Genet. Res., Camb. (2001), **77**, *pp.* 239–250. *With* 4 *figures.* Printed in the United Kingdom © 2001 Cambridge University Press 239

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

YAEL GROSJEAN[1], MARIA BALAKIREVA[2], LAURENCE DARTEVELLE[1] AND JEAN-FRANCOIS FERVEUR[1]*

[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 $86E_{1-2}$ is associated with the *Voila*^{1} allele that causes multiple phenotypes. Homozygous *Voila*$^{1/1}$ flies rarely reach adulthood and heterozygous *Voila*$^{1/+}$ 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, 1994*a*). 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, 1994*b*; 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

produce insertional mutagenesis by the direct effect of the *P* element with the possibility of driving the expression of a second UAS-transgene (Brand & Perrimon, 1993; Brand & Dormand, 1995). In spite of the popularity of this technique, very few data are available on the direct role that the PGal4 element plays in the genome, specifically on its mutagenic effects. It has been noted that *P* elements generally affect the expression of quantitative traits more than qualitative traits (Spradling & Rubin, 1983; Wilson *et al*., 1990). Very few studies have compared the phenotypic effects of genomic sequences of various sizes resulting from a series of partial excisions of a single *P* element transposon (Daniels *et al*., 1985; Delidakis & Kafatos, 1989; Lapie *et al*., 1993).

Here, we compare different excision strains derived from the Voila[1]-PGal4 enhancer-trap strain in order to genetically and molecularly dissect the pleiotropic effects of *Voila[l]* on (i) low larval viability in homozygotes of both sexes and (ii) abnormal courtship behaviour in heterozygous male flies (Balakireva *et al*., 1998, 2000). The two abnormal phenotypes were compared in a series of excision lines (Voila[exc]) that were derived by remobilization of the transposon from the Voila[1] strain. Rescue of the two phenotypes did not coincide in the same Voila[exc] strains, indicating they have different genetic bases. Furthermore, the fact that these phenotypes were not always rescued in an 'all-or-none' manner suggests that *Voila* controls both characters in a quantitative manner. Finally, as the cytological position of the PGal4 transposon insertion coincides with the position of the *prospero* gene ($86E_{1-2}$), we have started to investigate the molecular relationship between *Voila* and *prospero*.

2. Materials and methods

(i) Drosophila *strains and crosses*

All strains of *D. melanogaster* were maintained on standard cornmeal and yeast medium under a 12 h dark/12 h light cycle at 25 °C. A description of the chromosomes and mutations used in this study can be found in Lindsley & Zimm (1992). The *Voila[l]*-PGal4 insertion line in the *Voila* locus has already been characterized for adult expression and behaviour (Balakireva *et al*., 1998), and for pre-imaginal expression and behavioural defects (Balakireva *et al*., 2000). Since *Voila[l]* is a recessive lethal, the *Voila[l]* chromosome was maintained over TM3, a balancer chromosome carrying the dominant mutations *Stubble* and *Serrate* (*Sb*, *Ser*).

To generate derivative lines of *Voila[l]*, we used the scheme described by Cooley *et al*. (1988) to mobilize the *Voila[l]*-PGal4 transposon. Excision of the transposon was performed by crossing *Voila[l]*/TM3 females with males from a jump starter strain which provided

