

# Incomplete sister chromatid separation is the mechanism of programmed chromosome elimination during early *Sciara coprophila* embryogenesis

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## SUMMARY

Sex in *Sciara coprophila* is determined by maternally supplied factors that control the number of paternal X chromosomes eliminated during the syncytial embryonic divisions. Confocal microscopy and FISH demonstrate that the centromeres of the X chromosomes separate at anaphase and remain functional during the cycle in which the X chromosomes are eliminated. However, a region of the sister chromatids fails to separate and the X chromosomes remain at the metaphase plate. This indicates that failure of sister chromatid separation is the mechanism of chromosome elimination. Elimination of the X chromosomes requires the presence of a previously discovered Controlling Element that acts in *cis* during male meiosis. Using an X-autosome translocation, we demonstrate that the Controlling Element acts at-a-distance to prevent sister

chromatid separation in the arm of an autosome. This indicates that the region in which sister chromatid separation fails is chromosome-independent. Although chromosome elimination occurs in all somatic nuclei and is independent of location of the nuclei within the embryo, the decision to eliminate is made at the level of the individual nucleus. Programmed X chromosome elimination occurs at different cycles in male and female embryos. These observations support a model in which elements on the X chromosome are titrating maternally supplied factors controlling the separation of sister X chromatids.

Key words: chromosome elimination, sister chromatid separation, sex determination, imprinting, *Sciara*, *Bradysia*.

## INTRODUCTION

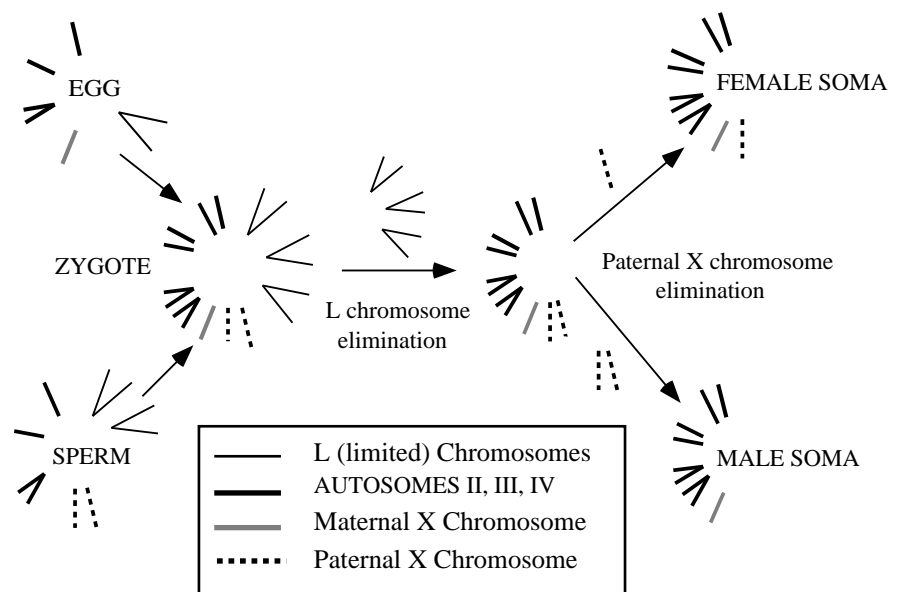
The accurate replication and segregation of chromosomes are defining features of the mitotic cell cycle. The structural and regulatory mechanisms governing these processes are conserved throughout the phyla. Exceptional cell cycles in which the chromosome content of the division products differs from the chromosome content of the parental cell serve as natural variants with which to explore the mechanisms of cell division. Meiosis is a particularly well known example. The diploid meiocyte undergoes two rounds of segregation without accompanying DNA synthesis and yields four haploid products. In the reductional division chiasmata joining the homologs serve the same function as the attachment between sister chromatids. Achiasmata chromosomes require a kinesin-like microtubule motor protein to keep the homologs together (Hawley et al., 1993; McKim and Hawley, 1995; Orr-Weaver, 1995).

Other exceptional cell cycles are those in which there is a developmentally programmed chromosome loss. For example, chromosome diminution, in which chromosome fragments are lost, is observed in nematodes and during the formation of the macronucleus of some ciliates (Tobler et al., 1992; Yao, 1989). At multiple stages in *Sciara coprophila* development, the programmed elimination of entire chromosomes occurs. Chromosome loss occurs during male meiosis, germline development and the early embryonic divisions (Gerbi, 1986). During

meiosis I in males, the paternal autosomes and X chromosome are eliminated. The meiotic spindle is monopolar and only the maternal haplotype and the heterochromatic L chromosomes move to the pole (Metz, 1938; Rieffel and Crouse, 1966; Crouse et al., 1971). In meiosis II, programmed X chromosome non-disjunction results in sperm bearing two maternally derived sister chromatids (Crouse, 1943, 1960a; Abbott et al., 1981). Chromosome elimination also occurs during the mitotic divisions of the germline stem cells of both sexes. In these cells a paternal X chromosome and a heterochromatic L chromosome are eliminated by expulsion from the prophase nucleus (Rieffel and Crouse, 1966). These observations suggest that chromosome elimination in male meiosis and in the germline stem cells occurs by distinct mechanisms.

This study focuses on the elimination of the X chromosomes, which occurs during the initial embryonic divisions in *Sciara* (see Fig. 1). Because of the exceptional pattern of chromosome segregation during male meiosis, fusion of the male and female pronuclei produces a nucleus containing a single maternal X chromosome and two paternal X chromosomes. Sex is determined by maternal factors that regulate whether one or both of the paternally derived X chromosomes are lost during the syncytial embryonic divisions. Loss of one paternal X chromosome produces a female XX soma while loss of both X chromosomes produces a male XO soma (Metz, 1938, and references therein). A *cis*-acting element mapping to the rDNA locus on the X chromosome, known as the Controlling Element

**Fig. 1.** Paternal X chromosomes and heterochromatic L chromosomes are eliminated during the syncytial divisions. The contributions of the sperm and the egg are unequal due to abnormalities in male meiosis. Zygotes of both sexes have the same karyotype, with three pairs of autosomes, one maternal X, two paternal X chromosomes and three pairs of L chromosomes. The L chromosomes are eliminated from the somatic nuclei when the germline differentiates. The sex of the embryo is determined by the loss of one paternal X chromosome to produce a female XX soma, or both paternal X chromosomes to produce a male XO soma. Later in embryogenesis, the germline of both sexes eliminates one paternal X chromosome and the number of L chromosomes is reduced to two. Adapted from Gerbi (1986).



(CE), is necessary for elimination of the X chromosomes during the syncytial divisions (Crouse, 1960b, 1977, 1979).

