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CHROMOSOMA

X Heterochromatin Subdivision and Cytogenetic Analysis in *Sciara coprophila* (Diptera, Sciaridae)*

II. The Controlling Element

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Abstract. The so-called controlling element (CE), which normally programs the curious behavior of the sex chromosome in this genus, has been localized in the short right arm of the polytene X in *S. coprophila*. The localization was accomplished by use of five X-autosome translocations whose break points define three blocks of heterochromatin ("heterochromomeres") extending from the X centromere to the very end (right) of the chromosome. The behavior of the translocation chromosomes at the crucial second spermatocyte division was examined and the "precocious" chromosome identified in all five cases. Then, knowing the heterochromomere make-up of each chromosome, the position of the CE could be mapped; it is located in heterochromomere H2, the same block of heterochromatin that contains 50% of the ribosomal RNA cistrons. – The question of whether the CE can manipulate any centromere in the nucleus has been only partially answered. It can manipulate translocation chromosomes which possess the centromere of the metacentric autosome (salivary chromosome IV) or that of the shorter rod (salivary chromosome II); but the longer rod (salivary chromosome III) whose proximal end, as seen in the polytene nucleus, is heavily laden with heterochromatin of its own, has not been brought under CE control. – In one of the translocations, T23, the precocious chromosome is a very large metacentric chromosome which resembles the "peculiar" V-shaped X of *S. pauciseta*. This peculiarity is not observed in the J-shaped precocious chromosome of T29. These points are discussed.

Introduction

Sex in *Sciara* is not determined at the time of fertilization by the chromosome complement of the zygote; all the eggs have the same chromosomal constitution and so do all the sperm. Each egg nucleus contains an X and three autosomes; and each sperm includes three autosomes and two genetically identical Xs. The sister X chromosomes which pass through the sperm to the zygote are

* Dedicated to Professor Hans Bauer on the occasion of his 75th birthday.

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subject, during the course of embryonic development, to irreversible genetic inactivation accomplished by physical removal of the chromosomes from the cell. The X transmitted by the egg is not thus subjected. The process whereby one homologue is predetermined to function differently from the other has been termed "imprinting" (Crouse, 1960, 1966). Selective elimination of X chromosomes from the nuclei of the *Sciara* embryo involves an identification or imprinting of the vulnerable chromosome. Using reciprocal translocations between the X and one or another of the autosomes, we were able to show that this identification does not pertain to the X as a whole but to only a very short region, which, in *S. coprophila,* is located in the proximal heterochromatin to the right of the centromere. Upon transfer of this region to an autosome, a program of behavior by the autosome was induced that was indistinguishable from the one enacted normally by the X (Crouse, 1960). Because this region could bring other chromosomal components, i.e., the centromeres, under its control, it was designated as *the controlling element* (hereafter CE).

The program of chromosome behavior regulated normally by the CE serves as the basic mechanism of sex determination in these flies and has been the object of genetic and cytological research extending over a period of years (for review see Metz, 1938). Stated briefly, this program involves three steps: (1) the inclusion of duplicate X chromosomes in the sperm nucleus; (2) the selective elimination of either one or both of them from the presumptive somatic nuclei of the developing embryo, thereby establishing the definitive complement of the two sexes – males being matroclinous and hemizygous for their sex-linked genes (X^mO) and females being biparental (X^mX^p); (3) the selective elimination of one paternal X from the germ cells of both male and female embryos.

The *first step* of the program, the inclusion of duplicate Xs in the sperm nucleus, is brought about by equational non-disjunction at the 2nd maturation division. The non-disjunction is accomplished by an advance of the double X to one pole of the spindle prior to anaphase separation of the other chromosomes. Because of its early arrival at the pole, Metz named it the "precocious" chromosome (Metz et al., 1926). This phenomenon of precocity is under CE control. A great deal more, however, in the way of coordinate chromosome activity is involved in insuring that the two Xs which enter the sperm are genetically identical; we find that not only is crossing-over prevented in the Sciara primary spermatocyte by failure of homologue pairing, but the entire 1st division is radically aberrant: the four paternal chromosomes move backward out of the cell, leaving only the maternal set and the supernumerary L chromosomes to undergo 2nd division.

The *second step,* the selective elimination of one or of both paternal Xs from the embryonic soma, occurs at anaphase of the 7th cleavage. The chromosomes which are eliminated begin their anaphase separation but fail to pull completely apart and are left lying in the middle of the spindle (DuBois, 1933).

