On the origin of metacentric, attached-X (A-X) chromosomes in *Drosophila melanogaster* males

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We describe here the isolation and cytogenetic characterization of a mutation *inseparabile* which generates in males a high frequency of A-X females. The mutation, segregating in low frequency in a laboratory stock, maps to cytological location 82F7–11 in the third chromosome. The mutation acts premeiotically in the male germ line. Disrupting the X chromosome centromeric heterochromatin suppresses the formation of A-X chromosome, implying that the mutation is involved in chromatid cohesion. The *inseparabile* mutation also affects disjunction of the chromosome 4 in males. We suspect that the mutation was responsible for the original A-X female found by L. V. Morgan in 1921.

chromosome disjunction | centromeric heterochromatin cohesion

Among the hallmark genetic discoveries on which classical genetics was founded, the discovery in 1921 by Lilian V. (Mrs. T. H.) Morgan (1) of an A-X chromosome in a *Drosophila melanogaster* female is the most remarkable. The female whose two X chromosomes were attached to one centromere occurred among the progeny of a mosaic fly which bred as a female. Based on the marker genes in the exceptional female, the A-X had to originate in a parental male gamete. In historical retrospect, the relevance of the A-X chromosome for the fundamentals of genetics is inestimable. First, the compulsory nondisjunction of A-X independently confirmed Bridges' (2) demonstration of the linkage of genes to chromosomes. Second, because A-X females carry a Y chromosome, this confirmed that in *D. melanogaster* the Y does not determine the maleness *per se*. Third, A-X provided a means for analyzing the meiotic mechanism of crossing-over via half-tetrad analysis (5). Fourth, because in crosses to A-X females the X chromosome of males is patroclinously inherited, the frequency of X chromosome mutation with a visible phenotype could be estimated. Fifth, the maintenance without selection of female sterile X chromosome mutants became possible. Sixth, on the basis of crossing-over in heterozygotes A-X females, the position of X chromosome genes *vis-à-vis* the centromere and telomere could be unambiguously determined.

Subsequent to Morgan's discovery, Sturtevant (3) and Stern, cited in ref. 4, independently found new spontaneous A-X chromosomes identical to the original and equally derived from a male parent. Because A-X chromosomes could be induced by x-rays in *D. melanogaster* females via chromatid or chromosome breakage (4), the rare, spontaneous, and sporadic formation of A-X in a male with one X chromosome presumably took place by a comparable event. What could not be determined is whether the formation of A-X in males was the by-product of a rare epigenetic replication error or whether there was some underlying genetic basis.

In this paper, we will document the serendipitous discovery of a third chromosome recessive mutation we call *inseparabile* (*ins*), which when homozygous in males regularly generates A-X chromosomes in a high frequency. In addition, we will submit cytogenetic evidence that *ins* induces the formation of A-X via chromatid cohesion in the centromeric heterochromatin of the X chromosome.

Materials and Methods

Table 1 includes a list of gene mutations and chromosomes described in the text. To assay A-X induction by *ins*, routinely single males were crossed to harems of *C(1)DX, y f* females and females homozygous for paternal X chromosome were sought. Presumptive A-X females were routinely progeny tested to establish the presence of A-X. The balancer chromosomes *Cy* and *TM3, Sb* were used to establish, where described in the text, homozygosity for the autosomes II and III, respectively.

For purposes to be described in the text, the short right arm of the X chromosome was replaced by a longer arm. The replacement, which includes the X regions defined cytologically as 16A1-A7,8 plus the heterochromatin of 20F and the ribosomal DNA, is marked with the mutant *B* and was derived from a B^s·*Y* (6). The chromosome genotype is designated *sc* z^v *ec*·B^s.

Nondisjunction of chromosome 4 was assayed by crossing males to *C(4)spa*^{pol} females and determining the occurrence of nullo-4 gametes as F_1 male and female *C(4)spa*^{pol} progeny.

Flies were cultured on a standard cornmeal, sugar, and Brewer's yeast medium at a room temperature of 23–24°C.

