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UNCONVENTIONAL ORGANIZATION OF AMPLIFIED ESTERASE B GENE IN INSECTICIDE-RESISTANT MOSQUITOES OF THE *CULEX PIPIENS* COMPLEX

D. HEYSE, J. CATALAN, E. NANCÉ,
J. BRITTON-DAVIDIAN AND N. PASTEUR[1]

Institut des Sciences de l'Evolution (CNRS URA 327),
Laboratoire de Génétique et Environnement,
Université de Montpellier II (Case Courrier 065),
34095 Montpellier 05, France

ABSTRACT. The amplified esterase B gene responsible for resistance to organophosphorus insecticides in *Culex* mosquitoes is shown to form a DNA "puff" in salivary gland polytene chromosomes. This "puff" is absent in susceptible mosquitoes lacking the amplified gene, and unlike all puffs known today, it is neither related to development nor involved in protein synthesis. The "puff" therefore corresponds to a special arrangement of the very large number of copies (possibly up to 250,000) of the 25–30-kb DNA fragment encompassing the esterase B structural gene in polytene chromosomes. In addition, the amplified and nonamplified esterase B gene(s) were found to be localized on chromosome 2L, and flanked by identical banding patterns, indicating the absence of an important chromosome rearrangement before, during, or after gene amplification.

INTRODUCTION

Among the organisms that have become resistant to pesticides and drugs by gene amplification, *Culex pipiens* Linn. is the only species with polytene chromosomes, and thus constitutes a unique material for a detailed study of the structural modifications associated with gene amplification. In this mosquito, resistance to organophosphorus insecticides (OP) may be achieved by the overproduction of an esterase B resulting from the amplification of the structural gene. Several independent amplifications have been identified, each involving a different allele of the structural gene (Mouchès et al. 1990, Raymond et al. 1991, Poirié et al. 1992, Xu et al. 1994, Vaughan et al. 1995). Resistance to OP and overproduction of the esterase B protein are inherited as a single Mendelian character (Georghiou et al. 1980, Wirth et al. 1990, Poirié et al. 1992), suggesting that all gene copies are clustered on a chromosome. The presence of the amplified esterase B1 gene in male gametes (Raymond and Pasteur 1989) and in meiotic chromosomes (Nancé et al. 1990), and the stability of the amplification over generations (Raymond et al. 1993) show that all copies of the amplification are transmitted from generation to generation. However, the amplified genes are not expressed in all tissues (Nancé 1991[2]), and it is not known whether certain cell lines lose their amplification during development, or whether the genes are simply not expressed in these tissues. Finally, there are no data on eventual chromosome rearrangements associated with gene amplification. The present investigation was undertaken to address some of these points.

MATERIALS AND METHODS

Two strains of *Cx. pipiens* were used: TEM-R (Georghiou et al. 1980), a *Cx. p. quinquefasciatus* Say strain with a 250-fold amplification of the esterase B1 allele (Mouchès et al. 1990), and MSE (Raymond et al. 1986), a *Cx. p. pipiens* strain that is lacking an amplified esterase B.

Salivary glands of 50–70 late 3rd- or 4th-instar larvae were dissected in 0.85% sodium chloride, and transferred to a test tube containing the same solution. After centrifugation (1 min at 300 × *g*), most of the supernatant was discarded and replaced by methanol : acetic acid (1 : 3 v/v) diluted in demineralized water (1 : 2 v/v). This solution was replaced by 3 ml of methanol : acetic acid (1 : 3 v/v) after 2 h. To increase chromosome spreading, the salivary gland suspension was then sonicated 3–4 sec (Sonifer 250, Branson, Sonotrode output = 1), and stored overnight at 5°C before preparing 2–3 slides by the air-drying method. Slides were stained with 2% Giemsa in 25 mM phosphate buffer (pH 6.88) for 2 min, rinsed twice rapidly in demineralized water, dried at 37°C for at least 1 h, and stored at −20°C until further processing.

In situ hybridization was performed as previously described (Nancé et al. 1990), using a

[1] To whom correspondence should be addressed.

