PGal4 excision reveals the pleiotropic effects of *Voila*, a *Drosophila* locus that affects development and courtship behaviour

**Yael Grosjean**, **Maria Balakireva**, **Laurence Dartevelle** and **Jean-Francois Ferveur**

1 Développement et Communication Chimique, CNRS-UMR 5548, Faculté des Sciences, Université de Bourgogne, 6, Bd Gabriel, 21000 Dijon, France
2 Centre des Sciences du Goût, 21000 Dijon, France

(Received 28 August 2000 and in revised form 27 November 2000)

**Summary**

In *Drosophila melanogaster*, the PGal4 transposon inserted at the chromosomal site 86E1.2 is associated with the *Voila* allele that causes multiple phenotypes. Homozygous *Voila* flies rarely reach adulthood and heterozygous *Voila* adult males display strong homosexual courtship behaviour. Both normal behavioural and developmental phenotypes were rescued by remobilizing the PGal4 element. Yet, the rescue of heterosexual courtship and of adult viability did not occur in the same strains, indicating that these defects have different genetic origins. Furthermore, many strains showed a partial rescue of both characters. Molecular analysis revealed that the PGal4 transposon is inserted upstream of the 5′UTR of the *prospero* gene. The excision strains with no detectable fragment of the PGal4 transposon remaining showed a rescued viability for homozygote adults. Moreover, the developmental period with the highest homozygote lethality was correlated with the size of PGal4 element that remained inserted at the *Voila* locus. This suggests a relationship between developmental viability and the amount of DNA inserted within the promoter of *prospero*.

**1. Introduction**

Mutations often cause pleiotropic abnormal phenotypes and one challenge of biology is to understand at what level (molecular, genetic, behavioural) the different functions of a given gene are intertwined (Hall, 1994a). Behavioural phenotypes are complex, often involving integration of many biological functions in the organism, and can thus reveal subtle alterations of developmental functions (Greenspan, 1995).

The study of courtship behaviour in *Drosophila* mutants is particularly useful for detecting subtle alterations of the ‘fixed action patterns’ that constitute the complex courtship behaviour and which are thought to be genetically controlled (Hall, 1994b; Goodwin, 1999). Before mating, the male exhibits a series of stereotypical sequences that depend upon the integrity of his nervous system, particularly on sensory systems (Sturtevant, 1915; Hall, 1977, 1979; Markow, 1987). However, *Drosophila* courtship is more complex than a stereotyped set of behavioural events (Greenspan & Ferveur, 2000). There is an active and reciprocal exchange of multi-sensory signals between the two sexual partners (Cobb & Ferveur, 1996) and the intensity of male courtship has been shown to be dependent upon the genotype of both partners (Ferveur & Sureau, 1996; Sureau & Ferveur, 1999). The genetic and neural bases of male courtship ritual have been intensively explored, but with the notable exception of fruitless (*fru*) very few studies were conducted on an extended series of mutant alleles affecting the same locus (Gailey & Hall, 1989; Taylor et al., 1994; Ito et al., 1996; Ryner et al., 1996; Villella & Hall, 1996; Villella et al., 1997), or with different protein motifs encoded by a single gene (Stanewsky et al., 1996).

A large choice of molecular and genetic tools in *Drosophila melanogaster* makes it possible to start unravelling the pleiotropic effects of mutant genes. The GAL4/UAS technique combines the ability to

* Corresponding author. Tel. +33 (0) 3 80 39 37 82 (office), +33 (0) 3 80 39 62 19 (lab). Fax: +33 (0) 3 80 39 62 89.
  e-mail: jean-francois.ferveur@u-bourgogne.fr

Downloaded from https://www.cambridge.org/core. IP address: 207.241.231.82, on 01 May 2019 at 20:28:10, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
https://doi.org/10.1017/S0016672301005006
The P element transposase (Robertson et al., 1988). Male progenies carrying the Voila\textsuperscript{1}-PGal4 transposon and the transposase-producing Δ2-3 chromosome were crossed to w\textsuperscript{+}/TM3 females. In order to obtain flies without the P transposon, males of the next generation were scored for the loss of the mini\textsuperscript{white}\textsuperscript{+} gene. Each Voila excision line derived from Voila\textsuperscript{1} resulted from a unique excision event on chromosome 3 that was subsequently maintained over a TM3 balancer chromosome.

Complementation analysis of Voila\textsuperscript{1} mutation was carried out with deficiencies covering the chromosomal region between 86C and 87C (for information about their breakpoints and origin, see Reuter et al., 1987). The restriction map of the Voila locus together with the site of PGal4 insertion is shown on Fig. 1. The lethality induced by two copies of Voila\textsuperscript{1} or Voila\textsuperscript{exc} was studied with a strain carrying the dominant adult marker Sh.

(ii) Behavioural assays

Courtship tests were done using a modified version of the protocol described in Ferveur & Sureau (1996). Briefly, all behavioural assays were carried out on 4-day-old subject males (kept alone after eclosion),
(iii) Developmental lethality

A procedure similar to Balakireva et al. (2000) was followed. Eggs were collected over 24 h and placed in vials at 25 °C. Between 35 and 40 h after the end of egg laying, the number of dead embryos was counted. Adults emerging from the pupal case were counted according to their genotype (nA), and the frequency of adult survival was estimated relative to the number of surviving embryos (nA/nE). The frequency of lethality during pupal life was also estimated (nP/nE). The occurrence of lethality during larval stages was thus the difference between the number of hatching embryos minus the number of individuals that reached (and died during) pupation and adulthood (nL = nE−[nP+nA]). The strains carrying the TM3 balancer chromosome are expected to yield an average of 25% dead embryos and our measure was weighted accordingly. We directly monitored the lethality of each genotype during adulthood on 2- and 7-day-old flies.