The *third step,* the selective removal of one paternal X from the germ line, occurs later in embryonic development, just after the germ cells have migrated from the pole plasm to the site of gonad formation. From each nucleus of approximately 30 germ cells, the X prochromosome passes directly through the nuclear membrane into the cytoplasm where it eventually loses its staining capacity and degenerates (Berry, 1941; Rieffel and Crouse, 1966).

It is of interest to note that the mode of X-chromosome elimination in the soma is quite different from the one in the germ line; the former takes place at anaphase of mitosis and the latter at a stage when the nuclear membrane is intact and the chromosomes are in the form of relatively large prochromosomal bodies. The same difference is observed with regard to the supernumerary Ls. These are large heterochromatic chromosomes found in the germ line of all but two of the cytologically known species of Sciara. In his classic paper on spermatogenesis cited previously, Metz noted that all of the Ls are conserved at the 1st meiotic division, passing

into the secondary spermatocyte with the set of maternal chromosomes. Because they are thus conserved during spermatogenesis, there must be a mechanism to prevent them from accumulating in the germ cells; and since they do not occur in somatic tissues, e.g., the larval salivary gland, there must also be a mechanism which removes the entire group from the soma. Their removal from the soma is the earliest of the chromosome eliminations; it occurs at 5th cleavage, each L failing to complete anaphase separation and thus being excluded from the daughter nuclei (DuBois, 1933). In the germ line the L's are removed at the same time and in the same manner as the paternal X, i.e., by passing directly through the nuclear membrane into the cytoplasm. Following this elimination each germ cell retains exactly 2 Ls; subsequently, during the course of germ cell proliferation, the number of Ls may become variable through mitotic nondisjunction (Rieffel and Crouse, 1966).

The experimental analysis of sex chromosome behavior in *Sciara* was greatly handicapped by the dearth of visible mutations in this genus (Crouse, 1949, 1961). The few sex-linked markers which were available were indispensable. For the most part the analysis was cytological and progress was slow.

The primary question to be resolved at the outset was whether the X could be subdivided without lethal effects. In the first set of experiments two species, *S. coprophila* and *S. reynoldsi*, were irradiated and 5 X-autosome translocations recovered. In each case only one of the exchange chromosomes was found to undergo non-disjunction at the 2nd spermatocyte division; the other one behaved like an autosome. In three of the five cases the precocious chromosome could be identified as the translocation chromosome having in its make-up the X centromere (Crouse, 1943).

The behavior of the translocation chromosomes subsequent to fertilization was more difficult to assess. Knowing what had occurred at the 2nd spermacyte division and, diagnosing the somatic complement (larval salivary glands) of the progeny, one could *deduce* what must have taken place in the nuclei of the presumptive soma: in every case the chromosome eliminated had to be the one which previously passed through the sperm in duplicate. Whether the same rule of elimination applied to the germ cells was not so readily answered. Finally, an examination of the actual elimination process was undertaken. This study required a very special kind of translocation; namely, one in which *both* translocation chromosomes could be positively identified in the germ cell nuclei. When such a translocation became available, the study was carried out. It revealed that one chromosome is removed from each germ cell, the time and mode of elimination being normal and the chromosome eliminated being clearly identifiable as a derivative of the precocious chromosome (Rieffel and Crouse, 1966).

The real break-through in the analysis came with the recovery and study of Oak Ridge translocation T1 (Crouse, 1960). The effect produced by T1 on chromosome behavior during spermatogenesis was unmistakable and enormously significant. This rearrangement had converted two rod-shaped chromosomes (X and autosome II) into a large metacentric and a very short rod. Polytene analysis showed that the rod was composed of a proximal segment of II terminated by a minute portion of heterochromatin from the end (proximal) of the X; the large metacentric included all of the X, except this heterochromatin, attached to the distal segment of II. In spite of the fact that the short rod lacked the X centromere and all other genetic components of the X except the tiny mass of heterochromatin, it was invariably the chromosome that underwent non-disjunction; the large metacentric behaved like an autosome. Interestingly, the X centromere had the capacity to effect normal mitotic division of the metacentric even though most, if not all, of the proximal heterochromatin had been transferred to the short rod. Perhaps even more remarkable was the fact that in its new position the heterochromatin could manipulate the autosomal centromere although these two interacting components were at opposite ends of the short rod, an arrangement quite unlike the one found normally on the X.

