Imprinting Capacity of Gamete Lineages in *Caenorhabditis elegans*

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ABSTRACT

We have observed a gamete-of-origin imprinting effect in *C. elegans* using a set of GFP reporter transgenes. From a single progenitor line carrying an extrachromosomal *unc-54::gfp* transgene array, we generated three independent autosomal integrations of the *unc-54::gfp* transgene. The progenitor line, two of its three integrated derivatives, and a nonrelated *unc-119::gfp* transgene exhibit an imprinting effect: single-generation transmission of these transgenes through the male germ line results in ~1.5- to 2.0-fold greater expression than transmission through the female germ line. There is a detectable resetting of the imprint after passage through the opposite germ line for a single generation, indicating that the imprinted status of the transgenes is reversible. In cases where the transgene is maintained in either the oocyte lineage or sperm lineage for multiple, consecutive generations, a full reset requires passage through the opposite germ line for several generations. Taken together, our results indicate that *C. elegans* has the ability to imprint chromosomes and that differences in the cell and/or molecular biology of oogenesis and spermatogenesis are manifest in an imprint that can persist in both somatic and germline gene expression for multiple generations.

Parent-of-origin effects refer to a set of phenomena in which an entire set or subset of the paternal and maternal genome are distinguished from each other in the progeny genome. One of the first described cases of parent-of-origin effects was by Helen Crouse (1960), who coined the term “imprinting” to describe the elimination of certain paternal chromosomes in *Sciara* flies (Crouse 1960). Today, the term genomic imprinting is often used to describe the monoallelic expression of a gene from either the paternal or the maternal chromosome, but not from both. Genomic imprinting exists in a diverse set of organisms that span different phyla, including mammals, plants, insects, and fish.

Insects show diverse imprinting phenomena. Perhaps one of the more extreme forms of imprinting is found in the mealybugs, in which the entire genome of one parent is epigenetically marked and silenced, rather than the silencing of individual paternal or maternal alleles. For example, the coccid mealybug does not possess sex chromosomes (Brown 1959, 1961); instead, male-ness is determined by the heterochromatization and silencing of the entire paternally derived genome (Brown and Nelsen-Rees 1961; Bongiorni and Prantera 2003). In the sciarid flies, the maternally derived and paternally derived chromosomes are distinguished from each other in the progeny genome. The somatic and germ-line development of these flies is driven by the selective loss of various paternal chromosomes in different tissues and at different stages of the life cycle (Goday and Esteban 2001). In *Drosophila*, manipulation of chromosomal environments can sometimes result in previously nonimprinted genes now being expressed in a parent-of-origin manner (Golic et al. 1998; Lloyd 2000). As in *Drosophila*, imprinting has not been shown to play a developmentally critical role in zebrafish; yet this organism has the capacity to methylate DNA in a parent-of-origin-specific pattern (Martin and McGowan 1995).

Although flowering plant development is drastically different from animal development, genomic imprinting has been observed to be an important feature of the plant life cycle (Alleman and Doctor 2000; Vinkenoog et al. 2003; Scott and Spielman 2004). Reproduction in flowering plants is characterized by a unique double-fertilization event. Each of two sperm cells, carried in the same pollen grain, fertilizes separate targets. One sperm cell fertilizes the haploid oocyte to become the zygote, while the other sperm cell fertilizes the diploid central cell to become the endosperm, a source of nutrients for the embryo. Of the handful of genes found to exhibit a parent-of-origin effect in plants so far, all affect development of the endosperm. Two well-characterized imprinted genes in *Arabidopsis* are *MEDEA* (Grossniklaus et al. 1998) and *FWA* (Soppe et al. 2000; Kinoshita et al. 2004).

By far the most extensively studied examples of genomic
imprinting have been in mammals. Early experiments involving translocations and nuclear transfer demonstrated the requirement for contribution of both parental genomes (i.e., Mann and Lovell-Badge 1987; Cattanach and Beechey 1990). As of December 2004, >70 murine genes have been listed on the Harwell Imprinting website to be imprinted (http://www.mgu.har.mrc.ac.uk/research/imprinting/imprin-viewdatagenes.html). Imprinting is critical for mammalian development, and defects in the imprinting process often lead to debilitating diseases (Walter and Paulsen 2003a). An interesting aspect of imprinting in the murine system is that many transgenes are also subject to imprinting. From work by multiple labs over many years, certain themes concerning transgene imprinting in mammals have emerged. Generally, passage through the female germ line results in decreased activity of the reporter transgene, ranging from partial (Kearns et al. 2000; Preis et al. 2003) to complete and irreversible silencing (i.e., Lau et al. 1999). Additionally, the expression imprint is correlated with DNA methylation levels, with the maternaly derived alleles generally being more methylated than the paternally derived alleles.

Although reports of parent-of-origin effects in other organisms have been abundant, accounts of parent-of-origin phenomena in Caenorhabditis elegans have been very rare. A screen for the requirement for biparental inheritance failed to uncover any evidence of whole-chromosome imprinting in C. elegans (Haack and Hodgkin 1991). Kelly and colleagues (Bean et al. 2004) recently reported the germ-line-sex-specific modification of the X chromosome in C. elegans. In their study, they observed a difference in chromatin state in the zygote between the spermatogenesis-derived and oogenesis-derived X chromosome. The differential chromatin marks persisted up to the 20-cell stage of embryogenesis (Bean et al. 2004). In this article, we present evidence that a set of unc-54 transgenes is expressed in a parent-of-origin manner in C. elegans and that the imprint persists into somatic development, but is reset upon passage through the opposite germ line. Equivalent levels of expression are obtained when the transgene is transmitted through hermaphrodite sperm compared to transmission through male sperm, suggesting that whatever process(es) acts to establish and/or maintain the imprint is dependent upon the gamete type.

MATERIALS AND METHODS

C. elegans strains and growth conditions: Worms were reared on Escherichia coli strain OP50 grown on nematode growth medium (MYOB) nutrient plates according to standard protocols (Brenner 1974). Worm strains used in the experiments are as follows: pha-1(e2123ts) III (Schinzel and Schinzel 1990), alp106- [lin15(+)] + [lin11: gfp] X (Reddien et al. 2001), unc-119[pha-1(e2123ts) III], PD3852 [ccIn3852 pha-1(e2123ts) III], PD3861 [pha-1(e2123ts) III], PD3862 [ccIn3862 I], PD3866 [ccIn3862 I; pha-1(e2123ts) III], PD3868 [ccIn3862 I; pha-1(e2123ts) III; him-5(e1467) V], PD3920 [dypl-5(e61) unc-13(e1091) I; pha-1(e2123ts) III], PD3924 [ccIn3862 dpy-5(e61) unc-13(e1091) I; pha-1(e2123ts) III], PD3928 [unc-13(e1091) I; pha-1(e2123ts) III], PD3936 [ccIn3852 dpy-17(e144) unc-69(e587) pha-1(e2123ts) III], PD3938 [ccIn3862 unc-13(e1091) I; pha-1(e2123ts) III], PD3990 [ccIn3862 dpy-5(e61) I; pha-1(e2123ts) III], PD3990 [ccIn3862 unc-13(e1091)/ccIn3862 dpy-5(e61) I; pha-1(e2123ts) III; him-5(e1467) V], PD3942 [ccIn3862 unc-13(e1091)/ccIn3862 dpy-5(e61) I; pha-1(e2123ts) III], PD3945 [dpy-17(e164) unc-69(e587) pha-1(e2123ts) III], PD3966 [ccIn3862 I; pha-1(e2123ts) III; him-5(e1467) V], PD4251 [ccIn4251 I; dpy-20(e1282) IV] (Fire et al. 1998), PD4838 [cex8438 sur-5: gfp]. Map positions for integrations edIs6 and lqk27 have not been determined. Strains were kept at either 16° or 25°, depending on whether they were pha-1(e2123ts) rescued, respectively. All genetic crosses were carried out at 23° unless otherwise specifically indicated. In experiments for which the transgene array was linked to recessive genetic markers, each cross was closely monitored to ensure continuous linkage between markers and the transgene array.