(iv) Statistical tests

For courtship tests, Clms (courtship index toward decapitated male object) of all Voila exc, of Voila1 and control w− strains were compared using ANOVA. Voila exc lines were then grouped according to the difference between their Clm value and the Clm of mutant and of control males. Within each group, strains were ranked according to the level of significance of these differences (see Table 1).

(v) Molecular techniques

Genomic DNA was isolated from whole flies (3- or 4-day-old males and virgin females) and homogenized in 100 mM NaCl, 100 mM Tris pH 7.6, 100 mM EDTA, 0.5% SDS. Purification was done according to Sambrook et al. (1989), using the DNA from approximately seven flies per lane.

The PGawB vector (Brand & Perrimon, 1993) was used to generate probes that were used to map the different fragments of the vector. They correspond to the following restriction fragments of the PGawB vector (Fig. 1): a 2.6 kb HindIII–XhoI DNA fragment corresponding approximately to ‘Gal4’, a 5 kb HindIII–XhoI DNA fragment corresponding to ‘mini-white’, and a 2.9 kb BamHI–PstI DNA fragment corresponding to ‘pBSK’. Fragments were then 32P-labelled by random priming with the Prime-a-gene kit (Promega).

Genomic DNA was digested with XhoI and HindIII. Electrophoresis and Southern blot analysis were performed according to Sambrook et al. (1989). Seven oligonucleotide primers belonging either to the genomic excision site flanking regions (a: CACACACACACACACACACATTTAT; a’: CTGCTTTTCTTCTGGCAATACC; b: ACTACAAACACAGCAGCAAGGAC; b’: GAACGACGTTCACTTACTGTTC; c: CAAAACGTCGTTCAGCCTC) or internal to the transposon (b’: AAGACAGTAGCTTCTAATTCGGA; c: CTAGCTAGAAGCTTGGCTACTCG; see Fig. 1) were used for PCR reactions in order to produce DNA the sequence of which was eventually determined (MWG-Biotech, Germany).

3. Results

(i) The PGal4 transposon causes the developmental lethality in Voila1 strain

To verify the involvement of the PGal4 transposon in the developmental lethality observed in the Voila1 strain, we induced the remobilization of this transposable element by crossing the Voila1 strain with a strain containing the transposase enzyme (see Section 2). This procedure yielded 61 stable excision lines (Voila exc). All Voila exc lines were tested for the presence and viability of homozygous Voila exc exc adults (Table 1, second column). Thirty-four lines produced viable and fertile homozygous flies (VB lines = adult viable) and 27 lines had no viable fertile adult homozygotes (LT lines = adult lethal). Therefore, the fact that adult viability was rescued following remobilization of the PGal4 element confirms that the transposon caused the developmental lethality of Voila1/1 individuals (Balakireva et al., 2000).

(ii) The developmental pattern of lethality varies between Voila exc strains

The developmental pattern of lethality was analysed in 18 strains belonging to both viability groups (11 Voila excUT and 7 Voila exc). For each Voila exc strain, the frequency of homozygote lethality was measured either indirectly (by estimating survivorship at the end...
Table 1. Viability of homozygotes and courtship behaviour of heterozygous males from various strains

| Strains   | Homozygote viability | CIm Mean ± SE | Difference in CIm compared with |
|-----------|----------------------|---------------|---------------------------------|
|           |                      |               | Voila1/TM3                      | w; +/TM3 |
| Mutant    | Voila1/TM3           | LT            | 42 ± 4.8                        |          |
| Control   | w; +/TM3             | VB            | 7 ± 2.3                         |          |
| Rescue group | Voila1/ex/TM3   |                |                                 |          |
| 2 (+)     | LT                   | 13 ± 4.2      | ***                             | NS       |
| 4         | LT                   | 18 ± 4.6      | ***                             | NS       |
| 5         | VB                   | 16 ± 5.4      | ***                             | NS       |
| 6         | VB                   | 18 ± 4.6      | ***                             | NS       |
| 7         | VB                   | 17 ± 3.8      | ***                             | NS       |
| 8 (+)     | LT                   | 18 ± 4.1      | ***                             | NS       |
| 9         | VB                   | 18 ± 4.1      | ***                             | NS       |
| 11 (+)    | LT                   | 8 ± 1.6       | ***                             | NS       |
| 13 (+)    | LT                   | 12 ± 2.4      | ***                             | NS       |
| 14 (+)    | VB                   | 21 ± 4.6      | ***                             | NS       |
| 15        | VB                   | 14 ± 4.4      | ***                             | NS       |
| 16        | VB                   | 17 ± 3.5      | ***                             | NS       |
| 18        | LT                   | 18 ± 3.8      | ***                             | NS       |
| 20        | VB                   | 20 ± 4.8      | ***                             | NS       |
| 23        | VB                   | 11 ± 2.5      | ***                             | NS       |
| 30        | VB                   | 11 ± 3.1      | ***                             | NS       |
| 37        | VB                   | 16 ± 3.8      | ***                             | NS       |
| 38        | LT                   | 21 ± 3.7      | ***                             | NS       |
| 43        | LT                   | 12 ± 3        | ***                             | NS       |
| 50        | LT                   | 16 ± 5.8      | ***                             | NS       |
| 52        | VB                   | 21 ± 3.8      | ***                             | NS       |
| 57        | VB                   | 11 ± 4.4      | ***                             | NS       |
| 59        | VB                   | 15 ± 5        | ***                             | NS       |
| 62        | VB                   | 10 ± 2.5      | ***                             | NS       |
| 68        | VB                   | 21 ± 4        | ***                             | NS       |
| 69 (+)    | LT                   | 20 ± 3.8      | ***                             | NS       |
| 70        | VB                   | 11 ± 3.3      | ***                             | NS       |
| 75        | LT                   | 14 ± 4.8      | ***                             | NS       |
| 19        | LT                   | 22 ± 4.4      | **                              | NS       |
| 24 (+)    | LT                   | 19 ± 4.8      | **                              | NS       |
| 29        | LT                   | 18 ± 4.6      | **                              | NS       |
| 39        | LT                   | 18 ± 7        | **                              | NS       |
| 41        | VB                   | 21 ± 5        | **                              | NS       |
| 54        | VB                   | 18 ± 7        | **                              | NS       |
| 64 (+)    | VB                   | 22 ± 3.8      | **                              | NS       |
| 21        | LT                   | 20 ± 7        | *                               | NS       |
| 25        | LT                   | 20 ± 6.6      | *                               | NS       |
| 31        | LT                   | 23 ± 4.7      | *                               | NS       |
| 45 (+)    | VB                   | 22 ± 3.5      | *                               | NS       |
| 58        | VB                   | 18 ± 6.5      | *                               | NS       |
| 60 (+)    | VB                   | 21 ± 6.3      | *                               | NS       |
| Intermediate group | |                |                                |          |
| 61 (+)    | LT                   | 23 ± 4.2      | **                              |          |
| 79 (+)    | LT                   | 28 ± 4.2      | *                               | **       |
| 3 (+)     | LT                   | 24 ± 4.3      | *                               | **       |
| 10        | VB                   | 27 ± 1.5      | *                               |           |
| 17 (+)    | LT                   | 25 ± 4.8      | *                               |           |
| 26 (+)    | VB                   | 27 ± 4.8      | *                               |           |
| 28 (+)    | VB                   | 24 ± 3.8      | *                               |           |
| 36        | VB                   | 25 ± 3.8      | *                               |           |
| 48        | VB                   | 24 ± 4.4      | *                               |           |
| 49        | VB                   | 23 ± 5.9      | *                               |           |
| 65 (+)    | VB                   | 25 ± 6.1      | *                               |           |
| 66        | LT                   | 27 ± 4.8      | *                               |           |
of embryonic, larval and pupal stages) or directly (in 2- and 7-day old adults).

