Copyright © 2001 by the Genetics Society of America

Protein Determinants of Insertional Specificity for the Drosophila *Gypsy* Retrovirus

Mariano Labrador and Victor G. Corces

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Manuscript received November 30, 2000
Accepted for publication March 29, 2001

ABSTRACT

The *gypsy* retrovirus invades the germ line of Drosophila females, inserting with a high frequency into the *ovo* locus. *Gypsy* insertion sites in *ovo* are clustered within a region in the promoter of the *ovo* gene that contains multiple binding sites for the OvoA and OvoB proteins. We found that a 1.3-kb DNA fragment containing this region is able to confer *gypsy* insertional specificity independent of its genomic location. The frequency of *gypsy* insertions into the *ovo* gene is significantly lower in wild-type females than in ovo^{D1} females. In addition, *gypsy* insertions in ovo^{D1} females occur during most stages of germ-line development whereas insertions in wild-type females occur only in late stages. This pattern of temporally specific insertions, as well as the higher frequency of insertion in ovo^{D1} females, correlates with the presence of the OvoA or OvoD1 proteins. The results suggest that *gypsy* insertional specificity might be determined by the binding of the OvoA repressor isoform to the promoter region of the gene.

ORIGINALLY considered as a long terminal repeat (LTR) retrotransposon, *gypsy* is currently classified as a retrovirus belonging to the family of errantivirus and is the first retrovirus described in insects (Boeke *et al.* 1999). The expression of *gypsy*, and as a consequence its infectivity and transposition, are controlled by the *flamenco* (*flam*) gene (Prud'homme *et al.* 1995). In the presence of a *flam* permissive allele, the amount of full-length *gypsy* RNA, as well as the amount of a spliced mRNA encoding the envelope protein, is increased significantly (Pelisson *et al.* 1994; Song *et al.* 1994). Transcription of full-length *gypsy* mRNA and expression of the envelope protein in homozygous permissive *flam* females occur mainly in the follicle cells surrounding the developing oocyte during stages 9–10 of oogenesis (Pelisson *et al.* 1994; Song *et al.* 1997). Mutations in the *flam* gene are necessary to produce significant levels of envelope proteins, suggesting that *gypsy* requires the envelope to infect the oocyte and subsequently integrate into the genome of next generation germ-line cells. It was shown that follicle cells produce infectious virus particles, capable of infecting larvae of a stock that lacks active *gypsy* elements (Kim *et al.* 1994; Song *et al.* 1994). However, there is also evidence suggesting that *gypsy* is capable of transposing in *flam*-permissive female offspring, even when the envelope gene is mutated and therefore no envelope protein is made (Chalvet *et al.* 1999).

Characterization of the factors controlling *gypsy* infection and transposition has always been based on a genetic assay provided by the ability of *gypsy* to insert with very high frequency into the *ovo* gene (Mevel-Ninio *et al.* 1989). However, the mechanisms determining such specificity remain largely unknown. Integration into *ovo* occurs with a frequency of ~10% among the offspring of mutant *flam* females crossed to ovo^{D1} males (Prud'homme *et al.* 1995; Mevel-Ninio *et al.* 1996). The function of the *ovo* gene product is cell autonomous and is necessary for the development of the female germ line and the normal progression of oogenesis (Oliver *et al.* 1987). The *ovo* gene encodes two proteins, OvoA and OvoB, which bind the promoter region of the *ovo* gene to repress or activate its transcription, respectively. Adult females homozygous for a null mutation of the *ovo* gene do not develop germ-line cells. The ovo^{D1} allele is caused by a point mutation that creates a new in-frame methionine codon in the 5′ region of *ovo*, adding an extra amino terminus domain that in the wild type is present only in the ovoA protein (Mevel-Ninio *et al.* 1996). The ovo^{D1} allele is dominant negative and causes female sterility even when heterozygous. The sterility is due to the expression of $Ovo^{D1}B$ protein, which is made at the same time of development as OvoB but has the repressor activity of OvoA; the presence of $Ovo^{D1}B$ is sufficient to arrest oogenesis at stage 4 (Lu *et al.* 1998). Insertion of *gypsy* into the ovo^{D1} allele in a heterozygous female reverts the phenotype to fertility, although the reversion occurs only in those germ cells in which *gypsy* is inserted into the ovo^{D1} sequence, preventing the expression of the $Ovo^{D1}B$ protein. The ability of *gypsy* to integrate specifically into *ovo* sequences was recently analyzed by Dej *et al.* (1998). These studies concluded that *gypsy* integrates in at least seven different target sites localized within a 200-bp sequence present in the promoter region of the

Corresponding author: Victor G. Corces, Department of Biology, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. E-mail: corces@jhu.edu

ovo gene. Close analysis of these sites reveals a very relaxed consensus sequence consisting of six alternating pyrimidines and purines. The weak conservation of the observed target sequence suggests that *gypsy* site-specific integration is not due to a direct interaction of the *gypsy* integrase with these sequences. Instead, insertional specificity is probably due to interactions with additional factors that remain so far undetermined.

Factors governing DNA integration into the genome are of particular importance to clarify the overall mechanisms of retrovirus infection. Understanding these mechanisms is also an invaluable tool for the development of genome manipulation and gene therapy technologies. The *gypsy* retrovirus of Drosophila shares properties with retroviruses and also with retrotransposons, since it is capable of transposing both vertically in the germ line of the females (Chalvet *et al.* 1999) and horizontally, using the envelope protein to produce infectious particles (Kim *et al.* 1994; Song *et al.* 1994, 1997). Such duality could be the evolutionary basis for the integration specificity into the *ovo* gene shown by *gypsy* and constitutes a unique feature among retroviruses and LTR retrotransposons from higher eukaryotes. This property provides a unique opportunity to study integration site specificity *in vivo* using the genetic and molecular tools available in Drosophila. Here we test whether the integration specificity of *gypsy* into *ovo* is related to the ability of *gypsy* preintegration complexes to interact with proteins that bind DNA sequences of the *ovo* gene. The results suggest a correlation between the developmental pattern of expression of OvoA and the timing of insertion of *gypsy* during germ cell differentiation. A role for OvoA in targeting *gypsy* to *ovo* is reinforced by the fact that *gypsy* insertions into *ovo* are not observed at the time when only OvoB protein is expressed in the germ line. Furthermore, a significant increase of *gypsy* insertions takes place in the presence of the Ovo^{D1}B protein, which is functionally equivalent to OvoA but is expressed with the same developmental pattern as OvoB.

MATERIALS AND METHODS

***P*-element-mediated germ-line transformation and *gypsy* insertion into the y^{+ovo} transgene:** The *yellow*-CaSpeR plasmid, containing all the coding and regulatory regions of the *yellow* (*y*) gene, was modified by adding a *Not*I site in the *Eco*47III site of *y* (Geyer *et al.* 1986). The *Not*I site was used to clone a PCR-generated fragment of the 5′ region of the *ovo* gene spanning nucleotides 12–1298 (Mevel-Ninio *et al.* 1996). The oligonucleotides used to amplify the *ovo* fragment were AGTT GGCCATGACCAACAGAGCGA at the 5′ end and CTCC CGCTCTGCGGGCT TCTCTTT at the 3′ end. *Not*I sites were added at the 5′ ends of both primers to facilitate cloning into the *yellow*-CaSpeR plasmid. Males from the ovo^{D1} stock were used to obtain the template genomic DNA for the PCR amplification. The resulting plasmid was called CasPeR y^{+ovo} and was used to microinject Drosophila embryos.

