Genomic Imprinting and Position-Effect Variegation in *Drosophila melanogaster*

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ABSTRACT

Genomic imprinting is a phenomenon in which the expression of a gene or chromosomal region depends on the sex of the individual transmitting it. The term imprinting was first coined to describe parent-specific chromosome behavior in the dipteran insect Sciara and has since been described in many organisms, including other insects, plants, fish, and mammals. In this article we describe a mini-X chromosome in *Drosophila melanogaster* that shows genomic imprinting of at least three closely linked genes. The imprinting of these genes is observed as mosaic silencing when the genes are transmitted by the male parent, in contrast to essentially wild-type expression when the same genes are maternally transmitted. We show that the imprint is due to the sex of the parent rather than to a conventional maternal effect, differential mitotic instability of the mini-X chromosome, or an allele-specific effect. Finally, we have examined the effects of classical modifiers of position-effect variegation on the maintenance and the establishment of the imprint. Factors that modify position-effect variegation alter the somatic expression but not the establishment of the imprint. This suggests that chromatin structure is important in maintenance of the imprint, but a separate mechanism may be responsible for its initiation.

GENOMIC imprinting is a phenomenon whereby a gene or a region of a chromosome is reversibly modified so that it retains a "memory" of its own genetic history. The term imprinting was originally coined to refer to the complex behavior of the X chromosome in the dipteran insect Sciara (Crouse 1960). The classical example of genomic imprinting involves a situation in which the activity of the imprinted gene or chromosome is determined by the sex of the parent that transmits it and the altered expression is limited to somatic tissue of the progeny; the germ-line DNA is not permanently altered. While imprinting was discovered in insects, imprinting has been most thoroughly described in mammals, where it appears to regulate the expression of developmentally important genes. Imprinting of both endogenous genes and transgenes has been well documented in mice (Sapienza 1989; Monk and Grant 1990; Surani 1998), resulting in the functional nonequivalence of the maternal and paternal genome. In humans a number of diseases are associated with either aberrant imprinting or the aberrant transmission of imprinted regions (Clarke 1990; Hall 1990). Genomic imprinting, however, is found in many other eukaryotes. These include sheep (Cockett *et al.* 1996), marsupials (Sharman 1971), maize (Kermicle and Alleman 1990), zebra fish (Martin and McGowan 1995a,b), and a variety of insects including the homopteran coccids (mealy bugs and other armored scale insects; Chandra and Brown 1975), the hymenopteran wasp, *Nasonia vitripennis* (Nur *et al.* 1988), the coleopteran beetle (the coffee berry borer beetle, *Hypothenemus hampei*; Brun *et al.* 1995), the dipterans, the fungus gnat Sciara (Crouse 1960), and the genetically well-characterized fruit fly *Drosophila melanogaster* (see Table 1 for references). The consequence of parental imprinting is far less drastic in many of these organisms. For example, gynogenic *D. melanogaster* are completely viable and fertile (Fuyama 1984), as are androgenic flies (Muller 1958; Komma and Endow 1995), suggesting that in these organisms the imprinted genes are not essential for early development. Likewise in zebra fish, the viability of parthenogenic (Streisinger *et al.* 1981) and androgenic (Corley-Smith *et al.* 1996) animals suggests that imprinting need not involve developmentally essential genes.

Imprinting phenomena have been recognized and studied in Drosophila for more than 50 years, albeit under the name of parental effects (Table 1). Curiously, all but one of the reported parental effects involved chromosome rearrangements that exhibit position-effect variegation (Table 1 and Figure 1). The one mutation not associated with position-effect variegation (*Uab*[1]) involves the Bithorax complex, which is regulated by chromatin-induced gene silencing (reviewed by Mihaly *et al.* 1998). Position-effect variegation is a process also intimately associated with changes in chromatin conformation. In position-effect variegation a fully functional euchromatic gene is variably inactivated as a consequence of its relocation adjacent to a broken

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TABLE 1

Imprinting (parental) effects in Drosophila

Chromosome	Direction of imprint[a]	Reference[b]
In(1)sc^{8} and *Dp(1;f)1187*[c]	Paternal	Prokofyeva-Belgovskaya (1947) Karpen and Spradling (1990)
Dp(1;4)w$^{m254.58a}$	Paternal	Spofford (1959, 1961); Hessler (1961)
In(1)w^{m2}[d]	Maternal	Hess (1970)
Dp(1;3)w^{VCO}	Maternal	Khesin and Bashkirov (1978)
In(1)w^{m4}	Maternal	Khesin and Bashkirov (1978)
In(3)Uab^{1}	Maternal[e]	Khuhn and Packert (1988)
T(1;2)dor^{var7}	Paternal	Demakova and Belyaeva (1988)
Dp(1;f)LJ9	Paternal	This work

[a] The direction of the imprint is arbitrarily listed as the parent producing offspring with mosaic or inactivated gene expression.

[b] In addition to these examples of genomic imprinting, the term genomic imprinting has also been applied to an apparently permanent change in gene activity (Dorn *et al.* 1993), a process called epimutation by Holliday (1987), and a nonparental chromatin effect in Drosophila (Bishop and Jackson 1996).

[c] *Dp(1;f)1187* is a derivative of *In(1)sc*^{8}.

[d] *In(1)w*^{m2} occurs in *D. hydei*; all others are found in *D. melanogaster*.

[e] As the imprinted homeotic transformations associated with *Uab*^{1} are likely due to a gain-of-function mutation, the direction of the imprint is defined as maternal, although the mutant phenotype is expressed only when the mutation is transmitted by the male parent.

segment of facultative (Cattanach and Perez 1970) or constitutive heterochromatin (Spofford 1976). The gene inactivation correlates with the adoption of heterochromatic morphology in the appropriate section of the polytene salivary gland chromosomes (Hartmann-Goldstein 1967). DNAse I studies also detect altered chromatin structure in variegating genes (Hayashi *et al.* 1990; Wallrath and Elgin 1995). Thus, most models of position-effect variegation propose that the genetic silencing is directly caused by the spread of heterochromatin, which packages the normally euchromatic region of DNA in such a way that the appropriate transcription factors cannot access the gene(s) located therein. An alternate model advocates that the genetic rearrangements associated with position-effect variegation could place the variegating reporter gene in a new compartment of the nucleus (Baker 1953; Hessler 1958; Wakimoto and Hearn 1990; Eberl *et al.* 1993; Talbert *et al.* 1994; Csink and Henikoff 1996). If one or more transcription factors essential for its expression are absent from this compartment, the gene would be silenced and chromatin structure would be altered as a consequence.

This article describes genomic imprinting of at least three genes on a mini-X chromosome in *D. melanogaster*. The imprinting phenomenon we describe has all of the, now classical, features of genomic imprinting: The imprint, once set, is mitotically stable, generating distinct clonal regions, but is reversed by passage through meiosis, and the imprinted region encompasses more than one gene and involves the formation of aberrant chromatin structures. Using this mini-X chromosome we have tested, and eliminated, a number of factors that might cause phenotypes that resemble genomic imprinting. Our findings suggest that the immediate cause of the imprinted expression of genes on the mini-X chromosome is parent-specific invasion of heterochromatin from an imprinted region of centric heterochromatin. To further assess the role of heterochromatin in the imprinting process, we have tested the stability of the imprint in this region using chemical, environmental, and genetic modifiers of position-effect variegation, all of which are believed to influence heterochromatin formation and integrity. These modifiers alter the somatic expression or maintenance of the imprinted state of these genes, but do not alter the initial decision of whether or not the gene is imprinted. This implies that altered chromatin structure is involved in the somatic memory of the imprint, but does not necessarily determine the imprint. Thus, the imprinting decision may be under independent genetic control.