We have used confocal microscopy and FISH to explore the mechanism of chromosome loss during the syncytial divisions. Our findings extend earlier work which demonstrated that the eliminated chromosomes were left at the metaphase plate (DuBois, 1932, 1933). We show that the centromeres of the eliminated X chromosomes separate at anaphase and remain active into telophase. However, a region of the sister chromatids fails to separate during anaphase and the chromosomes remain on the metaphase plate. This suggests that the mechanism of elimination is a failure of sister chromatid separation. Using a translocation, we demonstrate that the CE acts in *cis* and at-a-distance to prevent sister chromatid separation. Our studies also indicate that processes affecting chromosome elimination act at the level of individual nuclei and are independent of the position of the nuclei within the embryo.

We also demonstrate that male embryos usually eliminate X chromosomes at an earlier cycle than female embryos, and eliminate at multiple cycles. These observations support a model in which elements on the X chromosomes are titrating out maternally supplied factors controlling the separation of sister X chromatids.

## MATERIALS AND METHODS

### Strains and egg collection

*Sciara coprophila* (syn. *Bradysia coprophila* Lintner) strains 7298 and 6980 and the flies carrying the T1 translocation chromosome were kindly provided by Dr Susan Gerbi (Brown University, Providence, Rhode Island). In this species, separate sets of male and female embryos can be readily obtained because females have offspring of only one sex. Mothers of daughters are heterozygous for an X chromosome, called X', which is passed exclusively from mother to daughter. Mothers of sons are homozygous for the X chromosome (Metz, 1938; Crouse, 1977). In both 7298 and 6980 strains the X' chromosome is marked with *Wavy* and in the 6980 stock the X chromosome is marked with *swollen*. 0-12 hour collections were taken separately from X'X females and XX females to study the female

versus the male pattern of chromosome elimination. An important objection to this procedure is that an X'X female may occasionally produce sons and an XX female may occasionally produce daughters. Rieffel and Crouse (1966) were able to determine that these 'exceptional offspring' are due to non-disjunction of the X chromosome in female meiosis, so that the correct pattern of X chromosome elimination will nevertheless produce an offspring of the wrong sex.

To analyze the syncytial divisions, 1 hour egg collections were fixed and stained (see next section) after aging, in approximately 15 minute increments, from 0 to 13 hours at 22°C. The eggs were derived from X'X and XX females so that both female and male embryos could be analyzed.

### Fixation and staining

Embryos were fixed by a modification of the Mitchison and Sedat (1983) procedure and of procedures described elsewhere (Hiraoka et al., 1993; Sullivan et al., 1990). After dechorionation with 50% Clorox for 90 seconds, embryos were washed in 0.4% NaCl and 0.03% Triton X-100 and placed in heptane to fenestrate the vitelline membrane (Karr and Alberts, 1986). Embryos were fixed for in situ hybridization by adding an equal volume of 10-10-10 fixative (10% each of 37% formaldehyde, 5 M EGTa and 10× PBS in water) to the

**Table 1. Timing of the first 11 cycles**

Cycle	Hours AED (After egg deposition)	s.d.	Number of embryos
Syngamy	1.4	0.5	12
1	1.6	0.5	30
2	2.0	0.4	45
3	2.3	0.4	34
4	2.9	0.5	21
5	3.3	0.5	17
6	3.7	0.3	37
7	4.2	0.5	23
8	4.9	1.4	20
9	5.6	0.9	57
10	7.0	1.3	33
11	9.3	1.1	31
12	11.4	1.4	62

Nuclear cycles begin in interphase and end in telophase.

Hours AED is the mean age of the embryos collected at each cycle, determined as described in Materials and Methods.

heptane for 20 minutes, then removing the fixative and shaking with an equal volume of methanol to devitellinize. Embryos were fixed for immunofluorescent analysis by adding an equal volume of methanol and shaking to fix and devitellinize. Fixed embryos were stored in methanol and subsequently treated with RNase and stained with propidium iodide (Sigma, St Louis, MO) to visualize the DNA and with fluorescein-labeled antibodies to tubulin (Karr and Alberts, 1986).

### In situ hybridization

We constructed a DNA probe that hybridizes specifically to the *Sciara* rDNA on the X chromosome. Cloned *Sciara* rDNA was kindly provided by Dr Susan Gerbi. This probe was used for whole mount in situ hybridization, according to Hiraoka et al. (1993).

### Microscopy

Microscopy was performed using an Olympus IMT2 inverted photomicroscope equipped with a BioRad 600 laser confocal imaging system. The lenses used included the Olympus S Plan Apo 60 $\times$ , Oil, and the Olympus D Plan Apo 20 $\times$ , UV, Oil. Confocal images were imported into Adobe Photoshop 3.0, where composite plates were prepared. Fig. 3 (the cycle display) was the only image in which the embryos were edited: polar bodies and some condensed chromatin debris from chromosome loss were removed and the embryos were sized to make them the same length.

### Timing data

The data in Table 1 were generated from 1 hour collections of embryos aged between 0 and 13 hours, then fixed and stained. The embryos were assigned an age equal to the aging time plus 0.5 hours, and the nuclear cycle was determined by using confocal microscopy to count the nuclei. As the nuclear divisions during syncytial development in *Sciara* are synchronous (see Results), the nuclear cycle can be determined by estimating the total number of nuclei. For cycles 1-7, all the nuclei in the embryo were counted. In later cycles, optical sections were used to calculate the number of nuclei at the surface of the embryo. The nuclei of the germline fall out of synchrony at cycles 5-6 and the germline divides less often than the somatic nuclei (usually two cycles fewer between cycle 6 and cycle 11). Yolk nuclei (interior nuclei that do not migrate to the cortex or fall in from the cortex) lower the count of nuclei on the surface. Therefore the number of nuclei at the surface in the later cycles will be lower than the theoretical number (two raised to the cycle number at telophase). Nevertheless, the nuclear cycle could be determined by the method described above for almost all embryos.

The times listed in Table 1 were produced by taking the mean and standard deviation of the ages of the embryos for each cycle. The timing of fusion of the male and female pronuclei (two nuclei very close in the center of the embryo with decondensed polar bodies at the surface) was also tabulated. The high standard deviations indicate that these values are approximate and may vary from embryo to embryo.