Homozygous individuals from these Voila<sup>exc</sup> strains showed very different patterns of lethality during their pre- and post-metamorphic phases of development. As expected, homozygotes from all selected Voila<sup>VH</sup> lines (Fig. 2C) very frequently survived to the ‘2-day-old’ adult stage whereas most, if not all, homozygotes from the selected Voila<sup>LX</sup> lines died before reaching this stage (Fig. 2A, B). The peak of homozygote lethality shown by these Voila<sup>LX</sup> strains occurred at different developmental periods: Voila<sup>28</sup>, Voila<sup>8</sup> and Voila<sup>69</sup> died mostly during larval development (very similarly to Voila<sup>11/11</sup>). Voila<sup>23/24</sup> individuals died mostly during pupal development whereas lethality of Voila<sup>2/2</sup> and Voila<sup>27/27</sup> occurred constantly throughout most developmental stages from the larva to the 7-day-old adult. Homozygotes from Voila<sup>5</sup>, Voila<sup>12</sup> and Voila<sup>79</sup> lines, and to a lesser extent from Voila<sup>16</sup> and Voila<sup>41</sup> lines, showed high mortality during the first 2 days of adult life. Homozygous adult males in these lines were not fertile, probably due to their behavioural weakness during their brief adult life. However, homozygous females were fertile (e.g. Voila<sup>5</sup> and Voila<sup>41</sup> lines).

(iii) Relation between the pattern of lethality and the size of the inserted material at the Voila locus

First, the site of P Gal4 insertion was precisely mapped using primers corresponding to the 5′ pros coding region and to the pBSK fragment of the transposon (Vaessin et al., 1991; Brand & Perrimon, 1993). The insertion site was located at 216 bp from the coding region, upstream of the 5′ UTR (untranslated region) of the pros gene (Fig. 1). Furthermore, our sequence (data not shown) matches exactly the published pros sequence (Vaessin et al., 1991).

It was crucial to check that no genomic alterations occurred in the regions flanking the P Gal4 insertion point. Therefore, we used PCR to isolate and amplify the DNA covering 2 kb in the 5′ region and 1 kb in the 3′ region flanking the transposon. Primers were designed from the sequence of each genomic region as well as the transposon (Fig. 1). Resulting amplified fragments were compared with the respective fragments of the control strain. No alterations were found in either region for any of the 18 Voila<sup>exc</sup> strains (data not shown), indicating that the developmental defect does not depend upon the DNA surrounding the transposon but is a direct effect of the transposon.

Therefore, to evaluate whether the different profiles of homozygote lethality were related to genetic and/or molecular differences caused by imprecise excision, each Voila<sup>exc</sup> strain was analysed for the presence and size of an unexcised transposon sequence. Restricted DNA from flies of the 18 Voila<sup>exc</sup> strains was probed with the three principal genetic sequences (Gal4, mini-white and pBSK) carried by the P Gal4 transposon (Fig. 1; see Section 2). The size of each detected fragment was compared with that of the equivalent fragment probed in the Voila<sup>1</sup> control strain (Fig. 3).