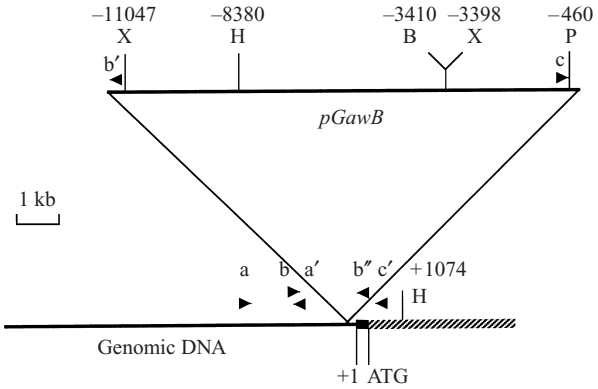

Fig. 1. Restriction map of the *Voila* locus. The triangle symbolizes the inserted PGal4 (PGawB) transposon. Restriction sites are X, *Xho*I; H, *Hin*dIII; B, *Bam*HI; and P, *Pst*I. The distance (in base pairs) of each restriction site from the +1 transcription site of the *pros* gene is indicated. The primers (see Section 2) used for amplification of sequences by PCR in several Voila[exc] strains are indicated by black arrowheads. Primers a and a′ were used for the amplification of the 5′ flanking region 1 kb from the *P* element insertion site. Primers b and b′ were used for the amplification of the 5′ flanking region close to the *P* element insertion site. Primers c and c′ for the amplification of the 3′ flanking region close to the *P* element insertion site and primers b and b″ were used in the lines 14, 26, 28, 45, 60, 64, 65 to obtain the genomic region left after excision. The black segment represents the 5′UTR of the *pros* gene and the hatched segment represents the translated *pros* region. The 3′ *PGawB* extremity was mapped at −216 and the ATG codon at +301.

the *P* element transposase (Robertson *et al*., 1988). Male progenies carrying the *Voila[l]*-PGal4 transposon and the transposase-producing *Δ2-3* chromosome were crossed to *w*; +/TM3 females. In order to obtain flies without the *P* transposon, males of the next generation were scored for the loss of the *mini-white*$^{+}$ gene. Each Voila excision line derived from *Voila[l]* resulted from a unique excision event on chromosome 3 that was subsequently maintained over a TM3 balancer chromosome.

Complementation analysis of *Voila[l]* 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[l]* or *Voila[exc]* was studied with a strain carrying the dominant adult marker *Sb*.

(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),

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

individually aspirated into an observation chamber (2·8 cm diameter, 0·5 cm height). After 10 min, a second 4-day-old Canton-S (Cs) male, used as the object fly, was introduced. The courtship index value (CI) is the percentage of time that the subject male spends in courting, during a 10 min observation period. CIm represents the courtship index toward control object male flies. Cs males were chosen as control objects because of their clear behavioural phenotype (Ferveur *et al.*, 1997). Each subject and object male was tested only once. Object males were always decapitated in order to prevent reciprocal courtship (Ferveur *et al.*, 1995). Decapitation was always performed 30 min prior to the experiment.

(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, CIms (courtship index toward decapitated male object) of all Voila^{exc}, of Voila[1] and control w^- strains were compared using ANOVA. Voila^{exc} lines were then grouped according to the difference between their CIm value and the CIm 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 *Hin*dIII–*Xho*I DNA fragment corresponding approximately to 'Gal4', a 5 kb *Hin*dIII–*Xho*I DNA fragment corresponding to 'mini-white', and a 2·9 kb *Bam*HI–*Pst*I DNA fragment corresponding to 'pBSK'. Fragments were then ^{32}P-labelled by random priming with the Prime-a-gene kit (Promega).

Genomic DNA was digested with *Xho*I and *Hin*dIII. 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: CACACAC-ACACACACACACATTTAT; a′: CTGCTTTTTC-TCTGGCAATACC: b: ACTACAAACACAGCAG-CAAGGAC; b″: GAACGACGATTCACTTTACT-GTTC; c′: CAAACAGCTCGTTCAGCATCT) or internal to the transposon (b′: AAGACAGTAG-CTTCATCTTTCAGGA; c: CTAGCTAGAGCTT-TGCGTACTCG; 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 Voila[1] strain*

To verify the involvement of the PGal4 transposon in the developmental lethality observed in the Voila[1] strain, we induced the remobilization of this transposable element by crossing the Voila[1] 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 *Voila$^{1/1}$* 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^{LT} and 7 Voila^{VB}). For each Voila^{exc} strain, the frequency of homozygote lethality was measured either indirectly (by estimating survivorship at the end