Plasmids and transgenic lines: A mixture of four plasmids was micro-injected (Mello et al. 1991) into pha-1(e2123ts) animals. Plasmid pC1 (Granato et al. 1994) contains the wild-type genomic pha-1 sequence without the 3′-UTR. Plasmids pPD95.93, pPD105.21, and pPD120.90 each have a 204-bp unc-54 promoter segment driving the GFP-coding region followed by the unc-54 3′-UTR. These three plasmids differ in their combinations of subcellular localization signals: pPD95.93 carries a nuclear localization signal and lacZ (yielding nuclear GFP); pPD105.21 carries a mitochondrial localization signal; and pPD120.90 carries a four-nuclear localization signals (yielding nuclear GFP). Transgenic lines derived from this mixture show a relatively uniform pattern of GFP within expressing cells. In standard transgenic lines, the unc-54 promoter provides mosaic expression in body muscles of C. elegans. Several independent transgenic lines with these characteristics were obtained, and one PD3815 was chosen for further analysis.

The transgene array in PD3815 (designated edIs3815) is inherited as an extrachromosomal element. Three integrated derivatives of this locus were obtained following treatment with EMS (Brenner 1974). These lines are designated PD3852, PD3861, and PD3862 and the corresponding integrated transgene loci as ccIn3852, ccIn3861, and ccIn3862.

Image capture: Animals of the desired genotype were sampled randomly and blindly (i.e., without knowledge of their GFP expression levels) and immobilized on eight-well glass slides (MP Biomedicals) in mounting solution (50 mM NaCl, 5 mM EDTA, 0.5 mM levamisole). Fluorescent images of live animals were captured using a chilled charge-coupled device camera (Nikon CDD300ET-RC camera). Neutral density filters were used whenever necessary to ensure linearity of signal. All measurements were carried out in the linear range of detection as assayed by proportionality between observed signal and transmission percentage of the neutral density filter. Identical instrument and software settings were used in all image capture sessions.

GFP quantitation: Quantitation of GFP levels was done according to the procedure outlined in Figure 1. We used two sources of constant fluorescence in normalizing sample populations: a uniformly labeled fluorescent bead standard (Molecular Probes, Eugene, OR) and a set of animals from a well-characterized and stable GFP-expressing line (PD4251). On the basis of these values and those for the sample population, we obtain a measure of GFP fluorescence. Analysis of fluctuations in the ratio between the reference line PD4251 and
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fluorescent bead standards provides a basis by which we can judge fidelity of the assay. In experiments carried out to date, the ratio between PD4251/beads is relatively constant (3.58 ± 0.55; *n* = 1180), giving a variability of ~15% (standard deviation/mean).

**Analysis of transgene DNA in transformed lines:** DNA from strains PD3815, PD3861, PD3862, PD3872, and PD3924 was extracted, digested with restriction enzymes (*Pst*I + *Age* I, *Pst*I + *Nco*I, *Pst*I + *Psp*OMI, *Mfe*I + *Psp*OMI), separated by agarose gel electrophoresis, visualized by ethidium bromide staining (where strong bands from the *pha-I* marker DNA were evident), transferred to membrane filters, and probed with a segment from the *unc-54* promoter. Strong signals consistent with copy numbers of 20–30 for the *unc-54* promoter were observed. Similar analysis of insertion *edIs6* demonstrated that this line also carried a high-copy-number tandem array (data not shown).

**Statistical analyses:** The *t*-test analysis was performed using two-tailed distribution and two-sample unequal variances (Pagano and Gauvreau 2000). We additionally subjected the data sets in Figures 5 and 7 to computer simulations to determine if the observed trends could occur by pure chance (see Appendix).

**RESULTS**

**Development of an assay for GFP quantitation of *C. elegans* populations:** Much of the work in this article involves comparing the relative GFP expression of genetically identical populations of animals, the only difference being the parental source of the reporter chromosome (sperm vs. oocyte) and the paired or unpaired character of the reporter-carrying chromosome in the parental generation. Measurement of relative GFP levels between two populations of animals presents a challenge. We obtained quantitative fluorescence data first by acquiring digitized images of each animal using a CCD camera system. On the basis of the experimental measurement of total fluorescence integrated over each sample image, we obtain a total signal. These signals are then corrected, as appropriate, by background subtraction to obtain an essentially quantitative comparison of different animal populations (Figure 1). Although some variation is inherent in this assay, we consider the results of the assay to be accurate to within an approximate variance of 20%.

**A fusion reporter for quantitative expression analysis:** We initially set out to screen for parent-of-origin effects

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**Figure 1.—GFP quantitation assay using NIH Image.** (A) Images of experimental PD4251 and N2 animals to be quantified were captured into NIH Image v1.6.3 followed by fluorescence measurements as described in C. (B) Fluorescent beads used as standard. Average fluorescence of three background areas was determined for a given fluorescent bead image. This background fluorescence was subtracted from the gross fluorescence signal of each of 10–30 beads (sampled randomly), followed by determination of the average fluorescence of the sampled beads. (C) Method of determining net, normalized signal for PD4251 and experimental animals. For each sample population whose fluorescence measurements were to be determined, we captured four sets of images: one set of the experimental animals, one image of a fluorescent bead standard, one set of PD4251 L4 animals, and one set of 5–10 N2 animals (autofluorescence control). For each N2 animal, net signal was determined by subtracting the average of three background values, multiplying by the area of the animal, and then dividing by the length of the animal. An average N2 value was then determined for each experiment. (We always used N2 animals of the same developmental stage as the experimental animal population. For example, for an experimental population consisting of L4 animals, we determined an N2 average using N2 animals at the L4 stage.) For each experimental animal, the net signal (*x*~ei~) was determined by subtracting the average of three background values plus the average N2 fluorescence, multiplying by the area (*A*~e,i~) of the animal, and then dividing by the length of the animal (*l*~e,i~). The net, normalized signal of each animal (*x*~ei~ normalized) is the ratio between *x*~ei~ and average of fluorescent beads. The same procedure was applied to determine fluorescence intensities of PD4251 animals.
on gene expression in *C. elegans* using an *unc-54::gfp* transpositional fusion reporter assay. The *unc-54* gene in *C. elegans* encodes the major myosin heavy chain of body wall muscles (Brenner 1974; Epstein *et al.* 1974; Mackenzie *et al.* 1978). Briefly, plasmids containing the *unc-54* promoter fused to GFP were micro-injected with a genomic clone of the *pha-1(+)* gene into L4 animals homozygous for the *pha-1(e2123ts)* mutation (Granato *et al.* 1994). At 16°C, the *pha-1(e2123ts)* mutation is permissive; at 23°C, 100% of *pha-1(e2123ts)* animals arrest as embryos or L1 larvae.