### Scoring fixed embryos for chromosome elimination

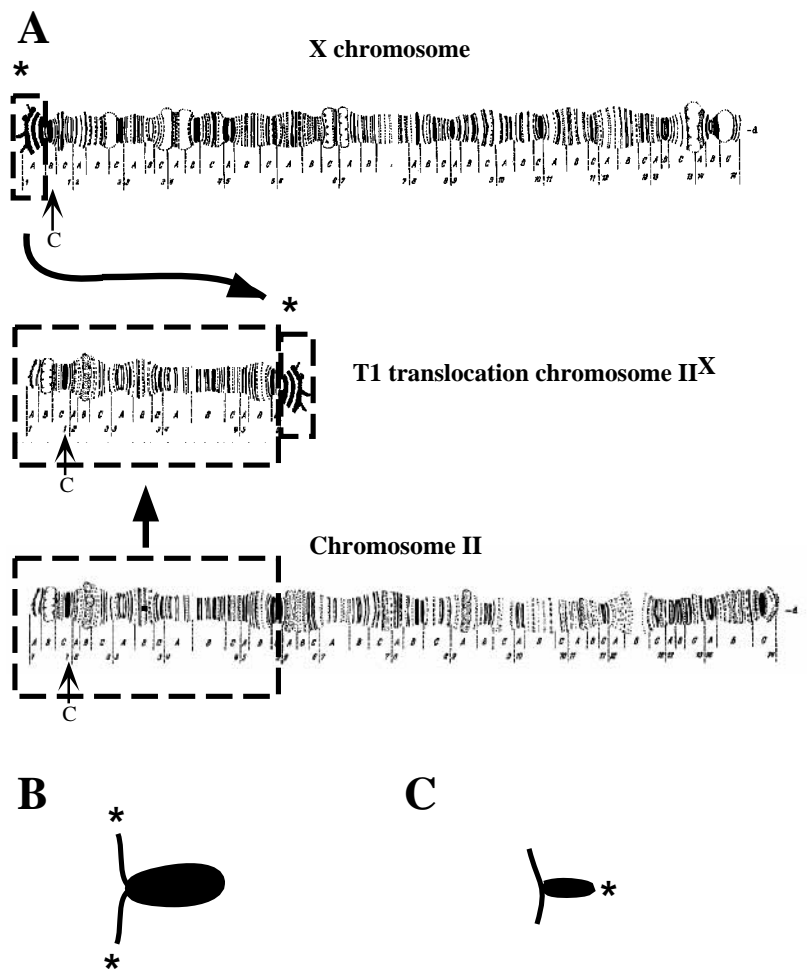
Nuclei were scored as losing chromosomes only if there was a gap between the chromatin at the metaphase plate and the daughter nuclei. To score elimination of X chromosomes in an embryo not hybridized

with the X chromosome probe, at least one putative X chromosome had to display the distinctive configuration of failed sister chromatid separation shown in Fig. 2B. In embryos hybridized with the X chromosome probe, chromatin between daughter nuclei with probe hybridized at two separate spots was scored as elimination of the X chromosome. This enabled us to score eliminations later in the cell cycle, when the chromosome arms were no longer stretched between the telophase daughter nuclei.

## RESULTS

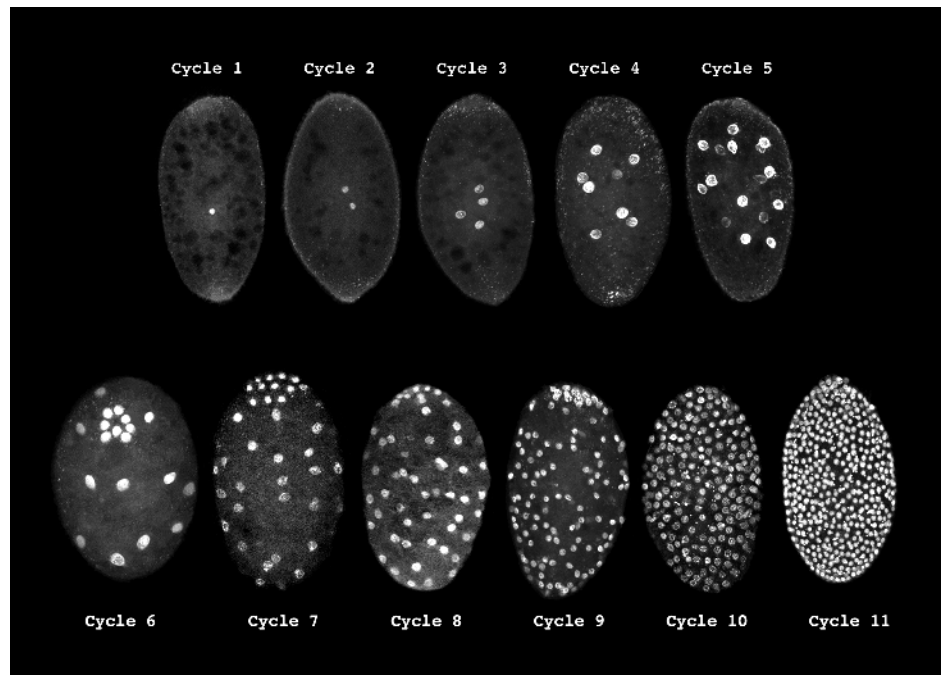
### The timing and nuclear migration patterns of the syncytial divisions of *Sciara coprophila*

To obtain a detailed description of the syncytial nuclear division pattern, we performed confocal analysis of fixed



**Fig. 2.** (A) The T1 translocation is the centromere and part of the long arm of chromosome II fused to a small region of the short arm of the X chromosome containing the CE embedded in the middle band of rDNA (\*). 'C' marks the centromeres of X, II, T1. (Adapted from the polytene illustration of Gabruszewicz-Garcia, 1964. Mapping data from Crouse et al., 1977). (B) The anaphase configuration of a normal X chromosome when it is eliminated. The rDNA probe (\*) localizes next to the centromere. Sisters fail to separate in a region on the long arm. (C) The anaphase configuration of the T1 translocation chromosome when it is eliminated. The rDNA probe (\*) localizes next to the telomere. Sisters fail to separate in a region derived from chromosome II.

**Fig. 3.** Distribution of the nuclei during prophase of the 11 syncytial cycles of *Sciara* embryogenesis: cycles 1 through 5 represent projections of the entire embryo and cycles 6 through 11 represent surface views. The germline nuclei are clustered at the posterior pole (top of each embryo) during nuclear cycles 6 and 7 (the cycle 6 embryo is tipped forward). After cycle 7, germ cells are interspersed with somatic nuclei at the posterior end. Embryos are stained for DNA with propidium iodide. Embryos average 200  $\mu\text{m}$  in length and 150  $\mu\text{m}$  in width.

