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Sex-linked peptidase-1 patterns in *Pleurodeles waltlii* Michah. (Urodele Amphibian) : genetic evidence for a new codominant allele on the W sex chromosome and identification of ZZ, ZW and WW sexual genotypes

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Summary. In *Pleurodeles waltlii,* peptidase-1 is a dimeric enzyme which has been shown to be controlled by two codominant alleles, Pep-1^A and Pep-1^B (Ferrier *et al.*, 1980, 1983), linked respectively to the Z and W sex chromosomes. The enzymatic patterns obtained after starch gel electrophoresis were used routinely to identify the sexual genotypes of animals reared in our laboratory. We describe here three new patterns that were encountered in standard females, in sex-reversed animals and in thelygenous females. The inheritance data indicated the occurrence of a third codominant allele, Pep-1^{β}, linked to the W sex chromosome. The discovery of this new allele does not impair peptidase-1 polymorphism as a reliable tool for ZZ, ZW and WW genotype identification.

The identification of the sexual genotype is essential for investigations on sexual differentiation. The salamander *Pleurodeles waltlii* has a ZZ/ZW genotypic sex determination (Gallien, 1954 ; Dournon *et al.*, 1984). However no sexual heteromorphism is visible on mitotic chromosomes. Till recently the identification of the sexual genotype was only possible in adults, after they were crossed with a partner whose sexual genotype was known and after the sex-ratio of the resulting offspring was established. Such a time-consuming method (about three years) can now be avoided since it was reported that peptidase-1 is sex-linked in *Pleurodeles waltlii* (Ferrier *et al.*, 1980, 1983) and in *Pleurodeles poireti* (Dournon *et al.*, 1984).

Like human peptidase-A, Pleurodeles peptidase-1 is specific for the valyl-leucine bond. In *Pleurodeles waltlii* it is a dimeric and polymorphic enzyme, coded by a couple of codominant alleles, Pep-1^A and Pep-1^B, which are localized on the Z and W chromosomes respectively. According to Ferrier *et al.* (1983), all standard males are Pep-1^A/Pep-1^A (Z_AZ_A), all standard females are Pep-1^A/Pep-1^B

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(Z_AW_B) and all gynogenetic females are Pep-1^B/Pep-1^B (W_BW_B). Cayrol *et al.* (1983) reported that females of the « 103 lineage » are exceptional due to the presence of a null allele on the W chromosome : standard females are Pep-1^A/Pep -1^0 (Z_AW_0) and gynogenetic females are Pep-1^0/Pep-1^0 (W_0W_0).

On this basis, peptidase-1 electrophoretic patterns were used routinely in order to identify the sexual genotype of each animal in our stock-breedings. However, during the course of our investigations, three new electrophoretic patterns were encountered among various lots of breeders. These observations led us to identify a new allele, designated Pep-1^β and localized on the W chromosome.

Materials and methods.

The salamanders *(Pleurodeles waltlii)* bred in two separate laboratories (see authors' adresses) were either standard animals : ZZ males and ZW females, or sex-reversed animals : ZZ neofemales obtained after estradiol treatment during larval stages (Gallien, 1951), and ZW or WW neomales resulting from rearing the larvae at 30-32 °C (Dournon and Houillon, 1984, 1985). Homogametic thelygenous WW females were obtained from crosses between ZW neomales and standard ZW females (Collenot, 1973, 1975 ; Dournon and Houillon, 1984).

In the present work, the peptidase-1 pattern was first studied in animals whose sexual genotype had been previously identified by studying the sex-ratio of their offspring. Then it was analyzed in their descendants. All the offspring were reared in standard conditions.

The enzyme was identified by horizontal 9 % starch gel electrophoresis of erythrocyte hemolysate according to the technique of Wright *et al.* (1976) using a Tris-versene-borate system (Siciliano and Shaw, 1976) at pH 8.2, 4 °C, under 10 v/cm and for 5 h. In several cases, peptidase-1 was identified after homogenization of a single embryo or after homogenization of the tail tip of a single larva. Peptidase-1 was revealed according to the method of Lewis and Harris (1967) using 0-dianisidine, snake venom powder *(Crotalus adamenteus)* and peroxidase.