[2] Nancé, E. 1991. Localisation chromosomique et expression tissulaire des gènes amplifiés codant les estérases impliquées dans la résistance aux insecticides organophosphorés chez *Culex pipiens*. Thèse de doctorat. Université de Montpellier 2, Montpellier, France.

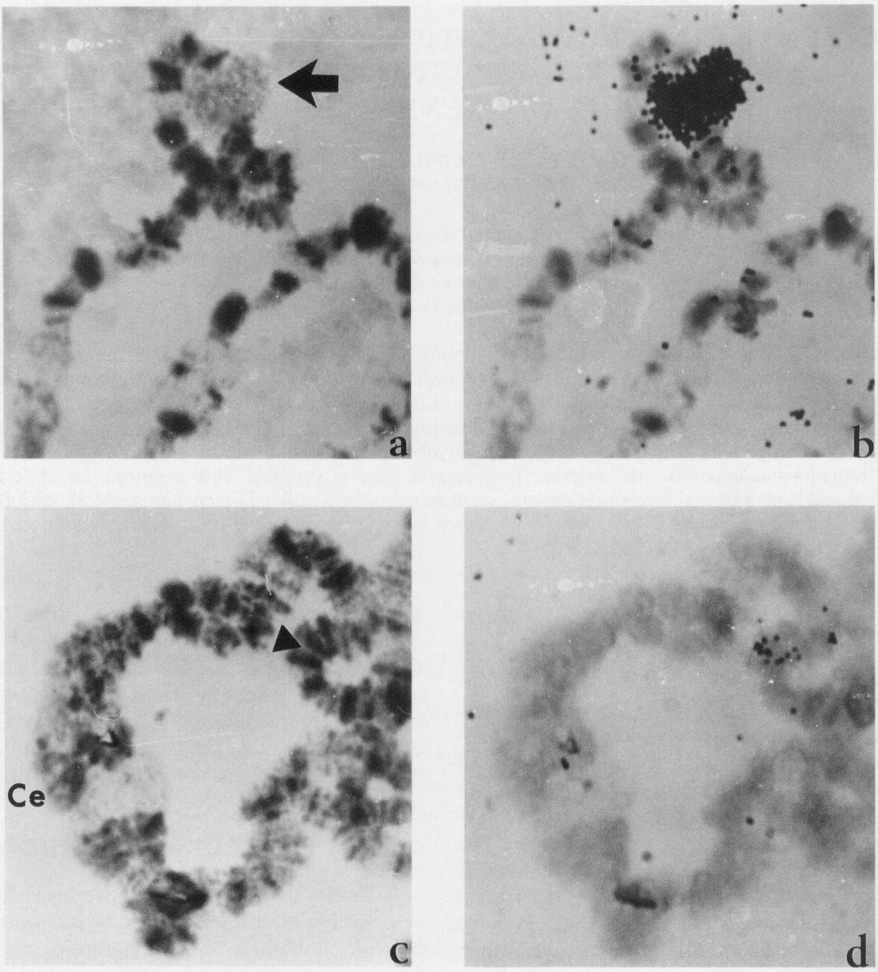

Fig. 1. *In situ* hybridization of salivary gland polytene chromosomes of *Culex pipiens* 4th-instar larvae with the esterase B1 cDNA probe. Each chromosome fragment was photographed before (a, c) and after (b, d) hybridization with the probe labelled with ^{3}H. a, b. TEM-R strain with an amplified esterase B gene (250 copies). c, d. MSE strain with no amplified esterase B gene. Note that the large pufflike structure present in TEM-R (arrow) is absent in MSE (triangle). *Ce* designates the centromere.

cDNA esterase B1 probe (Mouchès et al. 1990) labelled with either [^{3}H]adenine and thymidine or [^{32}P]adenine. Exposures were 3 wk and 2–5 days, respectively.

