The *Amylase* Gene Cluster on the Evolving Sex Chromosomes of *Drosophila miranda*

Sigrid Steinemann and Manfred Steinemann

Institut für Genetik, Heinrich Heine Universität Düsseldorf, Universitätsstrasse 1, D-40225, Düsseldorf, Germany

Manuscript received July 6, 1998
Accepted for publication September 14, 1998

ABSTRACT

On the basis of chromosomal homology, the *Amylase* gene cluster in *Drosophila miranda* must be located on the secondary sex chromosome pair, *neo-X* (*X2*) and *neo-Y*, but is autosomally inherited in all other Drosophila species. Genetic evidence indicates no active amylase on the *neo-Y* chromosome and the *X2*-chromosomal locus already shows dosage compensation. Several lines of evidence strongly suggest that the *Amy* gene cluster has been lost already from the evolving *neo-Y* chromosome. This finding shows that a relatively new *neo-Y* chromosome can start to lose genes and hence gradually lose homology with the *neo-X*. The *X2*-chromosomal *Amy1* is intact and *Amy2* contains a complete coding sequence, but has a deletion in the 3′-flanking region. *Amy3* is structurally eroded and hampered by missing regulatory motifs. Functional analysis of the *X2*-chromosomal *Amy1* and *Amy2* regions from *D. miranda* in transgenic *D. melanogaster* flies reveals ectopic AMY1 expression. AMY1 shows the same electrophoretic mobility as the single amylase band in *D. miranda*, while ectopic AMY2 expression is characterized by a different mobility. Therefore, only the *Amy1* gene of the resident *Amy* cluster remains functional and hence *Amy1* is the dosage compensated gene.

DROSOPHILA MIRANDA shows an unusual karyotype due to the fusion of an autosome to the *Y* chromosome (Dobzhansky 1935; MacKnight 1939; Steinemann 1982). The rearrangement led to a *neo-Y* chromosome, which already shows signs of genetic degeneration (Steinemann and Steinemann 1992), and initiated the transformation of the remaining, unfused partner chromosome into a *neo-X* chromosome, designated *X2* (Steinemann *et al.* 1996). Thus, in *D. miranda*, formerly autosomal genes are now located on a pair of sex chromosomes. It is generally assumed that *X* and *Y* chromosomes have evolved from a pair of originally homologous autosomes (for review, see Rice 1996). A so-called "primitive type" (White 1973) is represented, for instance, by the *X* and *Y* sex chromosome pair in Chironomids (Kraemer and Schmidt 1993). In *Megaselia scalaris* the sex chromosome pair is determined by the presence or absence of a male-determining factor *M* whereas the *X* and *Y* chromosomes are morphologically indistinguishable (*cf.* Traut 1994). A very early stage in the evolution of a *neo-Y*, *neo-X* chromosome pair is found in *Drosophila americana americana*. In *D. a. americana* the *neo-Y* shows no obvious signs of degeneration (Charlesworth *et al.* 1997) and no molecular evidence is found indicating dosage compensation of the *neo-X* chromosomal genes (Bone and Kuroda 1996; Marin *et al.* 1996). While the chromosome rearrangement in *D. a. americana* took place a few hundred thousand years ago (Throckmorton 1982), the separation of *D. miranda* from its next relatives *D. pseudoobscura* and *D. persimilis* occurred about 2 mya (Barrio *et al.* 1992). Population genetic theory predicts degeneration for a nonrecombining *Y* chromosome (*neo-Y* chromosome) via several mechanisms, such as sampling drift, genetic hitchhiking, background-trapping, Muller's ratchet, and mutational overload (for review, see Rice 1996). In addition to accumulating point mutations on the *Y*, a second phenomenon occurs, the conformational change from a euchromatic chromosome state into a heterochromatic one. Evolutionary changes during the process of sex chromosome differentiation in *D. miranda* are associated with massive DNA rearrangements on the *neo-Y* (Steinemann and Steinemann 1992; Steinemann *et al.* 1993; Lucchesi 1994) and changes in the *X2*-chromosomal chromatin (Bone and Kuroda 1996; Marin *et al.* 1996; Steinemann *et al.* 1996). We have chosen the species *D. miranda* as a model system to analyze the molecular bases of the evolutionary processes of *Y* chromosome degeneration and dosage compensation of the *X* chromosome (for review, see Steinemann and Steinemann 1998). Both evolutionary processes have led to dramatic structural changes in the sex chromosomes.

The *Amy* locus is on chromosome *2*R in *D. melanogaster*, 54A1-B1 (Bahn 1971a; Gemmill *et al.* 1985; Boer and Hickey 1986). On the basis of chromosomal homology the *Amy* genes must be located on the *X2* and *neo-Y* in *D. miranda* (Steinemann *et al.* 1986). Crosses be-

Corresponding author: Manfred Steinemann, Institut für Genetik, Heinrich Heine Universität Düsseldorf, Universitätsstr. 1, D-40225 Düsseldorf, Germany. E-mail: steinem@mail.rz.uni-duesseldorf.de

tween *D. miranda* lines showing different electrophoretic variants of amylase revealed no active amylase on the *neo-Y* chromosome (Steinemann *et al.* 1986; Norman and Doane 1990). Measurements of the α-amylase activity in males and females indicated that the *X2*-chromosomal locus is dosage compensated (Steinemann *et al.* 1986; Norman and Doane 1990). Autoradiographic studies (Strobel *et al.* 1978) and immunofluorescent staining with antibodies against H4.Ac16, a histone H4 isoform, which in Drosophila is preferentially located on the dosage compensated *X* chromosome in males, and antibody staining of the gene products of *maleless* (*mle*) and *male-specific lethal 1* (*msl-1)* suggest that the *X2* of *D. miranda* is already dosage compensated in great part (Bone and Kuroda 1996; Marin *et al.* 1996; Steinemann *et al.* 1996). We have cloned the *Amy* gene cluster from the *X2* chromosome of *D. miranda* and in addition from chromosome *3* of the two sibling species, *D. pseudoobscura* and *D. persimilis.* In their most recent article Da Lage *et al.* (1998) describe a gene from *D. melanogaster* related to the *Amy* gene. This gene was designated *Amyrel*. From *D. miranda* and its sibling species, *D. pseudoobscura* and *D. persimilis*, we obtained sets of *Amyrel* sequences, which are localized outside the *Amy* locus near the tip of the *X2* chromosome and chromosome *3*, respectively. From sequencing the *X2*-chromosomal *Amy* gene cluster it became clear that the *Amy3* gene, containing two large deletions, is structurally eroded. *Amy2* contains a complete coding sequence, but has a deletion in the 3′-flanking region. *Amy1* is structurally intact. Functional analysis of transgenic lines with ectopically integrated *X2*-chromosomal *Amy1* and *Amy2* regions allows us to address the question of whether each of the resident *Amy1*, *Amy2* genes is active or inactive.

We were unable to isolate clones derived from the *neo-Y* chromosome. *In situ* hybridizations reveal no *in situ* signal on the *neo-Y*. Genomic Southern analysis of the *Lcp* gene cluster from the *neo-X*/*neo-Y* chromosome pair showed, due to several insertions on the *neo-Y*, size differences between homologous male and female DNA fragments (Steinemann and Steinemann 1990, 1992). Long-range PCR and genomic Southern analyses from the *Amy* region with male and female DNA revealed no evidence for fragments of expected different sizes corresponding to a *neo-Y* copy. These results strongly suggest that the *Amy* gene cluster is already deleted from the *neo-Y* chromosome. Thus a relatively new *neo-Y* chromosome can start to lose genes even after only a couple of million years of evolution.