MATERIALS AND METHODS

All crosses were performed at 22° unless stated otherwise. Culture media was standard cornmeal/yeast/sugar media supplemented with tegosept as a mold inhibitor. Crosses were generally carried out in 8-dram shell vials with groups of three to five virgin females and two to five males per vial. Each vial was subcultured once at a 1-wk interval before the parents were discarded and each experiment was replicated four to six times in two different groups separated by at least 1 mo to control for environmental effects such as variation in media, temperature, humidity, and so forth. The results of the replicate experiments were combined as there were no differences between them.

Mutant strains: All mutant strains are described in Lindsley and Zimm (1992). The *Dp(1;f)LJ9* mini-X chromosome was

induced by X-ray deletion of most of the X chromosome euchromatin from *In(1)sc^{29}* (Hardy *et al.* 1984). The *In(1)sc^{29}* chromosome is an inversion between the tip of the X chromosome (1B) and the region proximal to the *garnet* gene (13A2-5), thus placing the *garnet* region near the tip of the X chromosome. The X-ray-induced deletion removed most of the euchromatin; only the euchromatic bands from 12A10 to 13A2 and the distal tip, 1A1 to 1B, remain. This region is appended to the heterochromatin of the X chromosome. The general structure and origin of the *Dp(1;f)LJ9* mini-X chromosome is diagrammed in Figure 1. Although not mentioned by Hardy *et al.* (1984), it may be assumed that the heterochromatin was broken by the X-ray treatment because the most proximal euchromatic genes are deleted and variegation for a number of genes adjacent to the heterochromatin is observed. The *Dp(1;f)LJ9* mini-X chromosome is carried as a free duplication balanced against an attached *XX*, *y*$^{-}$ and attached *XY*, *Bar y*$^{-}$ *g*^{l} chromosome in females and males, respectively. As the mini-X chromosome contains cytological division 12, a region that is diplo-lethal in males (Belote and Lucchesi 1980), the attached XY stock also carries the deficiency *g*^{l} on the X chromosome. The *Dp(1;f)LJ9* mini-X chromosome stock was generously provided by Dr. J. Waring.

Expression of *garnet*, *tiny*, and *narrow abdomen*: The level of expression of the genes *garnet (g)*, *tiny (ty)*, and *narrow abdomen (na)* was assessed visually using an arbitrary scoring system. Each fly was assigned a score of 0, 1/2, or 1 on the basis of whether it expressed an extreme mutant, a moderate mutant, or a wild-type phenotype, respectively. The value for the average phenotype was then obtained by dividing the cumulative score by the total number of flies. Thus the value is expressed as a proportion of 1 (full wild-type expression) plus or minus the standard error of the mean. A fly was scored as *ty* if its bristles appeared severely Minute. In practice this meant approximately half the length of regular bristles on the sibs. A fly was scored as *na* if the abdomen appeared exceptionally long and thin. As this is a rather subjective measurement the crosses were scored by one investigator (V.K.L.) always comparing flies grown under the same conditions (*i.e.*, on the same set of media at the same time). The crosses were scored as a single blind experiment. A fly was scored as mutant for *garnet* if its eyes were completely or extensively variegated. In addition to visual scoring, the expression of the *garnet* gene was quantified by measuring the amount of pigment in the eyes as described in Lloyd *et al.* (1997) and expressed as a percentage of wild-type gene expression.

Malpighian tubules: Malpighian tubules were dissected from wandering third instar larvae of the correct sex and genotype, placed in water, and immediately photographed. Wild-type Malpighian tubules are always uniformly pigmented, unless damaged in removal.

Crosses: *Parental-effect crosses:* Individuals with a maternally derived mini-X chromosome were generated by crossing *XX/Dp(1;f)LJ9* females to *y z^{a} g^{53d}/Y*, *na/Y* or *ty/Y* males to generate *g/Dp(1;f)LJ9* or *na/Dp(1;f)LJ9* or *g^{2} ty/Dp(1;f)LJ9* male progeny. In the first case, *yellow (y^{1})* is used to monitor the presence of the mini-X chromosome without bias as to eye phenotype, and *zeste (z^{a})* lightens the background *garnet* (*g^{53d}*) eye color. Individuals with maternally or paternally derived mini-X chromosomes will be designated as *Dp(1;f)LJ9*$^{\mathrm{MAT}}$ or *Dp(1;f)LJ9*$^{\mathrm{PAT}}$, respectively. Individuals with a paternally derived mini-X chromosome (*Dp(1;f)LJ9*$^{\mathrm{PAT}}$) were generated by mating attached XY males carrying the *Dp(1;f)LJ9* mini-X chromosome (*XY/Dp(1;f)LJ9)* to females homozygous for the same *yellow*, *zeste*, and *garnet* alleles described above or to *na/In(1)dl49* or *g^{2} ty/In(1)dl49* females. The genotypically identical *y z^{a} g53d/Dp(1;f)LJ9* or *na/Dp(1;f)LJ9* or *g^{2} ty/Dp(1;f)LJ9* males were compared to those resulting from the maternal cross. The crosses used most frequently to detect parental effects on expression of the *garnet* gene are diagrammed in Figure 1B. To generate female progeny with the mini-X chromosome the following crosses were performed: For the maternal cross, *y z^{a} g^{53d}/y z^{a} g^{53d}/Dp(1;f)LJ9* females were crossed with *y z^{a} g^{53d}/Y* males to produce *y z^{a} g^{53d}/y z^{a} g^{53d}/Dp(1;f)LJ9*$^{\mathrm{MAT}}$ female progeny. For the paternal cross, *y z^{a} g^{53d}/y z^{a} g^{53d}* females were crossed with *y z^{a} g^{53d}/Y/Dp(1;f)LJ9* males to produce the genotypically identical *y z^{a} g^{53d}/y z^{a} g^{53d}/Dp(1;f)LJ9*$^{\mathrm{PAT}}$ female progeny. *y z^{a} g^{53d}/Dp(1;f)LJ9*$^{\mathrm{PAT}}$ and *y z^{a} g^{53d}/Dp(1;f)LJ9*$^{\mathrm{PAT}}$*/Y* progeny were identified by fertility testing. Sterile males were assumed to be the *X/Dp(1;f)LJ9* genotype.

Meiotic and mitotic stability of the mini-X chromosome: The meiotic stability of the mini-X chromosome was monitored by examining the progeny of the *garnet* parental effect crosses (Figure 1B) for exceptional individuals. Mitotic stability was monitored by checking for yellow patches (loss of the mini-X chromosome), on a wild-type background, in the cuticle of the *y z^{a} g53d/Dp(1;f)LJ9* males.

Maternal effects: We tested for a direct maternal effect of the *garnet* gene by determining the amount of pigment in homozygous daughters (*y z^{a} g^{53d}/y z^{a} g^{53d}*) and hemizygous sons (*y z^{a} g^{53d}/Y*) derived from *garnet* homozygous *vs.* heterozygous females (*y z^{a} g^{53d}/y z^{a} g^{53d} vs. y z^{a} g^{53d}/+ + + ⊗ y z^{a} g^{53d}/Y*).

We tested for the effect of maternal-effect modifiers of position-effect variegation by comparing the genotypically identical *y z^{a} g^{53d}/Dp(1;f)LJ9*^{PAT} male progeny derived from females without the *Dp(1;f)LJ9* mini-X chromosome (Figure 1B, maternal cross) *vs.* females bearing but not transmitting the *Dp(1;f)LJ9* mini-X chromosome (*y z^{a} g^{53d}/y z^{a} g^{53d}/Dp(1;f)LJ9 ⊗ XY/Dp(1;f)LJ9*). As two mini-X chromosomes are lethal due to the presence of a male diplo-lethal region (Belote and Lucchesi 1980) and the attached XY chromosome disjoins normally from the free duplication, the resulting *y z^{a} g^{53d}/Dp(1;f)LJ9* progeny necessarily have a paternally derived mini-X chromosome as the *y z^{a} g^{53d}* chromosome is transmitted by the mother.

