3 depicts the precipitin pattern produced by
the Concepción population of *P. monacha-occidentalis* strain Ia (*Es-5°/°*). Note the fused twin arcs, one corresponding with the *a* allozyme (dotted line) while the other marks no functional allozyme. Similarly, track 4 illustrates the *Es-5°/°* pattern of *monacha-lucida* hybrids derived from strain Ia as described above. Here again, the sharp peak corresponds to the functional *d* allozyme; the shoulder on the right overlies no site of esterase activity. The precipitin arcs which do not overlie functional allozymes in tracks 3 and 4 (white arrows) mark the same relative position, which we assign to the product of the silent *Es-5°* allele.

It is important to note that precipitin arcs produced by the silent allele gene product and functional allozymes are completely fused, *i.e.*, no precipitin spurs are apparent. This reaction of "immunological identity" suggests a great deal of structural homology between the proteins, as there are apparently no antigenic determinants on the functional enzyme molecules which are not also present on the *Es-5°* protein.

**DISCUSSION**

The cumulative effects of silencing mutations, such as *Es-5°*, on the fitness of various clonal genomes of *P. monacha-occidentalis* are lessened by the nature of hybridogenetic reproduction. Recessive mutations in the *monacha* genomes are permanently sheltered in the heterozygous condition by the paternally derived *occidentalis* alleles. Thus the "ratchet mechanism" can only affect these fish to the degree that new deleterious mutations are partially dominant in their expression. When clonal genomes are rendered partially homozygous via the crossing protocol of Leslie and Vrijenhoek (1978, 1980), lethal effects were evident and quantifiable, but they could not be tied to individual genes or linkage groups. In this study we show that a potentially deleterious mutant, *Es-5°*, in Linkage Group I renders its protein product enzymatically inactive. Even though this carboxylesterase lesion is sheltered by the wild-type paternal allele, an accumulation of many such mutations might be costly in terms of unnecessary protein synthesis.

The *Es-5°* allele carried by clone Ia apparently is a mutant of the *Es-5′* allele carried by clone IIa which occurs in the next river to the south, the Rio Sonora. Angus (1980) concluded that clone Ia arose through mutations from clone IIa. He found that tissue grafts from "standard-bred" strains of Ia to IIa are accepted, but the reciprocal grafts are slowly rejected. These related strains have major histocompatibility differences with other strains of *P. monacha-occidentalis* from rivers to the south. Clone Ia also has a unique soluble muscle protein allele, *Mp-3°*, that distinguishes it from clone IIa and all other Poeciliopsis clones and species examined to date.

Our present finding of cross-reacting-material (CRM) associated with *Es-5°* suggests that this allele has undergone only slight mutational change, perhaps as little as a single amino acid substitution at or near its active site. It apparently has undergone no major shifts in electrophoretic mobility as compared with the *Es-5′* allele from which it arose. The mutation apparently has had little effect on the antigenicity of the *Es-5°* protein since it cross-reacts identically with anti-
serum directed against wild-type Es-5 allozymes. A silent esterase allele in *Drosophila melanogaster* was found to be CRM negative (R. Richmond, personal communication). It will be of interest to probe other species in this manner to determine the extent to which silent alleles produce potentially costly, non-functional proteins.

The physiological role of the *Es-5* carboxylesterase in *Poeciliopsis* remains obscure. The *Es-5* mutation renders its gene product enzymatically inactive under our assay procedures, using artificial esters that do not occur in normal metabolism. Presently, we do not know what the natural substrate is or how this mutation effects reactions with it. The metabolic significance of the carboxylesterase has been the subject of considerable speculation (see Krisch 1971). With the appropriate crosses, the silent *Es-5* allele can be rendered homozygous. Unless the condition is lethal in itself, the resulting carboxylesterase deficient strain could provide a useful tool for assessing the physiological role of this highly polymorphic enzyme system.

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**LITERATURE CITED**


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