MATERIALS AND METHODS

Fly strains and cloning of the *Amy* genes from *D. miranda* and the two sibling species: *D. miranda* MPI, *D. pseudoobscura* ST, and *D. persimilis* ST flies were cultured on standard Drosophila food at 18°. High-molecular-weight DNA from *D. miranda* and the two sibling species, *D. pseudoobscura* and *D. persimilis*, were isolated from a mixture of male and female flies according to Steinemann (1982). Genomic EMBL4 λ libraries from partial *Sau*3A (Boehringer Mannheim, Mannheim, Germany) digests were established and screened as detailed in Steinemann and Steinemann (1990). The filters were probed with pOR-M7, a cDNA clone from *D. melanogaster* OR, described in Figure 3 of Benkel *et al.* (1987). In earlier experiments we had screened a *D. melanogaster* library using the cDNA clone pMSa104 from mouse, described in Schibler *et al.* (1980, 1982). For detailed restriction mapping, the regions containing the *Amy* genes of the *X2* from *D. miranda* and of chromosome *3* from *D. persimilis* were subcloned into *pUC*18. Agarose gel electrophoresis and Southern blots were performed as detailed in Steinemann and Steinemann (1990). Selected regions of genomic male and female DNAs from *D. miranda* were amplified with the Expand Long Template PCR System (Boehringer Mannheim), as described by the manufacturers. The *Amy* region of the *X2* chromosome from *D. miranda* was sequenced on both strands from *M13mp18*/*19* subclones by the dideoxy chain termination method according to the protocol supplied with sequenase (United States Biochemical, Cleveland). Cloning and standard DNA techniques were carried out according to Sambrook *et al.* (1989).

Constructs and germ-line transformation: DNA fragments of interest were inserted into the polylinker of the CaSpeR vector (Pirrotta 1988). Cloning and germ-line transformations into *D. melanogaster* OR *w sn*^{w} (*cf.* Lindsley and Zimm 1992) embryos were performed as detailed in Steinemann *et al.* (1993). For embryo injections, constructs containing the following orientation of the *Amy1* and *Amy2* genes were chosen. The dmirAmy1 region used in AX1 starts at an artificial *Eco*RI site about 1.8 kb 5′ to the TATA-box motif and ends about 1.6 kb after the inferred poly(A) site with the *Eco*RI site left of the 3′ end of *Amy2*, total length 5085 bp (see Figure 1B). The dmirAmy2 region used in AX2 starts with the *Eco*RI site about 3.5 kb 5′ to the TATA-box motif, and extends 3′ to the *Eco*RI site, bordering the AX1 fragment, about 0.7 kb from the inferred poly(A) site, total length 5853 bp (see Figure 1B).

Computer analysis: The DNA database screening with BLASTN (Altschul *et al.* 1990) and the Genetics Computer Group Sequence Analysis Package (Devereux *et al.* 1984) was done using the updated GenBank/EMBL nucleotide Sequence Data Library (EMBL, Heidelberg, Germany; GenBank, NCBI, Washington, DC). DNA sequences were aligned using either MacMolly (Softgene, Berlin) or pileUp (GCG package) alignment programs. Critical sections were aligned by visual inspections. To clarify the arrangement of the *Amy3* gene from *D. miranda* we aligned the DNA sequence with the sequence from *D. pseudoobscura* Standard (ST) arrangement. For the alignment with the *D. miranda Amy2* deletion we used the *Amy2* DNA sequence from the *D. pseudoobscura* Chiricahua (CH) arrangement. The published sequence size from *D. pseudoobscura* ST was too short to match the end of the *D. miranda Amy2* deletion. In *D. pseudoobscura* and *D. persimilis* the third chromosome is polymorphic for gene arrangements that are the results of overlapping, paracentric inversions. On the basis of the breakpoints of the inversions these gene arrangements can be arranged in a phylogeny (Sturtevant and Dobzhansky 1936). Standard (ST), Santa Cruz (SC), and Chiricahua (CH) are representatives of different gene arrangements. Cladograms were performed using the PAUP program of Swofford (1993).

Chromosome *in situ* hybridization: λDmir1785 (containing the complete *Amy* region of *D. miranda*) and λDmir1792 (representative of the *Amy*-related clones) were labeled with Biotin-

16-dUTP (Boehringer Mannheim) by the nick translation reaction and hybridized at 58° overnight to alkali denatured chromosome squashes (Steinemann and Steinemann 1992). The slides were washed three times in 2× SSC at 53°. Signal detection followed the protocol for immunoperoxidase staining supplied with the DETEK I-hrp kit used (Enzo Diagnostics, New York). The intensity and contrast of the diaminobenzidine precipitate were enhanced using a silver diaminobenzidine enhancement kit (Amersham, Buckinghamshire, U.K.). Photomicrographs of the chromosome squashes were made with an Agfa Pan 25 film.

Fly homogenates and gel electrophoresis: Preparation of the fly homogenate, separation of the homogenate, and the visualization of the amylase enzyme activity were performed as detailed in Steinemann *et al.* (1986). For one sample, three flies were frozen in solid CO_2 and homogenized in a volume of 25 μl sample buffer (stock solution: mix 1 ml of 0.47 m Tris solution, adjusted with H_3PO_4 to pH 6.9, with 4 ml of 40% sucrose solution, 3 ml distilled water, and 40 μl of a 1% bromphenol blue solution). The homogenate was centrifuged for 30 min at 4° in an Eppendorf minifuge. A total of 20 μl of the supernatant was mixed with 3 μl 100% glycerol and applied directly to the gel. Polyacrylamide gel electrophoresis was performed according to Davis (1964). Crude extracts were separated on a 3.75% stacking and a 7.5% resolving gel (anode pole at the bottom) in the multiphasic buffer system A (Davis 1964). After the run, the gels were equilibrated for 10 min in 0.5 m Tris-HCl, pH 7.1 and then incubated for 90 min in a starch solution (1 g soluble starch and 220 mg $CaCl_2$, dissolved in 100 ml of boiling 0.1 m Tris-HCl, pH 7.5). The gel was washed twice in distilled water and stained for 2–5 min in a staining solution (300 mg KI and 130 mg iodine dissolved in 100 ml distilled water). As the AMY proteins from *D. miranda* and *D. melanogaster* OR could be clearly separated in the gel system that was used, we were able to monitor the expression of both genes in the same separation lane. We thus preferred the analysis of the expression of the *Amy* genes at the protein to the RNA level. Under the electrophoretic conditions used here, AMY1 and AMY2 revealed different mobilities. Zymograms were photographed with an Agfa Ortho 25 ASA film.

RESULTS

***Amylase* loci in *D. miranda* and the two sibling species *D. pseudoobscura* and *D. persimilis*:** From crosses between *D. miranda* lines, carrying *Amy* isozymes with different electrophoretic mobilities, it became clear that in *D. miranda* males only the *Amy* gene(s) from the *X2*-chromosomal locus encodes an active amylase enzyme. Estimates of the α-amylase activity in crude homogenates of male and female flies strongly suggest that the *Amy* gene(s) is dosage compensated in *D. miranda* (Steinemann *et al.* 1986; Norman and Doane 1990). In all other species, the *Amy* genes are autosomally inherited and hence not dosage compensated. We have cloned about 30 kb of the *Amy* region from the *X2* chromosome of *D. miranda*. The *X2*-chromosomal *Amy* region contains three stretches which reveal cross-hybridization with the *D. melanogaster* pOR-M7 probe, Figure 1A. As the 3′-fragments in these experiments always show stronger hybridization with this probe, the orientation of the *Amy* genes in the cluster could be derived from the intensity of the hybridization signals. The third stretch consists of a shorter, strongly labeled fragment indicating the observed deletions (see below). In addition, in *D. miranda* and the sibling species examined, *D. pseudoobscura* and *D. persimilis*, we obtained a second class of clones that showed a different restriction pattern when compared with the *Amy* gene cluster (not shown). These *Amyrel* clones are localized at a more distal site on the *X2* chromosome or chromosome *3*, respectively (see below).