Conventional salivary gland polytene chromosome cytology used aceto-lactic orcein as the stain.

Results

Genesis of the *ins* Mutation. In an intermittent but ongoing series of experiments designed to monitor intrachromosomal crossing-over in the X chromosome of *D. melanogaster* males, males of the genotype *sc z Dp(1;1)*w^+ *ec* are crossed to *C(1)DX, y f* and crossovers detected by the phenotypic reversion of *z* to z^+ (7). In one such cross, a single F_1 female of the phenotype *sc z ec* was found. Progeny testing this female demonstrated her to have A-Xs and cytology of her female progeny confirmed the genetics and established the Xs are attached precisely as in Morgan's A-X. Subsequently in a cross of *sc z Dp(1;1)*w^+ *ec* to *C(1)DX, y f*, a single male was recovered whose phenotype was not *z* but a variegated eye color denoted z^v. On progeny testing, the phenotype of this male bred true and a stock was established by crossing to *C(1)DX, y f* females. (Presumably a change in *Dp(1;1)*w^+ occurred producing the z^v phenotype.) Further crosses of these duplication males, to be designated *sc* z^v *ec*, to *C(1)DX, y f* established two points. In males, z^v can revert to z^+, indicating that z^v retained part of *Dp(1;1)*w^+. However, more importantly, in one cross of *sc* z^v *ec* males to *C(1)DX, y f*, three A-X females of the phenotype *sc z ec* were found. Additional crosses with lines derived by crossing the sibs to each of the three A-X females established that A-X females occurred regularly, albeit sporadically within the line. To explain this ongoing occurrence of A-X females, it was postulated that within the line, an autosomal recessive mutation was segregating which, by chance, became homozygous in some males and generated those

Abbreviations: A-X, attached-X; *ins*, *inseparabile*; wt, wild type.

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Table 1. Synopsis of mutants and special chromosomes used in text according to ref. 6

Symbol	Linkage	Phenotype
y	X-0.0	Yellow body color
sc	X-0.0	Scutellar bristles missing
z	X-1.0	Zeste eye color
w	X-1.5	White eye
ec	X-5.5	Echinus (rough) eye
f	X-56.7	Forked bristles
B	X-57.0	Bar eye
su(f)	X-65.9	Suppressor of *f*
Gl	3-41.4	Glued eye
Sb	3-58.2	Stubble bristles
spa^{pol}	4	Sparkling-poliert, smooth eye surface
$Dp(1:1)w^{+}$	X-direct tandem duplication of 3C1-4A	
w^{m4}	X-inversion from 3C2 to 20F	
X·B	X-an X segment from 16A1-centromere attached as short arm X	
C(1)DX	X-double X, complex linkage of two X chromosomes to centromere	
Cy	2-Curly wing; inversion in 2L and 2R	
TM3	3-Inversion in 3L and 3R	
C(4)	4-Two chromosome 4s attached to one centromere	

A-X females recovered among the progeny. To validate this postulate, crosses were made aimed at establishing a stock homozygous for the presumptive mutation.

This was done by making nine pair matings [males *sc* z^{v} *ec*, females *C(1)DX, y f*)] from flies in the segregating stock and screening their progeny for A-X females of the *sc* z^{v} *ec* phenotype. Among the nine, one pair produced three proven *sc* z^{v} *ec* A-X females among 44 male sibs. It was assumed that this parental pair and their F_1 progeny were homozygous for the postulated autosomal mutant. This line was designated *line 6*. (Parenthetically, from $F_1 \times F_1$ crosses among the progeny of the other pair matings, three more presumptive homozygous stocks were established.) Homozygosity for an autosomal mutant in *line 6* was confirmed by crossing single *sc* z^{v} *ec* males from this line to 10 unrelated *C(1)DX, y f* females. Five among the 10 males tested produced A-X female progeny numbering from one to three per parental male. An average of 300 male progeny was produced per cross and a total of 14 A-X females recovered. Comparable results were obtained with a second presumptive homozygous line, called *line 3*. When single *sc* z^{v} *ec* males of *line 3* were crossed to unrelated *C(1)DX, y f* females, six among 11 males tested produced A-X female progeny that varied from one to five per cross among a total of 2,207 male progeny.