The presence of the esterase B1 protein in salivary glands was investigated by immunohistochemistry on paraffin sections of whole 4th-instar larvae. Larvae were fixed for 24 h in Bouin–Dubosc, dehydrated, and embedded in paraffin. Paraffin sections were immersed 10 min in phosphate-buffered saline (PBS) buffer (0.1 M, pH 7.2), and 10 min in a 1/200 solution of sheep serum (Nordic Immunology) in PBS. Seventy-five milliliters of a 1/500 dilution in PBS of the esterase B1 rabbit antiserum (Mouchès et al. 1987), containing 2% of Triton X100, was deposited on each slide, and slides were stored at 5°C in a water-saturated atmosphere for 48 h. Slides were rinsed twice with PBS, incubated 45 min at room temperature with fluorescein-conjugated goat antirabbit antiserum (Diagnostic Pasteur) (1/200 in PBS), rinsed in PBS, and mounted in 2:1 glycerol:PBS. The coverslips were sealed with nail polish.

RESULTS

Structure of the amplified esterase B gene: Hybridization of salivary gland polytene chromosomes with the esterase B1 cDNA tritiated

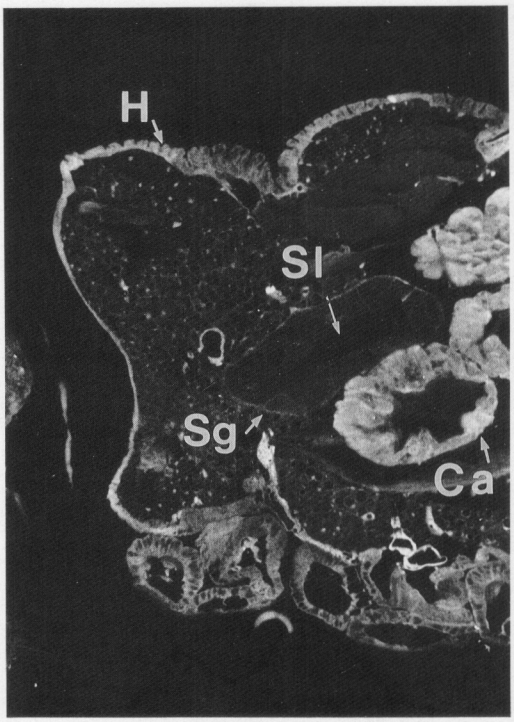

Fig. 3. Longitudinal section of the thorax of a 4th-instar larva of the TEM-R strain treated with esterase B1 rabbit antiserum and anti-rabbit goat antiserum linked to fluorescein. The tissues containing esterase B1 protein appear as white areas. Ca = ceca of the stomach, H = hypoderm, Sg = salivary gland cells, S1 = salivary gland lumen.

in control larvae from MSE or TEM-R strains treated with the goat anti-rabbit serum alone (results not shown). In contrast, a strong positive reaction was observed in cells of the alimentary canal (midgut and hindgut), Malpighian tubes, hypoderm, and in some neurones of TEM-R larvae, but no staining occurred in the salivary gland cells or lumen (Fig. 3), indicating that despite the large esterase B "puff" there is no more esterase B protein in the salivary glands of TEM-R than in those of MSE. This observation, associated with the fact that, in TEM-R larvae, the esterase B mRNA is only found in tissues showing a positive reaction with the esterase B1 antibody (Nancé 1991[2]) strongly suggests that the "puff" is not involved in gene transcription, in contrast to all puffs known so far, whether "normal" puffs (Ashburner 1978, Andrea and Thummel 1992) or Sciaridae DNA puffs (Breuer and Pavan 1955, Berendes and Lara 1975, Glover et al. 1982, Paço-Larson et al. 1992).

Chromosome localization of the amplified esterase B1: As well-spread whole polytene chromosomes could not be obtained owing to extensive anastomoses (Dennhofer 1968), chromosome identification had to rely on an easily recognized chromosome-specific structure. Figure 1c shows that hybridization of the esterase B1 cDNA probe occurred not far from a centromere in salivary gland chromosomes of MSE larvae, as did the TEM-R "puff" (Fig. 2b). Thus, the banding patterns of the centromeres were used to identify the chromosome carrying the esterase B gene using published photographs and line drawings of *Cx. pipiens* polytene chromosomes (Dennhofer 1968, Tewfik and Barr 1974).