P-element-mediated transformation was performed as described by Rubin and Spradling (1982). The CasPeR y^{+ovo} plasmid was injected into *y w; Sb [Δ 2-3]/TM6* embryos (Robertson *et al.* 1988) at a concentration of 0.5 μg/μl. Transgenic flies were identified by the rescue of the *white* phenotype. Two lines, *y w P[*y^{+ovo}*; *w^+*]*1.1 and *y w ; P[*y^{+ovo}*; *w^+*]*2.1, were selected among a total of five and genetically mapped to the *X* and second chromosomes, respectively. The *yellow* phenotype was completely rescued in all five lines.

Females homozygous for *y v f mal flam* were crossed to *y w P[*y^{+ovo}*; *w^+*]*1.1 and *y w ; P[*y^{+ovo}*; *w^+*]*2.1 males. The heterozygous female offspring with genotypes *y v f mal flam/y w P[*y^{+ovo}*; *w^+*]*1.1 and *y v f mal flam/y w; P[y* $^{+ovo}$*; *w^+*]*2.1/+ were individually crossed to *y* w^{67c} males. To detect *gypsy* insertions into the y^{+ovo} transgene, the offspring of this cross were systematically screened for individuals with a y^2-like phenotype. The y^2-like phenotype is expected because after insertion of *gypsy* the body and wing enhancers are blocked by the Su(Hw) insulator and cannot activate transcription of *y* in these tissues. Because the y^{+ovo} transgene is heterozygous in these females, only the offspring with a y^+ or a y^2-like phenotype were considered in all determinations of cluster size. As a positive control to test whether *flam*-permissive females were producing active virus particles, *y v f mal flam* females were crossed to ovo^{D1} males and the offspring were screened for fertile females as described below.

Analysis of *gypsy* insertions into *ovo* by PCR: Genomic DNA from single female crosses was extracted from 50–100 flies for the detection of *gypsy* insertions into *ovo* in wild-type females. DNA extraction was carried out using the potassium acetate quick prep as described in Dej *et al.* (1998). The primers used to amplify *gypsy* insertions were P3 CTTTGCCGAAAATATG CAATG and P1 CAACATGACCGAGGAGCGGTCATAAAC located in the 5′ and 3′ ends of *gypsy*, respectively, and P4 CGGCTTTTTCAGCGGCTAACC and P2 CTCCCGCTCTGC GGGCTTCTCTTT located in the *ovo* sequences flanking the *gypsy* insertion sites at the 5′ and 3′ sites, respectively (Figure 3). A combination of P1 with P2 or P4 will detect the insertion of *gypsy* in either orientation. The alternative combination of P3 with P4 or P2 can detect the same type of insertions and was used to confirm the results obtained with the first combination of primers. The same primers were used to clone and sequence the insertion sites of *gypsy* in the y^{+ovo} transgene. Approximately 100 ng of genomic DNA was used per PCR amplification. Conditions for this reaction were as follows: 91° (1 min), 65° (1 min), 72° (2–10 min) for 35 amplification cycles. Amplified DNA fragments containing *gypsy* insertion sites from the $y^{+ovo\text{-}gypsy}$ transgenes were cloned into the PCR 2.1 TA cloning vector from Invitrogen (Carlsbad, CA) and sequenced using an ABI 377 automated DNA sequencer.

Drosophila stocks, *ovo* reversion assay, and determination of *gypsy* insertion during development: All strains used in this work were kept at 25°. The *flam* stock was kept as *y v f mal flam/FM3*. ovo^{D1} males were maintained by crossing them to females carrying attached *X* chromosomes. The SS strain was used as a *flam*-permissive stock that does not carry active *gypsy* elements (Prud'homme *et al.* 1995). We used the ovo^{D1} reversion assay described in Prud'homme *et al.* (1995) to determine the *gypsy* activity of the *flam* stock. Five to 10 *y v f mal flam* virgin females were crossed with ovo^{D1} males and kept in the same vial for 3–5 days. After this time the flies were removed from the vials and the larvae were allowed to develop. Groups of 5–10 ovo^{D1}/ovo^+ females from the offspring were transferred to new vials and crossed to wild-type males. After several days, vials were examined for the presence of eggs or larvae. All the mothers in vials with eggs were dissected and the females displaying functional ovaries were considered fertile.

To determine the timing of insertion of *gypsy* during development, a similar procedure was carried out with some modi-

fications to ensure that ovaries were healthy and completely developed. Females homozygous for *y v f mal flam* were crossed to *v ovo*^{D1} males and to SS males as a control. Groups of five female offspring were crossed to wild-type males in fresh food containing a few grains of dry yeast. Vials were examined daily for the presence of eggs and those vials containing eggs were separated. After 5 days, all the females in egg-containing vials were dissected to identify those carrying functional ovaries. Females from vials lacking eggs were transferred to fresh food and the same process was repeated for another 5 days. The ovarioles from each functional ovary in fertile females were separated using tungsten needles and counted using a dissecting microscope. Ovaries containing 11 or more ovarioles were considered fully functional and were included in a single category. After 10 days, all females in vials lacking eggs were discarded and considered sterile. Statistica for Windows release 4.0 was used for the statistical analysis of the results.

RESULTS

Integration of *gypsy* into *ovo* is determined by local sequences contained within the gene: To understand the nature of the mechanisms responsible for the high rate of *gypsy* insertion into the *ovo* gene, we first asked whether the specificity is due to a local feature of *ovo* sequences or whether it depends on a general property of the gene or its flanking genomic sequences. A possible explanation for the insertional specificity of *gypsy* is that particular sequences in *ovo* attract proteins present in the preintegration complex. These proteins could be the *gypsy* integrase or any other protein component of the complex. A candidate for a targeting sequence is the *gypsy* insulator, which is located in the 5′ untranslated region and contains 12 binding sites for the Su(Hw) protein (Spana and Corces 1990). Since several *gypsy* insulators present in different chromosomal locations appear to associate together during interphase (Gerasimova and Corces 1998), it is possible that Su(Hw) proteins bound to the cDNA of *gypsy* in the preintegration complex could be targeted to other *gypsy* insulator sites in the genome. Interestingly, the analysis of DNA sequences adjacent to the insertion sites of *gypsy* in the *ovo* gene reveals the presence of putative Su(Hw) binding sites (data not shown). Thus it could be possible that *gypsy* insertion into *ovo* is due to the interaction of proteins binding the insulator sequences present in both the *gypsy* cDNA and the *ovo* gene. If this hypothesis were true, the putative binding sites in the *ovo* gene should bind Su(Hw) protein *in vivo* and the sequence containing such sites should act as an insulator.