In *in situ* hybridization experiments, clones of the *Amy* region from *D. miranda* labeled one site on the *X2* chromosome, Figure 2A. The label is restricted to one band in subdivision 27A using the cytogenetic map of Das *et al.* (1982). Using different λ clones and different *in situ* hybridization conditions we were unable to detect any signal on the *neo-Y*. On the other hand, *in situ* hybridizations with the cloned *Krüppel* (*Kr*) gene, which in *D. miranda* is located as well on the *neo-X*/*neo-Y* chromosome pair, show labeling, on both the *neo-X* and *neo-Y* loci (M. Steinemann and S. Steinemann, unpublished results). The *Amyrel* clones labeled one band at the distal tip of the *X2*, subdivision 22B, Figure 2B. A combination of both clones from *D. miranda* showed labeling of the *Amy* gene cluster and the *Amyrel* locus in *D. miranda* (Figure 3A) and the sibling species *D. pseudoobscura* and *D. persimilis* (Figure 3, B and C, respectively). Immunofluorescent stainings with antibodies against a histone H4 isoform, H4.Ac16, typically associated with the dosage compensated *X* chromosome in Drosophila males (Steinemann *et al.* 1996), and against the gene products of the *maleless* and the *male-specific lethal 1* genes MLE and MSL-1 (Bone and Kuroda 1996; Marin *et al.* 1996) stain chromosome regions along the *X2*. The *Amy* gene cluster is located in an H4.Ac16-enriched region of the *X2* chromosome. Anti-H4.Ac16 antibodies do not label the distal tip region of about 10% of the *X2* chromosome length (Steinemann *et al.* 1996). The *Amyrel* locus lies within this distal tip region. Thus we expect that, if these *Amyrel* sequences were transcribed in *D. miranda*, they should be autosomally regulated. In *D. pseudoobscura* ST, the *Amy* cluster is on chromosome *3*, division 73 and the *Amyrel* locus is at the distal tip, division 81 [Figure 3B; using the photographic map of Kastritsis and Crumpacker (1966)]. In *D. persimilis* ST, the *Amy* cluster is located on chromosome *3*, division 73 and the *Amyrel* locus at the distal tip, division 81 [Figure 3C; using the photographic map of Moore and Taylor (1986)]. Because of the resolution of the maps, a closer localization is not feasible. The *Amy* gene cluster and the *Amyrel* sequences are thus located at comparable positions along the *neo-X* (*X2*) of *D. miranda* and chromosome *3* of *D. pseudoobscura* ST and *D. persimilis* ST, respectively. Similar locations were reported in Norman *et al.* (1991). The *D. melanogaster* clone Dm1B, which was isolated during a screen of a genomic *D. melanogaster* library using the *Amy* clone

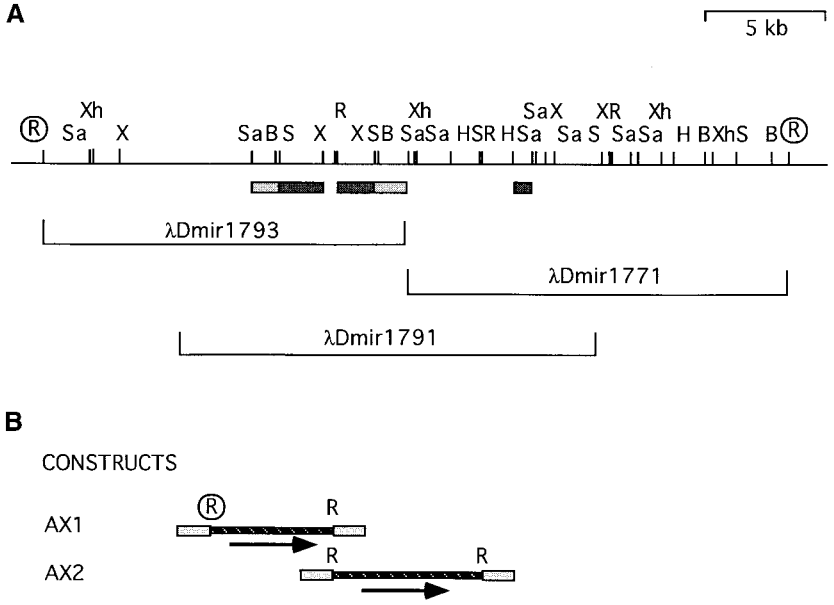

Figure 1.—(A) Restriction map of the cloned *D. miranda Amy* region from the *X2* chromosome. Fragments that cross-hybridize with pOR-M7 from *D. melanogaster* are stippled (strong hybridization, dark; weak hybridization, light). Recombinant λ phages covering the whole region are indicated. Restriction sites: S, *Sal*I; H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI; X, *Xba*I; Sa, *Sac*I; and Xh, *Xho*I. Circled *Eco*RI sites are artificial sites. (B) DNA fragments (hatched bars) were cloned into the *P*-element-derived vector CaSpeR (stippled bars), which utilizes the *white* (w^+) gene as visual marker. The 5′- to 3′-orientations of the *Amy1* and *Amy2* genes in the constructs are indicated with arrows. The constructs are orientated with the *white* gene on the right. The AX1 construct contains the *X2*-chromosomal dmirAmy1 and AX2, the dmirAmy2 fragment. In AX2 the inserted fragment is flipped horizontally with respect to the restriction map above (*cf.* materials and methods).

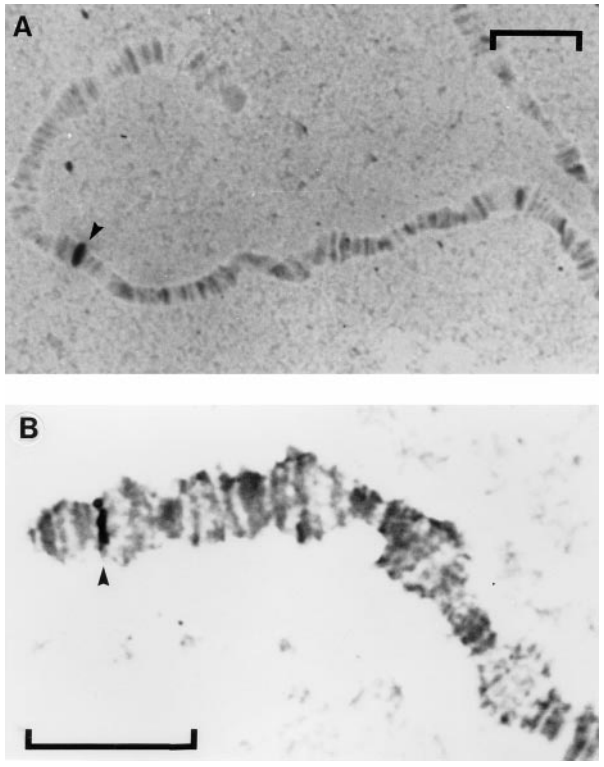

Figure 2.—*In situ* hybridization of biotinylated λ clones derived from the *Amy* region and from the *Amy*-related set of clones from *D. miranda* to the *X2* chromosome of *D. miranda*. The slides were stained in phosphate-buffered Giemsa solution. Arrowheads indicate the labeled sites. Bars, 20 μm. (A) Biotinylated λDmir1785, containing the complete *Amy* cluster, was hybridized to female polytene nuclei of *D. miranda*. The *X2* is labeled at a single site, subdivision 27A [using the cytogenetic map of Das *et al.* (1982)]. (B) Biotinylated λDmir1792 from the *Amy*-related set of clones was hybridized to female polytene nuclei of *D. miranda*. Only a single site is labeled in subdivision 22B on the *X2* chromosome of *D. miranda*.

pMSa104 from mouse (Schibler *et al.* 1980, 1982) as a probe, labels one site on *D. melanogaster 2*R, division 53, and not the *Amy* locus. In cross-hybridizations to *D. miranda* this clone labels only one band in subdivision 22B on the tip of the *X2* (not shown), exactly the same locus that is labeled with the set of *Amyrel* clones. We conclude that these loci are related on the basis of sequence similarities in both species.

DNA sequence analysis of the *X2*-chromosomal *D. miranda Amylase* gene cluster: Sequencing of the *D. miranda Amy* region revealed the structure of three genes (Figure 4A). For comparison they are aligned with the *Amy1-3* of *D. pseudoobscura* ST (Brown *et al.* 1990; Popadic and Anderson 1995) and *Amy-d* and *Amy-p* from *D. melanogaster* (GenBank accession numbers X04569 and L22724). The *D. miranda Amy* gene (dmirAmy) sequences are deposited in the EMBL gene bank, accession numbers *Amy1*, Y15603 DMAMY1; *Amy2*, Y15604 DMAMY2; and *Amy3*, Y15605 DMAMY3. *Amy1* and *Amy2* are arranged tail-to-tail (Figure 1A). *Amy3* is localized with respect to *Amy2* in a head-to-tail orientation. To determine the arrangement of the deletions in *Amy2* and *Amy3* in detail, we performed alignments with DNA sequences from *D. pseudoobscura* arrangements (*cf.* materials and methods). The *Amy1* gene is structurally intact. Alignment of the DNA sequences of *Amy2* and *Amy1* reveals for the *Amy2* gene, apart from an intact coding region, intact CAAT- and TATA-box motifs and an ATCAG motif for transcription initiation. Both genes are interrupted by an intron of 67 bp. The introns are located at identical positions, but diverge by the substitution of 4 bp. The intron size reported here corresponds to the result reported from Da Lage *et al.* (1996) using PCR-amplified fragments. *Amy2* is flanked on its 3′-side by a deletion of 273 bp that starts 1 bp in front of the inferred poly(A) signal (Figure 4A).

