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A Genomewide Survey Argues That Every Zygotic Gene Product Is Dispensable for the Initiation of Somatic Homolog Pairing in Drosophila

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ABSTRACT

Studies from diverse organisms show that distinct interchromosomal interactions are associated with many developmental events. Despite recent advances in uncovering such phenomena, our understanding of how interchromosomal interactions are initiated and regulated is incomplete. During the maternal-to-zygotic transition (MZT) of Drosophila embryogenesis, stable interchromosomal contacts form between maternal and paternal homologous chromosomes, a phenomenon known as somatic homolog pairing. To better understand the events that initiate pairing, we performed a genomewide assessment of the zygotic contribution to this process. Specifically, we took advantage of the segregational properties of compound chromosomes to generate embryos lacking entire chromosome arms and, thus, all zygotic gene products derived from those arms. Using DNA fluorescence *in situ* hybridization (FISH) to assess the initiation of pairing at five separate loci, this approach allowed us to survey the entire zygotic genome using just a handful of crosses. Remarkably, we found no defect in pairing in embryos lacking any chromosome arm, indicating that no zygotic gene product is essential for pairing to initiate. From these data, we conclude that the initiation of pairing can occur independently of zygotic control and may therefore be part of the developmental program encoded by the maternal genome.

AS the primary storehouse of the cell's genetic material, the nucleus must package the millions of bases that compose the genome into a miniscule volume, while simultaneously permitting the dynamic interplay between nuclear proteins and DNA elements that is required for faithful gene expression. The organization of chromosomes in three-dimensional space is an important factor in both of these functions (reviewed in Branco and Pombo 2007; Lanctôt *et al.* 2007; Misteli 2007). In general, chromosomes occupy discrete territories within the nucleus (reviewed in Heard and Bickmore 2007), with individual loci undergoing limited movement to reach a suitable environment for gene regulation. Furthermore, recent studies have shown that specific interactions can occur in *trans* between different chromosomes. For example, application of chromatin conformation capture (3C) technology to naive T-helper cells of mice has shown that the T_H2 locus control region on chromosome 10 physically interacts with the promoter region of the interferon-γ (*Ifng*) gene on chromosome 11 and that this interaction is important for robust *Ifng* expression (Spilianakis *et al.* 2005). Similar analyses have shown that the X-inactivation centers (XICs) of maternally and paternally derived X chromosomes become juxtaposed during random X inactivation in female murine embryonic stem cells (Bacher *et al.* 2006; Xu *et al.* 2006, 2007). In addition, several groups have used modified versions of 3C technology to survey the genome for sequences that associate with a specific chromosomal region, with multiple analyses reporting reproducible interactions between unlinked genomic segments (Ling *et al.* 2006; Lomvardas *et al.* 2006; Wurtele and Chartrand 2006; Zhao *et al.* 2006; Nunez *et al.* 2008). The ability of a genetic element on one chromosome to interact with a region on another chromosome raises the important question of specificity: How does a genetic element find and interact with another chromosomal locus in *trans*?

Although interchromosomal interactions have gained considerable interest in the recent literature, communication between chromosomes was first postulated over a century ago in Drosophila and other dipteran insects (Stevens 1908). Here, homologous chromosomes are intimately associated from end to end in virtually all cells of the organism, a phenomenon known as somatic homolog pairing (reviewed in McKee 2004). As originally suggested by Nettie Stevens (Stevens 1908) and demonstrated by Ed Lewis (Lewis 1954), somatic pairing in Drosophila can have a profound influence on gene expression; subsequent analyses have shown that this influence can be mediated by interhomolog enhancer–promoter interactions and by *trans* interactions between pairing-sensitive regulatory elements (reviewed in Morris *et al.* 1999; Duncan 2002; Kennison and

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Southworth 2002). Because pairing of homologous chromosomes is highly stable, somatic pairing in Drosophila provides an excellent model for understanding interchromosomal interactions and their effect on gene expression.

Prior genetic analyses in Drosophila have searched for mutations that disrupt pairing-sensitive gene regulation (*e.g.*, Lewis 1954; Gelbart 1982; Leiserson *et al.* 1994). While these and other genetic screens have been fruitful in uncovering chromosomal rearrangements that interfere with somatic pairing, we have yet to describe the molecular mechanism that mediates pairing, likely due in part to our reliance on phenotypes that are several steps removed from pairing *per se*. In addition, prior screens that relied on pairing-dependent gene regulation have been limited by technical considerations, including requirements for organismal viability, for mutations to act dominantly, and/or for pairing-sensitive phenotypes to be assessed one locus at a time. Thus, our understanding of pairing will benefit from whole-genome genetic screens that are based on a direct visualization of chromosome behavior.

One approach to better understand somatic pairing is to dissect how the process is initiated; since maternal and paternal chromosomes enter a newly formed zygote independently, mechanisms that identify and bring together homologous chromosomes may function as part of the developmental program. Following fertilization, the Drosophila embryo progresses through 13 synchronous mitotic cycles to create a syncitial blastoderm with thousands of nuclei arrayed just below the surface of the outer membrane (Foe *et al.* 1993). These mitotic divisions are initially just a few minutes long and then slow down during cycles 11–13 before finally pausing for at least 60 min during interphase 14, at which time the syncitial blastoderm cellularizes (Foe *et al.* 1993). The last few syncitial divisions are of particular interest, as studies using fluorescence *in situ* hybridization (FISH) to label specific chromosomal loci have shown that homolog pairing is first observed during this time, progressing to appreciable but locus-specific levels of pairing during the long interphase of cycle 14 (Hiraoka *et al.* 1993; Fung *et al.* 1998; Gemkow *et al.* 1998). For example, the highly transcribed and repetitive histone complex (HisC) is paired in ~70% of cycle 14 nuclei, while non-repetitive euchromatic regions are paired to a lesser degree in ~10–30% of nuclei. In addition, experiments using a panel of FISH probes that target 11 loci on a single chromosome arm support that pairing is initiated by multiple independent associations rather than by "zippering" the chromosomes from a discrete pairing initiation site (Fung *et al.* 1998).

What are the molecular events that initiate pairing in the blastoderm? Several genes have been postulated to play a role in pairing (see discussion), but the genetic requirements for the initiation of homolog pairing—and thus the molecular mechanism by which homologous chromosomes are first identified and aligned—have yet to be demonstrated. Interestingly, the onset of homolog pairing during the late syncitial mitotic cycles coincides with the critical period of embryogenesis when many aspects of the developmental program switch from maternal to zygotic control, known as the maternal-to-zygotic transition (MZT) (Figure 1). Zygotic transcription is first observed for a subset of genes as early as nuclear cycle 8, with robust widespread transcription being reached during cycle 14 (Edgar and Schubiger 1986; Erickson and Cline 1993; Pritchard and Schubiger 1996; ten Bosch *et al.* 2006; De Renzis *et al.* 2007; Lecuyer *et al.* 2007). Concomitant with the activation of the zygotic genome is the programmed degradation of thousands of maternally deposited RNAs (De Renzis *et al.* 2007; Tadros *et al.* 2007). Indeed, recent genomewide microarray studies have provided a high-resolution view of the regulation of specific transcripts during the MZT (Arbeitman *et al.* 2002; Pilot *et al.* 2006; De Renzis *et al.* 2007; Tadros *et al.* 2007), facilitating molecular analyses of the transition from maternal to zygotic control.