In the crossing procedure used, it should be noted that the frequency of A-X chromosomes is underestimated by one-half, because an A-X-bearing sperm fertilizing a *C(1)DX*-bearing ovum will be lethal. Additionally, the recovery of multiple A-X females from a single parental male implies that the A-X occur as a germ line premeiotic, mitotic event. Thus, an A-X chromosome may be replicated in a gonial cell before being incorporated into more than one spermatozoan.

Linkage of *ins*. Based on the data presented thus far, three tentative conclusions were drawn. The A-X chromosome arises from a failure of the chromatids to properly separate during premeiotic mitosis. The failed separation is associated with homozygosity of the mutant we call *ins*. The mutant *ins* is linked to one of the two large *D. melanogaster* autosomes. Linkage was determined by first separately homozygosing the II and III autosomes of *line 6* by using the *Cy* and *TM3, Sb* balancer chromosomes. Single *sc* z^{v} *ec* males homozygous for II or III were crossed to harems of 10 unrelated *C(1)DX, y f* females and their progeny scored. It will suffice to note here among 15 homozygous II chromosome males, one produced a single A-X female among 5,208 male progeny whereas among 15 homozygous III chromosome males, nine produced A-X female progeny varying from one to five per male among 5,214 male progeny. The conclusion is that *ins* is linked to III and not II; the single A-X female was the chance homozygosity of III segregating in the homozygous II stock.

More precise localization of *ins* entailed mapping to either the left or right arm of III. Females of the genotype *Gl Sb/ins; sc* z^{v} *ec/+* were obtained by an appropriate cross and backcrossed to *ins/ins; sc* z^{v} *ec* males. *Gl* or *Sb* crossover males were selected and tested individually to *C(1)DX, y f* females. Among 26 *Sb* males tested, four produced A-X females whereas among 21 *Gl* males, two produced A-X female progeny. Because *Gl* and *Sb* flank the centromere, it was tentatively concluded that *ins* is located between *Gl* and *Sb*, close to the centromere, more likely in III-R than III-L.

Deletions proximal to the centromere were then used to assign *ins* to III-R or III-L. Among the progeny of 14 males heterozygous for a III-L deficiency of the polytene chromosome segment 76B4–77B crossed to *C(1)DX, y f* females, no A-X females were recovered among 3,554 male progeny. However, one A-X female was recovered among 1,617 male progeny of 16 male progeny heterozygous for *ins* and a III-R deficiency for the chromosome segment 81F-83A. The data, while meager because of the poor fertility of the deficiency males, was deemed significant, and more precise mapping was undertaken within the 81F-83A region. Fifteen males heterozygous for *ins* and three deficiencies defined by losses of segments 81F3,5–82F5,7; 82D3,8–82F3,6; and 82F3,4–82F10,11 were tested as above for the generation of A-X females. The results are listed in Table 2 and demonstrate that only males heterozygous for the 82F3,4–82F10,11 loss

Table 2. Deletion mapping of *ins*; heterozygous males, *ins/Deletion* × *C(1)DX, y, f* females

Genotype of males	Number of males tested	Σ Male progeny	Σ A-X females
+/Y;*ins*/Df81F3-82F5,7	15	3,091	0
+/Y;*ins*/Df82D3,8-82F3,6	15	4,473	0
+/Y;*ins*/Df82F3,4-82F10,11	18	4,133	3 × 1 + 1 × 2*

*Three males produced one A-X female, and one male produced two A-X females.

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produced A-X females. These results delimit the location of *ins* to the region 82F7,11 of III-R.