Results and interpretation.

A) *Different patterns of peptidase-1.*

The different patterns of peptidase-1 observed in *Pleurodeles waltlii,* shown on figures 1 and 2, are represented on a diagram with the corresponding genotypes on figure 3. The number of individuals tested in each case is indicated in table 1.

1. *Patterns already described.*

a. *A single, thin and slow band* (Channel a, fig. 1 and 3). — This pattern corresponded to the homozygotic genotype Pep-1^A/Pep-1^A. It was observed in either males (54 animals) or sex-reversed neofemales (14 animals) ; all were Z_AZ_A individuals.

TABLE 1

Number of phenotypic males and females tested for peptidase-1 pattern in Pleurodeles waltlii. The individuals are classified according to their sexual and peptidase-I genotypes.

Sexual and peptidase-1 genotypes	Z_AZ_A	Z_AW_B	W_BW_B	Z_AW_β	W_BW_β	$W_\beta W_\beta$	Z_AW_0
No. of phenotypical males	54	107	([a])0	78	6	6	([b])
No. of phenotypical females	14	173	45	133	51	53	3
Total	68	280	45	211	57	59	3

([a]) W_BW_B males have not yet been obtained in spite of several sexual inversion attempts.
([b]) Sexual inversion of Z_AW_0 individuals has not yet been tried.

b. *A single, thin and slow band* (Channel g, fig. 3). — This band had the same localization as the former one. In quantitative electrophoresis it showed a weaker activity (Cayrol *et al.*, 1983). This pattern was observed in three ZW females (kindly supplied by Drs. Ferrier and Jaylet ; they were from the « 103 lineage ») ; it corresponded to the genotype Pep-1^A/Pep-1^0 ; these females were called Z_AW_0.

c. *Three distinct thin bands* (Channel b, fig. 1 and 3). — This pattern corresponded to the heterozygotic genotype Pep-1^A/Pep-1^B. The slow band corresponded to the homodimeric form AA, the middle one to the heterodimeric form AB and the fast one to the homodimeric form BB. This pattern was observed in either standard females (173 animals) or sex-reversed neomales (107 animals) ; all were Z_AW_B individuals.

d. *A single, thin and fast band* (Channel c, fig. 1 and 3). — This pattern corresponded to the homozygotic genotype Pep-1^B/Pep-1^B and was observed in 45 thelygenous females ; all were W_BW_B females.

In table 2, the sex-linkage of the codominant Pep-1^A and Pep-1^B alleles was verified from two crosses between genotypic females (crosses 1 and 2).

2. *New patterns.*

a. *A broad slow band* (Channel d, fig. 1 and 3). — It was situated between the levels of the homodimeric AA and the heterodimeric AB bands observed in Z_AW_B animals. This pattern was observed in either standard ZW females (133 animals) or sex-reversed ZW males (78 animals).

b. *A broad fast band* (Channel e, fig. 1 and 3). — It was situated between the levels of the heterodimeric AB and homodimeric BB bands observed in Z_AW_B animals. This pattern was observed only in WW animals : 51 WW thelygenous females and 6 WW neomales.