To test this hypothesis, a genomic copy of the *yellow* (*y*) gene from *Drosophila melanogaster*, containing a 1.3-kb DNA fragment from the 5′ region of *ovo* inserted between the *yellow* body and bristle enhancers (Figure 1A), was cloned into the CasPeR vector carrying *white* (*w*) as a reporter gene. This plasmid, named CasPeR y^{+ovo}, was microinjected into *y w; Sb [Δ 2-3]/TM6* Drosophila embryos. The 1.3-kb fragment inserted into the regulatory region of *y* corresponds to *ovo* sequences spanning nucleotides 12–1298 in the 5′ region of the gene (Mevel-Ninio *et al.* 1996). We refer to this sequence as the 5′*ovo* region. This fragment was selected because it contains all the putative Su(Hw) binding sites together with all seven *gypsy* insertion sites found previously in the *ovo* gene (Dej *et al.* 1998). Offspring with colored eyes were selected and examined for the pigmentation of body cuticle, bristles, and wing blades (Figure 1B). If the 1.3-kb fragment of *ovo* contains Su(Hw) binding sites, it will function as an insulator *in vivo*, causing a y^2-like phenotype similar to that in the *gypsy*-induced y^2 allele (Geyer and Corces 1992). The transgene conferred full expression of the *y* gene in all tissues, including body cuticle, wing blades, and bristles in five independent transgenic lines. This result indicates that functional Su(Hw) binding sites are not present in the 5′ region of the *ovo* gene.

It is possible that Su(Hw)-binding sequences targeting *gypsy* to *ovo* could be present outside the 5′*ovo* region used in this experiment. Targeting *gypsy* by Su(Hw) binding sites could also be achieved by targeting the integration complex to the general genomic region where *ovo* is found and subsequent selection of the *gypsy* insertion site by a preference for the consensus *gypsy* insertion sequence YRYRYR, where Y = pyrimidine and R = purine (Dej *et al.* 1998). To test this hypothesis, we examined whether *gypsy* can still insert with high frequency into the 5′*ovo* region in a genomic context different from that of the endogenous *ovo* gene. In this experiment, different genomic regions flanking the y^{+ovo} transgene provide such alternative genomic context. The rationale of the experiment is illustrated in Figure 1C. As mentioned before, flies containing the y^{+ovo} transgene show a wild-type *y* phenotype. It is well established that *gypsy* Su(Hw) binding sites located between body and wing enhancers and the promoter act as an insulator, blocking enhancer-promoter interactions and precluding transcription of *y* in the body cuticle and wing blades (Geyer and Corces 1992). Therefore, we expect that, after insertion of *gypsy* into the 5′*ovo* region present in the y^{+ovo} transgene, $y^{+ovo\text{-}gypsy}$ flies will display a y^2-like phenotype (Figure 1D). To induce *gypsy* mobilization, we crossed *y v f mal flam* females to *y w* males carrying the *P[*y^{+ovo}*; w*$^+$*]* 1.1 and *P[*y^{+ovo}*; w*$^+$*]* 2.1 transgenes in the *X* and in the second chromosome, respectively. We selected y^+ daughters of the genotype *y v f mal flam/y w P[*y^{+ovo}*; w*$^+$*]* 1.1 and *y v f mal flam/y w; P[*y^{+ovo}*; w*$^+$*]* 2.1/+ and crossed them individually to *y w*^{67c} males.

The offspring of individual females were screened for the y^2-like phenotype as a marker for *gypsy* insertion into the *5′ovo* region in the y^{+ovo} transgene (Figure 1D). The results of these experiments are shown in Table 1. Seven out of a total of 232 females carrying the *P[*y^{+ovo}*; w*$^+$*]*1.1 transgene gave rise to one or more individuals with a y^2 phenotype and 3 females out of a total of 79 carrying the *P[*y^{+ovo}*; w*$^+$*]*2.1 transgene produced offspring with a y^2 phenotype. The frequency of females producing y^2

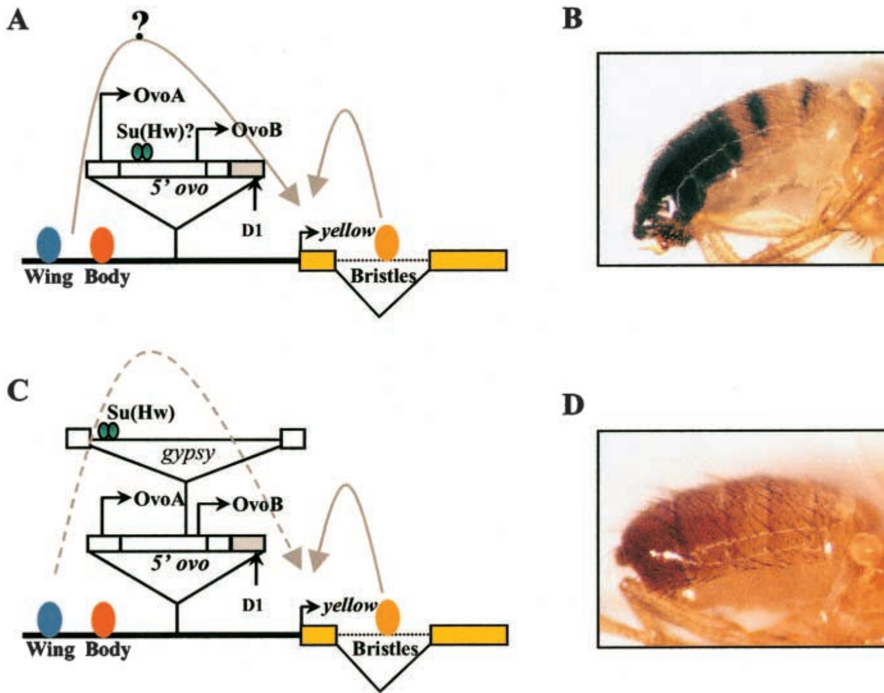

Figure 1.—Structure of transgenes and fly phenotypes. (A) Organization of the y^{+ovo} transgene showing the location of the 5′ *ovo* region. Wing, body, and bristle enhancers are indicated as ovals. Putative Su(Hw) binding sites in the 5′ *ovo* fragment would block the interactions between wing and body enhancers and the *y* promoter. (B) Body cuticle phenotype of a male of the genotype *y w P[*y^{+ovo}*; *w^{+}*]*1.1. The 5′ *ovo* fragment does not bind Su(Hw) *in vivo* and, therefore, fails to block the effect of the wing and body cuticle enhancers, resulting in normal pigmentation of the abdomen. (C) Structure of the $y^{+ovo\text{-}gypsy}$ transgene after *gypsy* insertion into the 5′ *ovo* region. After *gypsy* inserts into the 5′ *ovo* fragment, the *gypsy* insulator precludes the wing and body enhancers from activating transcription by the *y* promoter. The bristle enhancer is not affected by the insertion of *gypsy*. (D) Body cuticle from a male of the genotype *y w P[*$y^{+ovo\text{-}gypsy}$*; *w^{+}*]*1.1 obtained by *gypsy* insertion, showing a y^2-like phenotype.

in their offspring was 3% with the transgene in the *X* chromosome and 3.8% when the transgene was located in the second chromosome. To verify that the y^2-like phenotypes were due to insertions of *gypsy* into the 5′ *ovo* region of the transgene we established six independent y^2-like strains starting with a single male or female offspring. All strains showed Mendelian segregation of the reporter gene (w^+) located in the transgene, which was always associated with a y^2-like phenotype. We followed the procedure of Dej *et al.* (1998), using PCR to clone and sequence the *gypsy* insertion site in each of the six strains. The results demonstrate that *gypsy* insertions occurred in the same *gypsy* target sites found previously in the endogenous ovo^{D1} sequence (Dej *et al.* 1998). Figure 2 shows the comparison of the *gypsy* target sites found previously and the six insertion sites identified in this work. Out of the six insertions, only one was found in a new site, very close to the other seven previously described. From these results we conclude that the signals necessary to determine the specificity of *gypsy* insertion into *ovo* reside within the 1.3-kb sequence present in the 5′ region of this gene.