Our data suggest that Gal4 is still present in many Voila<sup>exc</sup> strains (2, 8, 11, 13, 24, 27, 69, 78 and 79). However, in strains 13 and 69, Gal4 seems to be

---

Table 1 (Cont.)

| Strains | Homozygote Viability | CIm Mean ± SE | Voila<sup>1</sup>/TM3 (mutant) | w; +/TM3 (control) |
|---------|----------------------|---------------|-------------------------------|-------------------|
| Mutant group | LT | 35 ± 5 | NS | *** |
| 27 (+) | LT | 35 ± 5 | NS | *** |
| 35 | LT | 35 ± 5 | NS | *** |
| 56 | LT | 35 ± 5 | NS | *** |
| 78 (+) | LT | 35 ± 5 | NS | *** |
| 51 | LT | 35 ± 5 | NS | *** |
| 67 | LT | 35 ± 5 | NS | *** |
| 12 | LT | 35 ± 5 | NS | *** |
| 63 | LT | 35 ± 5 | NS | *** |

The viability (VB) or the lethality (LT) of homozygous adults belonging to 61 Voila<sup>exc</sup> strains, to mutant Voila<sup>1</sup> and to control w; Cs strains is shown in the first column. The strains that were tested in detail for their developmental viability are indicated (+). All subject males tested for courtship behaviour were heterozygous and carried a similar TM3 balancer chromosome. The intensity of courtship (= courtship index of male subjects) was measured towards decapitated Canton-S male objects (= CIm). Courtship index is the percentage of time spent courting by the subject male during a 10 min observation period. Data shown are the mean ± SEM for n = 15–25. The CIm of all Voila<sup>exc</sup>/TM3, Voila<sup>1</sup>/TM3 and w; +/TM3 control males was tested with an ANOVA. The level of significance is indicated as follow: *** P < 0.001; ** P < 0.01; * P < 0.05; NS, not significant. Strains were grouped according to their effect (Rescue, Intermediate and Mutant). The courtship index with decapitated control females (CIf) was 74 ± 5 for mutant Voila<sup>1</sup>/TM3 males and 46 ± 6.5 for control w; Cs males (n = 20).
altered compared with Voila¹ (2-6 kb). The mini-white fragment was detected only in strains 3, 8 and 78 and its size seemed to be altered in all three strains. This result was expected because excision events were selected on the basis of the loss of white¹ function. Thus, the mini-white fragments that remain inserted in strains 3, 8 and 78 are likely to be non-functional. The pBSK fragment seems to be intact in strains 2, 3, 8, 24, 27, 61, 69 and 78, and has probably been altered in strain 79 (Fig. 3C).

It is possible that the differences in lethality among Voila²⁴⁴ strains are caused by size differences of the fragments that remained inserted at the chromosomal site 86E¹-². In fact, there is a relationship between the size of the remaining fragment and the developmental stage of lethality in homozygous individuals (Fig. 4). The Voila²⁴⁴ strains with larval lethality (as in Voila¹¹/¹) were those that generally retained the largest fragments (generally including most or all of the Gal4 and pBSK sequences). In addition, Voila⁷⁸ and Voila⁸ still carried...
Genetic dissection of Voila

The genomic material of the PGal4 transposon has been differently excised after remobilization in various Voila exc strains. Southern blots were performed on digested DNA from the Voila exc strains previously assayed for homozygote viability (see Fig. 2). Probes consisted of fragments of either Gal4 (A), mini-white (B) or pBSK (C) radioactively labelled (see Section 2). Because of the high number of strains to be tested, each series was divided over three separate gels, each one including DNA from the mutant Voila' (Voi), used here as a control. Arrows indicate the expected size for each fragment in Voila flies (HindIII + Xhol).

a portion of the mini-white sequence. The Voila27, Voila13 and Voila2 strains showed massive homozygous lethality during pupal and/or early imaginal stages. These three strains carried a smaller fragment of the transposon from which the mini-white sequence was completely excised. The size of the fragment detected in these three strains seems to be very close to that of the Voila 69 strain that displayed high larval lethality.

Homzygotes from the other Voila17 strains (Voila8, Voila11, Voila13, Voila64 and Voila79) died more frequently during early adulthood (Fig. 2B). Their inserted fragments are smaller than in Voila and in the previously described Voila17 strains (Figs. 3, 4), consisting of either an altered Gal4 sequence (in Voila11 and Voila13), a complete Gal4 sequence with an altered pBSK sequence (in Voila79), or a complete pBSK sequence (alone in Voila64, or combined with an incomplete mini-white sequence in Voila8).

(iv) The reproductive ability of homozygous adults varies between VoilaB strains

The seven strains with rescued homozygous adult viability (VoilaB) were also tested by Southern blot to check for the presence of the three fragments composing the PGal4 element (Fig. 3). None of these strains retained any detectable part of the three fragments, indicating that the complete rescue of homozygous adult viability correlates well with the absence of inserted material. However, in four VoilaB strains (26, 28, 60 and 65) we found a clear difference in the frequency of homozygous adults. In these strains, we first noted that after more than 50 generations the frequency of homozygous adult flies stabilized at around 30–35%. However, the fact that crosses of homozygotes within these strains yielded viable adult progeny suggests that homozygous flies were selectively disadvantaged in the presence of Voila exc TM3 heterozygotes. The fact that the three other VoilaB strains (14, 45 and 64) produced only homozygous adults after several generations indicates that the TM3 balancer has been eliminated. Overall, these observations suggest that the excised chromosome III provides a reproductive advantage over the TM3 balancer chromosome in the three later VoilaB strains, but not in the former ones. DNA sequencing in the region surrounding the PGal4 insertion point revealed that the four strains with disadvantaged homozygotes retained a small sequence of roughly 100 bp that remained inserted and which corresponds to the inverse terminal repeats of the P element (data...
The PGal4 transposon causes abnormal male courtship in Voila

As expected, Voila\textsuperscript{1}/TM3 males showed abnormal courtship behaviour because they actively courted both mature virgin females and males. First, we wanted to know whether the abnormal male courtship behaviour observed in Voila\textsuperscript{1}/TM3 male was caused by the PGal4 element. For the sake of clarity, the courtship index of Voila\textsuperscript{exc}/TM3 males from the 61 Voila\textsuperscript{exc} lines was measured in the presence of decapitated control males. We focused our attention on the intensity of the homosexual courtship index (Clm) because the relative increase in Clm between control and mutant males was much greater (> 500\%) than the relative increase in the courtship index towards control female flies (+ 61\%; see legend of Table 1). The Clm of Voila\textsuperscript{exc}/TM3 males from 61 Voila\textsuperscript{exc} lines towards decapitated control male target flies was noted (Table 1). The Clms of heterozygous Voila\textsuperscript{exc}/TM3 males of all Voila\textsuperscript{exc} lines were compared with the Clm of Voila\textsuperscript{d}/TM3 mutant subject males, and with the Clm of w;+/TM3 control subject males (Table 1).