From the T1 study it was evident that the program of sex chromosome behavior in these flies is controlled by an element (CE) in the proximal X heterochromatin, its location being to the right of the centromere and distal to the T1 exchange point. In the polytene nucleus (salivary gland) of Sciara there is no chromocenter; the centromeres are not morphologically distinguishable; and the X heterochromatin forms a conspicuous sticky mass of highly dispersive chromatic elements on the end of the chromosome. Mapping this region was of unusual interest since it was known to contain not only the CE but also the ribosomal RNA cistrons (Pardue et al., 1970). Construction of a map was not possible, however, without additional translocations having an exchange point in the region. A group of such translocations has been obtained, and, together with T1, it has been possible to decipher the chromomere make-up of the heterochromatin and to construct a cyto-genetic map of the region. A full description of the translocations, together with the data relating to centromere (Crouse, 1977) and rDNA (Crouse et al., 1977) localization, have been published previously. The purpose of this paper is to examine the behavior of the translocations during spermatogenesis and, knowing precisely the heterochromomere make-up of each chromosome, to identify the site of the CE. Although the behavior of the new translocations during oogenesis and at the time of elimination in the embryo are not dealt with in this paper, they have been examined, and it is known that the chromosome which is transmitted in duplicate by the sperm is the chromosome that undergoes elimination during development (see Crouse, 1960; Rieffel and Crouse, 1966; unpublished studies by the author).

Materials and Methods

1. The Translocations

The five reciprocal X-autosome translocations used in the present study have been described fully in the first paper of this series, together with details regarding their induction and propagation (Crouse, 1977). The diagram in Fig. 1a shows the exchange point of each translocation in relation to the X centromere and to the heterochromomeres H1, H2, and H3 which comprise the short right arm of the X. All of the translocations are transmissible through the germ cells of both sexes, and none is homozygous viable. In order to examine the behavior of a given translocation at the 2nd maturation division, it is necessary to use testes from males which inherit the translocation from their mother. This necessity derives from the fact that only the maternal set of homologues is retained by the spermatocyte at the end of the 1st division. Thus, the males used for testis preparations were the progeny of single female heterozygotes bred to normal males.

2. Testis Preparation

In *Sciara,* where the life of the adult fly lasts only a few days, it is not surprising to find that the germ cells of both sexes develop fairly synchronously. In some species the meiotic divisions in the male occur during late 4th larval instar; in others, during pupal life. *S. coprophila* falls into the latter category; consequently, we encounter a number of disadvantages: (1) It is not possible to examine the salivary glands and the testes of the same individual. This means that the polytene chromosomes cannot be used for diagnosing the presence or absence of a translocation; instead, this determination has to be made from the chromosomes in the germ cell nuclei, a

far more difficult assignment. (2) The fat bodies, which surround the testes in the larval stages and which are extremely useful in handling the gonads during dissection and transfer, have undergone disintegration to such an extent by the time of the meiotic divisions that the testes lie essentially naked in the body cavity; they are also unattached posteriorly because the genital ducts have not yet connected with the ejaculatory organ. Compounding the difficulties, the testes are perfectly spherical in shape at this stage and extremely fragile, each gonad consisting simply of a transparent epithelial membrane enclosing the cysts of synchronously-developing spermatocytes. Obviously the method of preparing this material for cytological study had to take into account the problem of handling the testes as well as other considerations: it had to be a short procedure so as to accommodate fairly large numbers of pupae, and it had to be one which would not disarrange the chromosomes appreciably in at least a large number of the cells.

A lactic-acetic orcein squash technic was developed which relies on the swelling of the germinal tissue in 45% acetic acid to rupture the gonads and thus release individual spermatocytes into the staining medium; no pressure at all is put on the coverglass. In practice the pupa is dissected in Shen Ringer on a slide which contains a *thin* coating of silicone; too much silicone hampers dissection, and too little makes it difficult to maneuver the testes from one fluid to the next. Upon recovery of the gonads, all debris is removed and the testes rinsed in a fresh drop of Ringer. The drop is reduced to zero volume and replaced quickly by Carnoy (3 parts absolute ethanol to 1 part glacial acetic acid). The Carnoy evaporates almost immediately. Additional drops can be added if necessary while the testes are brought close together on the slide. They are covered immediately with a small drop of 45% acetic acid, care being taken not to let them dry out in the process. While in the acetic acid, they are viewed constantly under the microscope. The acetic acid softens and swells the gonads. Finally the testis epithelium ruptures, releasing individual germ cells or clusters of cells (the acetic acid should be nearly depleted at this point). Quickly the testes are covered with a small drop of lactic-acetic orcein (1% orcein in equal volumes of lactic and glacial acetic), then separated slightly from each other, and the preparation gently covered with a siliconized coverglass (zero thickness, 18 mm square). When skillfully made, such preparations lack air bubbles and excess stain; and approximately half of the spermatocytes will be distributed in a single layer adjacent to or between the remnants of the two testes. These slides can be studied almost immediately, and some of them have maintained their excellent quality for 8–9 months. All of the data and photomicrographs presented here were obtained from such preparations.