Test for allele specificity of the imprinting effect: To determine if other *garnet* alleles showed the same pattern of imprinting effects, the *y z^{a} g^{53d}* chromosome was replaced with *y z^{a} g^{50e}*, *y e(g) cv g^{2}*, *g^{1}*, *g^{3}*, *g^{4} m*, *g^{61}*, or with *g^{S3}*.

Test for direct effect of Y chromosome: To determine if the presence of a Y chromosome would induce variegation of the *garnet* gene in the *Dp(1;f)LJ9* mini-X chromosome, females, with and without a Y chromosome, transmitting a normally nonvariegating, mini-X chromosome, were crossed to the *y z^{a} g^{53d}/Y* test males. The cross to produce females without a Y chromosome is as shown in Figure 1B (maternal cross). The cross to produce females with a Y chromosome is as follows: *XX/Dp(1;f)LJ9 ⊗ y z^{a} g^{53d}/Y → XX/Y/Dp(1;f)LJ9 ⊗ y z^{a} g^{53d}/Y → y z^{a} g^{53d}/Dp(1;f)LJ9*. *X/Dp(1;f)LJ9* and *X/Y/Dp(1;f)LJ9* progeny were separated by testing for fertility. Sterile males were assumed to be *X/Dp(1;f)LJ9*.

Effect of modifiers of position-effect variegation on imprinting: The effect of sodium butyrate and developmental temperature on the variegation (maintenance) and imprinting (establishment) on *garnet* expression was determined by replicating the parental-effect cross (Figure 1B) on media supplemented with various concentrations of sodium butyrate, or at 18°, 22°, 25°, and 29°, respectively. To test the effect of these modifiers on the variegation (maintenance of the imprint), the *y z^{a} g^{53d}/Dp(1;f)LJ9* progeny were raised under the experimental conditions. To test the effect of the modifiers on imprinting (establishment), the parents were raised under the experimental conditions and their untreated *y z^{a} g^{53d}/Dp(1;f)LJ9* progeny were assayed. To test the effect of butyrate and temperature on the expression of the *g^{50e}* allele an analogous set of experiments was done in which the *y z^{a} g^{53d}* chromosome was replaced

with *y z^a g^{50e}*. The effect of butyrate on the variegation of the conventional euchromatic variegator, *In(1)w^{m4}*, was used to monitor the effectiveness of the treatment. Variegation of *In(1)w^{m4}* males and females ranged from 1 ± 1 and 14 ± 3%, respectively, at 0 mm butyrate to 18 ± 6 to 23 ± 3% wild-type gene expression, respectively, at the test concentration of 200 mm butyrate. Higher concentrations were lethal. To monitor nonspecific effects (such as effect on fly or eye size) of both butyrate concentration and temperature, pigment levels of the phenotypically wild-type siblings were determined. Increasing levels of sodium butyrate did decrease both fly size and viability; pigment levels of the phenotypically wild-type XX/Dp siblings ranged from 98 ± 4% at 0 mm butyrate to 87 ± 3% at 200 mm butyrate. Hence, the experimental pigment values were adjusted by expressing pigment levels relative to these *garnet*$^+$ sibs reared on the same butyrate-containing media. No such effects were noted for the temperature experiments. However, there is limited information for the 29° series of paternal crosses as the *XY/Dp* males were generally sterile when raised at this temperature.

The effect of extra heterochromatin, in the form of an additional Y chromosome, was monitored by introducing an extra Y chromosome into *y z^a g^{53d}/Dp(1;f)LJ9* individuals in which the mini-X chromosome was derived either maternally or paternally. In the maternal cross *XX/Y/Dp(1;f)LJ9* ⊗ *y z^a g^{53d}/Y* → *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT} and *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT}*/Y*, these progeny were separated by fertility testing. Sterile males were assumed to be *X/Dp(1f)LJ9*. In the paternal cross *y z^a g^{53d}/Y/Dp(1;f)LJ9* ⊗ *y z^a g^{53d}/y z^a g^{53d}* → *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT} and *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT}*/Y*, progeny were separated by fertility testing and sterile males were assumed to be *X/Dp(1;f)LJ9*.

The effect of extra heterochromatin in parents was determined in four ways: by adding an extra Y chromosome in the male and female parents, both with and without the *Dp(1;f)LJ9* mini-X chromosome. In all cases the results were assessed by visual inspection and by microflourimeter pigment assays.

Set 1: To determine if an additional Y chromosome in the mini-X chromosome-bearing father affected imprinting, genotypically identical *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT} progeny were generated from *XY* + *Dp(1;f)LJ9* and in separate, concurrent crosses, *XYY* + *Dp(1;f)LJ9* fathers. [*XX/Dp(1;f)LJ9* ⊗ *XY* (any male) → *XX/Y/Dp(1;f)LJ9* ⊗ *XY/Dp* → *XY/Dp(1;f)LJ9* or *XY/Y/Dp(1;f)LJ9* ⊗ *y z^a g^{53d}/y z^a g^{53d}* → *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT}. *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT} progeny were distinguished from their *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT}*/Y* siblings by fertility testing.]

Set 2: To determine if an extra Y chromosome in mini-X chromosome-bearing mothers affected imprinting, genotypically identical *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT} progeny were generated from *XX* + *Dp(1;f)LJ9* and, in separate, concurrent crosses, *XXY* + *Dp(1;f)LJ9* mothers. [*XX/Dp(1;f)LJ9* ⊗ any male → *XX/Y/Dp(1;f)LJ9 vs. XX/Dp(1;f)LJ9* ⊗ *y z^a g^{53d}/Y* → *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT}. *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT} progeny were distinguished from their *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT}*/Y* siblings by fertility testing.]

Set 3: To determine if an extra Y chromosome has an effect in the male parent in the absence of the mini-X chromosome, genotypically identical *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT} progeny were generated from XY (no mini-X chromosome) and in separate, concurrent crosses, XYY (no mini-X chromosome) fathers. [*XY/0* ⊗ *XX/Y* → *XY/0 vs. XY/Y* ⊗ *y z^a g^{53d}/y z^a g^{53d}/Dp(1;f)LJ9* → *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT}. *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT} progeny were distinguished from their *y z^a g^{53d}/Dp(1;f)LJ9*^{MAT}*/Y* siblings by fertility testing.]

Set 4: To determine if an extra Y chromosome has an effect in the female parent unrelated to the mini-X chromosome, genotypically identical *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT} progeny were generated from *XX* (no mini-X chromosome) and in separate, concurrent crosses, from *XXY* (no mini-X chromosome) mothers. [*XY/Dp(1;f)LJ9* ⊗ *y z^a g^{53d}/y z^a g^{53d}* → *y z^a g^{53d}/XY vs. y z^a g^{53d}/y z^a g^{53d}* ⊗ *XY/Dp(1;f)LJ9* → *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT}. *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT} progeny were distinguished from their *y z^a g^{53d}/Dp(1;f)LJ9*^{PAT}*/Y* siblings by fertility testing.]

RESULTS

***garnet*$^+$ expression on the mini-X chromosome is imprinted:** The expression of the *garnet*$^+$ gene on the *Dp(1;f)LJ9* mini-X chromosome depends on its parental origin. When females carrying the mini-X chromosome (*XX/Dp(1;f)LJ9*) are crossed to *y z^a g^{53d}/Y males, the y z^a g^{53d}/Dp(1;f)LJ9* male progeny appear wild type. This is the expected phenotype because the mini-X chromosome carries the wild-type genes for *yellow* and *garnet*. In contrast, in the reciprocal cross (males carrying the

Figure 1.—Diagram of the structure and origin of the *Dp(1;f)LJ9* mini-X chromosome. (A) The top part diagrams the wild-type chromosome. The middle part shows the structure of the *In(1)sc^{29}* chromosome, and the bottom part shows the structure of the *Dp(1;f)LJ9* mini-X chromosome. The relative positions of the *narrow abdomen*, *tiny*, and *garnet* genes are shown. (B) The crosses used to generate males and females bearing maternally or paternally derived mini-X chromosomes and the results on expression of the *garnet*$^+$ gene are summarized. In all cases, the genotypes of the offspring being compared differ only in the parental origin of the mini-X chromosome. They are otherwise genotypically identical and isogenic. In most of the following experiments males of *y z^a g^{53d}/Dp(1;f)LJ9* were used to monitor the imprint.