Plasmid DNA populations injected into *C. elegans* are subject to a recombination process that in many cases leads to the formation of long, extrachromosomal tandem arrays that can be inherited from generation to generation in a non-Mendelian manner (Stinchcomb *et al.* 1985). When two or more plasmids are mixed, the extrachromosomal transgene array consists of tandem-mixed arrays of the two sequences, with a total copy number of 100–200. By rearing populations at 23°C, we select for *pha-1(+)* animals that harbor the transgene array. A single clonal line from the plasmid injections was selected for the initial analysis. This line, designated PD3815 (Figure 2), is viable at 23°C and exhibits detectable body wall muscle GFP activity.

For reasons that are not well understood, a large fraction of transgene arrays in *C. elegans* exhibit mosaic expression: expression in some but not all of the cells where expression would be expected on the basis of knowledge of the promoter used in the initial reporter construct (Fire and Mello 1995). Although a fraction of the mosaicism is due to mitotic loss of the extrachromosomal array, substantial mosaicism is observed even in the majority of transgenic lines harboring arrays that have integrated into the chromosome and thus should be present in every cell. Patterns of expressing and non-expressing cells tend to be random within a target tissue, with little or no adherence to lineal boundaries. The seemingly random pattern of GFP activity from array-based transgenes further argues against the mitotic loss of arrays as the sole cause of mosaicism. Instead, it appears that arrays are physically present but transcriptionally silenced in a large number of cells. Consistent with the hypothesized gene silencing, the level of reporter expression in transgenic strains carrying multicopy arrays is rarely a simple multiple of expression observed with rare single-copy integration events. Rather, it appears that the arrays are essentially in a silent state with rare copies (or whole arrays) undergoing a rare activation event.

To obtain a strain with a greater degree of uniformity than the original transgenic PD3815, we carried out an EMS mutagenesis, followed by a screen one to three generations later for animals with more uniform and increased level of GFP expression. Among a collection of candidates, we pursued the three with strongest expression. The resulting lines, designated PD3852, PD3861, and PD3862, exhibited increased overall expression combined with somewhat reduced mosaicism (Figure 2). Conceivably, the improved GFP activity in these three strains could result from a variety of different alterations. In the case of PD3852, PD3861, and PD3862 the critical alteration appears to be in the structure or genomic context of the transgene locus and not the induction of mutations in the genetic background that facilitate transgene expression. In particular, the array in each of these lines had become integrated into the genome. Integration of arrays is a very unusual event in *C. elegans* (Fire *et al.* 1991; Mello *et al.* 1991) and three independent unselected integrations would be extremely rare. It should be noted that not all integration events lead to the type of expression improvements seen with PD3852, PD3861, and PD3862 (Hsieh *et al.* 1999). The integra-
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**Figure 3.**—Initial observations of parent-of-origin effect and nonlinear expression ratios between array homozygotes and hemizygotes for *ccIn3852*, *ccIn3861*, and *ccIn3862*. (A) Experimental design used to determine the ratio of *A°/H11001* to *A*/H11001* expression in the three transgenic lines. Data are shown as both relative values normalized over *A°/H11001* (top number) and absolute values (bottom number, in parentheses). Unlike subsequent experiments described in this article in which L4 (larval stage) and adult animals were analyzed as separate data sets, this experiment combined both L4 and adult animals into the same data set. The results for *ccIn3861* were pooled from two independent experiments. The letter "A" in the crosses represents the integrated transgene array, and superscripted "o" and "s" indicate that the array was derived from oocyte and sperm, respectively. This convention is used throughout this article.

(B) Experimental design comparing relative expression between array hemizygotes and homozygotes. As in A, both L4 and adult animals were combined into a single data set.

The results for *ccIn3861* were pooled from two independent experiments. The letter "A" in the crosses represents the integrated transgene array, and superscripted "o" and "s" indicate that the array was derived from oocyte and sperm, respectively. This convention is used throughout this article. The Southern blot analyses similar to those of Stinchcomb et al. (1985) were used to examine structures of the original extrachromosomal transgene as well as the three integrated derivatives (see MATERIALS AND METHODS). As expected each of these lines contains a tandem array with sequences from *unc-54::gfp* and the *pha-1* marker. Each array appears to contain 20–30 *unc-54::gfp* copies interspersed among 50–200 copies of the *pha-1* marker plasmid. Although no differences in copy number or array structure were evident following restriction digestion with a representative group of enzymes, we cannot rule out the possibility that point mutations or subtle changes in configuration might have an affect on expression for the different integrated loci.

**Unexpected expression ratios from simple outcrosses:**

While outcrossing the three integrated *unc-54::gfp* strains from the mutagenized genetic backgrounds, we found an anecdotal correlation in some cases between parent-of-origin for the transgene and expression level. We further characterized this effect in outcrossed lines in experiments where a single transgene locus was introduced from either oocyte or sperm into a zygote, followed by quantitation of expression levels in the larval and adult stages. As shown in Figure 3A, we observed that two of the transgene loci (*ccIn3852* and *ccIn3862*) expressed more strongly when the transgene locus was introduced from the male parent (*i.e.,* through sperm). Expression was still observed following transmission through oocytes, but was seen at a significantly lower level. No evidence for a parent-of-origin effect was observed with the third array, *ccIn3861*. The parent-of-origin effect was a stable property of lines PD3852 and PD3862: consistent results were obtained with these lines in experiments carried out in several different generations following the establishment of the outcrossed line, while no parent-of-origin-dependent expression was observed at any point with PD3861 (Figure 3A). In addition to the observed parent-of-origin effect, we also observed an unexpectedly skewed ratio when comparing expression between homozygotes and oocyte-derived hemizygotes (Figure 3B). These skewed ratios presumably reflect the parent-of-origin effect combined with other (yet-to-be-characterized) consequences of homozygosity for the transgene.

Both integrated and extrachromosomal transgenes exhibit parent-of-origin expression patterns. In particular, the ratio of sperm-derived to oocyte-derived expression...
of ccIn3862 in line PD3815 was 2.2 (data not shown). Thus, integration is not a prerequisite for the imprinting of the unc-54::gfp transgene, since the extrachromosomal array ccEx3815 is also imprinted.

Several different processes could skew the ratio of expression levels in these experiments. There is certainly precedent for chromosomal imprinting from other systems. This would entail a mechanism in which the maternal and paternal chromosome sets provide different expression levels to the zygote. As an alternative, however, one must consider that the cytoplasmic contributions in the two crosses shown in Figure 3 are distinct. In the case of maternal acquisition of the transgene, the maternal cytoplasm is derived from an animal carrying the transgene. Products of the transgene locus (such as modulatory or aberrant RNAs) might be present in the oocyte and thereby modulate the subsequent activity of the reporter construct. Fortuitous transcripts of transgene loci have frequently been observed (Fire et al. 1991) and could potentially produce stable changes in gene expression, so this possibility cannot be ruled out without clear experimental tests. A second potential difference between the two crosses in Figure 3 relates to the meiotic pairing state of the transgene array in the parents of the assayed animals. In one case the parent is homozygous for the transgene array, hence an opportunity (at least) for pairing of the locus in the previous generation. In the other case the parent is hemizygous, suggesting the possibility of an unpaired state. Meiotic silencing of unpaired DNA has been demonstrated in Neurospora (Aramayo and Metzenberg 1996) and has been suggested for C. elegans (Bean et al. 2004).