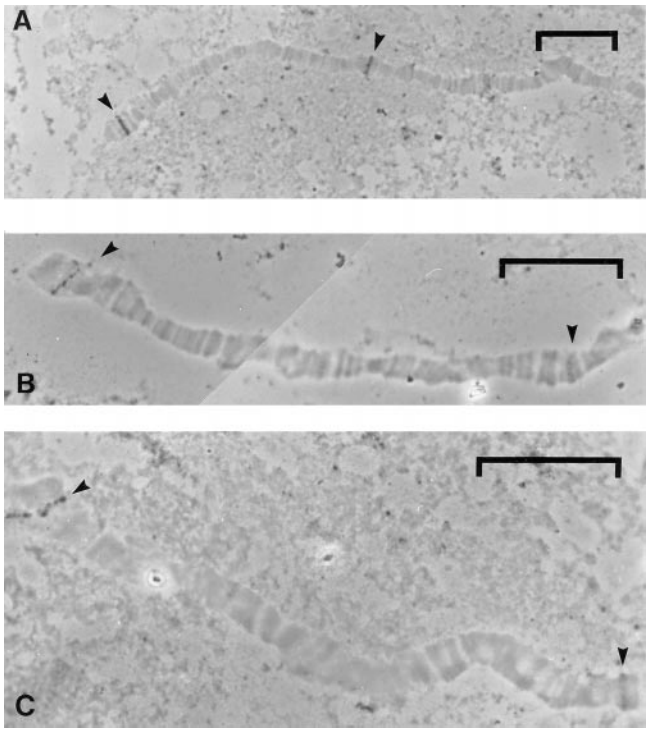

Figure 3.—*In situ* hybridization to *D. miranda* and the two sibling species with a mixture of two biotinylated λ clones derived from *D. miranda*, one clone containing the *Amy* cluster (λDmir1785) and the other clone taken from the set of *Amyrel* clones (λDmir1792). Two sites are labeled on each slide. The slides were stained in phosphate-buffered Giemsa solution. Arrowheads indicate the labeled sites. Bars, 20 μm. (A) *D. miranda X2* chromosome. The *Amy* cluster, subdivision 27A and the *Amyrel* locus, subdivision 22B [using the cytogenetic map of Das *et al.* (1982)] are labeled. (B) *D. pseudoobscura* autosome 3. The *Amy* cluster, division 73 and the *Amyrel* locus, division 81 [using the photographic map of Kastritsis and Crumpacker (1966)] are labeled. (C) *D. persimilis* autosome 3. The *Amy* cluster, division 73 and the *Amyrel* locus, division 81 [using the photographic map of Moore and Taylor (1986)] are labeled.

The size of this deletion is given with respect to the *Amy2* sequence of *D. pseudoobscura* CH (GenBank accession number U20336). Alignment of the *D. miranda Amy1* gene with the *D. pseudoobscura* ST *Amy1* gene shows extended similarities in the 5′- and 3′-flanking sequences (Figure 4A). Beside the CAAT- and TATA-box motifs, the 5′-upstream sequences of *Amy1* and *Amy2* from *D. miranda* are different (Figure 4A). A reasonable alignment of the 3′-flanking sequences after the deletion of *Amy2* is not possible with *Amy1*, but we obtain good sequence similarity with the corresponding 3′-flanking *Amy2* region of *D. pseudoobscura* (Figure 4A). It became obvious that the 3′-flanking sequences of *Amy1* and the putative corresponding sequences of *Amy2* from *D. miranda* already diverge 8 bp downstream of the poly(A) signal. In principle, *Amy1* and *Amy2* share only the transcribed sequences. The maximum parsimony analysis of the *Amy1* and *Amy2* genes using only the transcribed sequences (based on an alignment of 1658 nucleotides) from *D. miranda* and *D. pseudoobscura* (three arrangements: ST, SC, and CH) reveals that they cluster with one another and not with the putative orthologues (Figure 4B), thus indicating that the transcribed sequences have been homogenized within the species (*cf.* Shibata and Yamazaki 1995). However, the maximum parsimony analysis of the *Amy1* and *Amy2* genes including the 5′- and 3′-flanking sequences (based on an alignment of 2600 nucleotides) shows that the genes are clustered within each locus (Figure 4C). This clustering of species within genes indicates that the *Amy* duplications occurred before the divergence of the *D. pseudoobscura* and *D. miranda* species and that mechanisms of concerted evolution have not homogenized the *Amy1* and *Amy2* flanking sequences.

Amy3 reveals two large deletions, one of 445 bp and a second one of 872 bp in size, with respect to *Amy1* of *D. miranda* (Figure 4A). The arrangements of the *Amy3* deletions were clarified from the alignment of *D. miranda Amy3* with *Amy3* from *D. pseudoobscura* ST (Brown *et al.* 1990; Popadic and Anderson 1995; Figure 4A). This alignment shows that the 5′-nondeleted *Amy3* sequences from *D. miranda*, the CAAT- and TATA-box motifs, are conserved. The first deletion starts after the TATA-box and ends within exon 2. Exon 1 and the intron are completely deleted. The second deletion starts within exon 2 and ends shortly after the inferred poly(A) signal (Figure 4A). From exon 2 only a small part of 361 bp is left. Thus the *Amy3* gene of *D. miranda* is structurally hampered by missing regulatory motifs and coding sequences.

In contrast to the alignment of *Amy1* and *Amy2*, the sequence similarity shared between *Amy2* and *Amy3* of *D. miranda* extends further into the 5′-flanking (about 860 bp, not shown) and into the 3′-flanking sequences (about 180 bp; for *Amy2* the sequences are inferred from the alignment with *D. pseudoobscura* CH; see materials and methods). Upstream and downstream from this shared region the similarity drops off very quickly. Beyond this point the sequence similarities in the 3′-flanking regions are restricted to the *Amy2* genes (brick pattern) and the *Amy3* genes (checkerboard pattern) of *D. miranda* and *D. pseudoobscura* (Figure 4A). The intraspecific divergence between *Amy2* and *Amy3* in the compared 5′-flanking region is only 1.49%; the interspecific divergence is 4.17% (Table 1). Thus, the duplication that involves *Amy2* and *Amy3* of *D. miranda* encompassed, in addition to the transcribed region, several hundred base pairs of 5′- and 3′-flanking sequences. The extended flanking sequence similarity between *Amy2* and *Amy3* of *D. miranda* parallels the arrangements found in *D. pseudoobscura* (Brown *et al.* 1990; Popadic and Anderson 1995).

Expression of the *X2*-chromosomal *Amy* genes: Amylase is a monomeric protein. Thus in heterozygotes with alleles coding for different electrophoretic variants, two