The timing of pairing initiation during the MZT suggests two possible sources for factors that mediate pairing: factors may be maternally deposited prior to fertilization and/or generated by expression of specific genes from the zygotic genome. Put another way, pairing may be preprogrammed into the embryo from fertilization onward but prevented during the rapid

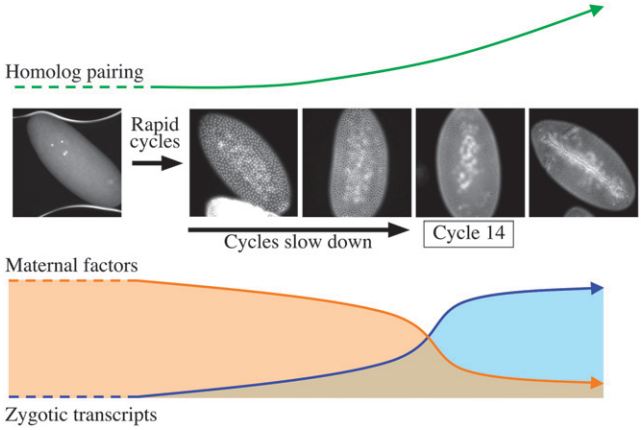

Figure 1.—Somatic homolog pairing initiates during the MZT. DAPI-stained embryos (center) show the progression of development during the syncitial blastoderm stage of embryogenesis. The time of development from fertilization to the onset of gastrulation (far right) is ~3 hr at 25° (Foe *et al.* 1993). Many maternal factors, which are deposited into the oocyte prior to fertilization, become depleted or are degraded during the later syncitial cycles (orange line). Other maternal factors perdure until later stages of development (not shown). In contrast, zygotic genes are initially silent and are first expressed during the late syncitial cycles (blue line). Previous studies have shown that homolog pairing (green line) initiates during the MZT (Fung *et al.* 1998; Gemkow *et al.* 1998).

early cell cycles or pairing may await the expression of one or more zygotic genes during the late divisions of the syncitial blastoderm. Indeed, prior genetic studies have analyzed other developmental processes that coincide with the MZT, including cell cycle regulation, cellularization, maternal RNA degradation, lipid droplet transport, and nuclear morphogenesis (Merrill *et al.* 1988; Wieschaus and Sweeton 1988; Pilot *et al.* 2006; De Renzis *et al.* 2007). Notably, each of these processes depends upon one or more zygotic transcripts for faithful execution, demonstrating the suitability of a genomewide analysis of zygotic requirements for the initiation of homolog pairing.

To gain insight into the initiation of homolog pairing and its coordination with the transition from maternal to zygotic control, we have addressed the requirement for zygotic transcripts in the onset of pairing by adapting a genetic strategy for the study of embryonic development used previously by Wieschaus and colleagues (Merrill *et al.* 1988; Wieschaus and Sweeton 1988; De Renzis *et al.* 2007). Specifically, we generated embryos lacking entire chromosome arms, and thus lacking all zygotic transcripts normally produced from those arms, allowing an efficient genomewide assessment of zygotic gene function. Using DNA–FISH to label specific chromosomal loci, we find that somatic pairing occurs in embryos lacking zygotic transcripts from any chromosome arm, implying that the initiation of homolog pairing is independent of zygotic control. We discuss the implications of this finding for the regulation of pairing in Drosophila and in other organisms.

MATERIALS AND METHODS

Drosophila stocks and culture conditions: Wild-type flies were Oregon-R (OR) unless otherwise indicated. Embryos lacking the X chromosome were created by crossing *C(1)DX y f* (attached-X) females to y^{3d}/Y males. Embryos lacking autosomal chromosome arms were generated from the following stocks obtained from the Bloomington Stock Center: *C(2L)RM-P1,* b^1*; C(2R)RM-S1,* cn^1 for the second chromosome, *C(3L)RM-P3,* $kni^{ri\text{-}1}$*; C(3R)-P3* for the third chromosome, and *C(4)RM,* ci^1 $ey^R/0$ for the fourth chromosome. Flies were maintained at 25° on standard Drosophila cornmeal, yeast, sugar, and agar medium with *p*-hydroxybenzoic acid methyl ester as a mold inhibitor (Morris *et al.* 1998). Embryos were collected at 25° on apple juice–agar plates with a dollop of yeast paste to feed adult flies.

Probes for FISH: Our analysis made use of two types of probes. To label repetitive sequences, we used oligonucleotide probes (Integrated DNA Technologies) with a 5′ fluorescent tag of Cy3, Cy5, or Alexa488. All oligonucleotides used in this study contained a mixture of DNA and locked nucleic acid (LNA) bases, which increases melting temperature relative to DNA alone (Silahtaroglu *et al.* 2003). For nonrepetitive euchromatic sequences, we generated fluorescently labeled probes via nick translation of a genomic DNA template according to the kit manufacturer's protocol (Vysis). For template DNA, we used genomic clones contained in P1 (Berkeley *Drosophila* Genome Project) or BAC (BACPAC Resource Center, Children's Hospital Oakland Research Institute) vectors or a pool of 10–12 ~1-kb PCR products that were generated from a span of 30–40 kb of a chromosomal locus (Williams *et al.* 2007).

The probes for the five regions used to assess pairing were: HisC, oligonucleotide AagCgcTcgAccAtcAccAgtC (B. R. Williams, unpublished observations) (where uppercase denotes an LNA base and lowercase denotes a DNA base); 359-bp repeat, oligonucleotide TttTccAaaTttCggTcaTcaAatAatCat (Hsieh and Brutlag 1979); dodeca satellite, oligonucleotide AcGg GaCcAgTaCgG (Williams *et al.* 2007); 28B, nick-translated P1 genomic clone DS01529 (Fung *et al.* 1998); and 62E, nick-translated pooled PCR products derived from the 62E chromosomal region.

Probes used to genotype embryos lacking each chromosome arm were the following: X, oligonucleotide CcAgTgCa GaAgAaAaTcAa targeting repetitive sequences in the 14A region; 2L, either the HisC or the 28B probes described above; 2R, nick-translated pooled PCR products derived from the 53F chromosomal region; 3L, nick-translated P1 DS02752 carrying genomic sequences from the 69C region (Dej and Spradling 1998; Williams *et al.* 2007); 3R, nick-translated BAC 32J3 carrying sequence from the 84A region; 4, nick-translated pooled PCR products from the 102 chromosomal region.

Fixing, staining, and imaging: We generally collected embryos for 1 hr and then aged them to a 2- to 3-hr developmental window, which corresponds roughly to interphase 14 (see below for more detailed staging). Occasionally, collections included earlier time points. For fixation, we modified an existing protocol designed to preserve nuclear structure (Gemkow *et al.* 1998; Bantignies *et al.* 2003). Following dechorionation, fixation, RNAse treatment, and prehybridization washes as previously described, embryos were mixed with hybridization buffer (50% formamide, 2× SSC, 10% dextran sulfate, 0.05% salmon sperm DNA) containing fluorescently labeled probes (50 nM oligonucleotide probes and/or ~1/8th–1/10th of a nick-translation reaction), denatured for 4 min at 91°, and incubated overnight (~16 hr) at 42°–45°. Hybridized embryos were washed in solution 1 (50% formamide, 2× SSC, 0.3% CHAPS) and then stepped through successive washes of 30, 20, and 10% formamide in PBT (PBS plus 0.1% Tween-20) before a final wash in PBT alone. Following the washes, nuclear envelopes were labeled with Alexa488- or Alexa647-conjugated wheat germ agglutinin (Molecular Probes) (Wilkie *et al.* 1999) by incubating embryos in a 1 μg/ml solution in PBT–Tr (PBS plus 0.3% Triton X-100) for 20 min followed by two 15-min washes in PBT–Tr. Embryos were mounted in Vectashield with DAPI (Vector Laboratories). For each embryo, a single region was chosen wherein all visible nuclei could be easily assayed, and then high-resolution three-dimensional images were collected and deconvolved using a Deltavision imaging system and Softworx software as described (Williams *et al.* 2007).

Staging and genotyping: The 2- to 3-hr collections used for most experiments should capture embryos in the final 10 min of cell cycle 13 and the first 50 min of cell cycle 14. Due to the time spent manipulating embryos during the dechorionation step, most embryos were aged ~5–10 min longer before development was stopped during fixation, and so very few cell cycle 13 embryos were observed. In these collections, and in those that included earlier time points, wild-type interphase 14 embryos were distinguished from embryos in prior cell cycles by the density of nuclei at the embryonic surface, which doubles during each division between cell cycle 10 and 14 (Foe *et al.* 1993). We defined the end of interphase 14 by the formation of the cephalic fold and gastrulation (~185 min after egg laying), and the rare embryos that had begun these developmental processes were not scored. Very rarely, we

observed germ-band extended embryos that were older than 3 hr of development, which arise when females hold fertilized eggs in their uteri prior to egg laying. These embryos were not scored.