Expression of ccIn3862 depends on the gamete-of-origin for the transgene chromosome: The suggestion of parent-of-origin effects with ccIn3852 and ccIn3862 led us to further investigate the genetic basis in greater detail. To determine the source of the observed skewing of expression ratios, we set up a series of crosses in which four variables were tested: parent of origin of the transgene array, pairing of the array in the parental generation, cytotype (cytoplasmic genotype of parental germline), and hemizygosity/homozygosity of the array in assayed progeny. We organized these experiments into the matrix shown in Figure 4. Eight different crosses yielded animals that were hemizygous for the transgene array, while four crosses yielded array homozygotes. Each intersection is an independent experiment; hence, 12 independent experiments were carried out to complete the matrix analysis. Note that we were careful to keep the genotypes of the hermaphrodite parents (rows) and male parents (columns) as consistent as possible (Figure 4B). We chose to analyze ccIn3862 over ccIn3852 because of the relative ease of constructing the necessary genetically marked derivatives of ccIn3862. ccIn3861 was not included in the analysis because it does not show a parent-of-origin effect.

The central question of our matrix experiments was whether, on average, two genotypically identical populations, the only difference being the parent-of-origin or pairing state in the parental germline of ccIn3862, differ in their level of GFP expression. The results of the matrix experiments are shown in Figure 5, with the accompanying statistical analysis. Each value in the matrix is a population average from numerous animals. Comparing each diametrically opposite pair (for example, A3 vs. C1), we saw that in all cases transmission from sperm gave greater expression than transmission from oocyte. This difference is statistically significant in all cases (Figure 5C) and for both adult and L4 animals alike. Significantly, the matrix data recapitulate results from a set of
Figure 5.—Results of the matrix experiment. (A and B) For each experiment, we separated the adult and L4 data sets in our analyses because the two stages showed a significant difference in the level of GFP expression, and we were concerned that treating both stages as a single data set would confound our analysis. Each number in the matrix is a population average after normalization over the fluorescent bead standard. Color schemes are as indicated for Figure 4. (C) Statistical analyses of the matrix data. The $t$-test column shows $p$ values in scientific notation ($i.e.$, 2.65E-07). We considered $p < 0.05$ to be statistically significant. Experiments C4 and D3 shared the same set of bead and PD4251 images. $n$, sample size; s.d., standard deviation; s.e.m., standard error of the mean.

Earlier experiments (Figure 3B) that detect nonlinear expression ratios between array homozygotes and hemizygotes. In the adult data set in Figure 5, GFP expression of array homozygotes (C3, C4, D3, D4) are at least twice that of any single array derived from hemizygous parents (C1, C2, A3, B3), although this difference is slightly
smaller when the single array was derived from homozygous parents (A4, B4, D1, D2). The L4 data set shows a similar trend.

We generated scatter plots of normalized expression values \([i.e., x_{i,\text{normalized}}]\) to determine the spread of the data points (Figure 6). As can be seen from the plots, each population displayed a range of GFP expression. Although there were outliers, the majority of the data points clustered around the population mean. It appears that for any given data set, L4 animals show less scattering than adults. This is probably due to the L4 being a more defined stage of development than the adult stage.

**ccdh3862** is expressed equivalently from male and hermaphrodite sperm: Because *C. elegans* sperm can be derived from either males or hermaphrodites, an intri-
Figure 7.—Comparison of ccIn3862 expression from male sperm vs. hermaphrodite sperm. (A–D) Experimental design and the resulting data analysis. Images of animals from all three crosses A, B, and C were captured during the same sitting and therefore shared the same set of fluorescent bead and PD4251 images. Only L4 animals were used in the analysis. (E) Histograms depicting the distribution of ccIn3862 expression in each data set. The vertical line indicates the population average for that data set. u, unc-13(e1091); d, dpy-5(e61); du, dpy-5(e61) unc-13(e1091).
Figure 8.—Test for pairing effects on ccIn3862 expression. (A–C) Experimental design to test for pairing effects on ccIn3862 expression. (D) Analysis for resulting data. Only the L4 data set is shown. The adult data set (data not shown) exhibits a trend similar to that of the L4 data set. (E) Histograms depicting distribution of ccIn3862 expression from each data set. The vertical line indicates the population average for that data set.

guing question is whether expression of ccIn3862 when derived from male sperm is equivalent to its expression when derived from hermaphrodite sperm. We sought to answer this question genetically by performing the experiment shown in Figure 7. Animals from progeny class A received ccIn3862 from the oocyte, whereas class B animals received ccIn3862 from male sperm. Class C animals were derived from selfing of F1 parents hemizygous for ccIn3862. Statistically, half of class C animals should receive ccIn3862 from the oocyte and half should receive ccIn3862 from hermaphrodite sperm. We rea-soned that since ccIn3862 in class C is a mixture of both sperm derived and oocyte derived, if male and hermaphrodite sperm expressed ccIn3862 equivalently, then the population average of class C should be intermediate, larger than that of class A but smaller than that of class B. This prediction is indeed borne out (Figure 7D). Additionally, when we combined classes A and B into a single class and determined the population average of the combined AB class, the combined AB average is indeed statistically equivalent to the average of class C. From these observations, we conclude that with respect
to expression of ccIn3862, we do not see evidence for a difference between male vs. hermaphrodite sperm transmission of ccIn3862 (Figure 7, D and E).

**Lack of an observed pairing effect on ccIn3862 expression:** It has been proposed in several systems that the pairing state of a locus in parental meiosis can be a determinant in setting the expression level of the subsequent generation. The experiments described in Figure 4 provide an initial indication of the relative contributions of pairing history and parental origin to expression level for the ccIn3862 transgene. Although the strongest apparent effect on expression of this transgene is from a parent-of-origin effect, an additional effect of pairing history needs to be considered. The data provide evidence that if such a pairing effect influences the expression of ccIn3862, this effect is rather limited in magnitude. The slight expression advantage among genotypically identical populations where ccIn3862 was transmitted from a homozygous state is within the margin of “noise” that we generally observe in these assays (Figure 5, A and B, columns C vs. D and rows 3 vs. 4). To obtain an additional measure of potential effects of pairing history on ccIn3862 expression, we performed the experiment shown in Figure 8. In Figure 8A, the male parent carried two unc-54::gfp transgenes integrated on different chromosomes: ccIn3862 on chromosome I and ccIn3852 on chromosome III. However, only progeny carrying ccIn3862 were used for fluorescence measurements. In Figure 8B, the male parent was homozygous for ccIn3862. In Figure 8C, the male parent was hemizygous for ccIn3862. Hence, the situation we have set up was a comparison of unc-54::gfp expression where the status of the array in the parental generation was two loci unpaired (Figure 8A) vs. one locus paired (Figure 8B) vs. one locus unpaired (Figure 8C). The results showed at most marginal differences between animals derived from the three crosses (within the 15% “noise” window that we generally accord the assays). These observations (and those of Figure 5) do not rule out pairing effects on transgene expression in C. elegans; rather, they indicate that any potential pairing effect on this particularly late-expressed transgene are relatively modest in magnitude.