In 41 Voila\textsuperscript{exc} lines males showed no significant difference in their Clm when compared with w;+/TM3 control males, indicating that the intensity of their homosexual courtship was normal (‘Rescue group’). The rescue of normal (= virtually absent) homosexual male courtship indicates that the PGal4 transposon inserted in the Voila\textsuperscript{1} strain caused the high intensity of homosexual courtship. However, the fact that the Clm yielded by male flies of these strains showed different levels of significance when compared with the Clm of Voila\textsuperscript{d}/TM3 mutant males indicates that the intensity of courtship varies.

Study of the Clm produced by males from the other 20 Voila\textsuperscript{exc} lines supports the hypothesis that the remobilization of PGal4 has not rescued normal male homosexual courtship in an ‘all-or-none’ manner (e.g. causing the absence or the high level of homosexual courtship). In 12 Voila\textsuperscript{exc} lines, the rescue of normal male homosexual courtship was partial, because Clm was significantly different from the indices produced by both control and mutant male flies (‘Intermediate group’). In eight other Voila\textsuperscript{exc} lines, the Clm values were not different from the Clm of Voila\textsuperscript{d}/TM3 males (‘Mutant group’). The variation in the level of significance observed between these mutant strains also indicates that some of these strains (12, 51, 63 and 67) have a weaker Clm than Voila\textsuperscript{d}/TM3 mutant males.

(v) The PGal4 transposon causes abnormal male courtship in Voila

As expected, Voila\textsuperscript{1}/TM3 males showed abnormal courtship behaviour because they actively courted both mature virgin females and males. First, we wanted to know whether the abnormal male courtship behaviour observed in Voila\textsuperscript{1}/TM3 male was caused by the PGal4 element. For the sake of clarity, the courtship index of Voila\textsuperscript{exc}/TM3 males from the 61 Voila\textsuperscript{exc} lines was measured in the presence of decapitated control males. We focused our attention on the intensity of the homosexual courtship index (Clm) because the relative increase in Clm between control and mutant males was much greater (> 500\%) than the relative increase in the courtship index towards control female flies (+ 61\%; see legend of Table 1). The Clm of Voila\textsuperscript{exc}/TM3 males from 61 Voila\textsuperscript{exc} lines towards decapitated control male target flies was noted (Table 1). The Clms of heterozygous Voila\textsuperscript{exc}/TM3 males of all Voila\textsuperscript{exc} lines were compared with the Clm of Voila\textsuperscript{d}/TM3 mutant subject males, and with the Clm of w;+/TM3 control subject males (Table 1).

In 41 Voila\textsuperscript{exc} lines males showed no significant difference in their Clm when compared with w;+/TM3 control males, indicating that the intensity of their homosexual courtship was normal (‘Rescue group’). The rescue of normal (= virtually absent) homosexual male courtship indicates that the PGal4 transposon inserted in the Voila\textsuperscript{1} strain caused the high intensity of homosexual courtship. However, the fact that the Clm yielded by male flies of these strains showed different levels of significance when compared with the Clm of Voila\textsuperscript{d}/TM3 mutant males indicates that the intensity of courtship varies.

Study of the Clm produced by males from the other 20 Voila\textsuperscript{exc} lines supports the hypothesis that the remobilization of PGal4 has not rescued normal male homosexual courtship in an ‘all-or-none’ manner (e.g. causing the absence or the high level of homosexual courtship). In 12 Voila\textsuperscript{exc} lines, the rescue of normal male homosexual courtship was partial, because Clm was significantly different from the indices produced by both control and mutant male flies (‘Intermediate group’). In eight other Voila\textsuperscript{exc} lines, the Clm values were not different from the Clm of Voila\textsuperscript{d}/TM3 males (‘Mutant group’). The variation in the level of significance observed between these mutant strains also indicates that some of these strains (12, 51, 63 and 67) have a weaker Clm than Voila\textsuperscript{d}/TM3 mutant males.

(vi) The rescue of adult viability is not correlated with the rescue of male courtship

The rescue of normal Clm shows that the PGal4 insertion causes the abnormally high level of homosexual courtship. However, the viability of homozygous adults was not rescued in all the Voila\textsuperscript{exc} lines that showed rescue of normal (= quasi-absent) male homosexual courtship. This can be seen in Table 1: the lethality character of homozygous adults (VB or LT) was evenly distributed among the three behavioural groups. Therefore, no relationship was found between the rescue of male courtship and the size or nature of the PGal4 fragment that remained inserted. In conclusion, there was no correlation between the rescue of the two defective phenotypes caused by the Voila\textsuperscript{d}-PGal4 element studied here.
4. Discussion

This study has focused on the genetic characterization of the Voila¹ strain that carries the PGal4 transposon. Two abnormal phenotypes have previously been shown to be associated with this P element: (i) lethality between larval and early imaginal stages in homozygotes, and (ii) strong homosexual male courtship behaviour in heterozygous adult males. Both phenotypes were previously mapped with a set of deletions covering the chromosomal region which corresponds to the site of PGal4 insertion (86E1; Balakireva et al., 1998, 2000). Here, we have shown that both normal phenotypes can be rescued following remobilization of the PGal4 transposon. The fact that the rescue of the two phenotypes did not necessarily occur in the same Voilaexc strains indicates that these characters have different genetic bases. Furthermore, neither behavioural courtship nor developmental phenotype was rescued in an ‘all-or-none’ manner.