In mutant embryos lacking chromosome arms, early interphase 14 embryos were differentiated from prior cycles by the density of nuclei at the surface of the embryo as for wild type. As the development of these embryos progresses, the normal morphology of regularly spaced nuclei is lost, and nuclei became misshapen, clumped, and lost from the surface into deeper planes of the embryo (Merrill *et al.* 1988; Wieschaus and Sweeton 1988). These morphological abnormalities were used as markers of late interphase 14, since embryos in earlier cell cycles do not show these characteristics. Because formation of the cephalic fold and gastrulation are generally defective in mutants lacking chromosome arms, we were unable to set an upper limit to cell cycle 14 analogous to that used for wild type. Since it was therefore not possible to verify that all mutant embryos were younger than the point when these processes should occur (~185 min), it is conceivable that some mutant embryos in our study are slightly older than their wild-type counterparts; however, we expect that these embryos are rare since very few of their developmentally normal siblings were observed to develop past gastrulation. Furthermore, because our goal was to assess zygotic gene function, any mutant embryo, regardless of being temporally beyond the developmental window used for wild type, that shows significant pairing has achieved this feat in the absence of zygotic gene function, supporting the conclusion of our study. We therefore expect our inability to observe head involution and gastrulation in mutant backgrounds to have no significant impact on our analysis.

To ensure accurate identification of embryos lacking each chromosome arm, all probes used for genotyping were tested for their efficacy by one of two strategies. For the two probes specific to the third chromosome (69C on the left arm and 84A on the right arm), embryos from the stock *C(3L); C(3R)* were double labeled with both probes using two different fluorophores; 50/50 of the embryos scored were stained by either one or both probes, with no evidence of false negatives. Furthermore, 23 embryos were labeled by both probes, 15 were negative for 69C and positive for 84A, and 12 were negative for 84A and positive for 69C, corresponding roughly to the expected Mendelian ratio of 2:1:1. Probes specific to the X, second, and fourth chromosomes were tested by hybridizing to wild-type embryos, which should result in 100% labeling in cell cycle 14 since all chromosomes are present in each embryo. For all four probes, all embryos were easily identified as positively stained, with no evidence for false negatives (probe 14A, 63/63 embryos labeled; probe 28B, 46/46; probe 53F, 78/78; probe 102, 36/36).

As a further assay of the efficacy of our probes and the behavior of the chromosomes used in this study, we tallied the numbers of positively and negatively stained cycle 14 embryos from a sampling of our experiments to determine whether we obtained the expected Mendelian ratios of embryos with and without a given chromosome arm. For each probe, we scored at least 100 embryos from two to five separate experiments (supplemental Table 4). For X, 2L, 2R, 3L, and 3R, we observed the expected ratio of ~1/4 of embryos from the appropriate compound chromosome stock to be lacking the chromosome arm of interest (also see Figure 2). In the case of our probe targeting the 102 region of chromosome 4, we observed a slightly higher than expected fraction of mutant embryos from C(4)RM parents (0.34 *vs.* 0.25 expected); because our probe is 100% effective in labeling wild-type embryos, we do not believe that this ratio reflects false-negative staining. Rather, due to the unique biology of this chromosome, which is small, gene poor, and largely heterochromatic, it may be that the compound fourth chromosome displays an altered pattern of segregation during meiosis, as has been observed for some other compound chromosomes (*e.g.* Dernburg *et al.* 1996b).

Scoring homolog pairing: For each probe, we scored a locus as paired when signals were overlapping or touching and as unpaired if signals appeared as separate foci. Occasionally, we observed three or four signals in one nucleus (for example, see 62E FISH in Figure 3C), likely indicating the separation of sister chromatids following replication of the genome at the beginning of interphase 14. Nuclei with three or four signals were scored as unpaired.

Because each of the five FISH probes used to score pairing produced a signal of slightly different size, the interhomolog distance required to resolve two separate nonoverlapping signals differed slightly from probe to probe. For the HisC and dodeca probes, signals scored as unpaired were separated by a distance $> \sim 0.5$–0.6 μm; for 28B and 62E, by ~0.4–0.5 μm; and for the 359-bp repeat, by ~1 μm. Importantly, these probe-specific distances did not vary between wild-type and mutant embryos, permitting the direct comparison of pairing levels for each locus between different backgrounds.

To verify our findings, we measured the distance between homologous loci for the HisC and the 62E regions in a subset of the wild-type and mutant cycle 14 embryos used in the study and arranged the data in percentile plots, which display all the distances between FISH signals in a single experiment regardless of the nucleus of origin (Williams *et al.* 2007) (supplemental Figure 1). Using this stringent method, nuclei with a single FISH signal that was roughly spherical in shape were given a measurement of 0 μm, while overlapping FISH signals that appeared elongated or dumbbell-shaped were treated as separate foci, and a measurement was made between the presumed centers of the two overlapping signals. We then determined the percentage of nuclei with paired homologs as defined by one of two criteria: (1) those with complete overlap of FISH signals (distance = 0 μm) or (2) those within 0.5 μm of their homolog, which is roughly the distance required to resolve the two FISH signals as nonoverlapping (supplemental Table 1). Using either of these criteria, we see equivalent or even greater levels of pairing relative to wild type in embryos lacking any chromosome arm, supporting the conclusion that zygotic gene function is not required for pairing to initiate.

To ensure that nuclei with a single FISH signal truly represented paired homologs and not, for example, inefficient hybridization that revealed only one of the two homologous loci, we compared fluorescent intensities of presumed paired FISH signals *vs.* unpaired FISH signals in wild-type and mutant backgrounds. Briefly, summed intensities of z-sections were projected into one plane with Softworx Explorer software, and the intensity of fluorescence in the relevant channel was measured for an area encompassing one FISH dot. For each embryo analyzed, we repeated this measurement for ~5 paired FISH signals and 10 unpaired FISH signals (*i.e.*, five nuclei of each category). We then subtracted the nonspecific background fluorescence, derived as the average intensity of four regions surrounding, but not including, each FISH signal, from each measurement to calculate an intensity score for each signal. For all five probes used in this analysis, the average intensity of paired signals was roughly twofold higher than that of unpaired signals (supplemental Table 2), with no significant differences between wild type and mutants, indicating that nuclei with a single FISH focus in fact result from overlapping homologs.

Scoring chance overlap of nonhomologous regions: To determine the percentage of FISH signals expected to overlap

by chance rather than by specific pairing of homologs, we stained wild-type cycle 14 embryos with two probes specific for nonhomologous regions and scored whether or not nonhomologous FISH signals were touching (supplemental Table 3). Due to the Rabl configuration of cycle 14 nuclei, the probes available for this analysis were limited to those that were roughly the same distance from their centromere; in light of this restraint, we restricted our scoring to those nonhomologous FISH signals that were in the same or adjacent planes of a three-dimensional image stack. Because this restriction was not used when scoring pairing of homologous regions, our analysis represents an overestimate of the rate of nonhomologous colocalization. In spite of this, nonhomologous regions were found to overlap at much lower rates than homologous regions. In the case of the 359-bp repeat, we expected this rate to be higher due to the larger size of its FISH signal; as expected, chance overlap of the 359-bp repeat with the dodeca satellite, which occupies the same territory near the top of the Rabl-configured nucleus, was 5- to 10-fold higher than that of probes to euchromatic autosomal regions, but still ~10-fold lower than the average pairing level of homologous 359-bp regions found in wild type and mutant backgrounds (Table 1).