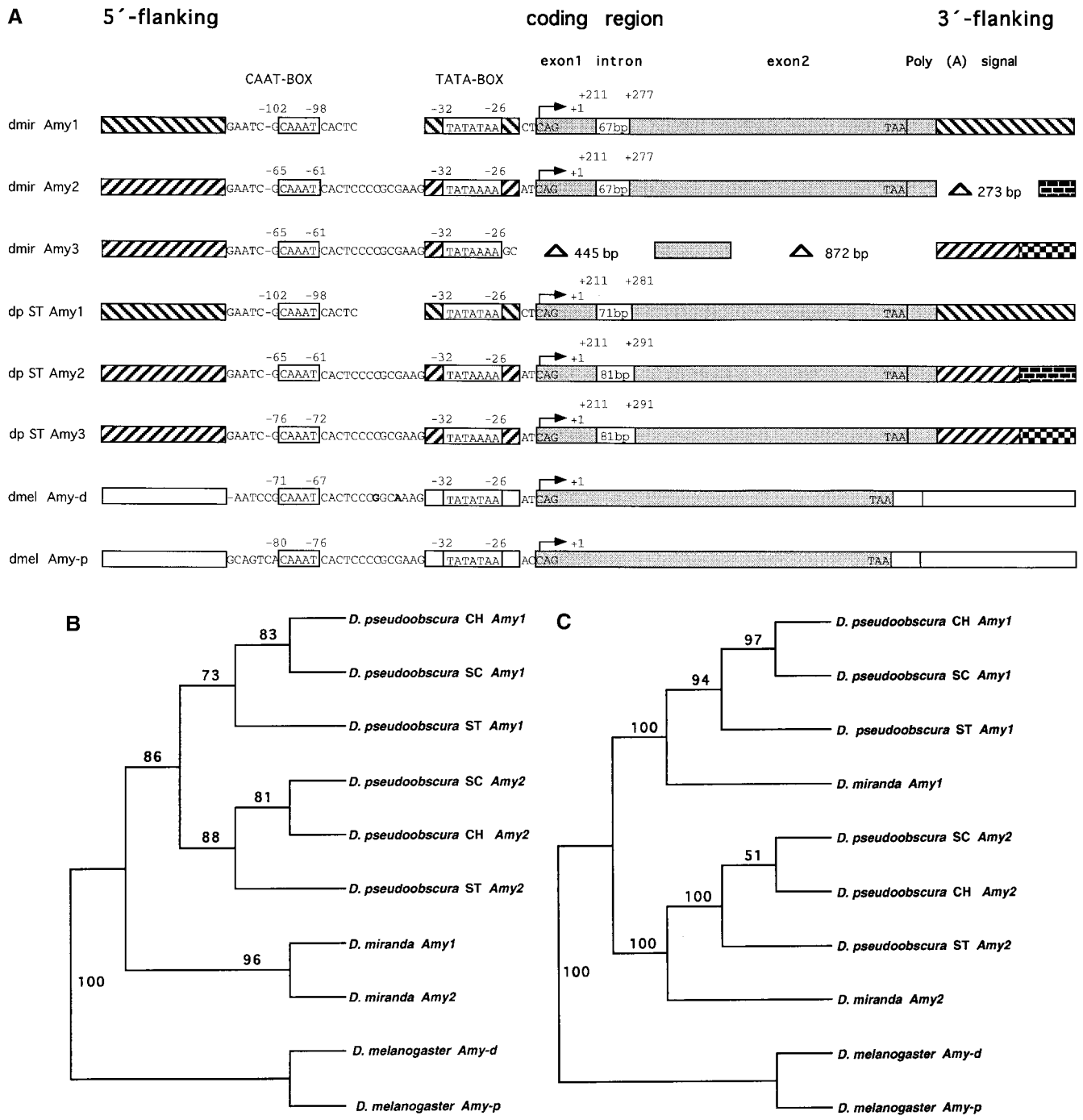

Figure 4.—(A) Schematic representation of the sequenced *X2*-chromosomal *D. miranda Amy* genes: *Amy1* (dmir Amy1: Y15603), *Amy2* (dmir Amy2: Y15604), and *Amy3* (dmir Amy3: Y15605) aligned with *D. pseudoobscura* ST *Amy1-3* (dp ST Amy1, dp ST Amy2, and dp ST Amy3) and *D. melanogaster Amy-d* (dmel Amy-d) and *Amy-p* (dmel Amy-p) sequences. The alignment reveals the arrangement of two large deletions (triangles) in the *D. miranda Amy3* gene and one deletion (triangle) in the 3′-flanking sequence of *Amy2*. The lengths of the *Amy2* and *Amy3* deletions are indicated. Each shading pattern represents a different homologous region: transcribed regions (stippled), trailer regions boxed (stippled), and introns boxed (white). The 5′- and 3′-flanking sequences are indicated by a striped, checkerboard, or brick pattern. From the *D. melanogaster* sequences the regions between the transcription start and the stop codon are shaded. +1, deduced transcription start (indicated with an arrow). The numbers detail the position of the introns and flanking regulatory motifs. (B and C) 50% majority rule bootstrap consensus trees of the *Amy1* and *Amy2* genes from *D. miranda* and *D. pseudoobscura* (three arrangements: ST, SC, and CH). The trees were rooted using *Amy-d* and *Amy-p* from *D. melanogaster* as outgroup. Numbers adjacent to each branch refer to bootstrap support from 100 replicates. (B) Transcribed sequences. (C) Transcribed and flanking sequences.

TABLE 1

Nucleotide substitutions in the 5′-flanking region of *Amy2* and *Amy3* from *D. miranda* and *D. pseudoobscura*

Region	Length[a] (bp)	No. of changes	Differences (%)	Corrected percentage divergence[b]
Between				
dmir Amy2/dmir Amy3	271	4	1.48	1.49
dmir Amy2/dp ST Amy3	271	11	4.06	4.17
dp ST Amy2/dp ST Amy3	271	4	1.48	1.49

[a] Upstream sequences including the TATA-box. *D. pseudoobscura* ST arrangement sequences from Brown *et al.* (1990).

[b] Corrected percentage divergence estimated as $d = \frac{3}{4}\ln(1 - \frac{4}{3}p)$, where p is the proportion of nucleotide sites that differ between the two sequences (Jukes and Cantor 1969).

bands are found. When the *Amy* locus of *D. miranda* is made homozygous and tested in different lines, from the pseudoobscura subgroup, *D. miranda*, *D. persimilis*, and *D. pseudoobscura*, only a single amylase isozyme was found (Norman and Prakash 1980). These observations suggest that only one *Amylase* gene is active. Therefore the question arises as to whether the *Amy1* or the *Amy2* gene in *D. miranda* produces an active amylase enzyme. DNA fragments containing the *X2*-chromosomal *Amy1* or *Amy2* gene from *D. miranda*, together with flanking 5′- and 3′- sequences (see materials and methods), were cloned into the *P*-element derived CaSpeR vector (Figure 1B). Transgenic lines of *D. melanogaster* with single insertions were tested. In seven tested transgenic lines containing the *Amy1* region (dmirAmy1) from the *X2* chromosome of *D. miranda* (AX1 construct), the *Amy1* gene is expressed, showing the same mobility as the *D. miranda* band (Figure 5). Two

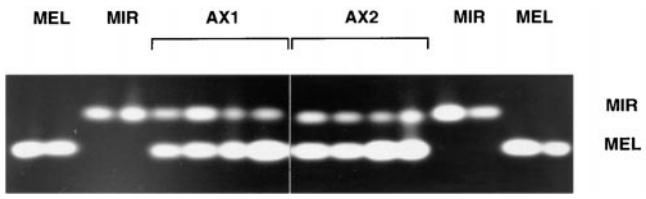

Figure 5.—Zymogram from transgenic *D. melanogaster* fly extracts. The protein extracts from three flies each, made homozygous for the *D. miranda* AX1 or AX2 constructs, are separated. For detection of the amylase activity see materials and methods. The *D. miranda* and *D. melanogaster* strains involved in cloning and transformation are used as markers. MEL, *D. melanogaster* OR *w sn*^{w}, the strain used for embryo injections; MIR, *D. miranda* MPI. In addition to the single band from the *D. melanogaster* OR strain (homozygous *Amy* locus) we obtain with all AX1 transformed lines an active AMY1 revealing the same mobility as the amylase in *D. miranda*. In five lines, the AX2 construct of the *Amy2* gene from *D. miranda* is not expressed (not shown). However, two exceptional lines show an active AMY2. The AMY2 reveals a faster mobility than the expressed amylase in *D. miranda*. Four samples from the same lines were applied, respectively. Because of variations in the applied fly extracts the intensity of the amylase bands varies from lane to lane.

of the lines show a reduced *D. miranda* amylase activity (not shown). In contrast, five transgenic lines transformed with the AX2 construct containing the *Amy2* region (dmirAmy2) from the *X2* of *D. miranda* indicate no *Amy2* gene expression (not shown). However, in two independent lines, the *Amy2* gene from *D. miranda* is expressed, but is characterized by a slightly different mobility than the amylase band in *D. miranda* (Figure 5). Therefore, only the *Amy1* gene of the resident *Amylase* cluster is expressed in *D. miranda* and hence the *Amy1* must be the dosage compensated gene.

***Neo-Y* chromosomal *Amy* locus:** From 35 analyzed clones, no *neo-Y*-chromosomal *Amy* clone was detected. In contrast to this finding, analyzing about the same amount of clones from the *Lcp* region, we detected several *neo-Y* chromosomal clones. *In situ* hybridization experiments showed no labeled site on the *neo-Y* using different clones from the *X2*-chromosomal *Amy* cluster. We thus wondered whether the *Amy* region is still present on the former homologous *neo-Y* chromosome, as we have found for the *Lcp1-4* cluster (Steinemann and Steinemann 1992; Steinemann *et al.* 1993). Long-range PCR, amplifying a fragment of about 9.9 kb from the *Amy* gene cluster in genomic male and female *D. miranda* DNAs, reveals a single band of the same size in both sexes, respectively (not shown). In Southern blots, equal amounts of restricted genomic male and female DNA from *D. miranda*, hybridized with the ^{32}P-labeled pOR-M7 probe from *D. melanogaster*, show two bands of the same size in males and females (Figure 6). Despite equally loaded lanes the signal strength in the male lanes is about half the intensity as in the female lanes. These results strongly suggest that the *Amy* cluster is deleted from the evolving *neo-Y* chromosome.