The first character which we have analysed genetically and molecularly is the pattern of viability of homozygotes during their pre-imaginal and early imaginal development. We measured the number of homozygous Voila¹voila exc individuals dying during each principal developmental stage, from embryonic development up to 7-day-old flies. Voila¹/¹ individuals generally died during larval development (Balakireva et al., 2000). The use of a TM3 fluorescent balancer (which makes it possible to score the genotype directly; Ferrandon et al., 1998) indicates that roughly half the homozygotes died before reaching their second larval instar and a third died before pupation (Y. Grosjean, unpublished observation).

In Voila¹VT strains, the escaping homozygous adults were not viable enough to produce progeny. In most of these Voila¹VT strains, the peak of lethality was delayed relative to the Voila¹ strain, occurring during either late larval, pupal, or even early imaginal development. Our results suggest that there is a relationship between the period of peak homozygote lethality and the size of the fragment that remained inserted at the original site of the Voila¹-PGal4 transposon (Fig. 4). However, several factors preclude a rigorous statistical analysis of the relationship between developmental lethality and the size of the inserted fragment. First, lethality did not occur in a linear manner because development is a succession of discrete steps of transformation. Second, we have not precisely measured the length of the inserted fragment (except for a few strains that were sequenced). Moreover, a similar degree of lethality (peaking in early imagos) seems to be caused by the combination of different sequences, the total length of which is roughly similar (see strains 79, 3, 11, 61 and 13; Figs. 3, 4). This result excludes the possibility that a transsplicing effect is involved in lethality. As the lethality phenotype was associated with gustatory problems in Voila¹/¹ larvae (Balakireva et al., 2000), we are currently investigating whether the variation in developmental lethality correlates with a quantitative variation in taste function and more specifically with abnormal food uptake by first and second instar larvae.

This PGal4 element is inserted at the chromosomal location (86E1) that corresponds to the site of the pan-neural gene prospero (pros; Doe et al., 1991). Given that this transposon is inserted at 216 bp upstream of the 5′UTR of pros, we suggest that the variation in developmental lethality among Voilaexc strains is a consequence of quantitative variations in the Pros product. We are currently investigating whether our various lethality phenotypes could result from alterations in the stability of the pros transcript or from lower levels of the Pros protein. No difference in either the pattern or the strength of Gal4 expression was noted between various Voilaexc strains (N. Gendre and R. F. Stocker, unpublished data). Complementation experiments combining Voilaexc with several available pros alleles (Deak et al., 1997) are in progress in order to understand the relationship between Voila and pros. The Voila¹ PGal4 strain shows a very similar embryonic expression pattern to that observed with pros¹⁄² (a PlacW enhancer-trap strain; Chu-Lagraff et al., 1991; Balakireva et al., 2000), but larval and pupal expression pattern are not available for pros. It is unlikely that Voila¹ insertion interferes with gene(s) in the opposite direction, because the nearest gene (KP78a) is located at 21 kb and it encodes a ubiquitously expressed protein kinase which is involved in cell-cycle regulation (Schulman et al., 2000).

Roughly half of the Voila¹exc lines showed rescued adult homozygous viability (Voila¹AB). However, in some of these strains the reproductive success of homozygous flies seems to be reduced when they are mixed with heterozygotes. Behavioural assays and particularly multiple-choice courtship tests are currently being undertaken to reveal whether homozygous flies are behaviourally disadvantaged when competing with same-sex heterozygous flies. We are trying to understand the molecular nature of the 100 bp fragment that remained inserted in some Voila¹AB strains. The sequence of this fragment barely corresponds to the feet of the transposon and could be the result of an internal rearrangement analogous to what has been described when hopping out transposon (Preston et al., 1996). Preliminary sequencing of some of the Voila¹exc strains indicates that Gal4 and pBSK share a common molecular sequence. The simultaneous presence of repeated sequences within the transposon could explain the increased frequency of imprecise excisions within the PGal4 transposon (Y. Grosjean, unpublished data). The present data reveal that the PGal4 element was cleanly excised in only...
three of 18 Voila\textsuperscript{exc} strains. If we exclude a possible bias caused by our sampling procedure, the high percentage of imprecise excision events found here (83\%) is not very far from the frequency reported in another study (75\% in [P-rosy]; Daniels \textit{et al.}, 1985). In conclusion, we found a clear relationship between the developmental defect and the amount of inserted DNA within the promoter of the \textit{pros} gene.

Male homosexual courtship behaviour is the second \textit{Voila}' phenotype that was rescued after remobilization of the PGal4 transposon. We focused on the intensity of male homosexual courtship (CIm) because this parameter differed more than 5-fold between control and mutant males. Of 61 strains, 41 exhibited a rescued CIm (= a low level of homosexual courtship); while the strains with a clean excision of the Gal4 transposon (14, 45 and 64) were among this group of rescued strains, so were other strains still retaining a PGal4 sequence. In a limited number of strains, the CIm was intermediate between the CIm of control and mutant strains, and in another small group of strains the CIm was not different from that of \textit{Voila}/TM3 mutant males (= a high level of homosexual courtship). The continuous variation in CIm indicates that the intensity of male homosexual courtship can be quantitatively controlled by various \textit{Voila} alleles. \textit{Voila}-PGal4 is strongly expressed in the mushroom bodies in the central nervous system and in the gustatory neurons of the legs and the proboscis, all of which are involved in pheromone perception and discrimination (Ferveur \textit{et al.}, 1995; Balakireva \textit{et al.}, 1998; Savarit \textit{et al.}, 1999). However, we do not know yet which part of the nervous system controls the quantitative variation of homosexual male courtship that occurs among the various \textit{Voila}\textsuperscript{exc} strains. We can exclude a general defect in locomotor activity because no correlation was observed between CIm and male locomotor activity in \textit{Voila}\textsuperscript{exc} strains (data not shown). We can also exclude the possibility that homosexual courtship depends upon the presence of the mini-white marker (Zhang & Odenwald, 1995), because no such relation was found in our \textit{Voila}\textsuperscript{exc} strains.