Sexing embryos to score X-specific pairing: The 359-bp repeat on the X chromosome is present in two copies in female embryos, but in only one copy on the single X chromosome in male embryos. While male embryos could theoretically be identified using FISH probes specific to the Y chromosome, our experimental strategy had already used four fluorescent channels to label the compound chromosome, the locus to be scored for pairing, all DNA, and the nuclear envelope, and we were not confident in the reliability of a fifth fluorescent signal. Thus, male embryos were identified as having 100% of their cycle 14 nuclei with a single FISH signal using the probe to the X-specific 359-bp repeat, while embryos with any number of nuclei containing two signals were scored as female. We confirmed the efficacy of this method of scoring by two independent criteria: first, we observed these two classes of embryos in an ~1:1 ratio as expected. For example, of 49 wild-type embryos analyzed in one experiment, 20 were scored female and 19 were male. Second, we quantified the intensities of signals from presumed male and female embryos by the summed intensity method described above. As expected, the paired FISH signals of presumed female embryos were twice the intensity of the single signals observed in presumed male nuclei (a ratio of 1.91, scoring 48 total nuclei from four male and four female embryos derived from two separate experiments), supporting this method for determining the sex of embryos.

RESULTS

Our goal is to better understand the initiation of homolog pairing in Drosophila. To do so, we tested the role of the zygotic genome using a high-throughput strategy that manipulates large genomic segments *in vivo* (Merrill *et al.* 1988; Wieschaus and Sweeton 1988; De Renzis *et al.* 2007). Specifically, we took advantage of a special class of chromosomes, called compound chromosomes, which can be used to generate embryos lacking entire chromosome arms in a predictable Mendelian fashion (Figure 2). For example, to generate embryos lacking second chromosome arms, we used the compound chromosomes C(2L) and C(2R), wherein C(2L) carries two copies of the left

TABLE 1

Summary of pairing in wild-type and mutant cycle 14 embryos

Probe	Wild type	Nullo-X	Nullo-2L	Nullo-2R	Nullo-3L	Nullo-3R	Nullo-4
359 (X chromosome)	65.6 ± 13.1 (2410)	—	66.5 ± 10.2 (910)	67.2 ± 15.8 (726)	65.4 ± 9.8 (1083)	70.8 ± 22.6 (1143)	69.3 ± 9.6 (937)
HisC (chromosome II)	70.6 ± 13.1 (1627)	59.4 ± 15.9 (2434)	—	—	66.5 ± 18.8 (1840)	70.4 ± 14.9 (1242)	ND
Dodeca (chromosome III)	17.7 ± 5.4 (2118)	18.8 ± 7.2 (1689)	20.4 ± 13.0 (588)	27.6 ± 12.3 (813)	—	—	24.7 ± 6.4 (1160)
28B (chromosome II)	15.2 ± 5.4 (777)	14.6 ± 6.3 (423)	—	—	10.5 ± 5.0 (588)	15.1 ± 11.6 (492)	ND
62E (chromosome III)	27.0 ± 6.9 (613)	ND	24.1 ± 8.4 (937)	30.0 ± 5.2 (650)	—	—	34.0 ± 4.4 (524)

Average pairing levels (percentage of nuclei with paired loci) ±SD are indicated for each combination of FISH probe and genotype, with the number of nuclei scored in parentheses. A "—" indicates combinations where the region to be assessed for pairing was located on the compound chromosome used for that experiment; these combinations were not scored.

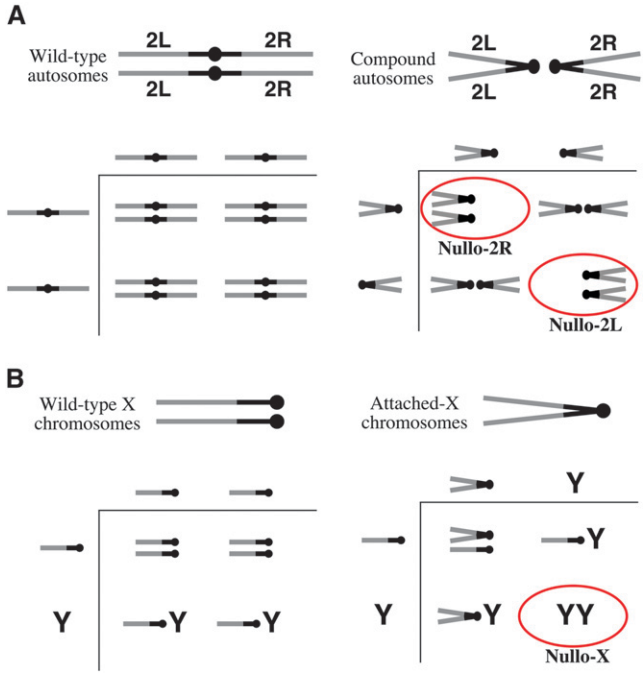

Figure 2.—Generating embryos lacking chromosome arms. (A) The structure of wild-type chromosome II homologs (left) and the compound chromosomes C(2L) and C(2R) (right). The predominantly euchromatic chromosome arms are shaded, while pericentric heterochromatin and the centromere are solid. The Punnett squares show that crosses between flies carrying wild-type chromosomes segregate to yield fully euploid progeny, while the compound chromosomes segregate to yield one class lacking the right arm of chromosome II (nullo-2R) and one class lacking the left arm of chromosome II (nullo-2L). The structurally analogous compound chromosomes C(3L) and C(3R) were used to generate embryos lacking the left or the right arm of chromosome III, while a compound chromosome carrying two entire copies of chromosome IV (the "dot" chromosome) was used to generate nullo-4 embryos. (B) Wild-type homologous X chromosomes (left) and attached-X chromosomes (right). Females carrying attached-X and Y chromosomes, when crossed to XY males, yield one class of progeny that lacks the entire X chromosome. The structure of the C(1)DX chromosome used in our analysis differs slightly from the figure shown here (Novitski 1954), but is identical in its pattern of segregation.

arm of chromosome 2 and C(2R) carries two copies of the right arm (Figure 2A). As such, flies carrying both C(2L) and C(2R) have the normal complement of chromosome II; however, when flies carrying these chromosomes are crossed together, one of the four classes of progeny lacks the left arm, while another class lacks the right arm (Figure 2A). Since these embryos arise from euploid mothers, the maternal contribution to their development is normal; however, because they lack a specific chromosome arm, the zygotic contribution to their development will lack all transcripts encoded by that portion of the genome. Similar compound chromosomes exist for the X (Figure 2B) and for the other two autosomes (chromosomes 3 and 4; not shown), allowing us to generate embryos lacking X, 3L, 3R, or 4 in addition to those lacking 2L or 2R. While our studies also address the Y, observations of somatic pairing in female cells lacking a Y automatically prelude the requirement of genes on this chromosome for pairing (Fung *et al.* 1998; Williams *et al.* 2007). In brief, our study constitutes a genomewide assessment of zygotic gene function in the initiation of pairing using just four parental genotypes.

As described below, we first quantified pairing in wild-type embryos using DNA–FISH, targeting several loci and thereby establishing a baseline for the remainder of our study. Then, using embryos collected from crosses of flies carrying compound chromosomes, we employed a two-color FISH assay to simultaneously identify embryos lacking a specific chromosome arm and score pairing levels on another chromosome in those same embryos. Importantly, because the overall development and cell cycle timing of embryos lacking any chromosome arm are indistinguishable from wild type for the first 13 synchronized divisions (Merrill *et al.* 1988; Wieschaus and Sweeton 1988), we were able to assay pairing during cell cycle 14 in each mutant background without the influence of prior gross developmental abnormalities. If zygotic gene products are required for pairing to initiate, we would expect to see reduced pairing levels in embryos lacking the chromosome arm, or arms, carrying the critical genes. However, if the initiation of pairing does not require the activity of any specific zygotic gene, we would anticipate that pairing levels would be unchanged in embryos lacking any chromosome arm.