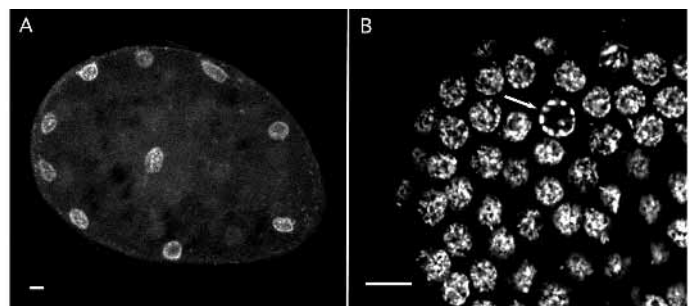


embryos fluorescently stained for their nuclei and microtubules. Previous studies demonstrated that the initial divisions of *Sciara coprophila* embryos are synchronous and syncytial, and that during these divisions the germline is established (DuBois, 1932, 1933). Using confocal analysis, we extend this work by observing that the nuclei in a syncytial embryo are in the same stage of the cell cycle (Fig. 3), or exist in a mitotic wave. In many embryos, the nuclei at the anterior pole are in telophase, the nuclei in the middle are in anaphase, and the nuclei at the posterior pole are in metaphase. This indicates that the syncytial division cycle often initiates at the anterior pole of the embryo and rapidly progresses towards the posterior end of the embryo. These mitotic waves are also observed during the syncytial divisions of *Drosophila* embryogenesis (Foe et al., 1993). Through nuclear density analysis of embryos examined with Nomarski optics, we find that cellularization occurs during interphase of nuclear cycle 11. Cycles 1-11 and their approximate times are shown in Fig. 3 and Table 1. Female meiosis is still in progress when the egg is laid, and is usually completed during the first hour after egg deposition (AED). Fusion of the male and female pronuclei occurs near the center of the embryo, at a mean time of approximately 1.4 hours AED. During nuclear cycles 2-6, the nuclei divide synchronously as an expanding ball, with some extension along the long axis of the egg. Unlike in *Drosophila melanogaster*, there is no distinct axial expansion stage in the smaller, rounder *Sciara* eggs (Zalokar and Erk, 1976).

The timing and pattern of migration is variable: nuclei arrive at the cortex as early as interphase of nuclear cycle 5 (16 nuclei) or as late as interphase of nuclear cycle 7 (64 nuclei) (Fig. 3, cycles 5-6). The migrating nuclei arrive at the posterior pole and the middle cortical region of the embryo before they reach the anterior pole. Although the nuclei that reach the cortex are initially evenly distributed, the syncytial blastoderm soon assumes a patchy appearance. The metaphase spindles of the cortical nuclei are parallel to the surface, but the axis is

randomly oriented, so that the daughter nuclei form lines and clumps until the surface of the embryo is filled at cycle 11 (Fig. 3, cycles 7-11). The cycles lengthen as the syncytial divisions progress (Table 1). After reaching the surface, mitotic waves (described above) are observed. These waves proceed rapidly and the nuclei at the extreme ends of the wave are never separated by more than half a cell cycle. A few embryos divide in a mitotic wave as early as cycle 6 and almost all do by cycle 9.

At the blastoderm stage there are a number of nuclei, known as yolk nuclei, in the interior of the embryo. Some yolk nuclei originate from nuclei left behind during migration. Fig. 4A depicts a cross section of a nuclear cycle 6 embryo at interphase with a nucleus in the center. However, most yolk nuclei originate from nuclei which have receded from the cortex. It is unusual to find interior nuclei before cycle 7, but by cycle 9 all embryos have interior nuclei. Some interior nuclei are located immediately under the cortical monolayer of nuclei, while others are located in the yolky interior of the embryo. It



**Fig. 4.** (A) Medial section of a cycle 6 embryo in which a nucleus has failed to migrate to the cortex. (B) Cortical view of the posterior of a cycle 11 embryo. Arrow marks a germ cell arrested in prophase among the somatic nuclei. Embryos are stained for DNA with propidium iodide. Bars, 10  $\mu\text{m}$ .

takes more than one cycle for a nucleus to recede from the cortex. Interior nuclei retain their centrosomes and have at least one normal mitosis, but eventually become polyploid (data not shown).

The nuclei that initially reach the posterior pole give rise to pole cells, the germline precursors. The number of nuclei and the cell cycle at which nuclei initially reach the pole varies between embryos. For example, the cycle 5 embryo shown in Fig. 3 has no nuclei at the posterior pole, while the cycle 6 embryo has eight clustered germline nuclei, indicating that four nuclei were at the pole at or before cycle 5. The pole nuclei cluster tightly in cycles 6 and 7 (Fig. 3). As in *Drosophila*, the germline nuclei cellularize at the pole. After cycle 7, the germline cells disperse and mix with the somatic nuclei (Fig. 4B). The cell cycle of the germline is slower than the cell cycle of the syncytial somatic nuclei. The germ cells stop dividing before cycle 12, when there are about 30 cells. Of 16 cycle 11 and cycle 12 embryos examined, the average number of pole cells was 29 (s.d. 1.8). The germ cells arrest in prophase with the chromosomes condensed on the nuclear membrane, like fingers grasping a ball (Fig. 4B).