In a preliminary study, the centromeres of the 3 chromosomes were located in MSE and TEM-R larvae. The patterns of bands and interbands of each centromere and its flanking segments could clearly be identified. On this basis, the site where the esterase B1 cDNA probe hybridized was identified as the left arm of chromosome 2 in both TEM-R and MSE mosquitoes.

Subsequent studies consisted of comparing the banding pattern of the chromosome segments flanking the area of hybridization. In the 2 strains, the following sequence could be observed from the centromere towards the chromosome tip (Fig. 4): 2 bands (a) followed by a large achromatic zone (b), 3 strongly stained bands (c), the pufflike structure in TEM-R or an interband in MSE (noted by a triangle on Fig. 4c), 2 close bands (d), a large amorphous zone with a band in its middle (e), 3 equidistant bands (f), and a long achromatic zone (g) that is probably equivalent to zone 35B–35D of Dennhofer (1968). The similar banding patterns, observed on each side of the esterase B gene(s) in TEM-R and MSE chromosome 2L, indicate that no major chromosome rearrangement has taken place before, during, or after the process of amplification. It further suggests that the "puff" in TEM-R corresponds to a single interband in MSE mosquitoes that have a single copy of esterase B gene.

DISCUSSION

In larval salivary gland polytene chromosomes of the highly resistant TEM-R strain, the amplified esterase B gene forms a large DNA pufflike structure that is not developmentally regulated, does not seem to be involved in protein synthesis, and is absent in the MSE strain with a single copy of the gene. These observations suggest that the TEM-R "puff" is related to the presence of multiple copies of the esterase B structural gene and of the DNA sequences that are coamplified with it (a total DNA sequence of 25–30 kb [Mouchès et al. 1990]). No such