Pairing in euploid embryos: To assess pairing in wild-type embryos, we first chose five regions of the Drosophila genome to serve as targets for DNA–FISH and thus as representative sites for pairing levels (Figure 3). To account for locus-specific effects, targets were chosen from the three major chromosomes and represented repetitive heterochromatic regions (the 359-bp repeat on the X chromosome and the dodeca satellite on chromosome III), a highly transcribed repetitive euchromatic region (HisC on chromosome II), and two regions of nonrepetitive euchromatin (polytene regions 28B on chromosome II and 62E on chromosome III). The analysis of both heterochromatic and euchromatic regions was an important factor in our experimental design, as heterochromatin has the ability to cluster in subnuclear compartments (Csink and Henikoff 1996; Dernburg *et al.* 1996a) and may therefore differ from euchromatin in the factors that can affect its pairing status. In addition, the five selected loci are distributed at different positions along the lengths of the chromosome arms such that they occupy varying positions in the Rabl-configured blastoderm nucleus (Figure 3B), accounting for potential effects of different subnuclear positions on pairing initiation. Finally, several of these loci were utilized in prior reports of pairing in the

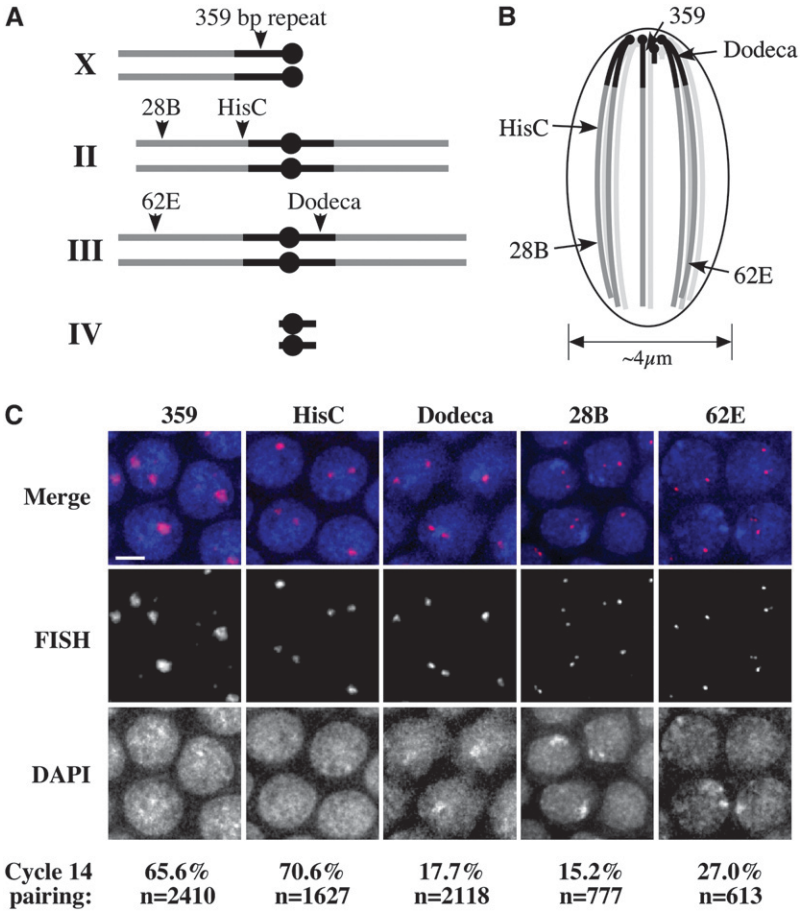

FIGURE 3.—Homologous loci pair at locus-specific levels during cycle 14 in wild-type embryos. (A) Schematic showing the chromosomes of *Drosophila melanogaster* (Y not shown) with chromosomal loci targeted by FISH probes indicated by arrows. Euchromatin is shaded, while pericentric heterochromatin and the centromere are solid. (B) Positions of FISH-targeted loci in cycle 14 nuclei. At this time of development, all chromosomes are in a Rabl configuration, with all centromeres facing the outer surface of the embryo and telomeres at the opposite side of the nucleus. (C) FISH staining of wild-type cycle 14 nuclei. All images represent compressed stacks of deconvolved z-sections. For each locus, the average pairing level is shown below the corresponding column, along with the total number of nuclei scored.

embryo (HIRAOKA *et al.* 1993; FUNG *et al.* 1998) and in cell culture (WILLIAMS *et al.* 2007), allowing us to compare our observations to existing data sets.

Wild-type OR embryos were subjected to fixation conditions designed to preserve nuclear structure (see MATERIALS AND METHODS), hybridized with a fluorescently labeled probe specific to one of the five regions above, and counterstained to highlight all DNA (DAPI) and the nuclear membrane (fluorescently labeled wheat germ agglutinin) (WILKIE *et al.* 1999). Embryos in interphase of cell cycle 14 were identified on the basis of several criteria, including elapsed time since egg deposition, the density of nuclei at the surface of the embryo, and nuclear morphology (see MATERIALS AND METHODS). Cycle 14 embryos were then subjected to high-resolution three-dimensional imaging, and the pairing status of the FISH target was assessed for ~50–150 nuclei from each embryo. We considered homologous loci to be paired when the hybridization signals were touching or overlapping and unpaired when two or more discrete signals were visible. A parallel analysis using precise distance measurements between FISH signals as previously described (WILLIAMS *et al.* 2007) was used for several experiments and produced data comparable to those described below (supplemental Figure 1; supplemental Table 1).

Consistent with previous observations (HIRAOKA *et al.* 1993; FUNG *et al.* 1998), we found the HisC to be the most highly paired of the five loci examined during cell cycle 14 (Figure 3C). Among 13 individual embryos scored, the percentage of nuclei with paired HisC loci ranged from 47 to 91%, with an average of 71% (n = 1627 nuclei). A parallel analysis of HisC pairing in a different wild-type background, Canton-S, provided similar results (average 74%, n = 1195 nuclei). Pairing levels for the 359-bp repeat on the X chromosome were also high, with 65.6% (n = 2410) of cell cycle 14 nuclei having paired homologs. Notably, pairing of the 359-bp repeat in female embryos confirms (FUNG *et al.* 1998; WILLIAMS *et al.* 2007) that genes encoded on the Y chromosome are not essential for pairing. In contrast to the high pairing levels observed for HisC and the 359-bp repeat, the euchromatic loci 28B and 62E and the dodeca satellite were paired to a lesser degree (Figure 3C), in line with previous reports of pairing levels in the range of ~10–30% for nonrepetitive euchromatic loci in cell cycle 14 nuclei (FUNG *et al.* 1998; GEMKOW *et al.* 1998) and of relatively low pairing levels for the dodeca satellite in cultured cells (WILLIAMS *et al.* 2007). Importantly, each locus was paired to a greater degree than one would expect by chance overlap of FISH signals, as determined by quantifying the overlap of nonhomolo-