#### L chromosomes are eliminated in cycles 5-6

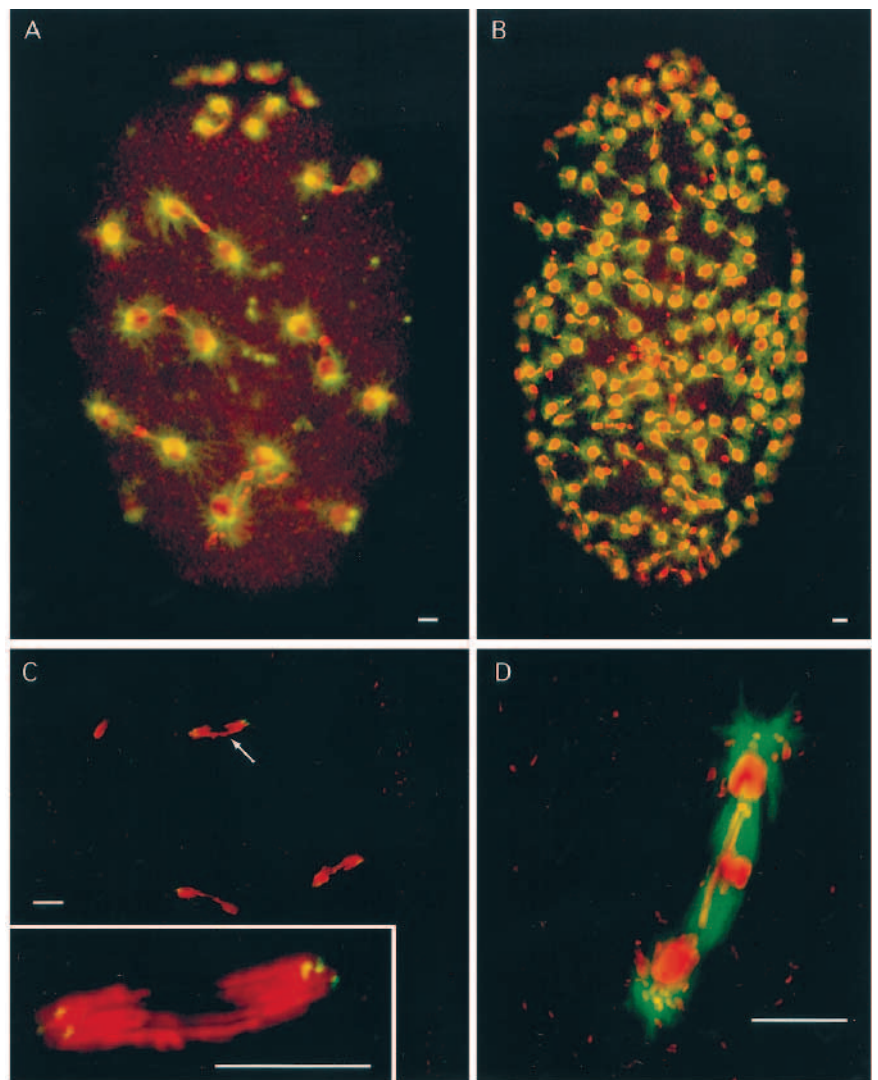
The elimination of the L chromosomes from the somatic nuclei usually occurs in nuclear cycle 6 (Fig. 5A). Of 18 embryos, we observed 2 (11%) and 16 (89%) were eliminating L chromosomes in cycle 5 and 6, respectively. We first observe chromosomes lagging in cycle 5, even before the nuclei reach the cortex (Fig. 5C). Through in situ hybridization with an rDNA probe that localizes next to the centromere of the X chromosome, we demonstrate that these lagging chromosomes are not X chromosomes, since the centromeres of the X chromosomes have already reached the poles (Fig. 2, Fig. 5C, inset). Our interpretation is that the L chromosomes lag in the division preceding their loss. Lagging chromosomes can be distinguished from eliminating chromosomes at late anaphase because the chromosomes forming the bridge are smoothly stretched out (Fig. 5C, inset). When chromosomes are eliminated the chromatin at the metaphase plate is bunched, and gaps appear between the material at the metaphase plate and the separated daughter nuclei (Figs 6B, inset, 7B).

Location of the nuclei at the cortex has been considered a requirement for chromosome elimination (DuBois, 1932, 1933). With improved fixation techniques and confocal microscopy, both cortical and interior nuclei in the process of eliminating chromosomes are observed. In embryos

eliminating L chromosomes at the cortex, interior nuclei also eliminate (Fig. 6A,B). In one unusual embryo, the L chromosomes were eliminated before any of the nuclei reached the surface (data not shown).

#### Males and females eliminate X chromosomes at different cycles

After the elimination of the L chromosomes in cycles 5-6, X chromosome elimination occurs in cycles 7-9. As with elimination of the L chromosomes, the X chromosomes are left at the metaphase plate (Fig. 5B). Cycle 7 embryos examined at prophase have all three X chromosomes (identified using the



**Fig. 5.** (A) Elimination of the L chromosomes in telophase of cycle 6, double-stained for DNA (red) and tubulin (green). The eliminated L chromosomes remain at the metaphase plate. (B) Elimination of the X chromosomes in telophase of cycle 9, double-stained for DNA (red) and tubulin (green). The eliminated X chromosomes remain at the metaphase plate. (C) A cycle 5 embryo in anaphase double-stained for DNA (red) and X chromosome centromeres (green). The arrow marks the nucleus shown in the inset. Inset: close-up of a nucleus with the L chromosomes lagging. The three X chromosomes have already arrived at the poles. (D) Telophase figure from a cycle 6 embryo eliminating L chromosomes, double-stained for DNA (red) and tubulin (green). The eliminated L chromosomes are stretched toward the daughter nuclei through the midbody. This nucleus is the one marked by the arrow in Fig. 6A. Bars, 10  $\mu$ m.

probe to the X chromosome described in Materials and Methods). The X chromosome probe does not hybridize with the chromosomes left at the metaphase plate in cycle 6 (data not shown), demonstrating that L and X chromosomes are eliminated separately. X chromosomes, like L chromosomes, are eliminated in both surface and interior nuclei (Fig. 6C,D).

The nuclear cycles in which the X chromosomes are eliminated differ in male and female embryos (Table 2). Females usually eliminate at cycle 9. Of 18 female embryos in the process of eliminating X chromosomes, 1, 1 and 16 were in cycles 7, 8 and 9, respectively. Males, on the other hand, display a more complex pattern of elimination: of 26 male embryos in the process of eliminating X chromosomes, 7, 10 and 9 were in cycles 7, 8 and 9, respectively. In matched collections of male and female embryos spanning cycles 7-9, we found approximately three times as many males in the process of eliminating X chromosomes as females [7.1% (8 of 113) of the male embryos and 2.6% (7 of 275) of the females were eliminating]. These data indicate that males routinely eliminate X chromosomes over multiple cycles.

The two paternal X chromosomes in each nucleus of a male embryo appear to be eliminated independently. Fig. 8A depicts a cycle 8 male embryo with neighboring nuclei losing either one or two X chromosomes. Fig. 8B shows another nucleus in the same embryo in which one X is being lost, but the other is only lagging. Asynchrony between neighboring nuclei is often observed in the cycles when chromosomes are eliminated. Of cycle 7 and cycle 8 male embryos eliminating X chromosomes, 2 of 9 and 5 of 10 embryos exhibited some nuclei eliminating both X chromosomes simultaneously. In cycle 9, only 1 of 7 embryos was eliminating both X chromosomes simultaneously in some nuclei.

The difference between male and female patterns of chromosome elimination may be the result of a difference in the concentration of a maternally supplied factor titrated by the nuclei. To eliminate the possibility that a difference in the volume of male and female eggs created a difference in the ratio of nuclei to cytoplasm, we measured the length and width of a cross section through the middle of each embryo, and found it to be the same for male and female embryos (data not shown).

#### Failure of sister chromatid separation as the mechanism of chromosome elimination

Failure to complete sister chromatid separation appears to be the mechanism by which the X chromosomes are left at the metaphase plate. Using FISH, we followed the behavior of the X chromosome during the syncytial nuclear cycles in which elimination occurs. We found that congression is normal, the centromeres of the X chromosomes are functional, and separation occurs early at anaphase. Fig. 7A shows early anaphase in an embryo at cycle 9, which is the last cycle at which chromosome elimination normally occurs.