Although the present data did not reveal a clear relationship between homosexual courtship and the amount of DNA inserted in the promoter of \textit{pros}, preliminary experiments performed with several \textit{pros} alleles support the hypothesis that this gene indeed controls courtship behaviour (M. Balakireva & J.-F. Ferveur, unpublished results). The future exploration of the courtship defect in \textit{Voila}\textsuperscript{exc} strains will be carried out using RT-PCR analysis, in order to determine whether different levels of homosexual courtship are related to the aberrant production of one or several transcripts, as in the \textit{fruitless} gene (Goodwin \textit{et al.}, 2000).

In conclusion, we have shown that the developmental and courtship defects described in the \textit{Voila}' strain are both caused by the PGal4 transposon, but that they are under separate genetic control. The new series of excision alleles will allow for a detailed genetic and molecular dissection of these two complex characters. Our preliminary molecular dissection suggests that the different peaks of developmental lethality are related to the size of inserted material, which could in turn induce a quantitative decrease in the Prospero protein that is normally required during different stages of development. We have not found any molecular correlate at the site of PGal4 insertion to explain the variation in male courtship. For this reason, we are currently undertaking the molecular dissection of the regulatory regions of the \textit{Voila}/\textit{pros} genomic region in order to elucidate the role of this locus in the control of male reproductive behaviour.

Matthew Cobb, Fawzia Baba-Aissa, Jean-Philippe Charles, Kathleen Siwicki and two anonymous referees are thanked for their comments on the manuscript. We also thank Josiane Alabouvette and Nathalie Sedano for their valuable technical help and advice. Funding was provided by the French Ministry of Research and Education (for Y.G.), by the Lilly Foundation and the Burgundy Region (for M. B.) and by the Centre National de la Recherche Scientifique (for L. D. and J.-F. F.).

**References**

Balakireva, M., Stocker, R. F., Gendre, N. & Ferveur, J.-F. (1998). \textit{Voila}, a new \textit{Drosophila} courtship variant that affects the nervous system: behavioral, neural, and genetic characterization. \textit{Journal of Neuroscience} \textbf{18}, 4335–4343.

Balakireva, M., Gendre, N., Stocker, R. F. & Ferveur, J.-F. (2000). The genetic variant \textit{Voila}\textsuperscript{+} causes gustatory defects during \textit{Drosophila} development. \textit{Journal of Neuroscience} \textbf{20}, 3425–3433.

Brand, A. H. & Dormand, E. L. (1995). The GAL4 system as a tool for unraveling the mysteries of the \textit{Drosophila} nervous system. \textit{Current Opinion in Neurobiology} \textbf{5}, 572–578.

Brand, A. H. & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. \textit{Development} \textbf{118}, 401–415.

Chi-Lagraff, Q., Wright, D. M., McNeil, L. K. & Doe, C. Q. (1991). The \textit{prospero} gene encodes a divergent homeodomain protein that controls neuronal identity in \textit{Drosophila}. \textit{Development Suppl.} \textbf{2}, 79–85.

Cobb, M. & Ferveur, J.-F. (1996). Evolution and genetic control of mate recognition and stimulation in \textit{Drosophila}. \textit{Behavioural Processes} \textbf{35}, 35–54.

Cooley, L., Kelley, R. & Spradling, A. (1988). Insertional mutagenesis in the \textit{Drosophila} genome with single \textit{P} element. \textit{Science} \textbf{239}, 1121–1128.

Daniels, S. B., McCarron, M., Love, C. & Chovnick, A. (1985). Dye-ogenesis-induced instability of \textit{rosy} locus transformation in \textit{Drosophila melanogaster}: analysis of excision events and the selective recovery of control element deletions. \textit{Genetics} \textbf{109}, 95–117.

Deak, P., Omar, M. M., Saunders, R. D. C., Pal, M., Komonyi, O., Szidonya, J., Maroy, P., Zhang, Y., Ashburner, M., Benos, P., Savakis, C., Siden-Kiamos, I., Louis, C., Bolshakov, V. N., Kafatos, F. C., Madueno,
E. Modolell, J. & Glover, D. M. (1997). P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: correlation of physical and cytogenetic maps in chromosomal region 86E–87F. Genetics 144, 1697–1722.

Deldakis, C. & Kafatos, F. C. (1989). Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. The EMBO Journal 8, 891–901.

Doc, C. Q., Chu-Lagriff, Q., Wright, D. M. & Scott, M. P. (1991). The prospero gene specifies cell fates in the Drosophila central nervous system. Cell 65, 451–464.

Ferrandon, D., Jung, A. C., Criqui, M., Lemaître, B., Uittenweiler-Joseph, S., Michaut, L., Reichhart, J. & Hoffmann, J. A. (1998). A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. The EMBO Journal 17, 1217–1227.

Ferveur, J.-F. & Sureau, G. (1996). Simultaneous influence on male courtship of stimulatory and inhibitory pheromones produced by live sex-mosaic Drosophila melanogaster: Proceedings of the Royal Society of London, Series B 263, 557–563.

Ferveur, J.-F., Störrktuhl, K. F., Stocker, R. F. & Greenspan, R. J. (1995). Genetic feminization of brain structures and changed sexual orientation in male Drosophila. Science 267, 902–905.

Ferveur, J.-F., Savarit, F., O’Kane, C. J., Sureau, G., Greenspan, R. J & Jallon, J.-M. (1997). Genetic feminization of pheromones and its behavioral consequences in Drosophila males. Science 276, 1555–1557.