The frequency of *gypsy* integration into *ovo* is lower in wild-type than ovo^{D1} females: In the course of performing the experiments designed to measure the frequency of *gypsy* insertion into the y^{+ovo} transgene, we carried out control experiments to determine the activity of *gypsy* in the *flam* strain by measuring the reversion rate of the ovo^{D1} allele. Fertile ovo^{D1}/ovo^{+} females arising in the offspring of a cross between ovo^{D1} males and *y f v mal flam* females occur after insertion of *gypsy* into the 5′ region of the ovo^{D1} locus in germ-line cells (Mevel-Ninio *et al.* 1989; Prud'homme *et al.* 1995). In a typical experiment, 86 revertant fertile females were found out of 926 ovo^{D1}/ovo^{+} females analyzed (Table 1). This result indicates that *gypsy* is actively transposing in the germ-line cells of these females. However, the frequency of insertions in the controls (9.3%) was significantly higher (χ^2 = 10.45; P = 0.0012) than the frequency of *gypsy* insertions in the y^{+ovo} transgene (3 and 3.8%). An impor-

TABLE 1

Frequency of *gypsy* insertion into y^{+ovo} transgenes

	Experiments			
Lines	A	B	C	Total (%)
Controls	37/406	26/291	23/229	86/926 (9.3)
P[y^{+ovo}*; *w^{+}*]*1.1(*X*)	3/140	1/57	3/35	7/232 (3)
P[y^{+ovo}*; *w^{+}*]*2.1(*2*)	2/52	1/27	—	3/79 (3.8)

Numbers represent fertile females in controls and females showing *gypsy* insertions in their offspring in y^{+ovo} transgenes (y^2 phenotypes) divided by the number of females in the sample. The percentage of *gypsy* insertions per sample is indicated in parentheses. A, B, and C are three independent experiments.

```
gtcgactgcaacagttggccatgaccaacagagcgagatggcgaatgcgtgatcggcatt   60
tgtaattgcacacactcctgtaattgcacaaacaacacatgcatgcacatacaaatatac  120
aaatgcaattacacactcgcaaagggccaagaaaaacacgcacacccacgccacaaagca  180

AAAACATACATATATATACACACACACACACACTACTCACTCACTTCGACAAAAAGCC    240
       A
TTGATCTTGATCGAAGTAACAACTTACAGTAACAGTGTTGTTGGTTTTCTTGCTTGCCTT  300

tttcgctgcacttgttttgttttaaagccaagacggctttttcagcggctaaccttgtct  360
       ovo A
ttgcccgtttgtctttgcacttgcacttgcaattggcgaatcgcaattgattaaaaaaa   420

GGCCACTTAATTTAACGTTTAACAAATCCGTTCGCTCAGCAAATGAACGTCAACAAAAAT  480
                                              M  N  V  N  K  N
GATCTTCGTAAGAATATCCGAGAGAGGGCGCTTTCAGCTTTGGTTTGAAAACAGCAGAAA  540
 D  L  R
AATAAAGCCGTTAAAATTGAATTTAACAGATTTTAATAAATAGTTTTAACTTAATGGTTC  600
AGCACATTTTAAATGTAACTGTTAATATGCATAGCCCTGTTTTAATTTTTAATTTCAAGT  660
                              8.2/8.1 (NEW)
TAATAACTTTTATTCACATCATACTCAGATAGTATATTTTAGTGTACTCTTTTGAGATAA  720

TTTCATCACTTGTTGATTCGTCGTTGGCAACTCTGCACCCACGACTACAGTTAGAATTAG  780
AATGACACACACCCATGGAATTGAAGAATGTAAGGAAGAAAGGGAGTGTGATCGAAAGTC  840
           ovo B
CGTTCCTTTTTACAGTTACATAGCAATCGTCCGAGCGAACGGACAGACAAATTTCTGAGA  900
           B                                                R
                                                       18.1 (C)
ATCGCACTTCTTTGCTTCTCTCATTTTCGGTGATTTTCCGTTGCTTTTTTATTGTGTGTG  960
  S  H  E  F  A  S  L  I  F                                CD
                                   10.1/10.2(G) /5.1(F)
CACTCGAAAGTTCTATTAGGTTCCACAGGGTTTTTATACATATGATTAATTCGTATTTAA  1020
                   E             FGH
AGTAACTTTGTGCACCTGATGCTAATTTAATTTTCTTCTCCTTTCTTGCAGAAAACTTTG  1080
```

Figure 2.—Binding sites for Ovo proteins and *gypsy* insertion sites in the *ovo* promoter region contained within the 1.3-kb 5′ *ovo* fragment. Highlighted sequences correspond to the Ovo recognition sequences as described by Lu *et al.* (1998). Bold underlined sequences indicate insertion sites A to H, as described by Dej *et al.* (1998). *Gypsy* insertion sites identified in this work are indicated with numbers above the previously described sites. All sites coincide with previous ones except 8.2 and 8.1, which define a new insertion site. Transcription start sites for RNAs encoding the OvoA and OvoB proteins are indicated. Numbers correspond to nucleotide positions starting at the zero nucleotide position of the *ovo* sequence (Mevel-Ninio *et al.* 1996).

tant distinction between control and experimental samples is that, in the former, *gypsy* insertions occur in ovo^{D1} sterile females, whereas in the latter, insertions occur in wild-type ovo^+ fertile females. To test whether the higher frequency observed in ovo^{D1} flies depends on the ovo^{D1}/ovo^+ genotype we performed an experiment to determine the frequency of *gypsy* insertions into the endogenous *ovo* gene in ovo^+/ovo^+ females.

Virgin females of the genotype *y v f mal flam* were crossed to ovo^+ males. Offspring females from this cross should carry *gypsy* particles produced in the ovaries of the *flam* mutant mothers and, therefore, insertions into the *ovo* gene should occur in their germ line with the same frequency as they occur in ovo^+/ovo^{D1} females. However, because these females are ovo^+ and perfectly fertile, insertions into the *ovo* gene of germ-line cells cannot be monitored using morphological or physiological traits. To detect these insertions we extracted genomic DNA from 50–100 progeny derived from the offspring of ovo^+/ovo^+ single female crosses and performed PCR amplifications using the primers indicated in materials and methods (see also Figure 3). These primers allow detection of *gypsy* insertions into the *ovo* gene in either orientation (Figure 3). Among 134 females, two positive samples were found, indicating that only 1.6% of the females produced offspring bearing *gypsy* insertions into the *ovo* gene. The same crosses were carried out in parallel but using ovo^{D1} males instead and detecting *gypsy* insertions by the reversion to fertility of ovo^{D1}/ovo^+ females. The results indicate that the *flam* females that produced 1.6% of progeny carrying *gypsy* insertions into *ovo* in an ovo^+/ovo^+ genotype produced 10% of progeny carrying *gypsy* insertions into *ovo* when the genotype is ovo^+/ovo^{D1}. This difference (1.6 *vs.* 10%) is similar to the difference found in the previous experiment, when comparing the frequency of *gypsy* insertions into the *ovo* gene in ovo^+/ovo^{D1} females (9.2%) with the frequency of insertions into the y^{+ovo} transgene (3 and 3.8%). Because the y^{+ovo} transgene is present in a strain with an ovo^+/ovo^+ genotype, the result suggests that the higher frequency of *gypsy* insertion into *ovo* might be due to the activity of the OvoD1 protein present only in ovo^+/ovo^{D1} females.