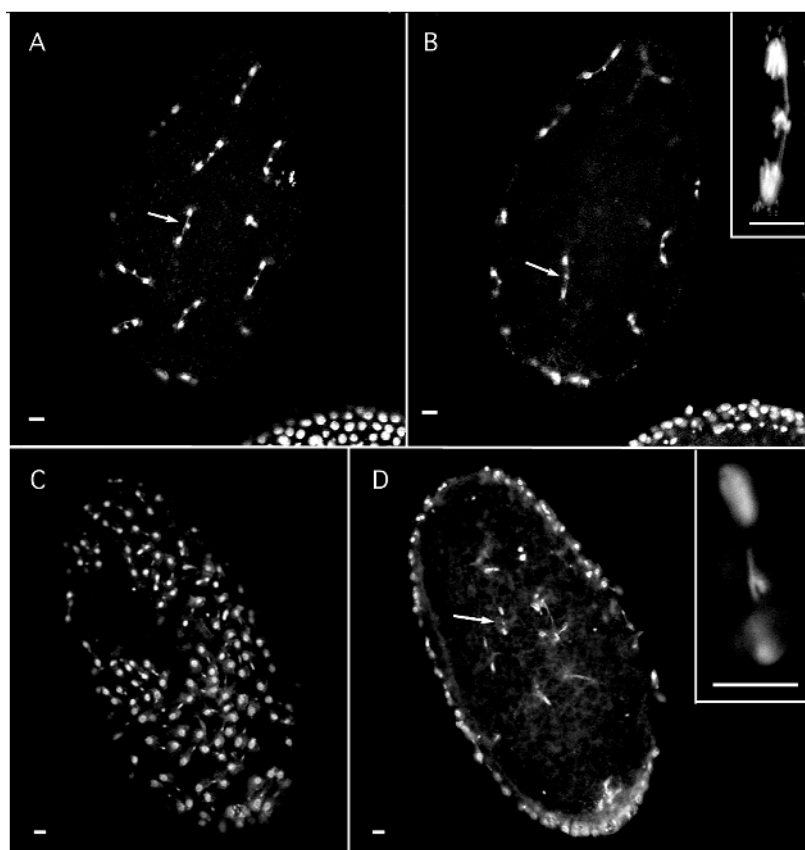
**Table 2. Sex differences in X chromosome elimination**

Cycle	Male embryos		Female embryos
	One X lost	Both X lost	
7	5	2	1
8	5	5	1
9	8	1	16

Probability that sex and cycle of elimination are independent is 0.00502 (Chi squared test). Probability that females eliminate randomly in cycles 7-9 is 0.00064 (Chi squared test).

Arrows mark two nuclei which still contain all three X chromosomes, one of which will be eliminated in this cycle. The normal anaphase separation of the centromeres of all three of the X chromosomes can be observed.

Failure of sister X chromosomes to completely separate is the first mitotic abnormality observed. Separation fails in a region of the long arm of the X chromosome. The centromeres and part of the chromosome adjacent to the centromere separate. Separation of the telomeric regions distal to the site of failed separation also occurs normally. Fig. 8C depicts an X



**Fig. 6.** Nuclei in the interior of the embryo eliminate X and L chromosomes. Surface (A) and medial (B) views of an embryo eliminating L chromosomes. The arrow in A marks the nucleus in 5D and the arrow in B marks the nucleus in the inset of B, and also in Fig. 5D. Inset: interior nucleus eliminating L chromosomes, showing the characteristic stretched chromosome arms. Surface (C) and medial (D) views of a female cycle 9 embryo eliminating one X chromosome per nucleus. The arrow in D marks the nucleus in the inset. Inset: interior nucleus eliminating an X chromosome, showing the characteristic shape of X chromosome elimination, with failure of sister separation. Embryos are stained for DNA with propidium iodide. Bars, 10  $\mu$ m.

chromosome in the process of being lost, in which the centromeres and telomeres are clearly separated.

The centromeres of the X chromosomes do not lose their attachment to the spindle during anaphase and the attached sister chromatids appear to remain under tension. Fig. 8 shows male and female embryos losing X chromosomes, which are stretched and under tension. Fig. 7B shows a female embryo in telophase. The centromeres of the eliminated X chromosomes are separated and stretched toward the daughter nuclei.

Two kinds of X chromosome non-disjunction are occasionally observed: breakage of the chromosome with unequal X chromosome fragments proceeding to each pole (data not shown), and loss of attachment by one centromere with both intact sister X chromatids migrating to one pole (Fig. 7C). These observations are consistent with a failure of sister chromatid separation as the mechanism of chromosome elimination.

In the case of the L chromosomes, we did not have a probe to identify the centromere, but during anaphase and early in telophase in the cycles in which they are eliminated, these chromosomes are bundled at the metaphase plate, with regions stretched toward the poles (Figs 5D, 6D). These observations indicate that the L chromosomes are also eliminated by a failure of sister chromatid separation.

### The site of failed sister chromatid separation is not specific to the X chromosome

We used an X chromosome translocation to investigate the possibility that a specific DNA sequence is the site where the sister X chromatids fail to separate. Elimination of the X chromosomes during the syncytial divisions requires the presence of a cis-acting element known as the CE, which was identified and mapped by analyzing elimination in a series of X-autosome translocations. Paternally inherited translocations bearing the middle band of rDNA from the X chromosome, containing the CE, are eliminated during the syncytial divisions (Crouse, 1960b, 1979; Rieffel and Crouse, 1966). In the T1 translocation shown in Fig. 2, the X chromosome telomeric region containing the rDNA serves as the telomere for a chromosome II missing the distal two thirds of its long arm. Previous studies show that this translocation is eliminated if inherited paternally (Crouse, 1979). We probed male embryos inheriting a normal maternal X and two paternal T1 chromosomes. The eliminated T1 chromosomes were left on the metaphase plate. The part of the sister chromatids which failed to separate came from chromosome II, not from the X chromosome. It is also notable that the centromere of the T1 translocation is from chromosome II. The probe hybridizes to the only part of the translocation chromosome which came from the X chromosome, located at the end of the sister chromatids left at the metaphase plate (Fig. 7D,E; compare to B). This demonstrates directly that the CE is able to control the separation of autosomal sister chromatids.

## DISCUSSION

### Failed sister chromosome separation as the mechanism of chromosome elimination

DuBois (1932) observed that chromosomes which are destined for elimination separate more slowly during anaphase and that

the sister chromatids never achieve complete separation. We demonstrate that during the nuclear cycles in which chromosomes are lost, congression of the chromosomes to the metaphase plate is normal. In addition, the centromeres of sister X chromosomes separate during early anaphase, indicating that they are functional (Fig. 7A). However, in late anaphase a region of the sister X chromatids remains attached. In telophase, when the midbody has formed on each side of the metaphase plate, the separated portion of each sister extends through the midbody (Fig. 7B). Our interpretation of these data is that the centromeres remain active but the sister X chromatids are mechanically prevented from moving to the poles because they fail to separate. If the centromeres had detached from the spindle before the end of anaphase, the separated portion of the sisters would have been pushed back to the metaphase plate by the polar ejection force. The effect of the polar ejection force is to position chromatin which is not attached to the spindle at the metaphase plate (Rieder and Salmon, 1994, and references therein). It is possible that the kinetochores remain attached to the spindle, but the agents responsible for the motion of the chromosomes to the pole (motor proteins at the kinetochore and/or disassembly of spindle microtubules) fail. Two observations argue against this interpretation: the appearance of stretching and tension in late anaphase (Fig. 5D), and the occasional non-disjunction in which both chromatids go toward one daughter nucleus (Fig. 7C).