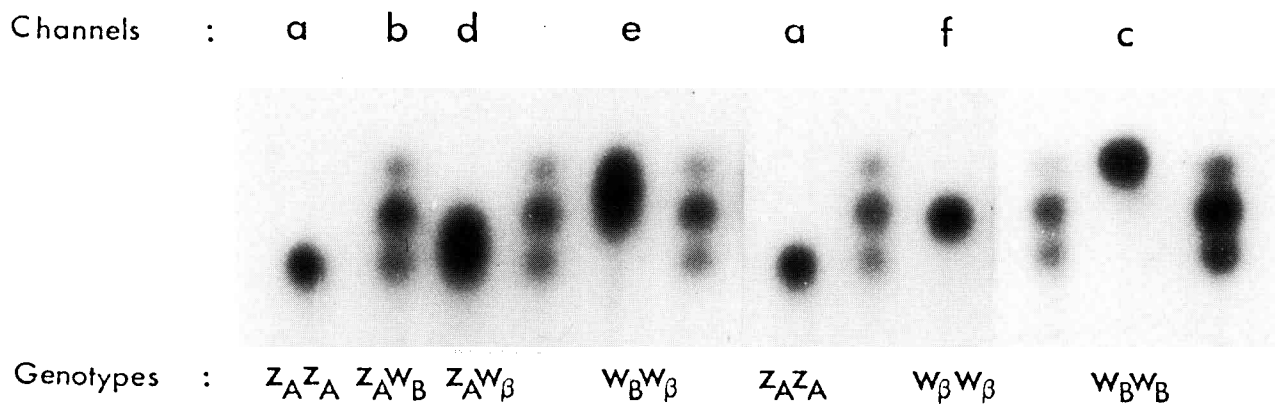

FIG. 1. — *Electrophoretic patterns of sex-linked peptidase-1 in* Pleurodeles waltlii.

TABLE 2

Genetical demonstration of the existence of allele β through non-standard crosses in Pleurodeles waltlii. For each cross, the parental sexual genotypes were known. Crosses 1 and 2 were used as controls. Z_AW_β, W_BW_β and $W_\beta W_\beta$ are the hypothetical peptidase-1 genotypes confirmed by analysis of the sex-ratio and peptidase-1 patterns of the offspring.

Parents Cross no :	Peptidase-1 patterns of the offspring — references of the channels —					
	a	b	c	d	e	f
1 ♂ Z_AW_B × ♀ Z_AW_B	11 ♂ (27.5 %)	18 ♀ (45.0 %)	11 ♀ (27.5 %)			
2 ♂ Z_AW_B × ♀ W_BW_B		28 ♀ (53.8 %)	24 ♀ (46.2 %)			
3 ♂ Z_AW_β × ♀ Z_AW_B	17 ♂ (28.8 %)	12 ♀ (20.4 %)		17 ♀ (28.8 %)	13 ♀ (22.0 %)	
4 ♂ Z_AW_β × ♀ W_BW_B		29 ♀ (72.5 %)			11 ♀ (27.5 %)	
5 ♂ Z_AW_β × ♀ Z_AW_β	22 ♂ (20.9 %)			57 ♀ (54.3 %)		26 ♀ (24.8 %)
6 ♂ Z_AW_β × ♀ W_BW_β		7 ♀ (-%)		7 ♀ (-%)	3 ♀ (-%)	1 ♀ (-%)
7 ♂ Z_AW_β × ♀ W_BW_β		24 ♀ (34.8 %)		16 ♀ (23.2 %)	12 ♀ (17.4 %)	17 ♀ (24.6 %)
8 ♂ Z_AZ_A × ♀ $W_\beta W_\beta$				(*) 10 (100 %)		
Sexual and peptidase-1 genotypes :	Z_AZ_A	Z_AW_B	W_BW_B	Z_AW_β	W_BW_β	$W_\beta W_\beta$

(*) The sexual phenotype is not mentioned because the peptidase-1 pattern was established individually for embryos or larvae before sexual differentiation occurred.

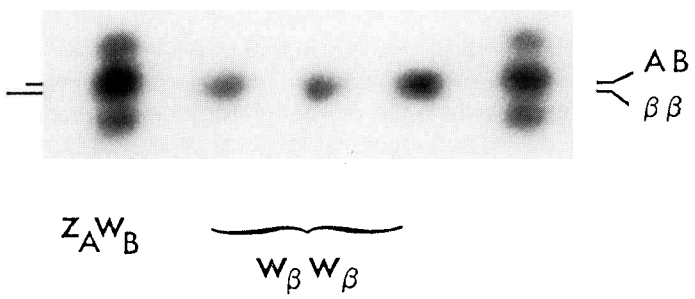

FIG. 2. — *Electrophoretic patterns of sex-linked peptidase-1 from Z_AW_B and $W_\beta W_\beta$ females.* The levels of the AB and ββ dimers are pointed. The ββ homodimeric band appears to be slightly slower than the AB heterodimeric band.

c. *A single thin band* (Channel f, fig. 1 and 3) which was slightly slower than the heterodimeric AB band (fig. 2). This new pattern corresponding to the homodimeric form ββ was observed in 57 individuals, 26 of which were issued from a cross between individuals exhibiting the slow broad band (table 2, cross 5).