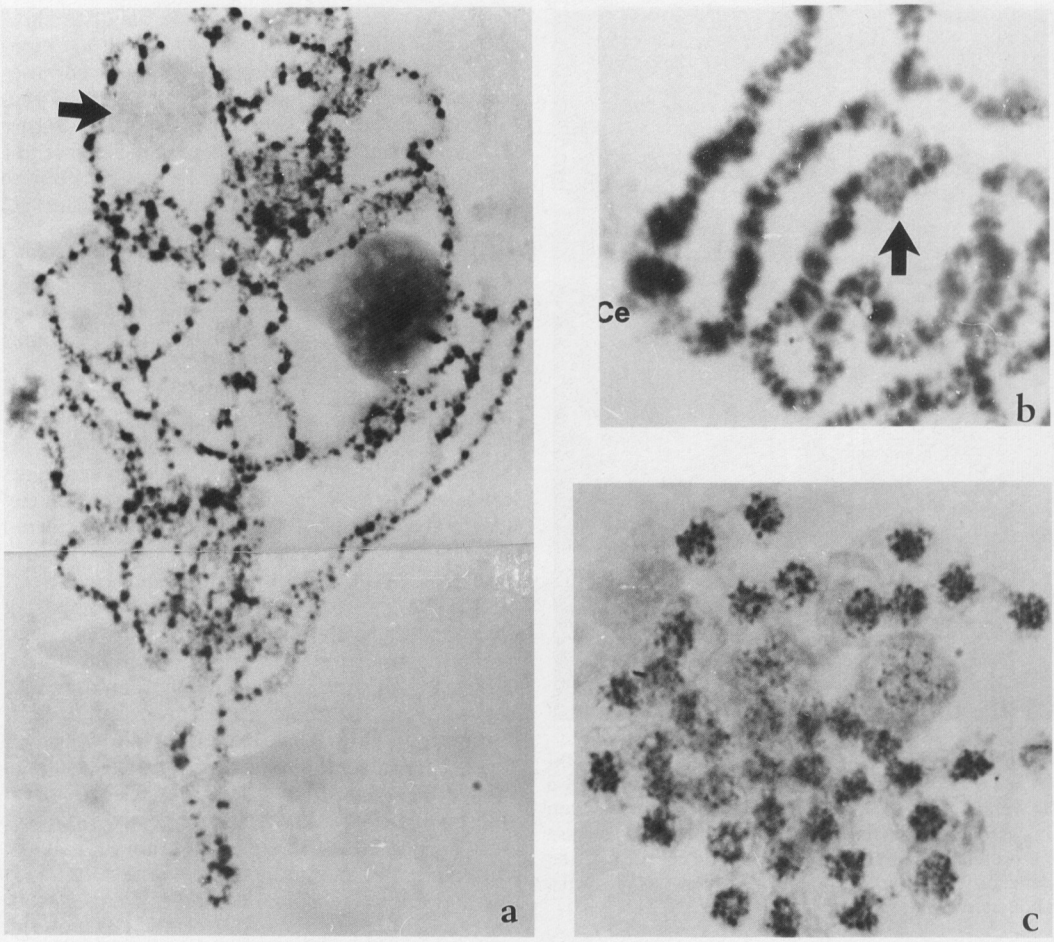

Fig. 2. Salivary gland polytene chromosomes of *Culex pipiens*. a. Polytene chromosome preparation of a 3rd-instar larva of the TEM-R strain showing the large pufflike structure (arrow) containing the esterase B1 amplified gene. b. Polytene chromosomes of TEM-R larvae showing that the pufflike structure containing the esterase B1 gene is near a centromere (*Ce*). c. Polytene chromosome preparation of a late 4th-instar larva showing the disintegration of chromosomes into chromatic bodies prior to pupation.

probe showed a large cluster of silver grains in TEM-R mosquitoes that carries a 250-fold amplification of the esterase B1 gene. Comparison of photographs of the same chromosome preparations, taken before and after *in situ* hybridization, clearly indicated that the silver grains were located on a large "puff" (Figs. 1a, 1b). This "puff" was absent in MSE larvae with a single copy of the esterase B gene (Figs. 1c, 1d).

The "puff" hybridizing with the esterase B1 cDNA probe was observed in all chromosome preparations from TEM-R 3rd- (Fig. 2a) and 4th-instar larvae (Figs. 1a, 1b, and 2b). It could not be studied in old 4th-instar larvae because the polytene chromosomes apparently disintegrate into a large number of small chromatic fragments before pupation (Fig. 2c). The TEM-R "puff", unlike puffs observed in other regions of the chromosomes (Fig 1), or in other Diptera (Berendes and Lara 1975, Ashburner 1978), presents a homogeneous aspect and its presence seems independent of developmental stages. Because salivary gland preparations were treated with RNase, the "puff" appears to be largely composed of DNA. To better understand its function, the tissue distribution of the esterase B1 protein was investigated on paraffin sections of whole 4th-instar larvae by immunocytochemistry, using a rabbit esterase B1 antiserum and the fluorescein-conjugated goat anti-rabbit antiserum of Mouchès et al. (1987). No evidence of the esterase B1 protein was found in MSE larvae: fluorescence was homogeneously distributed in all tissues and its intensity was as low as