Two supplementary facts can be added here to the results presented thus far. The first is that the induction of A-X chromosomes is not limited to the *sc* z^v *ec* X. In the mapping experiments described, the males used in crosses were invariably wild type (wt) and the A-X females recovered carried two wt chromosomes. Not surprisingly, this means that X chromosome mutation is not causally involved in the formation of A-X. A second fact germane to the data was the recovery in the several crosses described of F_1 *C(1)DX* females whose phenotype was not *y f* but y^+ *f*. Summarizing seven independent experiments and equating the number of females scored to that of the enumerated males, seven y^+ *f* females were recovered among *ca.* 37,000 females. For the most part, these females were poorly fertile; the few female progeny recovered were *y f*, demonstrating that they carried the *C(1)DX, y f* chromosome; their male progeny had the expected patroclinous phenotype. However, in two cases, females produced progeny. This demonstrates that the y^+ *f* phenotype was associated with a free X chromosome duplication derived from the male parent, because invariably the male parent X chromosome was y^+. Genetic analysis demonstrated that the free duplication rescued a telomere proximal deficiency of the X extending from the telomere to polytene chromosome section 1B4,9. The free duplication also rescued the *su(f)* mutation that maps to the base of the X at 20F. The other y^+ *f* females presumably carried a free duplication of comparable length but too large to permit the survival of males. Although the frequency is low, the regular recovery of y^+ *f* females shows that the *ins* mutation also generates deletions. Support for this conclusion comes from the results of experiments in which males *sc* z^v *ec*·B^s and homozygous *ins* were crossed to *C(1)DX, y f* females included among the 13,169 male progeny were five females of the phenotype y^+ *f* B^s, two females *y f* B^s, one male *sc* z^v w^+ *ec* and B^+, and one female *sc* z^v *ec* which on progeny testing proved to be A-X. Subsequent genetic analysis of the y^+ *f* B^s females demonstrated they carried a free X like those described above. Thus, the duplications rescued the telomere proximal deletion and *su(f)*. The *y f* B^s females also carried a free duplication, which did not rescue the aforementioned deletion nor a telomere proximal lethal mutation but did rescue *su(f)*. These results confirm that the *ins* mutation causes chromosome (chromatid) breakage and their significance will be discussed below.

The Reversion of w^{m4} by *ins*. Implicit in the synthesis of A-X chromosomes by *ins* is the idea that in some yet to be defined way, the centromeric heterochromatin region of the X chromosome is causally involved. One way to test this idea is to determine whether or not the production of A-X females is affected when the basal X heterochromatin is disrupted. Such a disruption occurs in the w^{m4} X inversion in which the heterochromatin is split, part moved to the X tip at section 3C and w^+ moved to the base juxtaposed to the residual basal heterochromatin at 20F. The molecular structural details are found in ref. 8. Three separate experiments were made in which males w^{m4} and homozygous *ins* were crossed to *C(1)DX, y f* females. Among a total of 15,063 males scored, no A-X females were recovered. Surprisingly, however, six females with a wt eye color were recovered. One female was accidentally lost, but the remaining were fertile, albeit poorly. Each female proved to be heterozygous; each produced two classes of male progeny (*ca.* half w^{m4} and half w^+, i.e., wt). Stocks were established from four w^+ females by crossing w^+ males to *C(1)DX, y f* females. To determine whether or not the w^+ chromosome is a genuine reversion of the w^{m4} phenotype, w^+ males from each putative reversion were crossed to A-X females lacking a Y chromosome. The resulting males lacking a Y chromosome were without exception w^+ in eye phenotype, as expected for the reversion of the w^{m4} position effect phenotype. They were also wt for the *bobbed* mutation associated with the loss of the ribosomal DNA proximal to the X centromere.