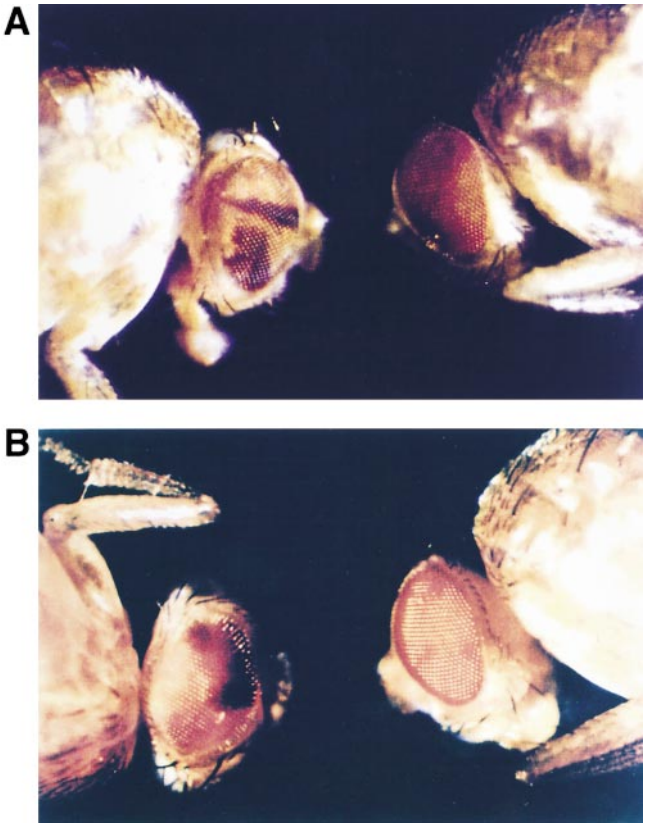

Figure 2.—The *garnet* phenotype in flies with paternally or maternally inherited *Dp(1;f)LJ9* mini-X chromosomes. (A) Two male flies of identical genotype: $y\ z^a\ g^{53d}/Dp(1;f)LJ9$. The fly on the left, with variegated eyes, has a paternally transmitted mini-X chromosome whereas the fly on the right bears a maternally transmitted mini-X chromosome. (B) Two female flies of identical genotype: $y\ z^a\ g^{53d}/y\ z^a\ g^{53d}/Dp(1;f)LJ9$. The fly on the left, with variegated eyes, has a paternally transmitted mini-X chromosome whereas the fly on the right bears a maternally transmitted mini-X chromosome. The crosses used to generate these flies are given in materials and methods. These phenotypes persist for one generation only. For example, both the females shown in B will transmit nonvariegating mini-X chromosomes regardless of whether they themselves show variegation for the *garnet* gene.

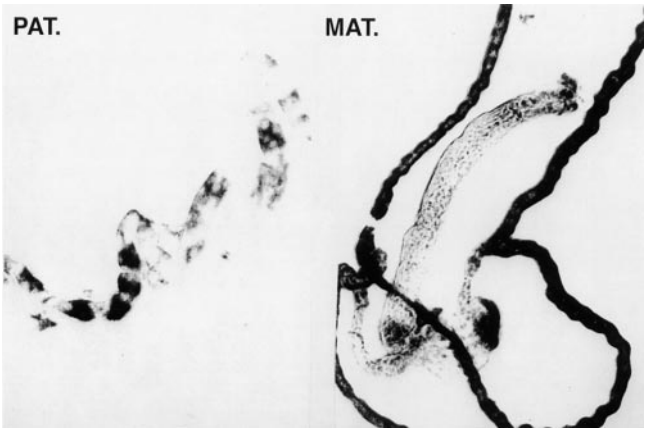

Figure 3.—The *garnet* phenotype in Malpighian tubules bearing maternally and paternally derived *Dp(1;f)LJ9* mini-X chromosome. (Left) A typical Malpighian tubule from a male of the genotype $y\ z^a\ g^{53d}/Dp(1;f)LJ9^{PAT}$. The dark areas are individual pigmented cells. The clear areas are regions in which no pigment is produced. (Right) A Malpighian tubule from a male of the identical genotype $y\ z^a\ g^{53d}/Dp(1;f)LJ9^{MAT}$. In this case, only one unpigmented region is seen, although usually there are none.

mini-X chromosome crossed to females of the same $y\ z^a\ g53d$ strain; Figure 1B), the wild-type *garnet* gene on the mini-X chromosome is variably silenced in the genotypically identical $y\ z^a\ g^{53d}/Dp(1;f)LJ9$ sons (Figure 2). The inactivation appears complete at the cellular level but not necessarily at the tissue level, so the phenotype is often a mosaic. In most cases the gene was expressed in zero, one, two, or three large wild-type spots ($garnet^+$) on the pale orange background color with occasional single pigmented ommatidium. The large spots appeared to correspond to clonally related regions of the eye (Janning 1970). The parent-dependent silencing of the *garnet* gene when the mini-X chromosome is derived from the paternal parent is seen in both male and female progeny (Figure 2, A and B) and persists for only one generation (data not shown). Therefore the expression of the wild-type *garnet* gene is dependent on the sex of the parent transmitting the mini-X chromosome and is reset by passage through the germ line. This situation constitutes a classical example of genomic imprinting.

The parent-dependent expression of *garnet* gene on this mini-X chromosome is not limited to the eye. Examination of Malpighian tubules in individuals bearing maternally *vs.* paternally derived mini-X chromosomes demonstrates that the expression of the $garnet^+$ gene in this tissue is also dependent on the parental origin of the mini-X chromosome. Malpighian tubules from individuals with a maternally derived mini-X chromosome usually have no unpigmented spots, although we have found up to three unpigmented regions in some individuals of this genotype. In contrast, the Malpighian tubules of genotypically identical individuals with a paternally derived mini-X chromosome show extensive variegation (Figure 3).

Imprinted expression of *narrow abdomen* and *tiny*: The imprinting effect is not restricted to the *garnet* gene. The closely linked genes *narrow abdomen* and *tiny* also show differential expression depending on the parental origin of the mini-X chromosome. When the mini-X chromosome was derived from the father, the wild-type gene on the mini-X chromosome showed variable mutant expression (Figure 4, A and B), whereas, when the mini-X chromosome was maternally inherited, the genotypically identical progeny (*na*/*Dp(1;f)LJ9* or *ty*/*Dp(1;f)LJ9*) were more wild type for body and bristle morphology, respectively. Thus the imprinting of the genes *narrow abdomen* and *tiny* is similar to that of the *garnet* gene with expression compromised when the mini-X

Figure 4.—Phenotype of *narrow abdomen* and *tiny* in flies bearing maternally or paternally derived *Dp(1;f)LJ9* mini-X chromosomes. (A) Two genetically identical flies bearing a mutation for the *narrow abdomen* (na^1) gene on the standard X chromosome and the wild-type allele of *narrow abdomen* on the mini-X chromosome (na^1/*Dp(1;f)LJ9*, na^+). The individual on the left bears a paternally derived mini-X chromosome whereas the genotypically identical fly on the right has a maternally derived mini-X chromosome. Arrows point to the abdomen, which is considerably more extended (more mutant) in the flies bearing a paternally derived mini-X chromosome. (B) Thoracic bristles from flies with a mutation for the *tiny* (ty^1) gene on the regular X chromosome and the wild-type *tiny* gene on the mini-X chromosome (ty^1/*Dp(1;f)LJ9*, ty^+). The individual on the left bears a paternally derived mini-X chromosome. The genotypically identical fly on the right bears a maternally derived mini-X chromosome. Arrows point to the thoracic bristles, which are smaller and finer (more mutant) in the flies bearing a paternally derived mini-X chromosome.

chromosome is transmitted by the male parent (Table 2). Interestingly, the magnitude of this effect (the relative difference in gene expression between maternally and paternally transmitted mini-X chromosomes) appears to diminish for the more distally located genes. Thus, the same genomic imprinting effect seems to encompass at least three genes in the small euchromatic portion of the mini-X chromosome and this effect appears to spread from the region of centric heterochromatin.