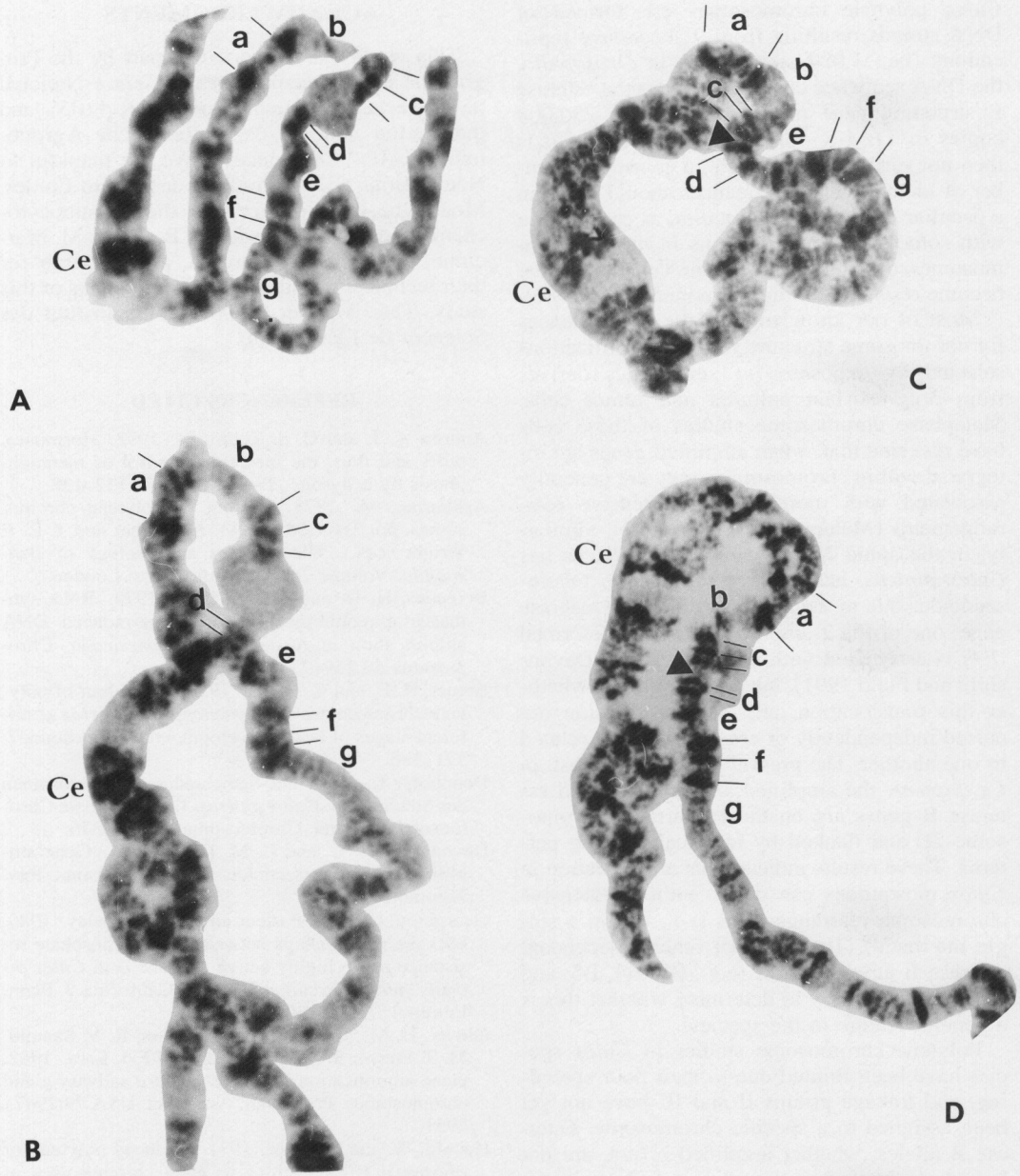

Fig. 4. Identification of polytene chromosome banding patterns flanking the pufflike structure containing amplified esterase B in TEM-R (A and B) and the zone of hybridization with the esterase B cDNA probe (noted by a triangle) in MSE (C and D). Homologous regions are identified by letters (a–g).

structure is observed in *Drosophila* salivary gland chromosomes for the multiple copies of rDNA or histone genes. In fact, ribosomal cistrons present a reduced polyteny in *Drosophila* giant chromosomes (Hennig and Meer 1971, Spear 1977), indicating that polytenization is regulated. Because multiple copies of ribosomal genes are observed in most eucaryote species, it is likely that regulation of polytenization for such genes is very ancient. In contrast, esterase B amplification is a recent phenomenon (30 years at the most), and there is no reason why regulation of polytenization should have coevolved with the amplification. If we assume that

Culex polytene chromosomes are formed of DNA strands resulting from 9 successive replications (i.e., 1,024 strands), as in *Drosophila,* the DNA sequence coamplified with the esterase B structural gene may exist in some 250,000 copies in TEM-R polytene chromosomes. It is then not surprising that such an enormous number of identical DNA sequences should result in a peculiar chromosome structure, as can be seen with some gene amplifications in mitotic chromosomes of cultured or tumor cells that have become resistant to drugs (Rovigatti et al. 1986).