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

Table 1. *Viability of homozygotes and courtship behaviour of heterozygous males from various strains*

			Homosexual courtship		
				Difference in CIm compared with	
	Strains	Homozygote viability	CIm Mean ± SE	*Voila*^{1}/TM3 (mutant)	w; +/TM3 (control)
Mutant	**Voila^{1}/TM3**	LT	42 ± 4·8		
Control	**w; +/TM3**	VB	7 ± 2·3		
	Voila^{exc}/TM3				
Rescue group	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 ± 7·3	**	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	*	*

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

Table 1 (*Cont.*)

			Homosexual courtship		
				Difference in CIm compared with	
	Strains	Homozygote Viability	CIm Mean ± SE	*Voila*^{1}/TM3 (mutant)	w; +/TM3 (control)
Mutant group	27 (+)	LT	35 ± 5·3	NS	***
	35	VB	35 ± 6·1	NS	***
	56	LT	35 ± 6·1	NS	***
	78 (+)	LT	31 ± 3·5	NS	***
	51	LT	31 ± 3·7	NS	**
	67	VB	30 ± 6·5	NS	**
	12	VB	28 ± 5·1	NS	*
	63	VB	28 ± 5·1	NS	*

The viability (VB) or the lethality (LT) of homozygous adults belonging to 61 Voila^{exc} strains, to mutant Voila[1] 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*^{exc}/TM3, *Voila*^{1}/TM3 and w; +/TM3 control males was tested with an ANOVA. The level of significance is indicated as follow: *** $P < 0{\cdot}001$; ** $P < 0{\cdot}01$; * $P < 0{\cdot}05$; 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*^{1}/TM3 males and 46 ± 6·5 for control w; Cs males ($n = 20$).

of embryonic, larval and pupal stages) or directly (in 2- and 7-day old adults).

Homozygous individuals from these Voila^{exc} strains showed very different patterns of lethality during their pre- and post-metamorphic phases of development. As expected, homozygotes from all selected Voila^{VB} lines (Fig. 2*C*) very frequently survived to the '2-day-old' adult stage whereas most, if not all, homozygotes from the selected Voila^{LT} lines died before reaching this stage (Fig. 2*A*, *B*). The peak of homozygote lethality shown by these Voila^{LT} strains occurred at different developmental periods: Voila^{78}, Voila^{8} and Voila^{69} died mostly during larval development (very similarly to Voila$^{1/1}$). Voila$^{24/24}$ individuals died mostly during pupal development whereas lethality of Voila$^{2/2}$ and Voila$^{27/27}$ occurred constantly throughout most developmental stages from the larva to the 7-day-old adult. Homozygotes from Voila^{3}, Voila^{13} and Voila^{79} lines, and to a lesser extent from Voila^{11} and Voila^{61} 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^{3} and Voila^{61} lines).

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

First, the site of PGal4 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 PGal4 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^{exc} 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^{exc} strain was analysed for the presence and size of an unexcised transposon sequence. Restricted DNA from flies of the 18 Voila^{exc} strains was probed with the three principal genetic sequences (Gal4, mini-white and pBSK) carried by the PGal4 transposon (Fig. 1; see Section 2). The size of each detected fragment was compared with that of the equivalent fragment probed in the Voila[1] control strain (Fig. 3).