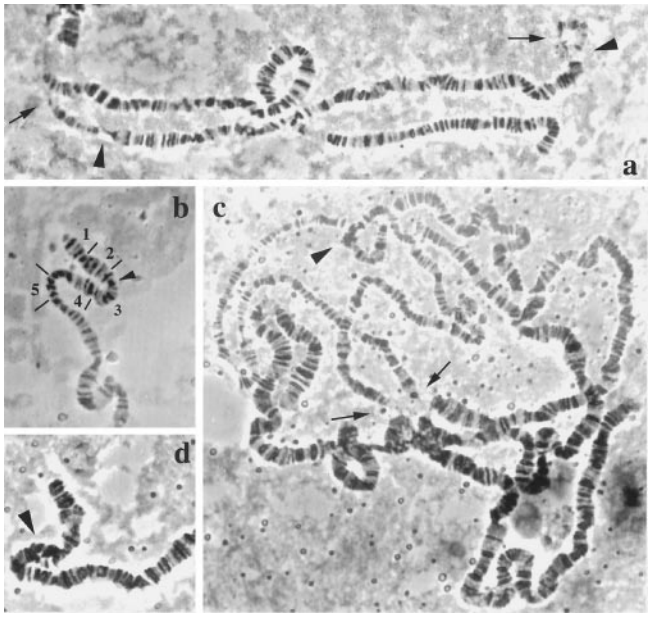

Fig. 1. Cytogenetic analysis of some selected w^+ reversions of w^{m4} described in text. (*a*) $w^{+(7)}/w^{m4}$ female; the $w^{+(7)}$ chromosome is a reinversion of w^{m4} and pairing occurs between 3C and 20F. As a result, the telomeric regions 1–3C appear at both sides of the X chromosome (arrows); 20F is on the left side of the picture. The arrowheads indicate the breakpoints in 3C. (*b*) $w^{+(7)}/Y$ male; the X chromosome shows the banding pattern of wt at the w^+ locus (arrowhead). (*c*) $w^{+(11)}/w^{m4}$ female; the $w^{+(11)}$ chromosome is also a reinversion. Here, the pairing occurs only at the telomeric region. Starting from 3C (arrowhead), the two homologues separate from each other over their entire length up to the chromocenter (arrows). (*d*) $w^{+(21)}/w^{m4}$ female; the $w^{+(21)}$ chromosome has the same sequence as w^{m4} with pairing along the entire length. However, at 3C1–2 (arrowhead), the difference between the two homologues is evident. This explains the visible small loop.

In addition to the w^+ females described, one near wt female was recovered. On progeny testing, this female produced two types of males: w^{m4} and near w^+ males designated $w^{+(21)}$ which exhibit a slight but distinct variegation. In contrast to w^{m4} males lacking a Y which are white eyed, $w^{+(21)}$ males without a Y are clearly variegated.

Because phenotypic reversion of w^{m4} position effects invariably accompanies reassociation of w^+ with euchromatin, the polytene chromosome cytology of each w^+ chromosome was undertaken. Cytology confirmed that the w^+ reversions are complete reinversions as judged by direct examination of their w^+ chromosomes and in heterozygotes with w^{m4} (Fig. 1) and the w^+ gene is relocated to its usual euchromatic position. Nonetheless, the w^+ reversions are not complete restorations. In the absence of a Y chromosome, the w^+ males are poorly viable, occurring in a ratio of 1 male:10 females. Additionally, homozygous w^+ females are sterile to poorly fertile. Females heterozygous for each w^+ reversion and a deletion which includes w^+ are viable and fertile. Presumably, reinversion is associated with some change in the centromeric heterochromatin not amenable to cytological observation. In contrast, females homozygous for the complete reinversion of w^{m4} synthesized by T. A. Grigliatti (personal communication) by using the rationale of Novitski (9) are fully fertile.

Disjunction of Fourth Chromosome by *ins*. To assess the effect of *ins* on disjunction of the fourth chromosome, a comparative small

experiment was carried and eight *sc* z^v *ec, ins/ins* males were crossed individually to harems of *C(4)spa*^{pol} females. As a control, 11 *sc* z^v *ec,* ins^+/ins^+ males were similarly crossed individually to *C(4)spa*^{pol} females. In the experiment series, four males each produced one *C(4)spa*^{pol} offspring, the product of a nullo-4 gamete in the male parent, among 2,234 diplo-4 progeny. No exception was found among the control diplo-4 progeny which numbered 2,694. Statistically, the difference is significant. A 2×2 contingency evaluation yields a χ^2 of 6.966 which with one degree of freedom equates to a $P < 0.0083$. Thus, *ins* affects the disjunction of chromosome four whether or not attached-4s are produced remains to be determined.