The imprint is a response to the sex of the parent: In Drosophila, the entirely heterochromatic Y chromosome is a potent modifier of position-effect variegation, as well as several other processes (Spofford 1976). Thus, it is possible that the imprinting observed in these experiments was due to a direct interaction between the Y chromosome and the mini-X chromosome in the male parent. Because, in *D. melanogaster*, the Y chromosome does not determine the sex of an individual, the impact of the Y chromosome on genomic imprinting can be separated from the sex of the parent. Specifically, if the Y chromosome causes the imprint, the progeny of XXY females should be imprinted. This was not the case; $y\ z^a\ g^{53d}$/*Dp(1;f)LJ9*^{MAT} (maternally derived mini-X chromosome) progeny from XXY females were not different from the genotypically identical progeny of XX females (94 ± 4 *vs.* 100 ± 3% wild-type pigmentation, respectively; Table 8, section 2). Thus, the expression of the genes on the mini-X chromosome depends on the physiological sex of the parent and not the presence or absence of a Y chromosome.

The imprinted expression of genes on the mini-X chromosome is not due to chromosome loss, maternal effects, or allele-specific interactions: The parent-specific silencing of the *garnet*$^+$ gene could be a consequence of genomic imprinting, or, alternatively, it could result from chromosome loss or masking of the mutant phenotype by a maternal effect, or it might merely reflect an allele-specific phenotypic oddity. Chromosome loss was monitored by testing the stability of the mini-X chromosome through both meiotic and mitotic cell divisions. We found that the mini-X chromosome disjoins regularly from an attached XY chromosome in males;

TABLE 2

Expression of the *garnet*$^+$, *tiny*$^+$, and *narrow abdomen*$^+$ genes on the *Dp(1;f)LJ9* mini-X chromosome

	Expression of genes on the mini-X chromosome		
	narrow abdomen (na)	*tiny (ty)*	*garnet (g)*
Maternally derived mini-X chromosome	0.67 ± 0.02	0.52 ± 0.02	0.86 ± 0.02
Paternally derived mini-X chromosome	0.51 ± 0.02	0.24 ± 0.02	0.06 ± 0.02

Expression of *garnet*$^+$, *narrow abdomen*$^+$, and *tiny*$^+$ in genotypically identical flies bearing either a paternally or a maternally derived *Dp(1;f)LJ9* mini-X chromosome. The level of variegation for three closely linked wild-type genes, *garnet* (position 44.4), *narrow abdomen* (position 44.5), and *tiny* (position 45.2), on the *Dp(1;f)LJ9* mini-X chromosome was assessed visually as described in the materials and methods. Data are given as mean ±SEM of wild-type expression. *n* for *narrow abdomen*: MAT cross = 397, PAT cross = 226. *n* for *tiny*: MAT cross = 317, PAT cross = 141. *n* for *garnet*: MAT cross = 267; PAT cross = 489. MAT, maternally derived; PAT, paternally derived. The crosses are described in materials and methods.

the nondisjunction rate is 0.1–0.3% ($n = 1546$). The rate of nondisjunction of the mini-X from an attached XX chromosome during meiosis in females is higher: 16% ($n = 745$). This presumably represents segregation via the aschiasmate (distributive) segregation system. As mitotic nondisjunction might generate patches of mutant tissue resembling those seen in the progeny of males transmitting the mini-X chromosome, we also examined the mitotic segregation of the mini-X chromosome. Because the mini-X chromosome is *yellow*$^+$, mitotic nondisjunction was monitored by looking for mosaic patches of *yellow* tissue in a wild-type background, in a fly bearing a mutant *yellow* gene on its normal X chromosome. Only two instances of mosaicism (both bilateral mosaics) were observed in over 10,000 flies. Therefore we conclude that mitotic nondisjunction does not occur at an appreciable rate, at least in the integument. These data indicate that the mini-X chromosome is transmitted faithfully through both meiosis and mitosis. Therefore, the mosaic expression of the *garnet*$^+$ gene in the eye and Malpighian tubules is probably not due to either meiotic nondisjunction or mitotic loss of the mini-X chromosome.

Maternal effects: The apparent repression of *garnet*, *narrow abdomen*, and *tiny* genes in those individuals who inherit the mini-X chromosome from their male parent could reflect a long perduring maternal effect rather than genomic imprinting. In this scenario, the *garnet*$^+$, *tiny*$^+$, and *narrow abdomen*$^+$ genes on the mini-X chromosome would have to variegate, more or less equally, regardless of parental origin, but the female parent would deposit wild-type product into the egg cytoplasm to mask this variegation in the affected offspring. As the only wild-type alleles of these three genes in these crosses are on the mini-X chromosome, the result would mimic a classical imprinted phenotype. While this is formally possible, it is highly unlikely in the crosses described here. This hypothesis requires that the *garnet*, *narrow abdomen*, and *tiny* genes all display maternal effects. The *garnet* gene is genetically well characterized, and of the more than 40 alleles examined, none has been reported to have a maternal effect (Lindsley and Zimm 1992). We confirmed that the allele used most extensively in this study (g^{53d}) does not show a maternal effect on eye pigmentation. Reciprocal crosses between the g^{53d} allele (including the same chromosome markers used in the imprinting crosses, *i.e.*, $y\ z^a\ g^{53d}$) and wild type were done. The amount of eye pigment in homozygous g^{53d} female and hemizygous g^{53d}/Y male progeny derived from g^{53d} homozygous mothers is 10 ± 1 and 12 ± 2% of wild type, respectively; whereas the same genotypes derived from heterozygous mothers have pigment levels of 4 ± 1 and 6 ± 1% of wild type, respectively. Thus there is no evidence that the presence of a wild-type copy of the *garnet* gene in the mother increases eye pigmentation in the progeny and we can conclude that the original parent-dependent difference in *garnet* expression is not caused by a persistent maternal effect of the *garnet*$^+$ gene.

Maternal-effect modifiers and physiological compensation: Another possible explanation for the imprinted effect is a form of physiological compensation. Such "physiological compensation" models have been proposed to account for the imprinted effect in maternal diabetes and maternal phenylketonuria and a paternal "imprint" in phenylketonuria, congenital hypothyroidism (Van Dyke and Weiss 1986), and Huntington's chorea (Bird *et. al.* 1974). These models propose that the mother can provide factors to the embryo that reduce the severity of the imprint—but only if these factors have been induced in the mother by the presence of the imprinted region or gene. In this case, this model would demand that if a female transmitted the mini-X chromosome, she would also be induced to make and transmit variegation-suppressing products to the zygote. To produce an imprinting-like phenotype, these products would have to exist unexpressed in the somatic tissue of the female parent with their expression triggered, or products activated, only in the egg, and only in the presence of the mini-X chromosome. Although this scenario seems somewhat unlikely, we tested this possibility because some variegation-modifying [*Su(var)*] mutations have been shown to have maternal effects (Grigliatti 1992; Sinclair *et al.* 1992). We generated progeny with a paternally derived, imprinted, mini-X chromosome from mothers who also possessed, but did not transmit, a mini-X chromosome. If the mini-X chromosome did stimulate the production and transmission of compensatory factors in the female parent, there should be no variegation of the *garnet* gene among their progeny. We found no evidence of such physiological compensation. Progeny with paternally derived mini-X chromosome ($y\ z^a\ g^{53d}/Dp(1;f)LJ9^{PAT}$), produced by non-mini-X chromosome-bearing mothers, had eye pigment levels that were 48 ± 2% of wild-type pigment. Genotypically identical progeny, but derived from mothers bearing, but not transmitting, a mini-X chromosome, had virtually identical levels of eye pigment (46 ± 9% wild-type pigment). Therefore the imprinting phenomenon is very clearly linked to the origin of the mini-X chromosome, and there is no evidence that it results from maternally deposited factors that modify the expression of variegating genes, directly or indirectly.