**Figure 9.—Consequences of long-term uni-gametic maintenance.** (A) Methods to ensure multigenerational inheritance of the transgene through a single gametic lineage. Assayed animals are indicated in dashed rectangular boxes. Double parallel bars (||) over dashed arrows indicate generations that are not shown. For every mating throughout the experiment, progeny classes were carefully noted to ensure that recombination had not occurred between the transgene array and the two recessive markers. Adu, ccIn3862 dpy-5(e61) unc-13(e1091); dpy-5(e61) unc-13(e1091). (B–D) Results of the germline maintenance experiment displayed as a scatter plot. The main experiment is shown in C. The transgene array was transmitted through the oocyte from F1 to F12, switched to sperm from F13 to F23, switched back to oocyte from F24 to F33, then back to sperm from F34 to F44. From F45 to F52, the array was alternately transmitted through each germline for one generation. At F12, a parallel experiment was splintered off from the main experiment in which the transgene array was kept continuously in the oocyte till F34 (D). A similar parallel experiment was performed for sperm transmission (B). Red indicates that the transgene array was oocyte derived, while purple indicates that it was sperm derived. Double parallel bars (||) between two data groups indicate nonconsecutive generations. Left of the long, dashed vertical line, note that the horizontal axis contains skipped generations. Note that the vertical scale is different for each graph. (E) Statistical analysis of the germline maintenance experiment. Color schemes are as indicated in B–D.
Resetting of the *ccIn3862* imprint after long-term maintenance in a single-gamete lineage: The experiments described up to this point have shown that resetting of the transgene can occur after a single-generation passage through the opposite germline. In these experiments, the transgene array had been initially kept in the hermaphrodite parent: it is only at the imprinting cross (where we performed reciprocal crosses) that we distinguished between the oocyte vs. sperm source of the transgene array. Since *C. elegans* hermaphrodites produce both sperm and oocytes, we would expect that a transgene kept in hermaphrodites has an equal chance at each generation of being sperm derived or oocyte derived. We thus expect the state of the hermaphrodite-derived transgene in such populations to reflect (in the long run) an average of the oocyte- and sperm-derived values. This is indeed seen in the experiment described in Figure 7, where the hermaphrodite-derived value (3.90 ± 1.67, cross C) is equal to the average of the oocyte- plus sperm-derived values (3.76 ± 1.96, crosses A and B combined).

It is possible using appropriate genetic markers to engineer the continued passage of a locus through a single germline (oocyte or sperm) for many generations. Several long-term genetic experiments to test the effects of long-term oocyte or sperm transmission were carried out using transgene *ccIn3862* (Figure 9). Figure 9C shows one example of expression levels from such an experiment. For the first 44 generations *ccIn3862* was transmitted through the oocyte or sperm lineage for 10–12 consecutive generations (where we performed reciprocal crosses) that we distinguished between the oocyte vs. sperm source of the transgene. Since *C. elegans* hermaphrodites produce both sperm and oocytes, we would expect that a transgene kept in hermaphrodites has an equal chance at each generation of being sperm derived or oocyte derived. We thus expect the state of the hermaphrodite-derived transgene in such populations to reflect (in the long run) an average of the oocyte- and sperm-derived values. This is indeed seen in the experiment described in Figure 7, where the hermaphrodite-derived value (3.90 ± 1.67, cross C) is equal to the average of the oocyte- plus sperm-derived values (3.76 ± 1.96, crosses A and B combined).

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The results in Figure 9 illustrate several properties of the gamete-specific effect. Most strikingly, the gamete-specific effect is somewhat cumulative. This was par-
Imprinting Capacity in *C. elegans*

All of these experiments indicate that *ccIn3862* that has been continually transmitted in one gamete line can acquire a state with some degree of meiotic stability. The locus thus appears to retain some memory of meiotic source reaching back at least a handful of generations. This has some interesting quantitative consequences. In particular, the results of long-term passage through the same germline followed by a single “switched” generation in Figure 9 can result in average values that are different from those starting from mixed populations of sperm- and oocyte-derived transgene loci (i.e., those in Figure 5). For example, following long-term sperm transmission and a single generation of oocyte transmission (Figure 9B, F45; Figure 9C, F3 and F6), the locus could retain activity higher than that for some of the earlier sperm-derived values (i.e., Figure 9C, F3–F6).

Some, but not all, additional transgenes are imprinted: We tested several additional GFP transgenes to see if they were also subject to a parent-of-origin effect. Of five non-*unc-54* transgenes tested, only *unc-119:gfp* (*edIs6*) exhibited a parent-of-origin effect (Figure 10). *unc-119:gfp* (*edIs6*) is a translational fusion that is seen strongly in the nervous system and faintly in a number of additional tissues (Maduro and Pilgrim 1995). This construct shows a reproducible difference when comparing animals with a sperm-derived transgene locus (higher expression) with animals that carry an oocyte-derived locus (lower expression).

**DISCUSSION**

In this study, we describe our analysis of parent-of-origin-specific imprinting with a set of GFP reporter transgenes in *C. elegans*. For one well-studied set of transgene constructs (*unc-54::gfp*) we observed imprinting in two of three independently integrated transgenic strains as well as in the progenitor extrachromosomal array from which the integrated lines were derived. Since imprinting effects on transgene expression in *C. elegans* had not been reported, these results were somewhat surprising. Further analysis of additional transgenic loci (integrated lines bearing different reporter constructs) indicated that the ability to imprint, although not widespread, was not limited to a single transgene construct or integration site.

The genetic analysis of imprinting for *unc-54::gfp* transgenes clearly indicates that *C. elegans* oocyte and sperm lineages have the capacity to differentially imprint parental chromosomes. Unlike certain imprinting events in other systems in which one parental allele is completely silenced, the imprinting we observed in our study was not an “on-off” situation. Because we are characterizing incomplete imprinting of a reporter transgene, our analysis is necessarily quantitative in nature. For an exemplary transgene *celn3862*, we found 1.5- to 2.0-fold greater expression in progeny that received the transgene from their fathers (sperm transmission) as particularly striking for sperm transmission, for which expression increased for multiple generations during continued passage (Figure 9C, F15–F25; F31–F32). In later generations, increases in expression appeared to slow or plateau (Figure 9B, F29–F35; Figure 9C, F33–F36). A multigenerational effect through oocyte transmission was also indicated, although this effect may require fewer generations than the maximal sperm effect (Figure 9B, F2–F5; Figure 9C, F14–F16).
Figure 10.—Tests for parent-of-origin effects in non-unc-54 reporter transgenes. (A) Experimental design. Because these lines were not in the 
pha-1(e2123ts) background, we used the strategy shown here. tra-2(q122) is a dominant mutation that renders hermaphrodites incapable of sperm production, essentially converting tra-2(q122) hermaphrodites into obligate females (Schedl and Kimble 1988). tra-2(q122) males are unaffected. For each transgene tested, both sperm-transmitted and oocyte-transmitted crosses were performed synchronously and images of F2 animals resulting from the crosses were captured in the same sitting. This allowed us to directly compare fluorescent values from the two crosses without the use of a fluorescent bead standard. Instead, values for $A_{S}/H11001$ were directly normalized relative to $A_{O}/H11001$. (B) Results. Each ratio in the right column is an independent experiment [i.e., two independent experiments for lin-11::gfp(nIs106)]. Asterisks (*) indicate that the experiment was performed at 20°C, rather than at 23°C. (C) Statistical analysis for unc-119:gfp(edIs6). Images from all three experiments for unc-119:gfp(edIs6) were captured using the same camera, but a different microscope was used for experiment 3.