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

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

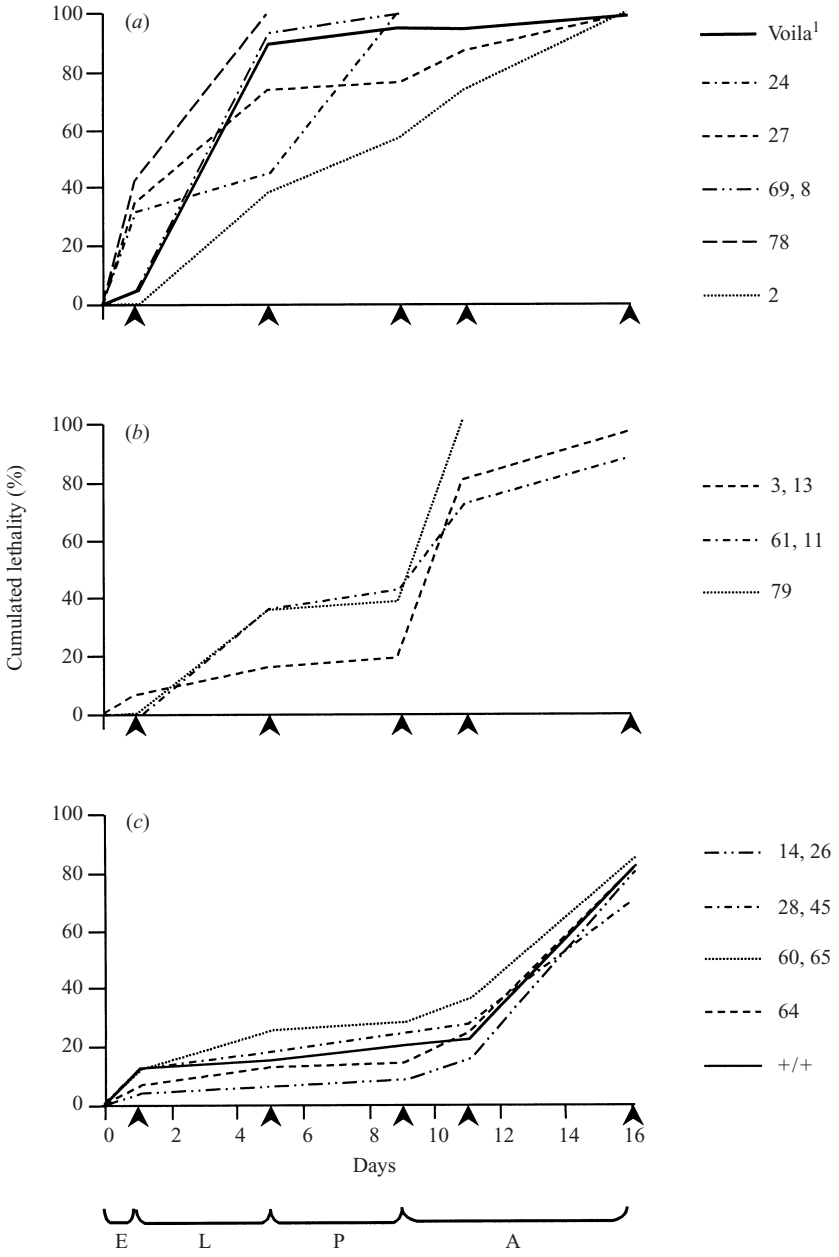

Fig. 2. Developmental lethality in homozygous flies of various Voila^{exc} strains, and of Voila^{1} and control strains. Cumulated lethality was estimated, or directly noted, at the end of each main developmental period (see arrowheads): embryonic (E), larval (L) or pupal (P) and in 2- and 7-day-old flies (for details see Section 2). For the sake of clarity, strains were grouped according to the stage of development during which lethality peaked: (*A*) larval or pupal, (*B*) early imaginal and (*C*) normally viable and fertile. $n = 104$–745 except for Voila^{14} (52) and Voila^{78} (38). Days were counted after egg-laying (at 25 °C).

altered compared with Voila^{1} (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 white^{+} 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. 3 *C*).