Allele-specific effects: Finally, to test the possibility that the *garnet* imprint phenotype reflects peculiar properties of the g^{53d} allele used in these studies, seven other *garnet* alleles (g^1, g^2, g^3, g^4, g^{50e}, g^{61}, g^{S3}) were examined. These all showed a similar parent-dependent mosaicism (Table 3 for g^{50e} and data not shown). Thus an allele-specific effect does not appear to explain the imprinted phenotype.

Position-effect variegation and imprinting: The preceding data rule out a number of more trivial causes for the parental effects on the expression of *garnet*.

TABLE 3

The effect of temperature on the expression of the *garnet*$^+$ gene on the *Dp(1;f)LJ9* mini-X chromosome

		Culture temperature			
		18°	22°	25°	29°
g53d	MAT	72 ± 8	73 ± 8	64 ± 6	39 ± 5
	PAT	44 ± 14	37 ± 13	36 ± 7	28 ± 4
g50e	MAT	70 ± 8	80 ± 9	71 ± 8	44 ± 7
	PAT	47 ± 11	50 ± 6	52 ± 9	35 ± 5

The effect of developmental temperature on the pigmentation of *y z^a g^{53d}/Dp(1;f)LJ9* males (top two rows) or *y z^a g^{50e}Dp(1;f)LJ9* males (bottom two rows). The origin of the mini-X chromosome is shown on the left (MAT, maternally derived mini-X chromosome; PAT, paternally derived mini-X chromosome). Expression of the *garnet* gene was monitored as described in materials and methods. Data are given as percentage wild-type *garnet* eye pigment expression ±SEM.

TABLE 4

The effect of butyrate concentration on the expression of the *garnet*$^+$ gene on the *Dp(1;f)LJ9* mini-X chromosome

	Butyrate concentration (mm)			
	0	100	150	200
MAT	87 ± 6	81 ± 4	71 ± 3	64 ± 6
PAT	40 ± 2	44 ± 5	38 ± 2	31 ± 2

The genotype of the test progeny genotype is *y z^a g^{53d}/Dp* in all cases. The origin of the mini-X chromosome is shown on the left (MAT, maternally derived mini-X chromosome; PAT, paternally derived mini-X chromosome). Expression of the *garnet* gene was monitored as described in materials and methods. Data are given as percentage wild-type *garnet* eye pigment expression, adjusted for nonspecific effects of butyrate ±SEM, as described in materials and methods.

Therefore we attribute the gene silencing associated with the paternal inheritance of the mini-X chromosome to traditional genomic imprinting.

Two questions arise. First, what causes the original imprint? And second, how is the genomic imprint propagated? There is no *a priori* reason why the initiating or determinative event and the propagation of the original imprint must be controlled by the same mechanism. The initiating event and its propagation are separated in time and tissue type; that is, the initiation presumably takes place in the germ line of the male or female parent, and the propagation occurs in the somatic tissues of the offspring. Thus each event might be expected to be controlled by a distinct mechanism. Therefore, we attempted to analyze both the initiation event of imprinting and its propagation independently, so we can ask if factors that affect the propagation of the imprint also affect the initiation and vice versa.

The clonal pattern of the *garnet* silencing that we observe in imprinted individuals is strongly reminiscent of position-effect variegation, as is the variable hypomorphic expression of the genes *tiny* and *narrow abdomen*. The distal centric heterochromatin was likely broken and deleted when the mini-X chromosome was generated because the most proximal euchromatic genes adjacent to the centric heterochromatin are deleted from the mini-X chromosome (data not shown). Thus, the mini-X chromosome might be expected to variegate for the genes, *g*$^+$, *ty*$^+$, and *na*$^+$. However, position-effect variegation does not usually show parental effects (Spofford 1976). To determine if the mosaic silencing was a result of position-effect variegation, we tested diagnostic modifiers of position-effect variegation. We examined the role of position-effect variegation and, by inference, chromatin structure in the initiation event in the parent, and in the somatic propagation of that decision in the imprinted offspring, separately. We focused on genomic imprinting of the *garnet* gene because it is the most visually dramatic and lends itself to precise measurement.

Three general groups of factors influence position-effect variegation: chemical factors such as sodium butyrate; environmental factors such as temperature; and genetic factors such as the presence of extra heterochromatin in the cell, usually in the form of an additional Y chromosome. Butyrate has been shown to inhibit histone deacetylases and thus presumably acts directly on chromatin structure (Mottus *et al.* 1980). The mechanism whereby developmental temperature affects position-effect variegation is unclear but it may act to control the rate of chromatin assembly (Spofford 1976). The Y chromosome has been postulated to modify position-effect variegation by competing for limited amounts of chromatin proteins that are normally involved in the formation of heterochromatin (Zuckerkandl 1974); however, it could also act by displacing the mini-X chromosome to a new nuclear compartment (Eberl *et al.* 1993; Talbert *et al.* 1994).

In every case tested, the factors that modified classical position-effect variegation also modified the variegation associated with the genomic imprinting of the mini-X chromosome (see Table 3 for the effect of temperature, Table 4 for the effect of butyrate, and Table 5 for the effect of extra Y-chromosome heterochromatin). The addition of butyrate to the medium and higher culture temperature during development influence the expression of the "imprinted" *garnet* gene. Curiously, the direction of the effect of both butyrate and temperature on the expression of the "imprinted" *garnet* is opposite to their usual effects on variegating euchromatic loci [high levels of butyrate and high temperatures generally suppress the inactivation; that is, they restore a more wild-type phenotype to variegating euchromatic loci (Spofford 1976)]. Both higher developmental temperature and sodium butyrate have the reverse effect on the variegation of the *garnet* gene on the mini-X chromosome; that is, they enhance the likelihood of gene repression. This enhancement, however, is typical of the effects of

TABLE 5

The effect of an additional Y chromosome on the expression of the *garnet*$^+$ gene on the *Dp(1;f)LJ9* mini-X chromosome

Genotype (assay)	X/Dp	X/Dp + Y
MAT (pigment)	79 ± 5	94 ± 4
(visual)	1.0 ± 0	1.0 ± 0
PAT (pigment)	37 + 9	99 ± 4
(visual)	0.07 ± 0.03	0.97 ± 0.03

The effect of an additional Y chromosome on the expression of the *garnet* gene in *y z^a g^{53d}/Dp(1;f)LJ9* males was assessed both by pigment assay and visually as described in materials and methods. The origin of the mini-X chromosome is shown on the left (MAT, maternally derived mini-X chromosome; PAT, paternally derived mini-X chromosome). Full, wild-type, *garnet* gene expression is 1.0 ± SEM by the visual estimation method and full *garnet* gene expression by the pigment assay method is 100% ±SEM wild-type pigment.