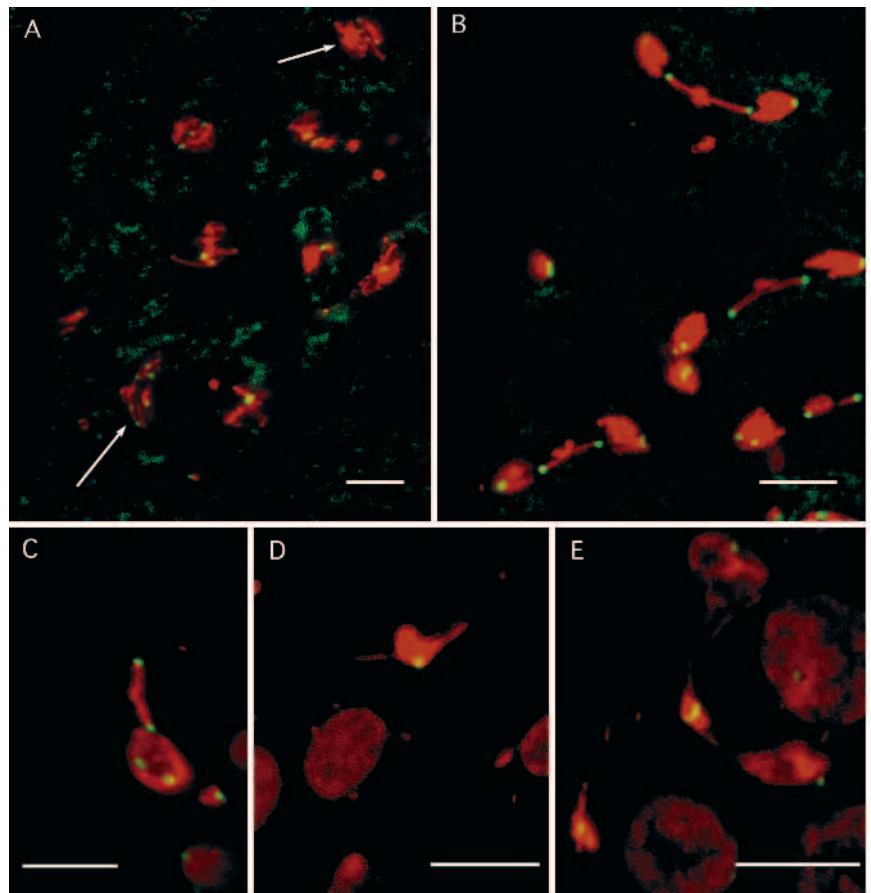
In the case of the L chromosomes, there was no probe to locate the centromere. However, the general appearance during anaphase and telophase is so similar to the elimination of X chromosomes that we suppose the mechanism is the same (Fig. 5D).

Although the elimination of the X chromosomes is a global process, the decision to eliminate occurs at the level of the individual nucleus. For example, in a single male embryo at a given nuclear cycle, some nuclei eliminate both X chromosomes, some eliminate one X chromosome, and some do not eliminate. Nuclei are also observed with an X chromosome at the metaphase plate, another X chromosome lagging, and the third already at the pole (Fig. 8B).

The failure of sister chromatid separation in *Sciara* is purely a defect in the separation of the chromatid arms and there is no effect on the separation of the centromeric regions. In *Drosophila*, there is evidence that sister chromatid separation is controlled separately at the centromeres and along the chromosome arms. The arms of the sister chromatids separate in meiosis I, while the centromeres separate at meiosis II (Sekelsky and Hawley, 1995; Orr-Weaver, 1995). The *ord* gene appears to be required for sister chromatid arm cohesion in meiosis, but not in mitosis (Orr-Weaver, 1995). The *Drosophila* genes *pimples* and *threeerows* are required for separation of the centromeres in mitosis, while the chromosome arms separate normally (Stratman and Lehner, 1996).

More generally, sister chromatid separation at anaphase has been shown to require ubiquitin-mediated proteolysis (King et al., 1995; Tugendreich et al., 1995; Irniger et al., 1995). There may be a protein attaching the sister chromatids which is destroyed by proteolysis at the anaphase transition (Holloway et al., 1993). Topoisomerase II activity is required to decatenate the sister chromatids (Shamu and Murray, 1992; Swedlow et al., 1993) and phosphatase activity is necessary for the transi-

**Fig. 7.** Embryos double-stained for DNA (red) and X chromosome centromeres (green). (A) Cycle 9 female embryo in early anaphase. Arrows mark early anaphase nuclei bearing three X chromosomes, one of which will be eliminated in telophase of this cycle. The centromeres of all three X chromosomes have separated normally. (B) An embryo eliminating a single X chromosome per nucleus. The X chromosomes being eliminated have the centromeres of the sister chromatids separated and are visibly under tension. The sister chromatids have failed to separate completely. The centromeres of the other two X chromosomes are already at the poles. (C) A possible non-disjunction during X chromosome elimination. Both X chromosomes have been pulled to a single pole. The daughter nucleus has two X chromosomes. This suggests that the centromeres remain attached to the spindle and are functional through the completion of anaphase. (D,E) depict the elimination of the T1 translocation chromosome. Embryos are double-stained for DNA (red) and the T1 telomere (green). The centromeres are stretched toward the daughter nuclei. The daughter nuclei are not in the plane of focus in D. The spindle is curved in E. The sister chromatids are attached in an area derived from chromosome II. Bars, 10  $\mu$ m.