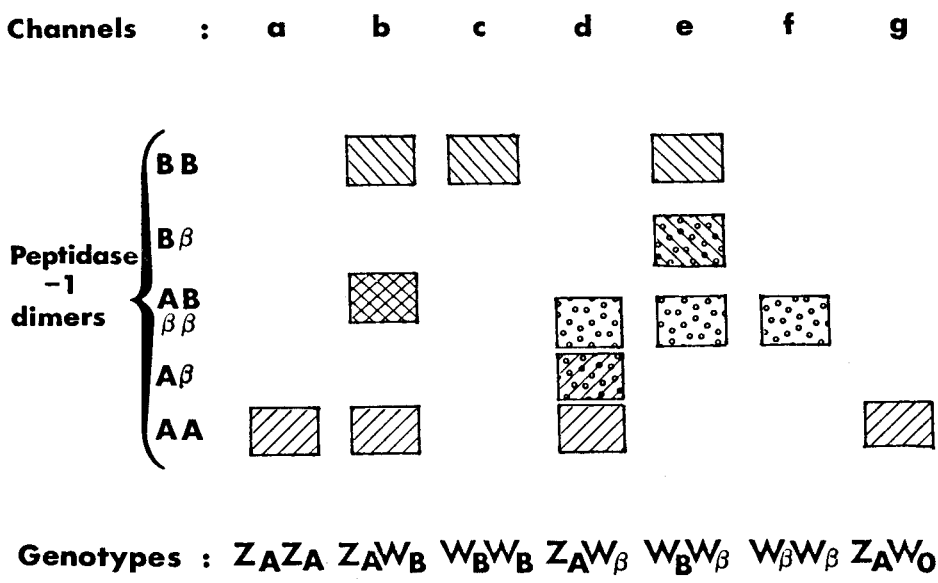

FIG. 3. — *Interpretative diagrams of the electrophoretic patterns of sex-linked peptidase-1 and its corresponding sexual and peptidase-1 genotypes in* Pleurodeles waltlii. These diagrams are theoretical : the patterns corresponding to the Z_AW_β and W_BW_β genotypes do not exhibit separate bands (fig. 1), and as for the Z_AW_0 genotype, the diagram does not take the gene dosage effect into account.

B) *Analysis of the new patterns ; genetical demonstration of the existence of a new codominant allele Pep-1$^\beta$.*

The patterns corresponding to the channels b and d were observed in standard ZW females and in ZW neomales issued from a cross between a standard ZZ male and a thelygenous WW female ([1]). Therefore the simplest explanation of the channel d pattern was to suppose the existence of an additional codominant allele, designated Pep-1$^\beta$ and different from the Pep-1^B allele described previously (Ferrier *et al.*, 1983).

According to this hypothesis, the broad slow band (Channel d) would correspond to the three dimeric forms : AA, Aβ and $\beta\beta$, and a cross between two animals exhibiting the broad slow band (hypothetically Z_AW_β individuals) would lead to offspring exhibiting the parental pattern or either of the other two patterns corresponding to the homodimeric forms AA and $\beta\beta$. This was what we found (cross 5, table 2). The existence of the allele Pep-1$^\beta$ was confirmed through the crosses 6 and 7 (table 2) between Z_AW_β male and W_BW_β female which led to 18 individuals exhibiting the pattern represented on channel f. The sexual and enzymatic genotypes of the 44 individuals exhibiting this pattern was $W_\beta W_\beta$.