Gailey, D. A. & Hall, J. C. (1989). Behavior and cytogenticis of fruitless in Drosophila melanogaster: different courtship defects caused by separate, closely linked lesions. Genetics 121, 773–785.

Goodwin, S. F. (1999). Molecular neurogenetics of sexual differentiation and behaviour. Current Opinion in Neurobiology 9, 759–765.

Goodwin, S. F., Taylor, B. J., Villella, A., Foss, M., Ryner, L. C., Baker, B. S. & Hall, J. C. (2000). Aberrant splicing and altered spatial expression patterns in fruitless mutants of Drosophila melanogaster. Genetics 154, 725–745.

Greenspan, R. J. (1995). Understanding the genetic construction of behavior. Scientific American 272, 72–78.

Greenspan, R. J. & Ferveur, J.-F. (2000). Courtship in Drosophila. Annual Review of Genetics 34, 205–232.

Hall, J. C. (1977). Portions of the central nervous system controlling reproductive behavior in Drosophila melanogaster. Behavior Genetics 7, 291–312.

Hall, J. C. (1978). Courtship among males due to a male-sterile mutation in Drosophila melanogaster. Behavior Genetics 8, 125–141.

Hall, J. C. (1979). Control of a male reproductive behavior by the central nervous system of Drosophila: dissection of a courtship pathway by genetic mosaics. Genetics 92, 437–457.

Hall, J. C. (1994a). The mating of a fly. Science 264, 1702–1714.

Hall, J. C. (1994b). Pleiotropy of behavioral genes. In Flexibility and Constraint in Behavioral Systems (ed. R. J. Greenspan & C. P. Kyriacou), pp. 15–27. Dalhem workshop report. New York: Wiley.

Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S. & Yamamoto, D. (1996). Sexual orientation in Drosophila is altered by the sator gene mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. Proceedings of the National Academy of Sciences of the USA 93, 9687–9692.

Lapie, P., Nasr, F., Lepesant, J. A & Deutsch, J. (1993). Deletion scanning of the regulatory sequences of the Fhp1 gene of Drosophila melanogaster using P transposase-induced deficiencies. Genetics 135, 801–816.

Lindsay, D. L. & Zimm, G. G. (1992). The Genome of Drosophila melanogaster. San Diego: Academic Press.

Markow, T. A. (1987). Behavioral and sensory basis of courtship success in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the USA 84, 6200–6204.

Preston, C. R., Sved, J. A. & Engels, W. R. (1996). Flanking duplications and deletions associated with P-induced male recombination in Drosophila. Genetics 144, 1623–1638.

Reuter, G., Gausz, J., Gyurkovics, H., Friede, B., Bang, R., Spierer, A., Hall, L. M. & Spierer, P. (1987). Modifiers of position-effect variegation in the region from 86C to 88B of the Drosophila melanogaster third chromosome. Molecular and General Genetics 210, 429–436.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. & Engels, W. R. (1988). A stable genomic source of P-element transposase in Drosophila melanogaster. Genetics 118, 461–470.

Ryner, L. C., Goodwin, S. F., Castrillon, D. H., Anand, A., Villella, A., Baker, B. S., Hall, J. C., Taylor, B. J. & Wasserman, S. A. (1996). Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene. Cell 87, 1079–1089.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Savarit, F., Sureau, G., Cobb, M. & Ferveur, J.-F. (1999). Genetic elimination of known pheromones reveals the fundamental chemical bases of mating and isolation in Drosophila. Proceedings of the National Academy of Sciences of the USA 96, 9015–9020.

Schulman, J. M., Benton, R. & St Johnston, D. (2000). The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. Cell 101, 377–388.

Spradling, A. C. & Rubin, G. M. (1983). The effect of chromosomal position on the expression of the Drosophila xanthine dehydrogenase gene. Cell 34, 47–57.

Stanewsky, R., Fry, T. A., Reim, I., Saumweber, H. & Hall, J. C. (1996). Bioassaying putative RNA-binding motifs in a protein encoded by a gene that influences courtship and visually mediated behavior in Drosophila: in vitro mutagenesis of nonA. Genetics 143, 259–275.

Sturtevant, A. H. (1915). Experiments in sexual recognition and the problems of sexual selection in Drosophila. Journal of Animal Behavior 5, 351–366.

Sureau, G. & Ferveur, J.-F. (1999). Co-adaptation of pheromone production and behavioural responses in Drosophila melanogaster males. Genetical Research 74, 129–137.

Taylor, B. J., Villella, A., Ryner, L. C., Baker, B. S. & Hall, J. C. (1994). Behavioral and neurobiological implications of sex-determining factors in Drosophila. Developmental Genetics 15, 275–296.

Vaessen, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. & Jan, Y. N. (1991). Prospero is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in Drosophila. Cell 67, 941–953.

Villella, A. & Hall, J. C. (1996). Courtship abnormalities caused by doublesex mutations in Drosophila melanogaster. Genetics 143, 331–344.

Villella, A., Gailey, D. A., Berwald, B., Ohshima, S., Barnes, P. T. & Hall, J. C. (1997). Extended reproductive roles of...
the fruitless gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. *Genetics* **147**, 1107–1130.

Voelker, R. A., Greenleaf, A. L., Gyurkovics, H., Wisely, G. B., Huang, S. & Searles, L. L. (1984). Frequent imprecise excision among reversions of a *P* element-caused lethal mutation in *Drosophila*. *Genetics* **107**, 279–294.

Wilson, C., Bellen, H. J. & Gehring, W. J. (1990). Position effects on eukaryotic gene expression. *Annual Review of Cell Biology* **6**, 679–714.

Zhang, S.-D. & Odenwald, W. F. (1995). Misexpression of the white (*w*) gene triggers male–male courtship in *Drosophila*. *Proceedings of the National Academy of Sciences of the USA* **92**, 5525–5529.