Compared to those receiving the same transgene from mothers (oocyte transmission). The most definitive imprinting assays are those in which activities of a single genetic locus (in this case a transgene) are compared under conditions where maternal and paternal "cytoplasmic" contributions are kept constant (each is hemizygous for the relevant transgene). We were able to take advantage of C. elegans genetics to construct such a situation. Thus, we could dissect a parent-of-origin effect under conditions where only the sperm or oocyte source of the transgene was varied.

How does the genome decide which regions or genes will be subject to imprinting? In mammals, ~80% of imprinted endogenous genes occur within clusters with other imprinted genes (Reik and Walter 2001; Verona et al. 2003). The occurrence of imprinted genes close together has been proposed to reflect coordinate control of these genes by a central imprint control region (ICR). For many imprinted genes in mammals, differential DNA methylation is observed at a CpG-rich region called the DMR (differentially methylated region). Studies in mice indicate a requirement for the DMR and other sequences in the proper temporal and spatial control of imprinted gene expression (i.e., Ainscough et al. 1997; Wutz et al. 1997; Thorvaldsen et al. 1998; Reinhart et al. 2002). Although the idea of coordinate control by an imprint control region seems elegant, the real picture is not so clear. First, what exactly marks a sequence of DNA for imprinting is not known. In the majority of imprinted clusters, certain genes within the cluster escape imprinting, and other genes within the cluster are imprinted only in specific tissues or at specific developmental stages. These observations suggest the existence of cis-acting sequences that may protect the genes from an imprinting effect or direct the temporal/spatial expression of an imprinted gene. Additional studies using transgenic mice indicate that integration into an imprinted cluster is not a requirement for the transgene to exhibit a parent-of-origin effect (Kearns et al. 2000). Given these complexities, one expects that the determination of an imprinted status of a given DNA sequence is likely a combination of many mechanisms. Studies in multiple genetic organisms have clearly demonstrated that local structure (either covalent or epigenetic) plays a critical role in genetic state. This is particularly readily observed with transgene insertions, where (at least for plants and mice) different copy numbers and/or array structures for the same transgene locus can display different levels of expression (i.e., Garrick et al. 1998; Day et al. 2000).
For unknown reasons, certain transgene constructs (including those driven by a minimal unc-54 promoter) are particularly susceptible to silencing in C. elegans. Thus, we were surprised to obtain three integrated derivatives of an unc-54::gfp transgene (ccIn3852, ccIn3861, ccIn3862) in which silencing had apparently been partially or completely lifted. Two of these (ccIn3852 and ccIn3862), along with the progenitor ccEx3815 construct, exhibit an imprinting effect (Figures 3 and 5). The observed lack of a parent-of-origin effect for ccIn3861 could conceivably reflect the internal structure of the transgene array (cis-acting sequences that render ccIn3861 resistant to imprinting), position in the genome (i.e., the integration site of ccIn3861 may not be susceptible to imprinting), or an insufficiently sensitive assay. In light of the fact that the extrachromosomal ccEx3815 is also imprinted, it seems less likely that a cis-acting sequence required to confer imprinting is present in the unc-54::gfp constructs that exhibit a parent-of-origin effect; instead, it may be that ccIn3861 has acquired a resistance to imprinting by virtue of its chromosomal environment. As all of our results are based on quantitation, it is certainly conceivable that a subtle parent-of-origin effect may have existed in ccIn3861 but was below the detection threshold of our assay.

Work in Drosophila has demonstrated that modulation of the chromosomal environment of certain genes can lead to these genes acquiring a parent-of-origin effect. Numerous inversions, translocations, and duplications have taken groups of genes out of their endogenous context and inserted them nearby or into heterochromatic regions. The result is that, in some cases, all genes on the displaced chromosomal segment now acquire a parent-of-origin effect (Lloyd 2000). Lloyd and others have shown that, among a group of displaced genes, the closer a displaced gene is to heterochromatin, the greater is its degree of imprinting compared to other more distal genes in the same group (Cohen 1962; Lloyd et al. 1999). Maggert and Golic (2002) have shown that the entire Y chromosome of Drosophila can confer an imprinting status to transgenes. Hence, in Drosophila, observed imprinting is invariably associated with heterochromatin.

Parent-of-origin imprinting may not be the only mechanism that modulates expression of transgene loci. In particular, we have found that homozygote expression of ccIn3852, ccIn3861, and ccIn3862 is somewhat greater than the expected sum of expression following independent sperm plus oocyte transmission (Figure 3B). Perhaps the nonlinear expression ratio is due to a combination of parent-of-origin and pairing effects. DNA-DNA pairing is an important feature of gene silencing in other systems, as seen with RIP and MSUD in Neurospora (Cambareri et al. 1989; Aramayo and Metzenberg 1996), transvection and pairing-dependent silencing in Drosophila (i.e., Lewis 1954; Henikoff and Dreesen 1989; Dorer and Henikoff 1997), and plants (Assaad et al. 1993; Matzke et al. 1994). Bean et al. (2004) suggest that pairing may be a critical feature of chromatin marks in the early C. elegans embryo. Although we have not observed a strong pairing effect on the later-expressed unc-54::gfp transgene described herein, it is important to note that a modest or earlier effect could have been missed.

How is the imprinted state established during gametogenesis and how is that state maintained through development of the progeny animal? To achieve monoallelic expression of a gene, the parents must establish imprints that mark the two parental alleles as distinct, and the progeny must then maintain the imprints in the somatic cell lineages (Delaval and Feil 2004). Tucker et al. (1996) indicate that (at least in mammals) germline passage is a requirement for the establishment and proper expression of imprinted genes, suggesting that establishment of the imprint occurs during gametogenesis where oogenesis and spermatogenesis differentially mark the maternal and paternal alleles, respectively. In mammals, this is evidenced by differential methylation at the DMRs. Nonhistone DNA-binding proteins as well as cis-acting sequences are important for directing differential methylation during oogenesis and gametogenesis (Perk et al. 2002; Yoon et al. 2002; Pant et al. 2003; Schoenherr et al. 2003; Fedoriw et al. 2004). Differential chromatin modifications also play an important role in the establishment of imprints (i.e., Xin et al. 2001).

Likewise, maintenance of the imprint (at least in mammals) likely involves multiple mechanisms, including maintenance of methylation by Dnmt1 (Bestor 2000; Howell et al. 2001), protection of unmethylated sites by DNA-binding proteins (Pant et al. 2003; Schoenherr et al. 2003; Fedoriw et al. 2004), differential chromatin modifications (Gregory et al. 2001; Fourrier et al. 2002; Yang et al. 2003), Polycomb group proteins (Mager et al. 2003; Otte and Kwaks 2003), and potentially noncoding RNAs (Fitzpatrick et al. 2002; Sleutels et al. 2002).