DISCUSSION

DNA sequences of *Amy1*, *Amy2*, and *Amy3* from *D. miranda*: In this article we present the complete DNA sequences of the small multigene family *Amy1*, *Amy2*, and *Amy3* from *D. miranda*. The described sequences

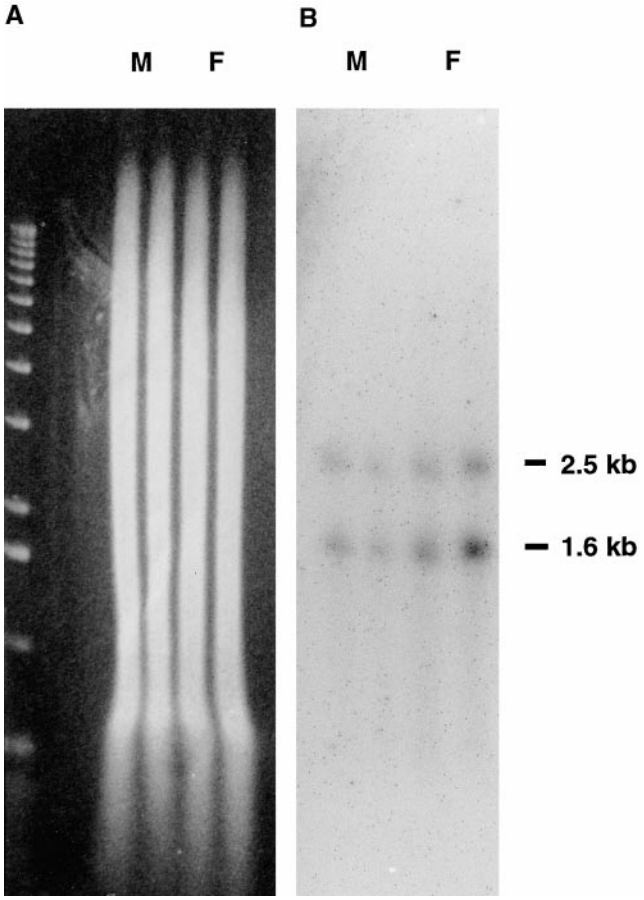

Figure 6.—Southern blot of *D. miranda* genomic male and female DNA probed with the pOR-M7. (A) *D. miranda* females carry two *X2* chromosomes, while males have an *X2* and *neo-Y* chromosome pair. Genomic male (M) and female (F) DNA was double-digested with *Eco*RI and *Bam*HI. Equal amounts, two lanes each, were applied and separated on a 0.7% agarose gel. As size marker the 1-kb ladder from BRL (Gaithersburg, MD) was used (left lane). (B) Southern blot from A, probed with the ^{32}P-labeled pOR-M7 from *D. melanogaster*. The genomic DNAs in the male (M) and female (F) lanes show the expected fragments of about 2.5 kb and 1.6 kb. Despite the applied equal amounts of DNA in the male and female lanes, the signal intensity is about half in males *vs.* females.

are cloned from the evolving *X2* chromosome. Database screens of the GenBank / EMBL gene bank revealed partial sequences from *D. miranda*. The reported sequence fragments of *D. miranda Amy2* (GenBank accession number U51236) and *Amy3* (GenBank accession number U51237) aligned with the sequences presented here appear to be a mixture of intermingled partial *Amy2* and *Amy3* sequences, respectively. The partial coding sequences of clone miranda1 (GenBank accession number AB003769) and clone miranda2 (GenBank accession number AB003770) seem to be sequences from *D. pseudoobscura* (intron length of 71 bp and base substitutions), and not from *D. miranda*. Alignment of the three *Amy* genes from *D. miranda* reveals a structurally intact *Amy1* gene and an *Amy2* gene with a complete coding sequence and a deletion in the 3′-region. *Amy1* and *Amy2* in *D. miranda* are arranged tail-to-tail and *Amy2* and *Amy3* head-to-tail, while *Amy-p* and *Amy-d* in *D. melanogaster* show a head-to-head orientation (Figure 7). Of interest is the observation that the short conserved flanking CAAT-box sequences (between CAAT- and TATA-box) of *Amy-d* and *Amy-p* show a more extended similarity to *Amy2* and *Amy3* than do those of *Amy1* (Figure 4A). The intergenic regions between *Amy-p* and *Amy-d* of *D. melanogaster* and *D. teissieri* contain open reading frames with sequence similarities to the *serpin* gene (Okuyama *et al.* 1997). Alignment of the *serpin* gene with the intergenic regions of *Amy1–Amy2* and *Amy2–Amy3* reveals no sequence similarity. Either the *serpin* gene is deleted from this position in *D. miranda* or it was inserted into *D. melanogaster* after the separation of the melanogaster and obscura groups.

The DNA sequence analysis reveals two large deletions within the *Amy3* gene, including the start of transcription and several hundred base pairs of coding sequences. Thus *Amy3* on the *X2* chromosome from *D. miranda* is structurally impaired and cannot be expressed. In *Amy2*, the 273-bp deletion starts 1 bp in front of the inferred poly(A) signal. Without the correctly positioned poly(A) signal, *Amy2* RNA might not be processed. On the other hand, further downstream sequences showing a poly(A) signal motif might be used (see below). In the coding region, there are 11 nucleotide differences among the *D. miranda Amy1* and *Amy2*; 6 represent synonymous substitutions and 5 nonsynonymous substitutions. The five replacement changes relative to the six silent ones could indicate an acceleration of replacement changes due to an inactive *Amy2* at its resident locus.

Expression and nonexpression of the resident *Amy* genes: The structural data provide no decisive answer as to whether in *D. miranda* the *Amy2* gene can produce a functional AMY2 enzyme or not. The *Amy3* gene cannot be expressed. Thus only the *Amy1* and *Amy2* genes are candidates as potentially active genes. In all tested germ-line transformed lines containing the *X2* chromosomal *Amy1* region from *D. miranda*, the *Amy1* gene is expressed with the same mobility as the amylase encoded by the resident *Amy* gene in *D. miranda*. In two lines carrying the AX1 construct, the *D. miranda Amy1* gene reveals low-level expression. It was shown by Bahn (1971b) that the *Amy* locus is sensitive to position-effect variegation (PEV). The reduced activities observed in these lines could be due to PEV effects induced from the ectopic sites of integration (*cf.* Henikoff and Matzke 1997). A more detailed analysis of these epigenetic effects has to be done. Five transgenic lines, containing the *Amy2* region, do not express the *Amy2* gene from the *X2* in *D. miranda*. However, in two independent lines, the *Amy2* gene is expressed. AMY2 reveals a slightly faster mobility compared with the AMY band in *D. mi-*