The timing of *gypsy* insertion during female germ-line development correlates with the expression pattern of Ovo isoforms: The 5′ region of the *ovo* gene contains several binding sites for Ovo proteins, suggesting an involvement of *ovo* in its own transcriptional regulation (Lu *et al.* 1998). Comparison of the location of *gypsy* insertion sites with that of binding sites for Ovo proteins shows that all *gypsy* insertion sites are located in close proximity to Ovo protein binding sites and are contained within the 5′ *ovo* region used to obtain the y^{+ovo} transgenic flies (Figure 2). The proximity between binding sites of Ovo proteins and insertion sites of *gypsy* in the *ovo* locus suggests the possibility of a functional correlation between the binding of Ovo proteins and *gypsy* insertion. Since the two protein products of *ovo,* OvoA and OvoB, bind to these sites and show distinct expression patterns and regulatory properties (Mevel-

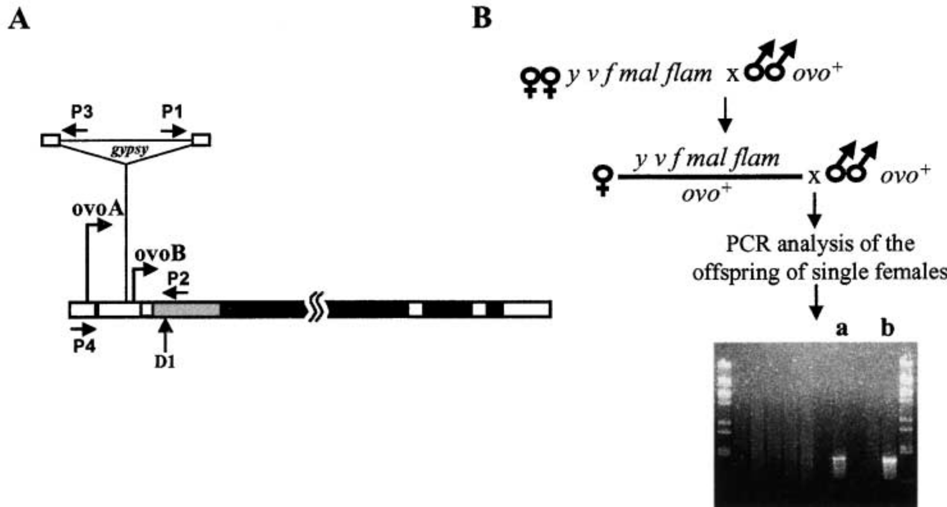

Figure 3.—PCR analysis of *gypsy* insertions in wild-type females. (A) Diagram representing a *gypsy* insertion in the 5′ *ovo* region. The positions of the P1, P2, P3, and P4 PCR primers are indicated. The location of the ovo^{D1} mutation (D1) is also indicated, although ovo^{D1} mutants were not used in this experiment. The same primers were used for cloning and sequencing the insertion sites shown in Figure 2. All insertions observed in Dej *et al.* (1998), except for one, were found between P4 and P2. (B) Experimental protocol for PCR amplification using the primers described in A. Lane a is an example of a positive signal in an experimental sample. Lane b is a control in which the PCR product was obtained from the offspring of an ovo^{D1} revertant female.

Ninio *et al.* 1996; Andrews *et al.* 2000), we decided to examine whether there is also a correlation between the expression of these two proteins and the timing of *gypsy* insertion. The OvoB protein is expressed from early stages of embryogenesis and throughout development and is necessary for the normal development of the germ line. The OvoA protein is expressed only during the later stages of oogenesis and antagonizes the function of OvoB by repressing transcription of the same genes whose expression is activated by OvoB (Mevel-Ninio *et al.* 1996; Andrews *et al.* 2000). To determine the time of *gypsy* insertion into *ovo* during female germ-line development we used the rationale depicted in Figure 4. The insertion of *gypsy* in a cell early during development of the female germ line will cause all or most stem cells in the germarium of each ovariole of the fully developed ovary to carry the same insertion (Figure 4A). An insertion at an intermediate time during development will result in only a few of the stem cells carrying the *gypsy* insertion (Figure 4B). Finally, an insertion at a very late stage of germ-line development will cause only a stem cell from a single ovariole in the whole ovary to produce oocytes carrying *gypsy* insertions (Figure 4C). An equivalent correlation was found when comparing the brood size of revertant ovo^{D1} females after somatic recombination induced by X rays at different stages of development (Perrimon 1984). Therefore, we can approximately determine the timing of *gypsy* insertion by counting the number of functional ovarioles in ovo^{D1}/ovo^{+} fertile females. Following a cross between *y v f mal flam* females and ovo^{D1} males, fertile ovo^{D1}/ovo^{+} female offspring were selected. After several days in fresh medium and in the presence of males, the ovaries of these females were dissected. A total of 118 ovaries dissected from 111 revertant females out of a total sample of 1219 ovo^{D1}/ovo^{+} females were analyzed (Table 2). The number of ovarioles in a fertile ovary is considered to be ∼15 (Spradling 1993). We counted the number of functional ovarioles in ovaries from ovo^{D1}/ovo^{+} revertant females and found that it varies between 1 and 11 or more (we considered ovaries with 11 or more ovarioles as a single class corresponding to a fully functional ovary). Figure 5A shows a histogram of the frequencies of ovaries containing a different num-

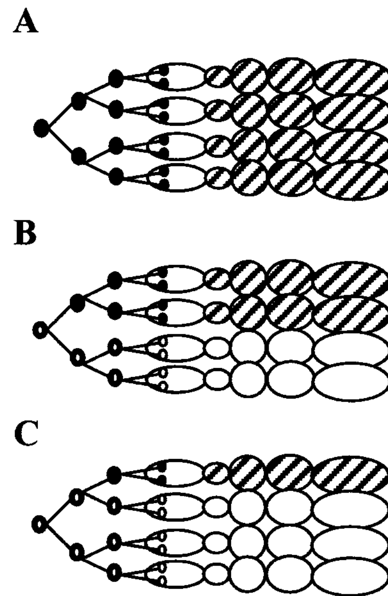

Figure 4.—Model representing cluster size in relation to time of insertion. (A) *Gypsy* insertion occurs early in the development of the female germ line. (B) Intermediate insertion time with only half of the ovarioles carrying a *gypsy* insertion. (C) Insertion of *gypsy* occurs late during germ cell differentiation. Only one ovariole produces oocytes arising from the cell where *gypsy* originally inserted. In this case, a single stem cell produces oocytes containing an insertion of *gypsy* into *ovo*.