While DNA methylation is an important feature of epigenetic gene silencing in mammals, plants, and fungi, DNA methylation has not been found to exist in C. elegans. The lack of DNA methylation does not preclude C. elegans from gene silencing activities. Genetic data in Drosophila are consistent with the hypothesis that histone modification serves the necessary role of localized information storage during imprinting (Joanis and Lloyd 2002). By analogy, our current working model is that the imprinting of C. elegans transgenes likely involves the establishment of metastable histone modification states during gametogenesis and the subsequent maintenance and expression of these epigenetic states during embryonic proliferation. Differences in chromatin state would conceivably result from a combination of activating histone modifications upon passage of the transgenes through the sperm and/or deactivating modifications upon transmission through the oocyte.
Because *C. elegans* is a hermaphroditic species, hermaphrodites undergo both spermatogenesis and oogenesis, whereas males undergo only spermatogenesis. Germ-line development in both sexes occurs under a program of temporal and spatial separation (L'Hernault et al. 1997; Schedl 1997; Singson 2001). The cytological processes of spermatogenesis and oogenesis are quite distinct. Spermatogenesis in both males and hermaphrodites occurs as a meiotic precursor cell in the gonad undergoes two rapid divisions, leaving the bulk of cytoplasm behind to produce four very compact spermatids. Hermaphrodite and male sperm are different, with male sperm larger in volume by twofold (LaMunyon and Ward 1998). Oocytes in *C. elegans* are large (50 × 30 × 30 μm) cells that have a 4n DNA content. Meiosis is completed in this species only after fertilization, with the meiotic spindle serving as the organizing center in generating cellular polarity. Genetic screens have identified diverse molecular players in gametogenesis and meiosis, some of which are sperm specific, some are oocyte specific, and some are involved in both processes (i.e., Hodgkin et al. 1979; Reinke et al. 2000). In general, oogenesis and spermatogenesis seem to have more unique than shared features. Most components that play a role in spermatogenesis are required in both hermaphrodites and males, although a small number of exceptions are required in only hermaphrodites (L'Hernault et al. 1988).

Our data indicate that expression of *ccIn3862* when it is transmitted through male sperm is equivalent to its expression when it is transmitted through hermaphrodite sperm (Figure 7). This result suggests that it is the sex of the gamete (i.e., oocyte vs. sperm) and not the chromosomal or physiological sex of the parent that is critical in establishing/maintaining an imprinted state of the transgene. Although hermaphrodite sperm and male sperm differ in size and competence for fertilization (male sperm out-compete hermaphrodite sperm for fertilization; LaMunyon and Ward 1997), our result suggests that the process of establishment and/or maintenance of this imprint is not substantially different between hermaphrodite sperm and male sperm. Interestingly, Bean et al. (2004) also found that the imprinted chromatin state of the X chromosome in early embryos of *C. elegans* is also dependent upon the sex of the gamete.

**How (and when) is imprinting reset upon repassage through the germline?** Maintenance of a constant expression profile in a population exhibiting genetic imprinting will be most effective if the organism has a mechanism to reverse any imprint in the subsequent generation. Numerous examples, particularly from studies in mammals, have demonstrated that passage of an imprinted transgene locus through the opposite germline for a single generation results in the resetting of the imprint (Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987). Although the relief of imprinting in subsequent generations may be the rule, there are exceptions where the imprint appears to be meiotically stable (i.e., Hadchouel et al. 1987; Lau et al. 1999; Kearns et al. 2000; Rakyan et al. 2003). In mice, maternal transmission of an imprinted locus is generally associated with decreased gene activity and hypermethylation of the transmitted DNA sequence. Comparison of the methylation status of DNA from somatic and germine tissues of male mice who had inherited their transgenes from their mothers indicated that DNA in somatic tissue was more heavily methylated than DNA in sperm (Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987). Although the methylation status of the oocyte was not determined, these studies indicated that the methylation status of the maternal parent was transmitted to the somatic cells of the male progeny, but was erased during male gametogenesis. Martin and McGowan (1995) recapitulated these findings in their work with transgenic zebrafish. Chaillet and colleagues provided evidence that a murine transgene had lost its parent-specific methylation patterns in primordial germ cells in both sexes. Sex-specific patterns began to emerge during both oogenesis and spermatogenesis. In the female germline, a female-specific methylation pattern was completely reacquired by late oogenesis, whereas male-specific patterns were completed only after fertilization (Chaillet et al. 1991).

Similar to the murine and zebrafish examples, we have observed repeatedly that the imprinted status of *C. elegans* transgenes can be at least partially reset in a single generation after passage through the opposite germline. Hence, as in the mammalian examples, gametogenesis in the opposite germline can be sufficient to reset the imprint. *C. elegans* apparently has a concerted mechanism for reactivation of transgenes during somatic development (Hsieh et al. 1999). This mechanism is most evident in examining a set of mutant strains (i.e., *tam-1* loss-of-function) in which expression of tandem array transgenes fails to reactivate in somatic lineages. It is certainly conceivable that the resetting of the parent-of-origin imprint during gametogenesis could share components or mechanistic features with the subsequent reactivation of transgene expression in somatic lineages.

Earlier observations with germline-expressed transgenes in *C. elegans* demonstrated that many are subject to a progressive and meiotically stable gene-silencing process over the course of several generations (Kelly and Fire 1998). The experiment shown in Figure 9 demonstrates that a meiotically stable state can be established for either a deactivated or an activated state through long-term transmission through the oocyte or sperm lineage, respectively. Furthermore, the meiotically stable state can be reversed by multigeneration passage through the opposite germline, the extent of reversal being a function of the amount of time the transgene array experiences gametogenesis in the opposite germline. This implies that gametogenesis in each generation is a fixed window of time in which each germline establishes its unique epigenetic marks. A very stable epige-
netic state, therefore, would require multiple generations to establish or reverse. In C. elegans, the mes genes (Kelly and Fire 1998) and the histone H1.1 variant (Jedrusik and Schulze 2001) are required for germ-line gene silencing. Loss-of-function mes mutations or H1.1 RNAi leads to desilencing of transgenes in the germ-line. Perhaps long-term, continual passage through each germ-line leads to the progressive removal and/or replacement of histone variants, resulting in an activated or deactivated state of the transgene array. The extent to which histone variant replacement occurs in C. elegans spermatogenesis is not known, but it is a common phenomenon that has been found in many organisms (Hennig 2003).