TABLE 6

Effect of parental developmental temperature on the imprinting of the *garnet*$^+$ gene of the *Dp(1;f)LJ9* mini-X chromosome

Parent culture temperature		Progeny culture temperature 18°	22°	25°	29°
18°	MAT	94 ± 3	89 ± 3	63 ± 5	33 ± 10
	PAT	42 ± 4	37 ± 2	50 ± 3	39 ± 2
22°	MAT	85 ± 4	87 ± 3	61 ± 2	49 ± 2
	PAT	27 ± 3	36 ± 3	54 ± 3	30 ± 3
25°	MAT	80 ± 4	96 ± 4	58 ± 3	54 ± 1
	PAT	30 ± 2	34 ± 4	27 ± 2	41 ± 2
29°	MAT	91 ± 5	92 ± 30	85 ± 4	59 ± 4
	PAT	ND	ND	ND	ND

The effect of developmental temperature on the pigmentation of *y z^a g^{53d}/Dp(1;f)LJ9* males. The origin of the mini-X chromosome is shown on the left (MAT, maternally derived mini-X chromosome; PAT, paternally derived mini-X chromosome). The rows give the effect of temperature on the progeny (effect on variegation) and the columns show the effect of temperature on the parents (effect on imprint). Expression of the *garnet* gene was monitored as described in materials and methods. Data are given as percentage wild-type *garnet* eye pigment expression ±SEM, as described in materials and methods. ND, not done.

the addition of butyrate and higher culture temperature on variegating heterochromatic genes (Spofford 1976) and the gene silencing induced by Polycomb-group proteins (Zink and Paro 1995). The Y chromosome is one of the most potent modifiers of the position-effect variegation (Spofford 1976). Therefore, we also examined the effect of Y chromosome aneuploidy on the genomic imprinting of the mini-X chromosome. The presence of an extra Y chromosome in the genome of the imprinted offspring (XY/mini-X^{PAT} *vs.* X/mini-X^{PAT}) essentially eliminates variegation of the *garnet* gene (Table 5). Thus, extra heterochromatin, in the form of an additional Y chromosome, effectively erases the expression or propagation of the imprint.

We next addressed the question of whether these modifiers of position-effect variegation influenced the initial generation of the imprint or simply the maintenance of the imprint. Because the imprinting of the mini-X chromosome is determined solely by the sex of the parent, we could address this issue by testing the effect of the various modifiers of position-effect variegation when present in the parents *vs.* their offspring. Interestingly, with the possible exception of butyrate, none of these factors perceptibly alters the imprint (see Table 6 for the effect of temperature, Table 7 for the effect of butyrate, and Table 8 for the effect of the Y chromosome). This is particularly evident in the case of an additional Y chromosome. Addition of extra heterochromatin, in the form of an extra Y chromosome in either the male or female parent, with or without the mini-X chromosome, had no impact on the genomic imprint. In contrast, the presence of an additional Y chromosome in the progeny can essentially abolish the manifestation of the genomic imprint (the variegation). Therefore, the presence of extra heterochromatin in the zygote either alters the somatic memory of the imprint or alters the expression of the imprinted gene(s) at the time of transcription. These data suggest that the somatic memory or propagation of the genomic imprint is maintained by the same process, or processes, which cause(s) position-effect variegation, likely chromatin formation. But the genomic imprint itself may be established by another process.

DISCUSSION

The *Dp(1;f)LJ9* mini-X chromosome of *D. melanogaster* exhibits genomic imprinting of at least three genes. The imprint is manifest as mosaic repression of these genes

TABLE 7

Effect of treating parents with butyrate on imprinting of the *Dp(1;f)LJ9* mini-X chromosome in the progeny

	Parental/Progeny concentration (mm) 0/0	0/200	200/0	200/200
MAT	87 ± 6	64 ± 6	81 ± 2	67 ± 3
PAT	40 ± 2	31 ± 2	43 ± 10	ND

The genotype of the test progeny genotype is *y z^a g^{53d}/Dp* in all cases. The origin of the mini-X chromosome is as given in the left column (MAT, maternally derived mini-X chromosome; PAT, paternally derived mini-X chromosome). Expression of the *garnet* gene was monitored as described in materials and methods. Data are given as percentage wild-type *garnet* eye pigment expression ±SEM, as described in materials and methods. ND, not done. The data in the control column (0 mm butyrate treatment for both progeny and parents) is taken from Table 4.

TABLE 8

The effect of an additional Y chromosome in parents on the imprinting of the *Dp(1;f)LJ9* mini-X chromosome

Parental genotype	*y z^a g^{53d}/Dp(1;f)LJ9* progeny phenotype: Pigment assay	Visual assay
1. Direct paternal effect *XY + mini-X vs. X/Y + Y + mini-X*		
XY + Dp male parent	41 ± 2	0.10 ± 0.02
XY + Y + Dp male parent	48 ± 3	0.12 ± 0.02
2. Direct maternal effect *XX/mini-X vs. XX/Y/mini-X*		
XX + Dp female parent	100 ± 3	0.92 ± 0.01
XX + Y + Dp female parent	94 ± 4	1.0 ± 0
3. Paternal Y-effect *XY vs. XYY ⊗ mini-X* females		
XY ⊗ Dp female parent	75 ± 10	0.73 ± 0.03
XY + Y ⊗ Dp female parent	81 ± 10	1.0 ± 0
4. Maternal Y-effect *XX vs. XXY ⊗ mini-X* males		
XX ⊗ Dp male parent	25 ± 5	0.05 ± 0.02
XX + Y ⊗ Dp male parent	34 ± 11	0.03 ± 0.03

The effect of an additional Y chromosome in the parents (effect on establishment of the imprinting) was assessed both by pigment assay and visually in *y z^a g^{53d}/Dp(1;f)LJ9* test males. Four tests were performed to monitor the effect of an additional Y chromosome in the parents—an effect in the male parent with (test 1) and without (test 3) the mini-X chromosome, and in the female parent with (test 2) and without (test 4) the mini-X chromosome. The origin of the mini-X chromosome is given on the left. Full, wild-type *garnet* gene expression is 1.0 by the visual estimate method and full *garnet* gene expression by the pigment assay method is 100% wild-type pigment. The details of the crosses are given in materials and methods.

when the mini-X chromosome is inherited from the male parent. In contrast, when the mini-X chromosome is inherited from the female parent, expression of these genes is essentially wild type, leading to no, or to a very low level of, mosaicism in the genotypically identical progeny. The genomic imprinting we observe with the mini-X chromosome is manifested as a mosaic of gene silencing. Indeed, this mosaicism is found in all previously reported examples of genomic imprinting in Drosophila (see Table 1 for references) as well as in maize (Kermicle and Alleman 1990), fish (Martin and McGowan 1995a,b), insects (Nur 1970, 1990), and mammals (Allen *et al.* 1988; Svensson *et al.* 1998). The gene silencing we observe for the three genes on the mini-X chromosome is graded in its severity and correlates with distance of the genes from the centric heterochromatin. The gene closest to the centric heterochromatin, *garnet*, shows the most extreme silencing or degree of genomic imprinting, seen as the greatest difference in phenotype between individuals with a maternally *vs.* paternally inherited mini-X chromosome. The genes *narrow abdomen* and *tiny* were also imprinted, but the frequencing of silencing diminished with increasing distance from the centric heterochromatin. This finding suggests that the genomic imprint is manifest and propagated by parent-dependent spread of heterochromatin from the centromeric region of the chromosome. Presumably the rearrangement that generated the mini-X chromosome placed these three genes (and others) sufficiently near a region in which the heterochromatin formation differs after passage through the male and female germ line. We have tested a number of factors to determine the cause of the genomic imprinting, that is, the determinative event that differentially "marks" the allele(s) or chromosome region in the parent. First, we have determined that the imprint responds to the physiological sex of the parent and is not associated with the presence or absence of the Y chromosome *per se*. Second, our data rule out the possibility that the *garnet* imprint is merely a consequence of chromosome loss or an allele-specific genetic curiosity. Third, we have shown that this parent-specific effect is not due to a masking of standard (nonimprinted) position-effect variegation by maternally deposited *garnet*$^+$ product, or the product of a variegation modifier. Finally, we have explored the role of heterochromatin in separate sets of experiments, both in the establishment and in the maintenance of the genomic imprint. We have found that the classical modifiers of position-effect variegation can alter the somatic manifestation of the imprint in the offspring, but they do not influence the initial acquisition of the imprint. Specifically, the presence of additional heterochromatin in the progeny completely abrogated the mosaic expression of the paternally imprinted mini-X chromosome, causing it to mimic the phenotype of a maternally transmitted mini-X chromosome. In contrast, none of these modifiers, including extra heterochromatin, had an impact when present in the parent. Therefore, modifiers of position-effect variegation appear to act on the somatic propagation, but not on the establishment, of the imprint. This implies that chromatin structure, specifically, heterochromatin-like structures, act as "somatic memory" of the gene silencing, or imprint, to ensure transmission in somatic cells. But a different mechanism may operate to cause the imprint in the parent.