Does *ins* Function in Females? It will suffice here to note that in a series of crosses designed to detect the occurrence of A-X females among the progeny of homozygous *ins* females, 12,879 females were scored with zero A-X.

Discussion

It is now possible to offer a genetic explanation for the origin of the A-X chromosomes found by Morgan, Sturtevant, and Stern (1, 2, 4). The isolation via inbreeding of *ins* segregating at low frequency in the genetic stocks described can account for the earlier sporadic occurrence of A-X. Thus, *ins* or an equivalent autosomal mutation present in stocks at a very low frequency by chance became homozygous in a male, thereby generating an A-X in each case cited above. It is unlikely that the genetic system generating a high frequency of A-X described by Morrison *et al.* (10) is responsible because two sites on the X chromosome were causally involved. The sporadic A-X described involved X chromosomes of diverse origin making identity to this system prohibitive. Of historical interest is the fact that A-X described by Sturtevant and Stern came from genetically related stocks. Thus, Sturtevant reported that a wt male derived from a homozygous *Bar* female and crossed to A-X females homozygous for *y* (Morgan's A-X) produced among its progeny one wt A-X female. A line derived by crossing the brothers of the wt A-X female to homozygous *y* A-X females produced yet another wt A-X female. By a noteworthy coincidence, the A-X found by Stern also originated in a homozygous *Bar* stock. In one cross of a *Bar* male crossed to a homozygous *y* A-X female, he recovered a homozygous *Bar* A-X female. Was an identical mutation segregating in the two *Bar* stocks responsible for the A-X? One can only speculate. Additionally, the two A-X females found by Sturtevant came from males related by descent. Did he, unknowingly, have a line segregating the A-X generating mutation? It seems likely.

How are the A-X chromosomes produced in males with a single X? The occurrence of A-X in clusters implicates a failure of sister chromatids to properly disjoin during premeiotic mitotic gonial cell division in the male germ line. In normal cell division, cohesive forces keep the sister chromatids together until at metaphase, these forces are dissolved, and disjunction occurs. "The robust cohesion at centromeres may be due more to their heterochromatic nature than their ability to form attachments to the mitotic spindle" (11). Presumably, A-X arise because the dissolution of cohesive forces (the complex *cohesin* of refs. 11 and 12), centered in the X heterochromatin, is delayed by *ins* and X chromatids are unresolved until the next cell division. The failure of w^{m4} males homozygous for *ins* to produce A-X chromosomes implies that an intact heterochromatic region is prerequisite for *ins* to exert its influence. The near complete reinversion of w^{m4} by *ins* can be explained by assuming that resolution of the chromatids involves separation in their heterochromatin plus pairing during mitosis of the separated heterochromatin in a loop-like manner. A separation in the separated but paired heterochromatin segments plus appropriate repair of the broken chromatid could lead to the infrequent occurrence of reinversion. The reinversion is not exact. Males with the reinverted X but without a Y are poorly viable and homozygous females are sterile to poorly fertile. Presumably some loss of heterochromatin occurred during reinversion, a loss which is compensated for by the Y chromosome. The heterochromatin loss does not involve the ribosomal region of heterochromatin because reinversion males without a Y and homozygous females show no signs of *bobbed* in phenotype, a characteristic of ribosomal heterochromatin deletions.

It is all too obvious that the genetic events described here are phenomenology of more than passing interest, but inexplicable by further cytogenetic experimentation. Presumably the biochemical resolution of the *cohesin* complex will supply an answer to the question of how chromatids disjoin. We believe a molecular delineation of the *ins* mutation will contribute to the solution.

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