What mechanistic or evolutionary process(es) select for (or against) imprinting phenomena? While imprinting serves a developmentally important role for certain groups of organisms such as mammals, plants, and certain insects, there are other organisms that have clearly demonstrated the ability to imprint parental DNA, yet for which imprinting has not been found to be developmentally essential. Viable animals having both copies of one or more chromosomes from only one parent have been generated in Drosophila (Muller 1958; Fuyama 1984; Komma and Endow 1995), zebrafish (Streisinger et al. 1981; Corley-Smith et al. 1996), and C. elegans (Haack and Hodgkin 1991). Parthenogenesis (i.e., Atchley 1977) and androgenesis (i.e., McKone and Halpern 2003) can occur naturally in certain animal and plant species. Clearly, the ability to imprint DNA does not necessitate its use in development. Since the conception of the parental conflict theory (Moore and Haig 1991) to explain the evolutionary significance of genomic imprinting in mammals, numerous other hypotheses that attempt to explain the origin and evolution of imprinting in a broader scope to include non-mammalian systems and in systems that can imprint DNA but where imprinting apparently is not essential have emerged (Haig and Trivers 1995; Hurst 1997; McGowan and Martín 1997; Lloyd 2000; de la Casa-Esperon and Sapienza 2003; Walter and Paulsen 2003b).

We do not know the extent to which parent-of-origin affects the expression of endogenous C. elegans genes. Certainly a strong argument that such effects are either subtle or rare can be made. Three decades of genetic experiments with semidominant genetic markers in C. elegans have failed to yield any known examples where the parent-of-origin for a particular locus affects its expression. Although many of these genetic studies of semidominant loci were not directed toward finding such effects, they would certainly have been detected if such an effect were universal.

A second approach to looking for imprinting in C. elegans has been to generate diploid animals in which both copies of a given chromosome are derived from the same parent (Haack and Hodgkin 1991), from either the male parent or hermaphrodite parent. Such animals have been produced for the X chromosome and each of the five autosomes. In each case the resulting animals can be viable and fertile. Although these experiments rule out an essential role for imprinting of any single locus or chromosome in C. elegans, the experiments do not rule out subtle effects on phenotype or (even more significantly) quantitative effects on viability.

A third approach to detecting parent-of-origin effects on native genes in C. elegans has been taken by the lab of Bill Kelly (Bean et al. 2004). They have examined the overall modification state of the X chromosome during gametogenesis and just after fertilization. Strikingly, the spermatogenesis-derived X chromosome (in both males and hermaphrodites) shows a strong heterochromatin-like imprint (i.e., lack of dimethylated H3-Lys4 and di-acetylated H3-Lys9/Lys14). Although the differential modification of the X chromosome in response to genetic history (as observed by Bean et al. 2004) has some similarity with the imprinting-modulated gene expression that we observe, we note several differences: First, we observe at most a modest effect of parental sex and pairing state relative to a much more substantial difference reflecting gamete-of-origin (sperm vs. oocyte). This is distinct from the X-chromosome-pairing dependence observed by Bean and colleagues. Second, the transgene imprinting that we observe appears distinct in terms of timing. In particular, the X chromosome imprint disappears from the embryo by the 20-cell stage and, hence, might not result in any transcriptional difference between the paternally derived and maternally derived chromosomes. In our case, the parent-of-origin effects that we observe are active much later in the life of the animal (unc-54 is not expressed until just prior to the final division of the myogenic precursors at a stage with several hundred cells) and thus represents a transcriptional difference between maternally vs. paternally derived chromosomes. The timing could indicate a fundamentally different mechanism (i.e., a dependence on independent chromatin modifications) or a common mechanism that derives from some relatively stable chromatin modification that is distinct from those tested with the antibody probes that have been used by Bean and colleagues to date.

In conclusion, we have developed a quantitative assay to measure average GFP expression of C. elegans populations. We have found that among genotypically identical animals, those that receive an unc-54::gfp transgene from sperm show, on average, a reproducible greater expression of the transgene compared to animals that receive the same transgene from oocyte. Moreover, when the transgene was kept in the same gamete lineage for multiple, consecutive generations, the transgene acquired a more stable imprint that required multigeneration passage through the opposite germline to completely reset. Since the parent-of-origin effect was observed for multiple unc-54::gfp transgenes and for a non-unc-54 GFP transgene,
the ability of C. elegans to imprint DNA may not be limited to any unique transgene or integration site. Furthermore, the fact that hermaphrodite sperm and male sperm express an imprinted transgene equivalently suggests that the source of the imprinted may be the gamete type and not the physiological sex of the animal.

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LITERATURE CITED


Bartolomei, S. M. 1997 Transgene repeats and the ability of C. elegans to imprint DNA may not be limited to any unique transgene or integration site.


APPENDIX

TABLE A1

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<td>8.01</td>
<td>9.706</td>
<td>6</td>
<td>9,994</td>
</tr>
<tr>
<td>C1(ad)</td>
<td>11.41</td>
<td>9.709</td>
<td>6</td>
<td>9,994</td>
</tr>
<tr>
<td>A4(ad)</td>
<td>7.63</td>
<td>14.099</td>
<td>0</td>
<td>10,000</td>
</tr>
<tr>
<td>D1(ad)</td>
<td>17.80</td>
<td>14.062</td>
<td>0</td>
<td>10,000</td>
</tr>
<tr>
<td>B3(ad)</td>
<td>7.62</td>
<td>8.778</td>
<td>128</td>
<td>9,872</td>
</tr>
<tr>
<td>C2(ad)</td>
<td>9.83</td>
<td>8.790</td>
<td>128</td>
<td>9,872</td>
</tr>
<tr>
<td>B4(ad)</td>
<td>11.08</td>
<td>13.729</td>
<td>0</td>
<td>10,000</td>
</tr>
<tr>
<td>D2(ad)</td>
<td>16.98</td>
<td>13.734</td>
<td>0</td>
<td>10,000</td>
</tr>
<tr>
<td>C4(ad)</td>
<td>28.68</td>
<td>31.668</td>
<td>0</td>
<td>9,807</td>
</tr>
<tr>
<td>D5(ad)</td>
<td>33.45</td>
<td>31.684</td>
<td>0</td>
<td>9,807</td>
</tr>
</tbody>
</table>

Sperm Equiv.

A 2.45 3.761 0 10,000 0
B 5.19 3.763 0 10,000 0
A 2.45 3.313 0 10,000 0
C 3.90 3.313 0 10,000 0
B 5.19 4.394 0 10,000 0
C 3.90 4.397 0 10,000 0
AB 3.76 3.821 3 7,246 2,753
C 3.90 3.823 3 2,755 7,245

The four columns on the right show the means of the randomized data sets and the frequency each randomized mean is equal to, greater than, or less than the experimental mean. The computer simulation results show that the observed differences are statistically significant for all the paired data sets listed, except for the bottom row, where AB is supposed to be statistically equivalent to C (see Figure 7). Frequencies in the bottom row (AB vs. C) do not add up to exactly 10,000 due to rounding errors in computer floating-point arithmetic.


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Figure A1.—We used computer simulation to test the observed statistical significance of data presented in Figure 5 (matrix experiment) and Figure 7 (3862SE, bottom four rows). Two experimental data sets whose averages were to be compared were pooled. The pooled data set was divided into two random data sets; each randomized data set contained the same number of data points as the original two data sets. Comparison of the averages of the two randomized data sets allowed us to determine statistical significance between the two original sets. If the difference between the two original data sets was statistically significant, then the averages of the randomized data sets should lie between the averages of the two original data sets (i.e., the averages of the randomized data sets should not equal the averages of the experimental data sets). Each paired data set was subjected to 10,000 iterations. Each iteration consists of the pooling of the two experimental data sets, generation of the two randomized data sets from the pool, and determination of the averages of the two randomized data sets.