It is possible that the differences in lethality among Voila^{exc} strains are caused by size differences of the fragments that remained inserted at the chromosomal site $86E_{1-2}$. 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 Voila^{exc} strains with larval lethality (as in *Voila$^{1/1}$*) were those that generally retained the largest fragments (generally including most or all of the Gal4 and pBSK sequences). In addition, Voila^{78} and Voila^{8} still carried

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

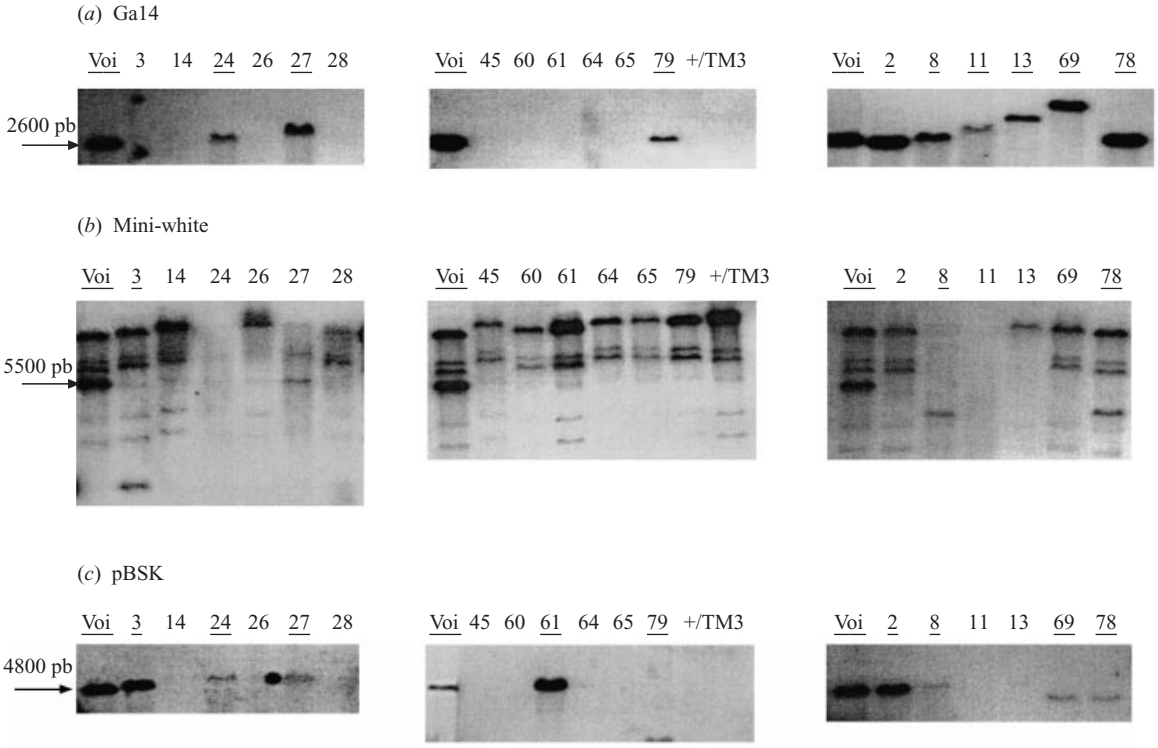

Fig. 3. The genomic material of the PGal4 transposon has been differently excised after remobilization in various Voila^{exc} strains. Southern blots were performed on digested DNA from the Voila^{exc} strains previously assayed for homozygote viability (see Fig. 2). Probes consisted of fragments of 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*^{1} (*Voi*), used here as a control. Arrows indicate the expected size for each fragment in Voila[1] flies (*Hind*III + *Xho*I).

a portion of the mini-white sequence. The Voila^{27}, Voila^{24} and Voila^{2} 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.

Homozygotes from the other Voila^{LT} strains (Voila^{3}, Voila^{11}, Voila^{13}, Voila^{61} and Voila^{79}) died more frequently during early adulthood (Fig. 2*B*). Their inserted fragments are smaller than in *Voila*^{1} and in the previously described Voila^{LT} strains (Figs. 3, 4), consisting of either an altered Gal4 sequence (in Voila^{11} and Voila^{13}), a complete Gal4 sequence with an altered pBSK sequence (in Voila^{79}), or a complete pBSK sequence (alone in Voila^{61}, or combined with an incomplete mini-white sequence in Voila^{3}).