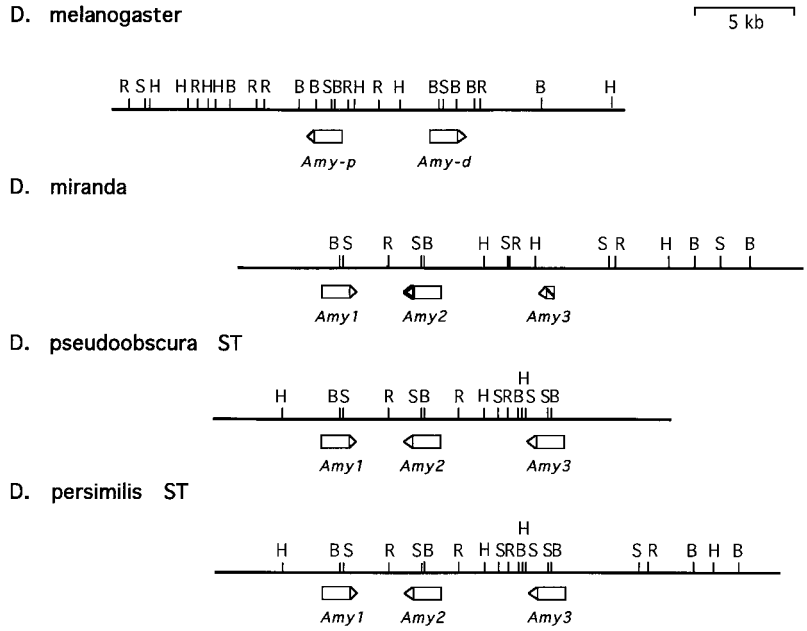

Figure 7.—Organization of the *Amy* gene cluster in *D. melanogaster*, *D. miranda*, *D. pseudoobscura* ST, and *D. persimilis* ST. Size and orientation of the *Amy* genes are indicated by rectangles and open arrows. The *D. melanogaster* arrangement is taken from Boer and Hickey (1986) and the *D. pseudoobscura* ST from Brown *et al.* (1990). The size and orientation of the *D. persimilis Amy* genes is deduced from restriction fine mapping. The *Amy3* gene of *D. miranda* (striped) is, because of two large deletions, structurally hampered. In the *Amy2* gene of *D. miranda* a deletion starts 1 bp in front of the poly(A) signal (bold open arrow) whereas all other parts of the gene are perfectly intact. Of interest is the different orientation of the *D. melanogaster Amy-p* and *Amy-d* genes with respect to the arrangement in *D. miranda* and the sibling species. For orientation only some of the mapped restriction sites are indicated: S, *Sal*I; H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI.

randa. In all transgenic *D. melanogaster* lines AMY1 shows the same mobility as in *D. miranda*; therefore posttranscriptional modifications, being responsible for the slight mobility difference of AMY2, could be excluded. The five nonsynonymous substitutions must be responsible for the slight mobility difference observed between AMY1 and AMY2. Thus, only the *Amy1* gene of the resident small multigene family is active in *D. miranda* and hence must be the dosage compensated gene. The reason for nonexpression of the *Amy2* gene at its resident position is unclear. Either it cannot make use of a further downstream poly(A) signal motif (see above), or the 3′-deletion has removed a necessary regulatory element. If it can make use of a further downstream poly(A) signal motif, the *Amy2* gene might be silenced by epigenetic effects at the resident *Amy* locus. The remaining two transgenic lines that do express the construct may be due to fortuitous positive effects compensating the putative missing regulatory element or due to chromatin effects at the ectopic integration sites.

Basic arrangement of *Amy* gene cluster in the pseudoobscura subgroup: The number of structurally intact *Amy* genes can vary between different *D. pseudoobscura* arrangements. In the *D. pseudoobscura* ST arrangement the structure of all three *Amy* genes is intact (Brown *et al.* 1990; Figure 7). The *Amy3* gene, however, has a stop codon at amino acid 157 that shortens the deduced protein to 31.6% of its normal length. In the *D. pseudoobscura* SC arrangement, *Amy1* and *Amy2* are structurally intact and *Amy3* has a large deletion including 5′-flanking and coding sequences. In the *D. pseudoobscura* TL arrangement, *Amy2* and *Amy3* show large deletions upstream from the start of transcription reaching into the coding region (Popadic *et al.* 1996). The basic arrangement of three *Amy* genes in the *pseudoobscura* group (Figure 7) seems to be prone to deletions in the *Amy3* and *Amy2* genes, while selection pressure keeps the *Amy1* gene structurally intact. For mechanisms (unequal crossing over and/or gene conversion) that may be homogenizing the gene family, the extended shared flanking regions of *Amy2* and *Amy3* could have some relevance for the apparent instability of the *Amy* locus in the pseudoobscura group.

Deletion of the *neo-Y Amy* gene cluster: *D. miranda* females carry two *X2* chromosomes, while males have an *X2* and a *neo-Y* chromosome. Detailed analysis of the *neo-Y* chromosomal *Lcp1-4* loci revealed a large tandem duplication that is several kilobases in length (Steinemann and Steinemann 1993), short deletions, a massive accumulation of transposable elements, and silencing of flanking *Lcp* genes (Steinemann and Steinemann 1992; Steinemann *et al.* 1993). The fate of the three *Amy* genes on the degenerating *neo-Y* is different from *Lcp1-4*. *In situ* hybridizations, long-range PCR, and Southern blot analysis of genomic male and female DNA strongly suggest that the *Amy* gene cluster has been deleted during the degeneration process in progress. This finding shows that a relatively new *neo-Y* chromosome can start to lose genes and hence gradually lose homology with the *neo-X*, even after about 2 million years of evolution. The molecular mechanism(s) generating the deletion is unclear. On the basis of the target site duplication associated with the 221-bp deletion we have detected in the *neo-Y* chromosomal *Lcp4*, we concluded that the null allele there is generated by insertion/excision mutagenesis (Steinemann and Steinemann 1992). This might be one possible mechanism responsible for the generation of the deletion of the *Amy* loci on the *neo-Y*. The mechanisms discussed in the context of *neo-Y* chromosome degeneration in *D. miranda*

could be relevant to studies of mammalian *Y* chromosome evolution. In the addition-attrition hypothesis, the original *X* and *Y* have been enlarged by cycles of autosomal addition to one partner, recombination onto the other, and continuing attrition of the compound *Y* (for review, see Graves 1995). Drosophila males have achiasmate meiosis. Because of the absence of recombination in *D. miranda* males we assume that the degeneration process of the *neo-Y* will be faster than the progressive degradation of the pseudoautosomal region in mammals. Plant sex chromosomes have evolved recently on a geological time scale. For example, *Silene latifolia*, a dioecious plant, shows a heteromorphic sex chromosome pair. The *X*-linked *MROS3* gene has a homologue in the nonpairing region of the *Y* chromosome that has degenerated as a result of nucleotide deletion and accumulation of repetitive sequences (Guttman and Charlesworth 1998). These findings corroborate the described mechanisms for *Y* chromosome degeneration in *D. miranda* (Steinemann and Steinemann 1992; Steinemann *et al.* 1993). Investigating the enigma of *Y* chromosome degeneration we could demonstrate in our *neo-Y*/*neo-X D. miranda* model system, apart from point mutations, three mechanistic principles involved in *Y* chromosome degeneration: (1) accumulation of transposable elements and silencing of flanking resident genes, (2) tandem duplications, and (3) deletions of loci. This greatly strengthens the classic argument that true *Y* chromosomes have evolved from ancestors that were originally homologous to the *X* by a process of gradual erosion.

We thank Donal A. Hickey for the pOR-M7 clone, U. Schibler for the pMSa104 clone, C. Kraemer for support with the long-range PCR, and Mrs. K. Wildhagen for preparing the photomicrographs. We thank the anonymous reviewers for constructive criticism of this article. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Ste 266/4-1.

LITERATURE CITED

Altschul, S., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

Bahn, E., 1971a Cytogenetical localization of the *Amylase* region in *Drosophila melanogaster* by means of translocations. Hereditas **67:** 75–78.

Bahn, E., 1971b Position-effect variegation for an isoamylase in *Drosophila melanogaster*. Hereditas **67:** 79–82.

Barrio, E., A. Latorre, A. Moya and F. J. Ayala, 1992 Phylogenetic reconstruction of the *Drosophila obscura* group, on the basis of mitochondrial DNA. Mol. Biol. Evol. **9:** 621–635.

Benkel, B. F., S. Abukashawa, P. H. Boer and D. A. Hickey, 1987 Molecular cloning of DNA complementary to *Drosophila melanogaster* α-amylase mRNA. Genome **29:** 510–515.

Boer, P. H., and D. A. Hickey, 1986 The alpha-amylase gene in *Drosophila melanogaster*: nucleotide sequence, gene structure and expression motifs. Nucleic Acids Res. **14:** 8399–8411.

Bone, R. J., and M. I. Kuroda, 1996 Dosage compensation regulatory proteins and the evolution of sex chromosomes in *Drosophila*. Genetics **144:** 705–713.

Brown, C. J., C. F. Aquadro and W. W. Anderson, 1990 DNA sequence evolution of the amylase multigene family in *Drosophila pseudoobscura*. Genetics **126:** 131–138.

Charlesworth, B., D. Charlesworth, J. Hnilicka, A. Yu and D. S. Guttman, 1997 Lack of degeneration of loci on the *neo-Y* chromosome of *Drosophila americana americana*. Genetics **145:** 989–1002.