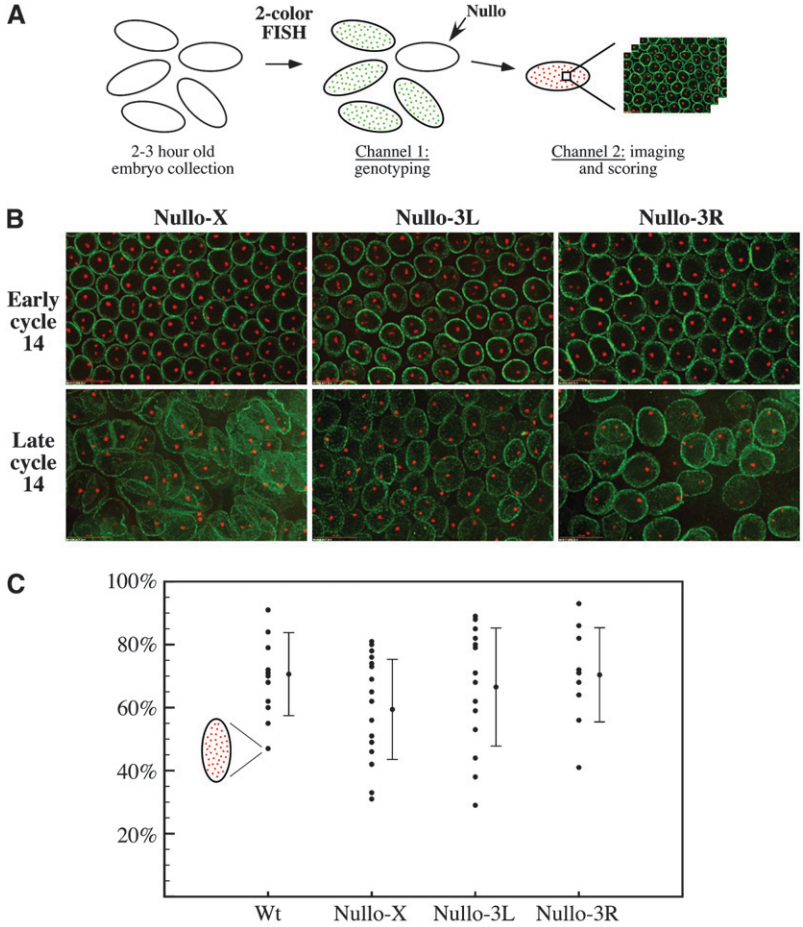

FIGURE 4.—The HisC pairs in the absence of zygotic transcripts from X, 3L, or 3R. (A) Strategy for assaying pairing in embryos lacking chromosome arms. Staged cycle 14 embryos were stained with one probe that was specific to the compound chromosome of interest, allowing us to identify embryos that were missing the chromosome arm, and a second probe that targeted one of the five loci shown in Figure 3, which was used to score pairing. Importantly, the probe used to assess pairing did not target the compound chromosome in any experiment, and thus pairing was always scored on a structurally wild-type chromosome. (B) Cycle 14 embryos lacking the entire X chromosome (nullo-X), the left arm of chromosome III (nullo-3L), or the right arm of chromosome III (nullo-3R). FISH targeting the HisC is shown in red, and the nuclear envelope is highlighted in green. Early in cycle 14, morphology is relatively normal (top row), while late cell cycle 14 is characterized by a loss of wild-type morphology (bottom row). (C) Quantitation of pairing in wild-type and mutant cycle 14 embryos. Each point in a vertical column represents the percentage of nuclei with paired HisC loci in one embryo of the given genotype; points with error bars represent the average pairing level of all embryos of the indicated genotype ±1 SD.

gous loci (supplemental Table 3); thus, each of the five loci examined reaches a characteristic pairing level in wild-type cell cycle 14 nuclei.

The HisC pairs in the absence of zygotic genes from X and III: We next evaluated the ability of the HisC on chromosome II to pair in embryos lacking either the X chromosome (nullo-X) or the left or right arm of chromosome III (nullo-3L and nullo-3R, respectively), each of which encodes ~20% of the Drosophila genome. To carry out this analysis, we used our two-color FISH strategy to identify embryos lacking a given chromosome arm and then to determine the level of HisC pairing in those embryos (Figure 4A). We assessed pairing on only structurally wild-type chromosomes and not on the compound chromosomes themselves. For each mutant background analyzed in this experiment and those described below, we tallied the number of embryos carrying the chromosome arm of interest and those lacking it to ensure that the expected Mendelian ratio was obtained (supplemental Table 4).

Remarkably, despite the absence of thousands of zygotic transcripts, FISH signals from the HisC were paired to a high degree during cell cycle 14 in each of the three mutant backgrounds analyzed (Figure 4B). To compare HisC pairing levels for each genotype, we quantified pairing as described above for wild-type embryos and graphed the percentage of nuclei with paired HisC signals for each embryo analyzed (Figure 4C; Table 1). In general, pairing levels in mutant backgrounds showed more embryo-to-embryo variability relative to wild type, which is not unexpected, given the dramatic developmental and physiological disruption in these embryos. Importantly, there was no significant difference between the pairing levels of wild-type embryos and those of the mutants ($P > 0.05$, two-tailed Mann–Whitney test); thus, pairing of the HisC proceeds in the absence of any zygotic transcript normally produced from the X chromosome or the left or right arm of the third chromosome.

Embryos lacking chromosome arms enter interphase 14 with correct timing and morphology, but begin to appear abnormal as the cell cycle progresses, with nuclei taking on irregular shapes and eventually falling into the depths of the embryo (Figure 4B) (MERRILL *et al.* 1988; WIESCHAUS and SWEETON 1988). We were curious as to whether pairing between homologous HisC loci is maintained during these changes or, alternatively, whether it is disturbed by the gross morphological disruptions observed in late cell cycle 14. To address this, we binned embryos lacking X, 3L, or 3R into two

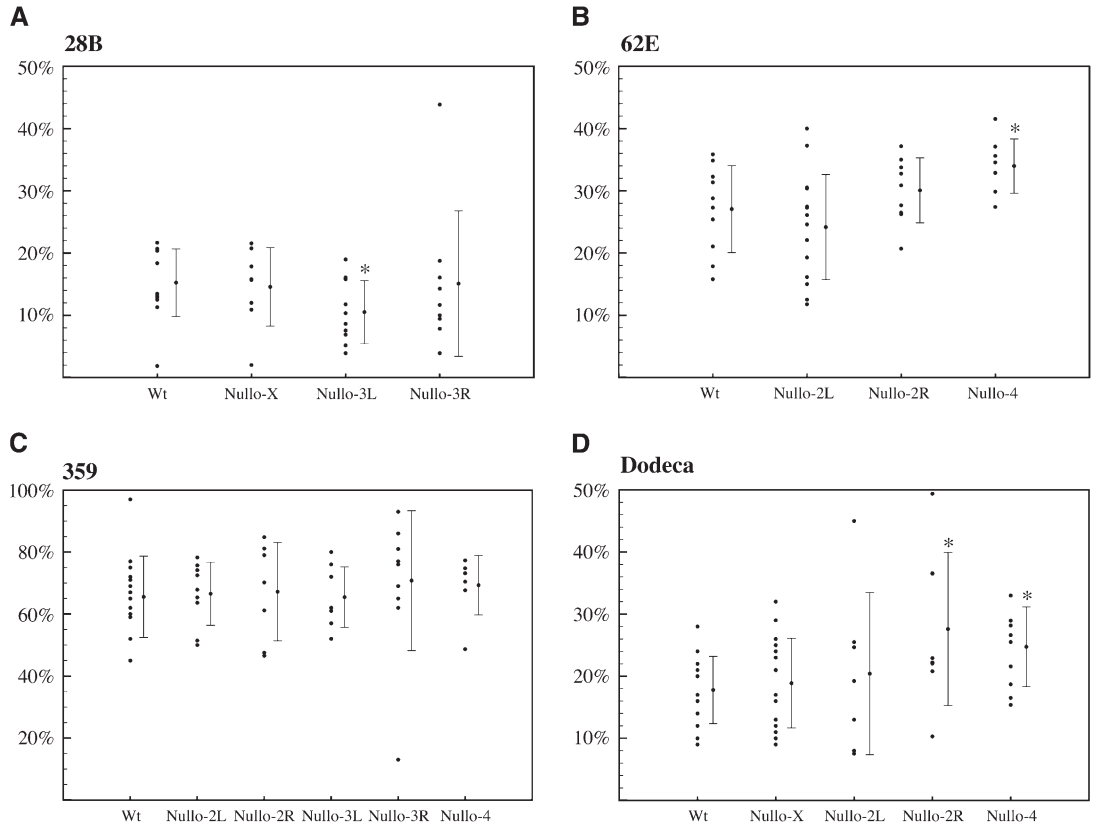

FIGURE 5.—Widespread pairing in the absence of any chromosome arm. Graphs display cycle 14 pairing levels as assayed by FISH targeting the euchromatic regions 28B (A) and 62E (B) and the heterochromatic regions containing the 359-bp repeat (C) and the dodeca satellite (D). Each point in a vertical column represents the percentage of nuclei with paired loci in one embryo of the given genotype; points with error bars represent the average pairing level of all embryos of the indicated genotype ±1 SD. Average pairing levels were significantly higher than wild type for the 62E region in nullo-4 embryos ($P = 0.04$) and for the dodeca satellite in nullo-2R ($P = 0.02$) and nullo-4 ($P = 0.02$) embryos. Although pairing levels of the 28B region were significantly lower than wild type ($P = 0.03$) in nullo-3L embryos, all data points from this experiment fell within the wild-type range, and a comparison to the larger data set of wild type, nullo-X, and nullo-3L showed no significant difference ($P = 0.06$). Even taking into account instances where pairing levels differ from wild type, it is clear that pairing is supported in embryos lacking any chromosome arm.

categories: those with normal nuclear morphology, representing early cell cycle 14, and those with abnormal morphology, representing late cell cycle 14. If pairing contacts are lost as embryos die, we would expect to see a drop in the percentage of nuclei with paired signals in later embryos. However, in the three mutant backgrounds combined, we observed a higher average level of pairing for HisC FISH signals from the later group (75% of nuclei with paired signals, $n = 2035$) relative to the earlier embryos (58% paired, $n = 3368$), consistent with the increase in HisC pairing that has been reported for wild-type embryos progressing through cell cycle 14 (FUNG *et al.* 1998). Thus, paired HisC loci remain associated and may continue to form new contacts during the dramatic changes in nuclear morphology and loss of physiological homeostasis in mutant embryos.