TABLE 2

Number of ovarioles per revertant female

	flam	SS
Females	1219	1770
Revertant females	111	6
% revertant females	9.1	0.3
Revertant ovaries	118	6
% reversions of ovo^{D1}	9.7	0.3
Average no. ovarioles	3.9	—
% ovaries with 1 ovariole	16.3	—

ber of functional ovarioles. The most abundant class coincides with ovaries containing a single functional ovariole, which we considered as the result of *gypsy* insertion at a late stage. The least frequent class coincides with ovaries containing 11 or more functional ovarioles and corresponds to *gypsy* insertion at earlier stages of germ-line development. These results suggest that *gypsy* can insert into *ovo* at a wide range of stages of germ cell development in ovo^{D1}/ovo^{+} females. Interestingly, the probability of having a certain number of ovarioles increases significantly in a linear manner from $P = 0.017$ for 11 or more ovarioles to $P = 0.14$ for a single ovariole, with a regression coefficient of $r = -0.96$ and $P < 0.0001$ (Figure 5B).

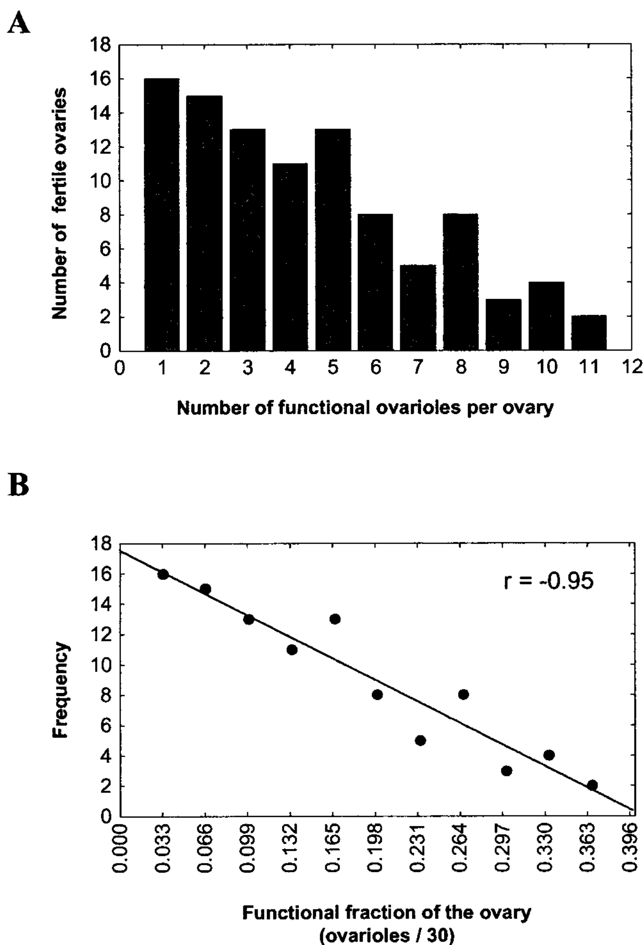

Figure 5.—Distribution of the number of ovarioles and cluster sizes after *gypsy* insertion into *ovo.* (A) Histogram showing the distribution of functional ovarioles per revertant ovary. (B) Correlation between the fraction of functional ovarioles and the frequency of each event. Note that the cluster size obtained for y^2-like flies using the transgene roughly corresponds to 0.033, the same frequency obtained after dividing a single ovariole by a total of 30 per ovary.

To determine whether *gypsy* insertions into the *ovo* gene in wild-type females occur at similar stages and frequencies as in ovo^{D1}/ovo^{+} females, we used the same rationale as in the previous experiment. For this purpose, we monitored the proportion of flies carrying *gypsy* insertions in the y^{+ovo} transgene ($y^{+ovo\text{-}gypsy}$ with a y^2-like phenotype) in the offspring of single female crosses. As in the previous experiment, a large cluster of flies with a y^2-like phenotype indicates an insertion into the transgene at an early stage (as in Figure 4A), whereas a small cluster indicates a later insertion during germline development (as in Figure 4C). Considering that each of the two ovaries of a healthy female contains ~15 ovarioles, and assuming that each ovariole contributes equally to the pool of oocytes, each ovariole should be responsible for ~1/30 of the eggs laid by a female. Table 3 shows the size of the clusters of flies with a y^2-like phenotype observed from each female producing y^2-like individuals in its offspring. We estimated the values for such clusters under the hypothesis that more than one ovariole was participating in the cluster (number of ovarioles divided by 30 times the observed number of offspring). Table 3 shows the expected numbers of y^2-like individuals among the total offspring if 1, 2, or 3.9 ovarioles were present in one ovary (*i.e.*, 1/30, 2/30, or 3.9/30 of the offspring; 3.9 is the average number of functional ovarioles obtained in the experiment using ovo^{D1} females). The observed *vs.* expected value of χ^2, considering all values together, shows that the y^2-like flies arose with a frequency not significantly different from 1/30 ($\chi^2 = 7.97$; $P = 0.5372$), whereas the differences are statistically significant when the expected values for 2 and 3.9 ovarioles are compared to the observed values (see Table 3). This result suggests that insertions of *gypsy* occur only during the latest stages of germ-line development in wild-type females, contrary to what is found in ovo^{D1} heterozygous females, where *gypsy* inserts also at early stages of development. This conclusion can be rationalized in the context of the developmental expression of the *ovo*-encoded proteins. In particular, the results show an increasing amount and a broader distribution of *gypsy* insertion events during development in the presence of the dominant negative $Ovo^{D1}B$ protein.

DISCUSSION

Site-specific integration of the retroviral genome can be mediated by protein-protein interactions between preintegration complexes and proteins that bind DNA

TABLE 3

Cluster size of y^2-like phenotypes in y^{+ovo} transgenes after crosses with *flam* permissive females

Cluster size	Frequency	$E_{1/30}$*	$E_{2/30}$**	$E_{3.9/30}$***
		$P[y^{+ovo}; w^+]1.1(X)$		
2/72	0.028	2.4	4.8	9.4
1/86	0.012	2.7	5.7	11.8
1/51	0.013	1.7	3.4	6.6
3/199	0.015	6.6	13.3	25.9
2/62	0.032	2.1	4.1	8.1
2/54	0.037	1.8	3.6	7
1/65	0.015	2.2	4.3	8.5
		$P[y^{+ovo}; w^+]2.1(2)$		
1/164	0.006	5.5	10.9	21.3
2/46	0.043	1.5	3.1	6
2/50	0.04	1.7	3.3	6.5

E represents the expected size for clusters of y^2-like flies considering that 1 (1/30), 2 (2/30), or 3.9 (3.9/30) ovarioles yield adult flies with a *gypsy* insertion in the y^{+ovo} transgene. The values of χ^2 when the expected cluster size is compared to the observed cluster size are (*) $\chi^2 = 7.97$, $P = 0.54$; (**) $\chi^2 = 29$, $P = 0.0006$; (***) $\chi^2 = 80.62$, $P < 0.00001$.

or are associated with chromatin (Bushman 1994; Kirchner *et al.* 1995; Bushman and Miller 1997; Zhu *et al.* 1999). Su(Hw) is a good candidate to mediate interactions leading to *gypsy* insertional specificity, but our results suggest that this protein is not directly involved in determining *gypsy* insertional specificity into *ovo*. This conclusion is based on the observation that a DNA fragment from the 5′ region of the *ovo* gene, where *gypsy* inserts with high frequency, does not display the properties of an insulator and therefore does not bind Su(Hw) protein *in vivo*. Alternatively, the ability of *gypsy* to insert specifically into the *ovo* gene could be developmentally regulated by the expression of the Ovo proteins, since the frequency of germ-line insertion events of *gypsy* is affected by the *ovo* genotype of the female. *Gypsy* insertions are significantly more frequent and occur along different stages of germ-line development in ovo^{D1} females, whereas they are less frequent and occur only at late stages in wild-type females. Since the number of *gypsy* integration complexes depends on maternal inheritance (Chalvet *et al.* 1999), and the *flam* mutant females used in experiments involving ovo^{D1} and wild-type females are the same, we conclude that the insertion frequency is determined by the influence of Ovo proteins on the insertion process. This conclusion is also supported by the observation that Ovo protein binding sites are found in close proximity to *gypsy* insertion sites in the *ovo* gene.