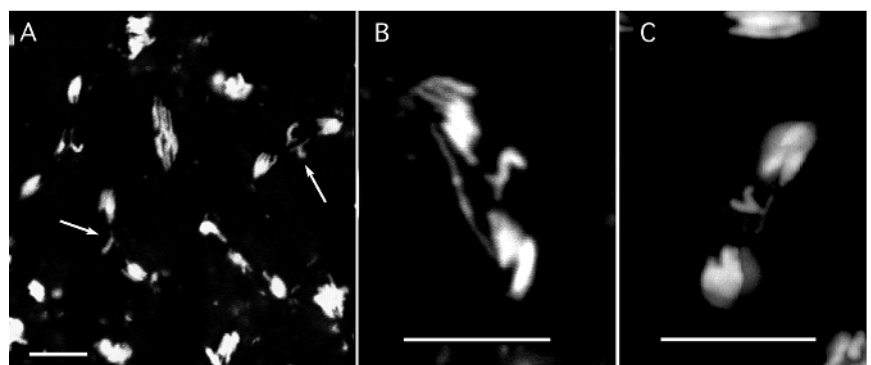
tion to anaphase (Wolniak and Larsen, 1992; Vandre and Wills, 1992). For example, the *Drosophila* gene *aar* (abnormal anaphase resolution) encodes the 55 kD regulatory subunit of the protein phosphatase 2A (Mayer-Jaekel et al., 1993). Zygotic *aar* mutants display asynchronous and failed sister chromatid separation in neuroblasts. Embryos from females that provide a defective *aar* protein display the same defects in the syncytial divisions. It is likely that the programmed elimination of the X chromosomes in *Sciara* embryos involves the regulation of one or more of these activities in a chromosome-specific manner. The effect of the CE, discussed below, suggests a model in which a region of the X chromosome is less accessible to these activities.

#### The CE acts in *cis* and at-a-distance to prevent sister chromatid separation

Paternally inherited translocations bearing the CE located in the middle band of rDNA from the X chromosome are eliminated during the syncytial divisions (Crouse, 1960b, 1979; Rieffel and Crouse 1966). The site of failed sister chromatid separation is not the CE itself, which is adjacent to the centromere, but a distal region on the long arm of the X chromosome. Our results using the T1 translocation indicate that the

site of failed sister chromatid separation is chromosome-independent and that the CE can act in *cis* and at-a-distance to prevent autosomal chromosome regions from separating during anaphase.

The CE on the maternal X chromosome has no effect on sister chromatid separation. The effect of the CE on the paternal X chromosomes is heritable and manifests itself after



**Fig. 8.** (A,B) Males do not lose the same number of chromosomes in each nucleus and there is asynchrony in the cell cycle between neighboring nuclei. (A) Male embryo losing two X chromosomes per nucleus in some nuclei (right arrow) and a single X chromosome in others (left arrow). (B) Another nucleus in the embryo shown in A, where one X chromosome has separated its sister chromatids early in anaphase and the other has not. (C) Female embryo losing a single X chromosome per nucleus. The telomeres of the sister chromatids are clearly separated. Embryos are stained for DNA with propidium iodide. Bars, 10  $\mu$ m.



7-9 zygotic divisions. A heritable chromatin structure which makes a site susceptible to the failure of sister separation is an attractive candidate to mark specific chromosomes for elimination. This structure could influence catenation of sister chromatids, which must be resolved by topoisomerase II at metaphase, or it could protect a protein tethering the sisters from proteolysis. The hypothesis of a heritable chromatin structure involved in sister chromatid separation is also consistent with the elimination of the L chromosomes, which precedes the elimination of X. Both maternal and paternal L chromosomes are eliminated (Crouse et al., 1971; Rieffel and Crouse, 1966). The hypothetical chromatin structure may be native to the L chromosomes, yet require the presence of the CE during male meiosis to form on other chromosomes.

### A cytoplasmic factor determines the pattern and timing of chromosome elimination

The number of X chromosomes eliminated is a property of the egg. The paternal X chromosomes from the sperm are identical and both are marked for elimination by the CE. A single male can fertilize two females, one a mother of daughters and the other a mother of sons. In spite of receiving an identical pair of X chromosomes, one set of eggs will eliminate a single paternal X chromosome and the other set of eggs will eliminate both paternally derived X chromosomes. Our observations extend previous studies by demonstrating that the nuclear cycle at which elimination occurs is also determined by the egg. Males eliminate their X chromosomes primarily during nuclear cycles 7, 8 and 9 and females primarily during nuclear cycle 9, and chromosome loss occurs in interior as well as in cortical nuclei.

These data strongly suggest that a maternally supplied diffusible factor determines the pattern of X chromosome elimination during the syncytial divisions. The amount of this factor in the egg determines the sex of the embryo. This factor may be involved in separating the sisters or in holding them together. The simplest model would assume a single maternal factor necessary for sister chromatid separation, which is titrated by the X chromosomes at each division cycle. Males have less of this separation factor, so X chromosomes are lost earlier in males than in females. This loss occurs as early as cycle 7, in which 256 paternal X chromosomes must separate. The number of paternal X chromosomes that are not eliminated doubles in successive cycles, causing repetitive X chromosome elimination until all the paternal X chromosomes are lost. Females have enough separation factor until there are 1024 paternal X chromosomes to separate in cycle 9, when they lose a single paternal X chromosome. A factor holding sister chromatids together does not fit with this model because, if males have more of the factor (as they must, since they lose chromosomes earlier), females should not lose chromosomes at all during later cycles, when there are more chromosomes to titrate the factor. In this model, the L chromosomes are lost early by both sexes because they require so much more of the separation factor than the X chromosomes.

A different kind of model would involve activation at a specific nuclear cycle. For example, a maternal factor holding the sisters together could be activated at different nuclear cycles in males and females. Repetitive loss by males would occur because the factor is activated earlier and remains active for three cycles. The degradation of the maternal *pim* tran-

script, required for the separation of sister chromatid centromeres after cycle 13 in *Drosophila*, is an example of the nuclear cycle-dependent activation of factors regulating sister chromatid separation (Stratman and Lehner, 1996).

These simple models do not explain why females lose one X chromosome from all nuclei, rather than losing one or two from some, and none from others, on a random basis. In addition to other mechanisms, dosage compensation may kill cells derived from nuclei that eliminate the wrong number of X chromosomes. In fact, the large number of nuclei that recede into the interior of the embryo during nuclear cycles 11-12 (data not shown) may include nuclei in which X chromosome elimination has not occurred or has occurred improperly. Such a mechanism exists during the cortical divisions of the *Drosophila* embryo to eliminate the products of an abnormal division (Sullivan et al., 1990, 1993).

### Failed sister chromatid separation as a mechanism for chromosome elimination is specific to the syncytial divisions

In cells undergoing conventional cytokinesis, chromosomes left at the metaphase plate would be trapped in the cleavage furrow. For example, mammalian cells treated with topoisomerase II inhibitors form massive chromosome bridges during anaphase and telophase. The chromosomal material in the bridge remains in the path of cleavage furrow formation and is unevenly segregated to the daughter nuclei, rather than being eliminated from both (Gorbowski, 1994). This may be the primary reason for other mechanisms of chromosome elimination being employed in cells that undergo conventional cytokinesis.

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