No zygotic gene product is essential for the initiation of pairing: We were unable to use HisC to assess the role of second-chromosome-encoded zygotic genes in the initiation of pairing, as the locus is lost altogether or present on a structurally altered chromosome in nullo-2L and nullo-2R embryos, respectively. We therefore chose the 62E region on chromosome III to continue our analysis. In addition, as the biology of the HisC may not be representative of the genome as a whole due to its highly repetitive and highly transcribed nature, we conducted further experiments using a probe to the nonrepetitive euchromatic region 28B, located on chromosome II. Analysis of these two additional regions combined allowed us to survey every chromosome arm for a role in pairing of nonrepetitive euchromatin.

Consistent with our observations for the HisC, loss of X, 3L, or 3R did not preclude pairing of the 28B locus, as many embryos demonstrated pairing well above that expected by chance (Figure 5A). Similarly, the 62E region showed substantial pairing in the absence of 2L, 2R, or chromosome IV (Figure 5B), indicating that mechanisms for identifying and pairing homologs remain in place in the absence of zygotic transcripts from

each of these chromosome arms. Only the pairing of 28B in a nullo-3L background was found to be significantly lower than its wild-type counterpart ($P = 0.03$); importantly, this significance is lost when the comparison is made to the larger combined data set of wild-type, nullo-X, and nullo-3R embryos ($P = 0.06$), supporting that nullo-3L embryos are competent for pairing at wild-type levels. Our analysis also showed that the distribution of 62E pairing in nullo-4 embryos was significantly higher than that of wild type ($P = 0.04$); in this case, the significance remains ($P < 0.05$) when compared to pairing of the same region in all other backgrounds combined. It is as yet unclear whether this instance of higher than expected pairing is biologically significant or may be accounted for by variables within our experimental system (see DISCUSSION).

Finally, we extended our analysis to two heterochromatic regions, the 359-bp repeat and the dodeca satellite, to ask whether this different type of chromatin structure may depend on any zygotic genes for its pairing initiation. For the 359-bp repeat, we scored pairing in embryos lacking each of the autosomal arms, while the dodeca satellite was analyzed in embryos lacking X, 2L, 2R, or IV. Consistent with our analyses of euchromatic regions, embryos from each experiment showed competence for pairing (Figure 5, C and D). Similar to the 62E region, dodeca pairing levels were significantly higher in a nullo-4 background ($P = 0.02$) and also in nullo-2R embryos ($P = 0.02$) relative to wild type. However, pairing was not significantly lower than wild type for either heterochromatic locus in any mutant background tested, indicating that mechanisms for pairing heterochromatic regions remained functional in all mutant backgrounds tested. In sum, our analysis shows that homologous chromosomes can initiate their pairing in the absence of any zygotically transcribed gene.

DISCUSSION

Recent technological advances have expanded our capacity to identify and characterize interchromosomal interactions, but the manner by which chromosomes and/or chromosomal regions are recognized and brought together remains a mystery. Here we focused on Drosophila to better understand the conditions that initiate somatic homolog pairing. Our strategy took advantage of the segregational properties of compound chromosomes to generate embryos lacking entire chromosome arms and, thus, all zygotic gene products encoded on those arms. Analysis of these embryos showed that no single zygotic gene product is required for the initiation of pairing during the long interphase of cell cycle 14 as assayed by DNA–FISH. This result was true regardless of whether we assayed heterochromatic or euchromatic regions or whether we analyzed loci that pair to a high or low degree during cycle 14. From these data, we propose that the onset of pairing does not await the expression of critical factors from the zygotic genome; rather, the capacity to pair homologous chromosomes is likely an integral property of embryos as they enter the MZT.

Another interpretation of our data is that, in fact, the initiation of pairing does require zygotic gene functions, but that these essential functions are redundantly encoded in the genome. Our approach of removing entire chromosome arms decreases the likelihood of this scenario: Because pairing initiates in the absence of any chromosome arm, all redundant functions would have to be encoded on separate chromosome arms; otherwise, they would be removed concurrently in mutant embryos. Further rebutting the possibility of redundancy, we have used a compound chromosome that carries two entire second chromosomes (NOVITSKI *et al.* 1981) to analyze pairing in the absence of all zygotic transcripts from chromosome II, accounting for ~40% of the genome, and still do not observe a significant difference in pairing levels relative to wild type (J. R. BATEMAN, unpublished observations). Our data, however, do not exclude the intriguing possibility that pairing could be driven by the concurrent action of a very large set of maternal and/or zygotic factors that constitute redundant systems and therefore confound mutational analyses. Examples of such mechanisms for pairing would include the multimerization of diverse chromosomally bound proteins along the length of two homologs (*e.g.*, WILLIAMS *et al.* 2007), the action of a variety of enhancers on homologous promoters in *trans* (LEE and WU 2006), and/or the activation of zygotic transcription throughout the genome (COOK 1997; also see below).

Additionally, due to the pattern of segregation displayed by compound chromosomes, embryos lacking one chromosome arm are expected to be polyploid for another chromosome arm. For example, embryos lacking C(2L) will likely carry two copies of C(2R) and therefore twice the normal diploid dosage of zygotically expressed genes on 2R (Figure 2). In one sense, this is advantageous as it maintains the total DNA content of mutant nuclei at a level comparable to wild type and therefore conserves the nuclear volume through which chromosomes must search for a homologous partner. While it is conceivable that the extra dose of one chromosome arm could influence the pairing of a locus on another chromosome, it seems unlikely that this would compensate for loss of a critical zygotic gene product such that pairing levels are restored to wild-type levels. We therefore do not believe that the issue of ploidy can fully explain our observations.

Finally, the technology used to generate compound chromosomes (see ASHBURNER *et al.* 2004) can cause regions tightly linked to centromeres to remain associated with both the left and the right arms of compound autosomes, and it is therefore possible that small segments of the genome have escaped our analysis. Indeed,

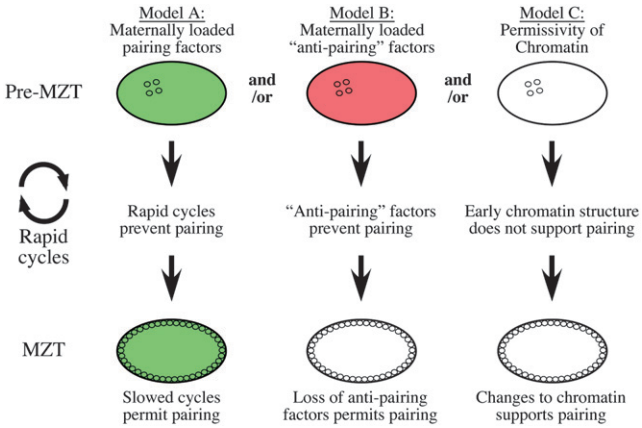

FIGURE 6.—Models for the initiation of pairing. The timing of pairing initiation during the MZT could be accounted for by at least three models, any combination of which may occur simultaneously. In model A, factors that promote pairing are provided maternally (green), but are prevented from acting during early embryogenesis due to the rapid mitotic cycling. As the mitotic cycles slow during the MZT, these factors persist, allowing homologs to form stable contacts. In model B, maternal loading of factors that prevent pairing (red) precludes interactions between homologs during early cycles; loss of these factors during the MZT permits pairing to initiate. In model C, structural aspects of chromatin during early embryogenesis prevent pairing from occurring until the MZT, when conformational changes to chromatin (for example, via the initiation of zygotic transcription) create a permissive substrate for pairing.