Most of our knowledge of the consequences for chromosome structure of gene amplifications selected by exposures to xenobiotics derives from drug-resistant cultured and tumor cells. Metaphase chromosome studies of these cells have revealed that, when amplified genes are integrated within chromosomes, they are generally associated with more or less extensive rearrangements (Melera and Biedler 1991). Similarly, in the aphid *Myzus persicae,* which, as has *Culex pipiens,* has become resistant to OP insecticides due to the amplification of an esterase gene, one of the 2 amplified esterases described (E4) is associated with a translocation (Devonshire and Field 1991), but it is not known whether this translocation and the amplification occurred independently or are in some way related to one another. The present study shows that, in *Cx. pipiens,* the amplified and nonamplified esterase B genes are on the left arm of chromosome 2L and flanked by identical banding patterns. These results indicate that amplification in *Culex* mosquitoes can occur without extensive chromosome rearrangements (i.e., within a single interband). The study of other independent esterase B amplifications (e.g., B2, B4, B5, and others) is necessary to determine whether this is a general feature in the species.

Polytene chromosome studies in *Culex* species have been limited due to their poor spreading, and linkage groups II and III have not yet been assigned to a specific chromosome. Esterase B alleles, whether amplified or not, are not linked to the *yellow larva* gene, which is the only widespread mutation of linkage group II available in laboratory reference strains. The esterase B gene (also named *Est-2*) has, therefore, been attributed to linkage group III (Pasteur et al. 1981). Our *in situ* hybridization study, by assigning the esterase B structural gene to chromosome 2L, is the first report of a chromosomal identification of linkage group III. It is believed that the knowledge of the genome structure of important vector species, such as *Cx. pipiens,* will progress in the future as *in situ* hybridization investigations develop.

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REFERENCES CITED

Andres, A. J. and C. S. Thummel. 1992. Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. Trends Genet. 8:132–138.

Ashburner, M. 1978. Puffing of polytene chromosomes, pp. 316–395. *In:* M. Ashburner and T. R. F. Wright (eds.). The genetics and biology of *Drosophila,* Volume 2b. Academic Press, London.

Berendes, H. D. and F. J. S. Lara. 1975. RNA synthesis: a requirement for hormone-induced DNA amplification in *Rhynchosciara americana.* Chromosoma 50:259–274.

Breuer, H. E. and C. Pavan. 1955. Behaviour of polytene chromosomes of *Rhynchosciara angelae* at different stages of larval development. Chromosoma 7: 371–386.

Dennhofer, L. 1968. Die Speicheldrüsenchromosomen der Stechmücke *Culex pipiens.* I. Der normale Chromosomenbestand. Chromosoma 25:365–376.

Devonshire, A. L. and L. M. Field. 1991. Gene amplification and insecticide resistance. Annu. Rev. Entomol. 36:1–23.

Georghiou, G. P., N. Pasteur and M. K. Hawley. 1980. Linkage relationships between organophosphate resistance and a highly active esterase B in *Culex pipiens quinquefasciatus* Say from California. J. Econ. Entomol. 73:301–305.

Glover, D. M., A. Zaha, A. D. Stocker, R. V. Santelli, M. T. Pueyo, S. M. De Toledo and F. J. Lara. 1982. Gene amplification in *Rhynchosciara* salivary gland chromosomes. Proc. Natl. Acad. Sci. USA 79:2947–2951.

Hennig, W. and B. Meer. 1971. Reduced polyteny of ribosomal RNA cistrons in giant chromosomes of *Drosophila hydei.* Nature New Biol. 233:70–72.

Melera, P. W. and J. L. Biedler. 1991. Molecular and cytogenetics analysis of multidrug resistance-associated gene amplification in Chinese hamster, mouse sarcoma, and human neuroblastoma cells, pp. 117–145. *In:* I. B. Roninson (ed.). Molecular and cellular biology of multidrug resistance in tumour cells. Plenum Publ. Corp., New York, NY.