The *ovo* gene encodes two protein products, OvoA and OvoB, both containing the same zinc finger domains and therefore recognizing exactly the same DNA-binding sites (Garfinkel *et al.* 1994; Lee and Garfinkel 2000). The OvoB protein is expressed during all stages of germ-line development, including oogenesis, and it is necessary for the normal development of the germ line (Garfinkel *et al.* 1992; Mevel-Ninio *et al.* 1996). OvoA protein probably antagonizes the function of OvoB, repressing transcription of the same genes activated by OvoB including the *ovo* gene itself (Lu *et al.* 1998; Andrews *et al.* 2000). In the ovo^{D1} allele, translation of the ovoB transcript starts prematurely and the resulting protein has an additional amino-terminal domain ordinarily present only in the OvoA protein and responsible for its repressive activity (Mevel-Ninio *et al.* 1996; Andrews *et al.* 2000). Therefore, the OvoB product of the ovo^{D1} mutation ($Ovo^{D1}B$) is functionally equivalent to the OvoA protein but it is expressed earlier in development. Expression of $Ovo^{D1}B$ is equivalent to expression of OvoA and results in sterility of ovo^{D1} females by arresting oogenesis at stage 4.

The low frequency of *gypsy* insertions into the *ovo* locus in wild-type females correlates with the absence of the OvoA protein during most of the female germ cell development. Our results suggest that *gypsy* insertion events in wild-type females take place only during late stages of gonadal development. This conclusion is supported by the observation that y^2-like phenotypes due to *gypsy* insertion into the y^{+ovo} transgene occur at frequencies expected if the insertion takes place in a stem cell from a single ovariole, indicating that insertions never occur before this stage. In contrast, ovo^{D1} revertant females show ovaries containing a wide distribution of functional ovarioles. The presence of a high number of ovarioles in an ovary is a consequence of an early insertion event, whereas a single ovariole represents an insertion during the very last division of the stem cells in the germarium (Figure 4). Our interpretation of these results is that *ovo* is an open target for *gypsy* insertion only when the OvoA protein, or the equivalent $Ovo^{D1}B$, is present in the germ cells. In wild-type females, nonmaternal OvoA protein is present only at the end of gonadal development, whereas the expression of $Ovo^{D1}B$ occurs throughout germ cell development in ovo^{D1} females.

An alternative explanation for the observed results could be that the germ cells carrying a *gypsy* insertion in the *ovo* locus of ovo^{D1} mutants actually become phenotypically wild type and therefore outcompete the ovo^{D1} mutant cells during the process leading to the population of the ovary. This competition could explain why most revertant ovaries in ovo^{D1} females contain several ovarioles but fails to explain the large number of ovaries with a single ovariole. Also, the frequency distribution of the number of ovarioles per revertant ovary in ovo^{D1} females contradicts such explanation. The smaller the number of ovarioles in a revertant ovary from an ovo^{D1} female, the higher the frequency among revertant ovaries. If insertions took place only at a particular stage, the outcompetition would not produce a linear distribution of the frequencies. Rather, it would result in a

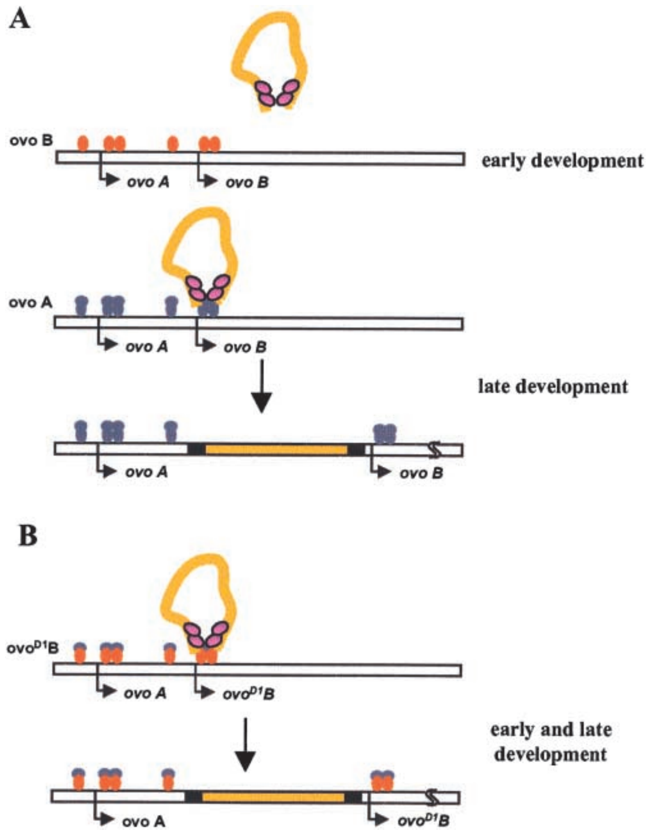

Figure 6.—A model to explain *gypsy* insertion site specificity. The cDNA of the *gypsy* preintegration complex is bound at the ends by integrase. (A) *Gypsy* does not target the *ovo* gene early in development, when the OvoB protein is present in the 5′ region of *ovo*. *Gypsy* is targeted to the promoter region of the gene only in the presence of OvoA at late stages of germ cell development. (B) Targeting of *gypsy* in *ovo*^{D1} mutants occurs in the same manner as in wild type, but earlier in development due to the presence of the Ovo^{D1}B protein.

normal distribution with an average that would correlate with the time at which insertions occur: the later the insertion the smaller the average. Additional observations supporting our interpretation are provided by experiments using X rays to induce somatic recombination in the germ line of Drosophila females during different developmental stages in wild-type as well as in *ovo*^{D1} females (Wieschaus and Szabad 1979; Perrimon 1984). Irradiation during early embryonic development, including blastoderm, results in a low frequency of large clones of recombinant oocytes, whereas irradiation during third instar larva, pupa, or adult flies causes very small clones with a higher frequency (Wieschaus and Szabad 1979). These results in wild-type females, where outcompetition is ruled out, are equivalent to the results obtained in experiments where *gypsy* inserts in *ovo* in an *ovo*^{D1} mutant female.

Current models to explain how *gypsy* reaches the germ-line cells of the offspring of *flam* females suggest that *gypsy* virus particles are present in the posterior region of the embryo before cellularization, providing an additional explanation for the linear distribution mentioned above (Song *et al.* 1997). Once the cytoplasmic membrane surrounds the nucleus of the pole cells, *gypsy* particles should be found in the cytoplasm of these cells. At this point the *gypsy* preintegration complexes must reach the nucleus to integrate into the genome. Among retroviruses, only lentiviruses have developed a mechanism to cross the nuclear envelope (Zennou *et al.* 2000). All other retroviruses depend on cell division to reach the nuclear DNA. Since it is very possible that *gypsy* also requires cell division to enter the nucleus, the probability of insertion into *ovo* will be higher at later stages of development, when more cell divisions have taken place. We should then expect a significant correlation between the size of the clusters of revertant ovarioles and the frequency of such clusters only if integration occurs with the same probability along all developmental stages. If insertion of *gypsy* depends on the activity of the OvoA protein this condition is met only in the case of the *ovo*^{D1} mutant.