Our data suggest that the mechanism that establishes the original genomic imprint may differ from the mechanism that propagates the imprint in the somatic tissues of the affected offspring. One way to determine the mechanism by which the genomic imprint is established in Drosophila would be to isolate genetic modifiers of the imprinting process. Some progress has been made in detecting genes that influence imprinting in mice (Allen *et al.* 1988; Babinet *et al.* 1990; Cattanach and Beechey 1990; DeLoia and Solter 1990; Reik *et al.* 1990; Surani *et al.* 1990; Engler *et al.* 1991; Foreijt and Gregurova 1992; Sapienza *et al.* 1992). However, many of the genes that are imprinted in mammals act early in embryogenesis and influence important developmental processes. Thus, one might expect that muta-

tions in genes that affect imprinting in mammals are likely to be both pleiotropic and lethal. However, it should be possible to isolate such modifiers in Drosophila, because it appears that the imprinted region is within heterochromatin, far from most genes, and wild-type function of the marker gene, *garnet*, is not essential. We have done some preliminary studies that show that the severity of the *Dp(1;f)LJ9* imprint can be influenced by altering the X chromosome present in the female parent; by switching the X chromosome we can reduce the expression of the *garnet* gene on a maternally derived mini-X chromosome from 100 ± 3% to 80 ± 3% of wild-type pigment. Paternal imprinting was unaffected. While these data are preliminary, they suggest that X-linked modifiers of imprinting exist and can be identified. The isolation and study of such genes in Drosophila should prove useful for understanding the mechanism(s) of genomic imprinting in insects and its relationship to imprinting in other organisms.

The underlying basis and mechanism of genomic imprinting, among invertebrates as well as vertebrates, is of considerable interest. We focused on the role of heterochromatin in both the initiation and somatic propagation of the imprint for several reasons. The clonally variegated phenotype of the imprinted *garnet* gene and the variable hypomorphic expression of the *narrow abdomen* and *tiny* genes are highly reminiscent of position-effect variegation, a process generally associated with aberrant heterochromatin formation and spread. Also, the gradient of gene silencing, from proximal to distal, away from the centromeric heterochromatin, resembles the spreading of gene silencing observed in position-effect variegation (Spofford 1976). Furthermore, genomic imprinting is associated with heterochromatin formation in a number of different organisms. For example, imprinting in the coccids and Sciara involves heterochromatinization of entire chromosomes (Chandra and Brown 1975) as does X-chromosome inactivation that is imprinted in both eutheran mammals (Takagi and Sasaki 1975; West *et al.* 1977) and marsupials (Sharman 1971). Paramutation, an epigenetic phenomenon possibly related to imprinting, is associated with altered chromatin structure in maize (Patterson *et al.* 1993). Aberrant chromatin structure has also been proposed as the causal feature leading to misexpression of the *fmr1* gene responsible for fragile X mental retardation (Laird 1987) and a number of other recent studies have implicated chromatin structure in imprinting in mammals (Sasaki *et al.* 1992; Bartolomei *et al.* 1993; Ferguson-Smith *et al.* 1993; Koide *et al.* 1994; Bruiting *et al.* 1995; Lyko *et al.* 1997, 1998; Nicholls *et al.* 1998; Svensson *et al.* 1998).

In the case we report here it is clear that the genomic imprint is a property of the placement of these genes near an imprinted region of centric heterochromatin on the mini-X chromosome, rather than an intrinsic property of the genes themselves, because none of these genes show parent-dependent expression in their normal chromosomal position (Lindsley and Zimm 1992). Imprinting acquired as a result of placing normally non-imprinted genes into an imprinted region of the genome has been documented for transgenes in mammals (DeLoia and Solter 1990; Reik *et al.* 1990). The graded and polar nature of the imprint (Table 2) and the response of the *garnet* variegation to butyrate and temperature suggest that the imprint is transmitted, if not imposed, on these genes as a consequence of being placed near to an interrupted segment of heterochromatin. The manifestation of the genomic imprinting could reflect differences in formation and/or spread of heterochromatin-like packaging into the marker genes. The observation that high temperature and butyrate can induce a weak mosaic expression of *garnet* in individuals with a maternally transmitted mini-X chromosome (Tables 3 and 4), suggests that it is the extent of heterochromatic invasion, rather than the formation of heterochromatin *per se*, that is dependent on the sex of the parent. Furthermore, assuming position-effect variegation is associated with altered packaging of chromatin, whether directly or as a consequence of being placed into a different compartment of the nucleus, it appears that this segment of the chromosome is packaged differently, at least in the somatic tissue of the offspring, after passage through the male *vs.* the female germ line. In the *Dp(1;f)LJ9* mini-X chromosome the *garnet* gene is placed extremely close to the heterochromatic breakpoint (Hardy *et al.* 1984), so it is not surprising that *garnet* becomes a sensitive marker for genomic imprinting. The 12A-D region around the *garnet*$^{+}$ gene on the mini-X chromosome is peppered with reiterated sequences such as the *Stellate* sequences (Hardy *et al.* 1984), multiple copies of *tRNASer 4-7* genes, and a Y-chromosome satellite repeat (Leung *et al.* 1991). Dorer and Henikoff (1994) have provided evidence that repeat sequences may nucleate gene-silencing events associated with position-effect variegation. However, this observation does not resolve the question of how or why the heterochromatin forms differently depending on parental origin of the mini-X chromosome.

In eutherian mammals and plants, DNA methylation has long been known to be linked to gene inactivation. While DNA methylation patterns correlate with genomic imprinting and are essential for maintenance of the imprint (Li *et al.* 1993), in at least one case, it has been shown that the decision to inactivate a gene occurs several days before DNA methylation is evident (Lock *et al.* 1987). Thus methylation is likely to be involved in propagation of the "silenced" state during development in mammals, rather than to be the cause of genomic imprinting. Detectable levels of methylation do not occur in Dipteran insects (Bird and Taggart 1980; Urieli-Shoval *et al.* 1982; Patel and Gopinathan 1987) and thus methylation is unlikely to be responsible for the imprint, either its initiation or its somatic propagation.

However, other DNA or chromatin modifications could provide an equivalent mechanism. Chromatin formation may serve a similar function in Drosophila to DNA methylation in mammals; that is, it may be responsible for transmitting the memory of the initial imprint in the somatic tissue of the offspring. Because altered chromatin conformation is also associated with imprinting in mammals, and because a mammalian imprinting signal is recognized as a gene-silencing element in Drosophila (Lyko *et al.* 1997, 1998), it is tempting to speculate that imprinting is not only an ancient and conserved form of gene silencing but also a similar chromatin-based mechanism that might operate in mammals as an early maintenance mechanism before the onset of methylation. Despite the considerable similarities between imprinting phenomena in Drosophila and other organisms, it remains unclear if the genomic imprinting reflects one ancient and conserved process, or a number of distinct mechanisms that result in a similar phenotype. This question can be resolved only by a genetic and molecular analysis of imprinting in a variety of organisms.

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