Mouchès, C., M. Magnin, J. B. Bergé, M. de Silvestri, V. Beyssat, N. Pasteur and G. P. Georghiou. 1987. Over-production of detoxifying esterases in organophosphate-resistant *Culex* mosquitoes and their presence in other insects. Proc. Natl. Acad. Sci. USA 84:2113–2116.

Mouchès, C., Y. Pauplin, M. Agarwal, L. Lemieux, M. Herzog, M. Abadon, V. Beyssat-Arnaouty, O. Hyrien, B. Robert de Saint Vincent, G. P. Georghiou and N. Pasteur. 1990. Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. Proc. Natl. Acad. Sci. USA 87:2574–2578.

Nancé, E., D. Heyse, J. Britton-Davidian and N. Pasteur. 1990. Chromosomal organization of the amplified esterase B1 gene in organophosphate-resistant *Culex pipiens quinquefasciatus* Say (Diptera, Culicidae). Genome 33:148–152.

Paço-Larson, M. L., J. C. de Almeida, J. E. Edström and H. Sauaia. 1992. Cloning of developmentally amplified sequence in the DNA puff C4 of *Bradysia hygida* (Diptera: Culicidae) salivary glands. Insect Biochem. Mol. Biol. 22:439–446.

Pasteur, N., A. Iseki and G. P. Georghiou. 1981. Genetic and biochemical studies on the highly active esterases A′ and B associated with organophosphate resistance in the *Culex pipiens* complex. Biochem. Genet. 19:909–919.

Poirié, M., M. Raymond and N. Pasteur. 1992. Identification of two distinct amplifications of the esterase B locus in *Culex pipiens* (L.) mosquitoes from Mediterranean countries. Biochem. Genet. 30:13–26.

Raymond, M. and N. Pasteur. 1989. The amplification of B1 esterase gene in the mosquito *Culex pipiens* is present in gametes. Nucleic Acid Res. 17:7116.

Raymond, M., A. Callaghan, P. Fort and N. Pasteur. 1991. World-wide migration of amplified insecticide resistance genes in mosquitoes. Nature 350: 151–153.

Raymond, M., E. Poulin, V. Boiroux, E. Dupont and N. Pasteur. 1993. Stability of insecticide resistance due to amplification of esterase genes in *Culex pipiens*. Heredity 70:301–307.

Raymond, M., D. Fournier, J. M. Bride, A. Cuany, J. B. Bergé, M. Magnin and N. Pasteur. 1986. Identification of resistance mechanisms in *Culex pipiens* (Diptera: Culicidae) from southern France: insensitive acetylcholinesterase and detoxifying oxidases. J. Econ. Entomol. 79:1452–1458.

Rovigatti, U., D. K. Watson and J. J. Yunis. 1986. Amplification and rearrangement of Hu-*ets*-1 in leukemia and lymphoma with involvement of 11q23. Science 232:398–400.

Spear, B. B. 1977. Differential replication of DNA sequences in *Drosophila* chromosomes. Am. Zool. 17:695–706.

Tewfik, H. R. and A. R. Barr. 1974. The salivary gland chromosomes of *Culex pipiens* L. Mosq. News 34:47–54.

Vaughan, A., M. Rodriguez and J. Hemingway. 1995. The independent gene amplification of electrophoretically indistinguishable B esterase from the insecticide-resistant mosquito *Culex quinquefasciatus*. Biochem. J. 305:651–658.

Wirth, M. C., M. Marquine, G. P. Georghiou and N. Pasteur. 1990. Esterases A2 and B2 in *Culex quinquefasciatus* (Diptera: Culicidae): role in organophosphate resistance and linkage studies. J. Med. Entomol. 27:202–206.

Xu, J., F. Qu and W. Liu. 1994. Diversity of amplified esterase B genes responsible for organophosphate resistance in *Culex quinquefasciatus* from China. J. Med. Coll. PLA 9:20–23.