The specificity of gypsy integration into *ovo* might be mediated by interactions between the amino-terminal domain of the OvoA protein and a component of the *gypsy* integration complex. This interaction might involve the repressive domain of OvoA and the *gypsy* integrase (Figure 6) in a similar manner to the *Ty5* integrase and Sir4 proteins (Zhu *et al.* 1999). Alternatively, *gypsy* integration might be mediated by the repressive activity of OvoA or Ovo^{D1}B on the chromatin structure of the *ovo* gene. Experiments to distinguish between these two alternatives are in progress.

We thank Kelly Baxter for her help during the cloning of the vector CaSpeR *yellow*. This work was supported by Public Health Service award GM56022 from the National Institutes of Health.

LITERATURE CITED

Andrews, J., D. Garcia-Estefania, I. Delon, J. Lu, M. Mevel-Ninio *et al.*, 2000 OVO transcription factors function antagonistically in the *Drosophila* female germline. Development **127:** 881–892.

Boeke, J. D., T. H. Eickbush, S. B. Sandmeyer and D. F. Voytas, 1999 *Metaviridae*. Springer-Verlag, New York.

Bushman, F. D., 1994 Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. Proc. Natl. Acad. Sci. USA **91:** 9233–9237.

Bushman, F. D., and M. D. Miller, 1997 Tethering human immunodeficiency virus type 1 preintegration complexes to target DNA promotes integration at nearby sites. J. Virol. **71:** 458–464.

Chalvet, F., L. Teysset, C. Terzian, N. Prud'homme, P. Santamaria *et al.*, 1999 Proviral amplification of the *gypsy* endogenous retrovirus of *Drosophila melanogaster* involves env-independent invasion of the female germline. EMBO J. **18:** 2659–2669.

Dej, K. J., T. Gerasimova, V. G. Corces and J. D. Boeke, 1998 A hotspot for the Drosophila *gypsy* retroelement in the *ovo* locus. Nucleic Acids Res. **26:** 4019–4025.

Garfinkel, M. D., A. R. Lohe and A. P. Mahowald, 1992 Molecular genetics of the *Drosophila melanogaster ovo* locus, a gene required for sex determination of germline cells. Genetics **130:** 791–803.

Garfinkel, M. D., J. Wang, Y. Liang and A. P. Mahowald, 1994 Multiple products from the shavenbaby-*ovo* gene region of *Drosophila melanogaster*: relationship to genetic complexity. Mol. Cell. Biol. **14:** 6809–6818.

Gerasimova, T. I., and V. G. Corces, 1998 Polycomb and trithorax group proteins mediate the function of a chromatin insulator. Cell **92:** 511–521.

Geyer, P. K., and V. G. Corces, 1992 DNA position-specific repression of transcription by a *Drosophila* zinc finger protein. Genes Dev. **6:** 1865–1873.

Geyer, P. K., C. Spana and V. G. Corces, 1986 On the molecular mechanism of *gypsy*-induced mutations at the yellow locus of *Drosophila melanogaster*. EMBO J. **5:** 2657–2662.

Kim, A., C. Terzian, P. Santamaria, A. Pelisson, N. Prud'homme *et al.*, 1994 Retroviruses in invertebrates: the *gypsy* retrotransposon is apparently an infectious retrovirus of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **91:** 1285–1289.

Kirchner, J., C. M. Connolly and S. B. Sandmeyer, 1995 Requirement of RNA polymerase III transcription factors for in vitro position-specific integration of a retrovirus like element. Science **267:** 1488–1491.

Lee, S., and M. D. Garfinkel, 2000 Characterization of *Drosophila* OVO protein DNA binding specificity using random DNA oligomer selection suggests zinc finger degeneration. Nucleic Acids Res. **28:** 826–834.

Lu, J., J. Andrews, D. Pauli and B. Oliver, 1998 *Drosophila* OVO zinc-finger protein regulates *ovo* and *ovarian tumor* target promoters. Dev. Genes Evol. **208:** 213–222.

Mevel-Ninio, M., M-C. Mariol and M. Gans, 1989 Mobilization of the *gypsy* and *copia* retrotransposons in *Drosophila melanogaster* induces reversion of the ovo^D dominant female-sterile mutations: molecular analysis of revertant alleles. EMBO J. **8:** 1549–1558.

Mevel-Ninio, M., E. Fouilloux, I. Guenal and A. Vincent, 1996 The three dominant female-sterile mutations of the *Drosophila ovo* gene are point mutations that create new translation-initiator AUG codons. Development **122:** 4131–4138.

Oliver, B., N. Perrimon and A. P. Mahowald, 1987 The *ovo* locus is required for sex-specific germ line maintenance in *Drosophila*. Genes Dev. **1:** 913–923.

Pelisson, A., S. U. Song, N. Prud'homme, P. A. Smith, A. Bucheton *et al.*, 1994 *Gypsy* transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila flamenco* gene. EMBO J. **13:** 4401–4411.

Perrimon, N., 1984 Clonal analysis of dominant female-sterile, germline-dependent mutations in *Drosophila melanogaster*. Genetics **108:** 927–939.

Prud'homme, N., M. Gans, M. Masson, C. Terzian and A. Bucheton, 1995 *Flamenco*, a gene controlling the *gypsy* retrovirus of *Drosophila melanogaster*. Genetics **139:** 697–711.

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

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of *Drosophila* with transposable element vectors. Science **218:** 348–353.

Song, S. U., T. Gerasimova, M. Kurkulos, J. D. Boeke and V. G. Corces, 1994 An env-like protein encoded by a *Drosophila* retroelement: evidence that *gypsy* is an infectious retrovirus. Genes Dev. **8:** 2046–2057.

Song, S. U., M. Kurkulos, J. D. Boeke and V. G. Corces, 1997 Infection of the germ line by retroviral particles produced in the follicle cells: a possible mechanism for the mobilization of the *gypsy* retroelement of *Drosophila*. Development **124:** 2789–2798.

Spana, C., and V. G. Corces, 1990 DNA bending is a determinant of binding specificity for a *Drosophila* zinc finger protein. Genes Dev. **4:** 1505–1515.

Spradling, A. C., 1993 Developmental genetics of oogenesis, pp. 1–70 in *The Development of Drosophila melanogaster*, edited by M. Bate and A. Martinez Arias. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wieschaus, E., and J. Szabad, 1979 The development and function of the female germ line in *Drosophila melanogaster*: a cell lineage study. Dev Biol. **68:** 29–46.

Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier *et al.*, 2000 HIV-1 genome nuclear import is mediated by a central DNA flap. Cell **101:** 173–185.

Zhu, Y., S. Zou, D. A. Wright and D. F. Voytas, 1999 Tagging chromatin with retrotransposons: target specificity of the *Saccharomyces Ty5* retrotransposon changes with the chromosomal localization of Sir3p and Sir4p. Genes Dev. **13:** 2738–2749.

Communicating editor: M. J. Simmons