([1]) Thus female died before identification of its peptidase-1 pattern.

According to these results, the broad fast band (Channel e) corresponded to three dimeric forms : $\beta\beta$, βB and BB.

In crosses 3 to 7 (table 2), the enzymatic patterns of the offspring confirmed the mendelian inheritance of the three sex-linked alleles Pep-1^A, Pep-1^B and Pep-1^β.

Finally, the genetic demonstration of the existence of β allele carried by the W sex chromosome was further confirmed after cross 8 (table 2). This cross between a thelygenous WW female exhibiting the pattern of channel f and a standard Z_AZ_A male led to offspring which all exhibited the slow broad band. Therefore it appears that the slow and the fast broad bands included three dimers : respectively AA, Aβ, $\beta\beta$ and the $\beta\beta$, Bβ, BB characterizing Z_AW_β and W_BW_β individuals.

Discussion.

In spite of several attempts to modify the starch gel electrophoresis method in order to separate three subbands in the fast and slow broad bands, resolution was not sufficient to clearly see three subbands in the patterns of heterozygotic Aβ and Bβ individuals. Fortunately, the availability of non-standard breeders made it possible to demonstrate the genetical existence of the allele β by obtaining homozygotic $\beta\beta$ individuals. A heterozygotic Aβ offspring exhibiting the slow broad band was obtained from homozygotic breeders : a standard AA male and a thelygenous $\beta\beta$ female (cross 8). A symetrical demonstration has not yet been achieved for the fast broad band due to the lack of mature homozygotic $\beta\beta$ or BB neomales.

The strikingly different patterns obtained with our electrophoresis technique in Z_AZ_A, Z_AW_0, Z_AW_B and Z_AW_β individuals show that the new allele β differs from the peptidase-1 alleles previously described (Ferrier *et al.*, 1983 ; Cayrol *et al.*, 1983). Unlike the null allele characteristic of the « 103 lineage », the allele which was detected in the separate stock-breedings of our laboratories does not appear to be limited to a particular lineage ; among the 491 ZW individuals randomly tested, 280 were Z_AW_B and 211 were Z_AW_β. It would be interesting to look for these different alleles and for any new one in *Pleurodeles sp.* obtained from other laboratories and in the wild.

The results from the different crosses show that the alleles are inherited with the sex chromosomes in a mendelian fashion. It is worth noting that the proportions of the sexual and peptidase-1 genotypes are in accordance with theoretical expectations. Thus, identification of the peptidase-1 pattern is a reliable tool to use in establishing both peptidase-1 and the sexual genotypes of any individual, whether adult, larva or embryo. The early detection of the enzyme is relevant to embryological and biochemical investigations in which the sexual genotype is implicated.

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Résumé. *Zymogrammes de la peptidase-1, enzyme liée au sexe chez* Pleurodeles waltlii *(Amphibien Urodèle) : démonstration génétique de l'existence d'un nouvel allèle codominant lié au chromosome W et identification des génotypes sexuels ZZ, ZW et WW.*

Chez *Pleurodeles waltlii,* Ferrier *et al.* (1980, 1983) ont démontré que la peptidase-1 est une enzyme dimérique qui dépend de 2 allèles codominants, Pep-1^A et Pep-1^B, liés respectivement aux chromosomes sexuels Z et W. Le génotype sexuel des Pleurodèles issus de nos élevages a été identifié en routine à partir des zymogrammes obtenus après électrophorèse en gel d'amidon. Au cours de ces identifications trois types nouveaux de zymogrammes ont été observés chez des femelles standard, des individus au phénotype sexuel inversé et des femelles thélygènes. L'analyse des zymogrammes des descendants de telles femelles a montré l'existence d'un nouvel allèle codominant, Pep-1^{β}, lié au chromosome sexuel W. La découverte de Pep-1^{β} n'invalide pas l'utilisation du polymorphisme de la peptidase-1 comme moyen fiable d'identification des génotypes sexuels ZZ, ZW et WW.

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