(iv) *The reproductive ability of homozygous adults varies between Voila^{VB} strains*

The seven strains with rescued homozygous adult viability (Voila^{VB}) 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 Voila^{VB} 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 Voila^{VB} 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 Voila^{VB} 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

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

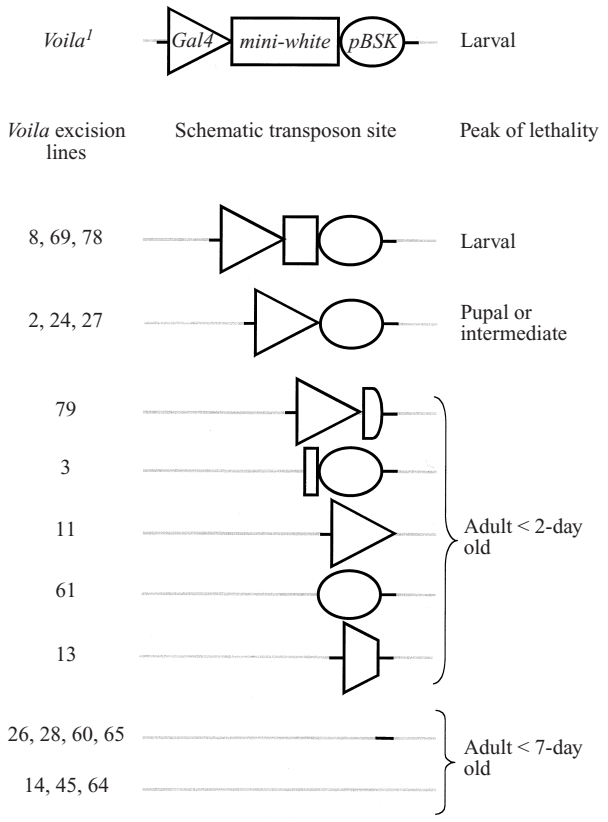

Fig. 4. Relationship between developmental lethality and the size of inserted DNA. On the basis of data produced by developmental lethality (Fig. 2) and on the approximate size of the genomic material still inserted at the site of insertion (Fig. 3), there seems to be a relationship between the size of the inserted DNA and the developmental period during which homozygote lethality peaks.

not shown). Conversely, in lines 14, 45 and 64, the PGal4 element has been completely excised. In conclusion, these data strongly suggest that there is a relationship between the developmental period during which the viability defect occurred and the amount of genetic material that remained inserted in chromosomal site $86E_{1\text{-}2}$ after remobilization of the *Voila*^{1}-PGal4 transposon.

(v) *The PGal4 transposon causes abnormal male courtship in* Voila^{1}

As expected, *Voila*^{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*^{1}/TM3 male was caused by the PGal4 element. For the sake of clarity, the courtship index of *Voila*^{exc}/TM3 males from the 61 Voila^{exc} lines was measured in the presence of decapitated control males. We focused our attention on the intensity of the homosexual courtship index (CIm) because the relative increase in CIm 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 CIm of *Voila*^{exc}/TM3 males from 61 Voila^{exc} lines towards decapitated control male target flies was noted (Table 1). The CIms of heterozygous *Voila*^{exc}/TM3 males of all Voila^{exc} lines were compared with the CIm of *Voila*^{1}/TM3 mutant subject males, and with the CIm of *w*; +/TM3 control subject males (Table 1).

In 41 Voila^{exc} lines males showed no significant difference in their CIm 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^{1} strain caused the high intensity of homosexual courtship. However, the fact that the CIm yielded by male flies of these strains showed different levels of significance when compared with the CIm of *Voila*^{1}/TM3 mutant males indicates that the intensity of courtship varies.