Da Lage, J.-L., M. Wegnez and M.-L. Cariou, 1996 Distribution and evolution of introns in *Drosophila* Amylase genes. J. Mol. Evol. **43:** 334–347.

Da Lage, J.-L., E. Renard, F. Chartois, F. Lemeunier and M.-L. Cariou, 1998 *Amyrel*, a paralogous gene of the amylase gene family in *Drosophila melanogaster* and *Sophophora* subgenus. Proc. Natl. Acad. Sci. USA **95:** 6848–6853.

Das, M., D. Mutsuddi, A. K. Duttagupta and A. S. Mukherjee, 1982 Segmental heterogeneity in replication and transcription of the *X2* chromosome of *Drosophila miranda* and conservativeness in the evolution of dosage compensation. Chromosoma **87:** 373–388.

Davis, B. J., 1964 Disc electrophoresis-II: method and application to human serum proteins. Ann. NY Acad. Sci. **121:** 404–427.

Devereux, J., P. Haeberli and O. Smithies, 1984 A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:** 387–395.

Dobzhansky, Th., 1935 *Drosophila miranda*, a new species. Genetics **20:** 377–391.

Gemmill, R. M., J. N. Levy and W. W. Doane, 1985 Molecular cloning of α-amylase genes from *Drosophila melanogaster*. I. Clone isolation by use of a mouse probe. Genetics **110:** 299–312.

Graves, J. A. M., 1995 The origin and function of the mammalian Y chromosome and Y-borne genes—an evolving understanding. Bioessays **17:** 311–320.

Guttman, D. S., and D. Charlesworth, 1998 An X-linked gene with a degenerate Y-linked homologue in a dioecious plant. Nature **393:** 263–266.

Henikoff, S., and M. A. Matzke, 1997 Exploring and explaining epigenetic effects. Trends Genet. **13:** 293–295.

Jukes, T. H., and C. R. Cantor, 1969 Evolution of protein molecules, pp. 21–132 in *Mammalian Protein Metabolism*, Vol. 3, edited by H. N. Munro. Academic Press, New York.

Kastritsis, C. D., and D. W. Crumpacker, 1966 Gene arrangements in the third chromosome of *Drosophila pseudoobscura*. J. Hered. **57:** 151–158.

Kraemer, C., and E. R. Schmidt, 1993 The sex-determining region of *Chironomus thummi* is associated with highly repetitive DNA and transposable elements. Chromosoma **102:** 553–562.

Lindsley, D. L., and G. G. Zimm, 1992 *The Genome of Drosophila melanogaster*. Academic Press, San Diego.

Lucchesi, J. C., 1994 The evolution of heteromorphic sex chromosomes. Bioessays **16:** 81–83.

MacKnight, R. H., 1939 The sex-determining mechanism of *Drosophila miranda*. Genetics **24:** 180–201.

Marin, I., A. Franke, G. J. Bashaw and B. S. Baker, 1996 The dosage compensation system of *Drosophila* is co-opted by newly evolved *X* chromosomes. Nature **383:** 160–163.

Moore, B. C., and C. E. Taylor, 1986 *Drosophila* of Southern California. III. Gene arrangements of *Drosophila persimilis*. J. Hered. **77:** 313–323.

Norman, R. A., and W. W. Doane, 1990 Dosage compensation and dietary glucose repression of larval amylase activity in *Drosophila miranda*. Biochem. Genet. **28:** 601–613.

Norman, R. A., and S. Prakash, 1980 Variation in activities of amylase allozymes associated with chromosome inversions in *Drosophila pseudoobscura*, *D. persimilis* and *D. miranda*. Genetics **95:** 187–209.

Norman, R. A., H. M. Dunstan and W. W. Doane, 1991 Amylase gene family homologies in *D. melanogaster* and *D. miranda*. Dros. Inf. Serv. **70:** 157–160.

Okuyama, E., H. Tachida and T. Yamazaki, 1997 Molecular analysis of the intergenic region of the duplicated *Amy* genes of *Drosophila melanogaster* and *Drosophila teissieri*. J. Mol. Evol. **45:** 32–42.

Pirrotta, V., 1988 A survey of molecular cloning vectors and their uses, pp. 437–456 in *Vectors*, edited by R. L. Rodrigues and D. T. Denhardt. Butterworth-Heinemann, Boston.

Popadic, A., and W. W. Anderson, 1995 Evidence for gene conversion in the amylase multigene family of *Drosophila pseudoobscura*. Mol. Biol. Evol. **12:** 564–572.

Popadic, A., R. A. Norman, W. W. Doanet and W. W. Anderson, 1996 The evolutionary history of the Amylase multigene family in *Drosophila pseudoobscura*. Mol. Biol. Evol. **13:** 883–888.

Rice, W. R., 1996 Evolution of the Y sex chromosome in animals. Bioscience **46:** 331–343.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schibler, U., M. Tosi, A.-C. Pittet, L. Fabiani and P. K. Wellauer, 1980 Tissue-specific expression of mouse α-Amylase genes. J. Mol. Biol. **142:** 93–116.

Schibler, U., A.-C. Pittet, R. A. Young, O. Hagenbüchle, M. Tosi *et al.*, 1982 The mouse α-Amylase multigene family: sequence organization of members expressed in the pancreas, salivary gland and liver. J. Mol. Biol. **155:** 247–266.

Shibata, H., and T. Yamazaki, 1995 Molecular evolution of the duplicated *Amy* locus in the *Drosophila melanogaster* species subgroup: concerted evolution only in the coding region and an excess of nonsynonymous substitutions in speciation. Genetics **141:** 223–236.

Steinemann, M., 1982 Multiple sex chromosomes in *Drosophila miranda*: a system to study the degeneration of a chromosome. Chromosoma **86:** 59–76.

Steinemann, M., and S. Steinemann, 1990 Evolutionary changes in the organization of the major LCP gene cluster during sex chromosomal differentiation in the sibling species *Drosophila persimilis*, *D. pseudoobscura* and *D. miranda*. Chromosoma **99:** 424–431.

Steinemann, M., and S. Steinemann, 1992 Degenerating Y chromosome of *Drosophila miranda*: a trap for retrotransposons. Proc. Natl. Acad. Sci. USA **89:** 7591–7595.

Steinemann, M., and S. Steinemann, 1993 A duplication including the *Y* allele of *Lcp2* and the *TRIM* retrotransposon at the *Lcp* locus on the degenerating *neo-Y* chromosome of *Drosophila miranda*: molecular structure and mechanisms by which it may have arisen. Genetics **134:** 497–505.

Steinemann, M., and S. Steinemann, 1998 Enigma of *Y* chromosome degeneration: *neo-Y* and *neo-X* chromosomes of *Drosophila miranda* a model for sex chromosome evolution. Genetica **102/103:** 409–420.

Steinemann, M., S. Steinemann and J. Fakler, 1986 Differential expression and dosage compensation of the α-amylase gene in *Drosophila miranda*. Experientia **42:** 577–579.

Steinemann, M., S. Steinemann and F. Lottspeich, 1993 How Y chromosomes become genetically inert. Proc. Natl. Acad. Sci. USA **90:** 5737–5741.

Steinemann, M., S. Steinemann and B. M. Turner, 1996 Evolution of dosage compensation. Chromosome Res. **4:** 185–190.

Strobel, E., C. Pelling and N. Arnheim, 1978 Incomplete dosage compensation in an evolving Drosophila sex chromosome. Proc. Natl. Acad. Sci. USA **75:** 931–935.

Sturtevant, A. H., and Th. Dobzhansky, 1936 Inversions in the third chromosome of wild races of *Drosophila pseudoobsura*, and their use in the study of the history of the species. Proc. Natl. Acad. Sci. USA **22:** 448–450.

Swofford, D. L., 1993 PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Illinois Natural History Survey, Champaign.

Throckmorton, L. H., 1982 The *virilis* species group, pp. 227–296 in *The Genetics and Biology of Drosophila*, edited by M. Ashburner, H. L. Carson and J. N. Thomson. Academic Press, London.

Traut, W., 1994 Sex determination in the fly *Megaselia scalaris*, a model system for primary steps of sex chromosome evolution. Genetics **136:** 1097–1104.

White, M. J. D., 1973 Sex chromosomes, pp. 129–146 in *The Chromosomes*, Ed. 6. Chapman and Hall, London.

Communicating editor: S. Henikoff