FISH has shown that the dodeca satellite, located immediately adjacent to the centromere on 3R, is present on both C(3L) and C(3R) (CARMENA *et al.* 1993). Although sequences adjacent to the centromeres of Drosophila are largely repetitive and heterochromatic, a small number of genes are encoded within these regions (reviewed in YASUHARA and WAKIMOTO 2006). Furthermore, we cannot exclude the possibility that some repetitive heterochromatic sequences may themselves be involved in pairing homologous chromosomes, perhaps through the generation of small RNAs (reviewed in GREWAL and ELGIN 2007) that function as part of a global pairing machinery. Nevertheless, our data show that the vast majority of zygotically expressed genes are dispensable for the initiation of pairing.

A developmental timer for pairing: Because pairing can initiate in the absence of any specific zygotic gene product, our analysis suggests that factors required for pairing are present throughout early embryogenesis. Why, then, do homologous chromosomes not pair during the cell cycles prior to the MZT? An explanation put forth previously suggests that early mitotic cycles are so rapid that there is insufficient time between mitoses for homologous chromosomes to establish stable contacts (FUNG *et al.* 1998; GEMKOW *et al.* 1998; also see FUNABIKI *et al.* 1993; GOLIC and GOLIC 1996; GUBB *et al.* 1997) (Figure 6). While this is supported by the observation that progression through anaphase can partially disrupt pairing (FUNG *et al.* 1998), alternative models are also possible. For example, pairing might be prohibitive to the extremely rapid succession of DNA synthesis and mitoses that occur during the earlier cycles of embryogenesis and thus may be specifically prevented by maternally provided "anti-pairing" factors (Figure 6). Such factors, which could exist as proteins, protein/RNA complexes, or chromosomal marks, may then be depleted or removed either before or during the MZT to allow pairing to initiate once the embryo has progressed beyond the requirement for rapid expansion of DNA content. A removal of chromosomal marks at this point in development would be reminiscent of the early stages of mouse embryogenesis, where the paternal genome is actively stripped of methylation marks shortly after fertilization and prior to the MZT (MAYER *et al.* 2000; OSWALD *et al.* 2000). Notably, this demethylation event likely precedes the pairing of XICs (BACHER *et al.* 2006; XU *et al.* 2006) that is associated with the initiation of X inactivation in female mouse embryos. Although a direct relationship between these two events has not been demonstrated, an intriguing possibility is that erasure of chromatin marks on the paternal X is important for establishing counting and choice in murine X inactivation.

One other explanation for the timing of pairing initiation during the MZT reserves a role for the actual process of zygotic transcription, if not for zygotic gene products themselves. Specifically, a previous model for homolog pairing postulated that the pattern of gene activity along the length of a chromosome facilitates interactions between homologs either by folding the chromosome fibers into similar conformations (COOK 1997) or perhaps by enlisting transcription factories (OSBORNE *et al.* 2004) to bring homologous genes together. According to this model, the initiation of pairing during the MZT would be a direct effect of the changes to chromatin conformation associated with activation of zygotic transcription at this time (Figure 6). Indeed, the Rabl configuration of cycle 14 nuclei would assist homologous associations by this mechanism, allowing for a direct comparison of active and inactive genes along linear chromosome fibers. Because our analysis involved assessing pairing on one chromosome while removing the genes of another, our findings do not address this possibility. Prior reports have considered a role for transcription in pairing—either by examining HisC pairing in the transcriptionally quiescent pole cells (FUNG *et al.* 1998) or by comparing pairing of the bithorax complex in cells where these genes are active to those in which they are silent (GEMKOW *et al.* 1998)—and have found no evidence in support of the model. However, a more thorough investigation may be warranted by, for example, assessing pairing in the presence of chemical inhibitors of RNA polymerases and/or maternal mutations that alter global patterns of zygotic transcription.

What pairs homologous chromosomes? While a molecular machinery that pairs homologous chromosomes has yet to be identified, molecules that promote long-distance interactions in the nucleus have been considered as possible candidates for this function, including members of the Polycomb group (Buchenau *et al.* 1998; Saurin *et al.* 1998; Bantignies *et al.* 2003; Vazquez *et al.* 2006) and insulator proteins such as CTCF (Ling *et al.* 2006; Zhao *et al.* 2006; Xu *et al.* 2007) and Su(Hw) (Gerasimova *et al.* 2000; Byrd and Corces 2003; Kravchenko *et al.* 2005; Fritsch *et al.* 2006; but see Golovnin *et al.* 2008). Another candidate to consider is topoisomerase II (Top2), whose inhibition was recently shown to disrupt pairing in Drosophila cell culture (Williams *et al.* 2007). Notably, each of these molecules can be directly assessed for a role in the initiation of pairing in the Drosophila embryo by removing or interfering with their maternally derived components during the MZT either through the use of maternal-effect mutants or via injection of inhibitory chemicals and/or double-stranded RNAs. However, it is not entirely clear how these proteins could account for the specificity of homolog pairing, as they would theoretically be just as likely to bring together nonhomologous chromosomal regions as homologous sequences. It will therefore be important in the future to clarify whether candidate mutations that disrupt homolog pairing represent genes that are specific to a homolog pairing mechanism or, alternatively, whether they also affect nuclear colocalization independently of sequence homology.

Although our analysis was focused on identifying factors required to initiate pairing, it is notable that the loss of some chromosome arms led to a statistically significant increase in the level of pairing of some loci at cycle 14 (Figure 5). It is possible that these increases resulted from effects that are not directly related to a pairing mechanism; for example, the reduced genetic complexity of nuclei lacking large segments of the genome may facilitate the search for a homologous partner. However, it is also possible that the observed increase in pairing reflects the loss of zygotically transcribed factors that limit the rate of pairing initiation and/or stabilization. The existence of such factors would be consistent with prior suggestions that pairing of homologous sequences may be an intrinsic low-energy property of chromatin (Lee *et al.* 2004) and can be negatively regulated (Williams *et al.* 2007). This notion may be further supported by a recent study of ligand-dependent interchromosomal associations in human cells, where genes that respond to estrogen were found to physically associate upon stimulus with the hormone (Nunez *et al.* 2008). Curiously, despite a high degree of colocalization between nonhomologous estrogen-responsive genes, homologous chromosomal regions were never found associated, consistent with the existence of a global repression of homolog pairing in these cells. Such a repressive mechanism may exist in many organisms to allow transient interchomosomal interactions to regulate specific epigenetic and developmental phenomena or perhaps as a means to restrict gene conversion and subsequent loss of heterozygosity by favoring the use of a sister chromatid rather than a homolog as a template for repair.

Interactions between chromosomes have been known for over a century (Montgomery 1901), with pairing of homologous chromosomes being well established as an integral step of meiosis in most sexually reproducing organisms. Furthermore, physical interactions between homologous sequences have been either directly observed or implicated in many epigenetic phenomena, including transvection (reviewed in Duncan 2002; Kennison and Southworth 2002), paramutation in plants and in mice (reviewed in Chandler and Stam 2004; Grant-Downton and Dickinson 2004), repeat-induced point mutation and methylation induced premeiotically (reviewed in Galagan and Selker 2004), meiotic silencing of unpaired DNA (Aramayo and Metzenberg 1996; Shiu *et al.* 2001), meiotic sex chromosome inactivation (reviewed in Turner 2007), and X inactivation (Marahrens 1999; Bacher *et al.* 2006; Xu *et al.* 2006). The recent characterization of interactions between nonhomologous sequences (Ling *et al.* 2006; Lomvardas *et al.* 2006; Simonis *et al.* 2006; Spilianakis and Flavell 2006; Zhao *et al.* 2006; Nunez *et al.* 2008) further highlights the importance of regulating contacts between chromosomal regions. By identifying mechanisms that control homology-dependent and -independent interchromosomal interactions in Drosophila and in other organisms, we will greatly enhance our understanding of the regulation of gene expression and better appreciate the importance of the three-dimensional organization of the genome.

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