Study of the CIm produced by males from the other 20 Voila^{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^{exc} lines, the rescue of normal male homosexual courtship was partial, because CIm was significantly different from the indices produced by both control and mutant male flies ('Intermediate group'). In eight other Voila^{exc} lines, the CIm values were not different from the CIm of *Voila*^{1}/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 CIm than *Voila*^{1}/TM3 mutant males.

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

The rescue of normal CIm 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^{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*^{1}-PGal4 element studied here.

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

4. Discussion

This study has focused on the genetic characterization of the Voila[1] 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 ($86E_{1-2}$; 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 Voila^{exc} 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 *Voila*$^{exc/exc}$ individuals dying during each principal developmental stage, from embryonic development up to 7-day-old flies. *Voila*$^{l/l}$ 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 Voila^{LT} strains, the escaping homozygous adults were not viable enough to produce progeny. In most of these Voila^{LT} strains, the peak of lethality was delayed relative to the Voila[1] 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 *Voila*^{l}-PGal4 transposon (Fig. 4). However, several factors precludes a rigorous statistical analysis of the relationship between developmental lethality and the size of the inserted fragment. First, lethality did not occured 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 transplicing effect is involved in lethality. As the lethality phenotype was associated with gustatory problems in *Voila*$^{l/l}$ 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 ($86E_{1-2}$) 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 Voila^{exc} 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 Voila^{exc} strains (N. Gendre and R. F. Stocker, unpublished data). Complementation experiments combining Voila^{exc} with several available *pros* alleles (Deak *et al*., 1997) are in progress in order to understand the relationship between *Voila* and *pros*. The *Voila*^{l} PGal4 strain shows a very similar embryonic expression pattern to that observed with pros^{139} (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 *Voila*^{l} 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 Voila^{exc} lines showed rescued adult homozygous viability (Voila^{VB}). 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 Voila^{VB} 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 Voila^{exc} 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

https://doi.org/10.1017/S0016672301005006 Published online by Cambridge University Press

three of 18 Voila^{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 *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 *pros* gene.

Male homosexual courtship behaviour is the second *Voila*^{1} 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 *Voila*^{1}/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 *Voila* alleles. *Voila*^{1}-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 *et al*., 1995; Balakireva *et al*., 1998; Savarit *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 Voila^{exc} strains. We can exclude a general defect in locomotor activity because no correlation was observed between CIm and male locomotor activity in Voila^{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 Voila^{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 *pros*, preliminary experiments performed with several *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 Voila^{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 *fruitless* gene (Goodwin *et al*., 2000).

In conclusion, we have shown that the developmental and courtship defects described in the Voila^{1} 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 *Voila*/*pros* genomic region in order to elucidate the role of this locus in the control of male reproductive behaviour.

Matthew Cobb, Fawzia Baba-Aïssa, 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). *Voila*, a new *Drosophila* courtship variant that affects the nervous system: behavioral, neural, and genetic characterization. *Journal of Neuroscience* **18**, 4335–4343.

Balakireva, M., Gendre, N., Stocker, R. F. & Ferveur, J.-F. (2000). The genetic variant *Voila*^{1} causes gustatory defects during *Drosophila* development. *Journal of Neuroscience* **20**, 3425–3433.

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

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

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

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

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

Daniels, S. B., McCarron, M., Love, C. & Chovnick, A. (1985). Dysgenesis-induced instability of *rosy* locus transformation in *Drosophila melanogaster*: analysis of excision events and the selective recovery of control element deletions. *Genetics* **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* **147**, 1697–1722.

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

Doe, C. Q., Chu-Lagraff, 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., Lemaitre, B., Uttenweiler-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**, 967–973.

Ferveur, J.-F., Störtkuhl, 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 cytogenetics 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. (1994*a*). The mating of a fly. *Science* **264**, 1702–1714.

Hall, J. C. (1994*b*). 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 *satori* 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 *Fbp1* gene of *Drosophila melanogaster* using *P* transposase-induced deficiencies. *Genetics* **135**, 801–816.

Lindsley, 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., Villela, 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.

Vaessin, 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 anomalies 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.