Results

The chromosome complements shown diagrammatically in Figure 1 b are based on measurements made on salivary gland nuclei. Nevertheless, they accurately represent the mitotic chromosomes observed in the testis. According to the diagram, the only case in which the two translocation chromosomes are fairly similar in length and in arm ratio is T70; in the other four, the chromosomes differ markedly as judged on both criteria. Actually, in all five cases it is possible to identify the precocious chromosome with certainty: in T23 and T70 it is X^A, the translocation chromosome with the X centromere; and in the remaining three (T1, T29, and T32) it is A^X, the chromosome with the autosomal centromere. In presenting the data, some of the translocations are treated more fully than others depending on the issues they raise. The objective here is not to document the full course of germ cell maturation in each case but rather to identify the precocious chromosome (hereafter PC) for each translocation and bring the data to bear on a variety of problems.

The behavior of the PC in both normal and translocation material is recorded in the photomicrographs (Figs. 2–24) and in Table 1. The counts given in

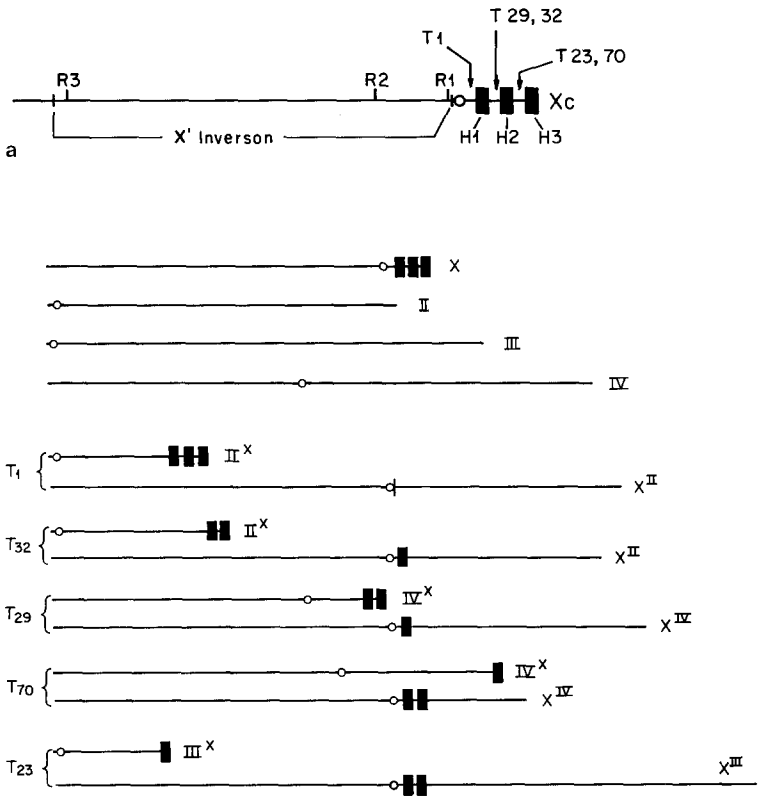


Fig. 1. a Diagram of the polytene X of *S. coprophila* showing the exchange point of each translocation in relation to the centromere (clear circle) and the three heterochromatic chromomeres (heterochromomeres) H1, H2, and H3. For clarity the size of the heterochromomeres has been very much exaggerated. The position of the three repeat regions (R1, R2, R3) and the X′ inversion are also indicated. Based on Crouse (1977). **b** Diagram of the normal and translocation chromosomes showing centromeres and relative arm lengths. The exchange between autosome and X is marked by the position of the heterochromatin except in the case of the T1 chromosome X^{II}; this chromosome contains the X centromere but lacks all three heterochromomeres; its exchange point is marked by a short vertical line just to the right of the centromere

the Table were made only after the full course of spermatogenesis had been studied in each kind of material.

Additional precautions were taken to further reduce the possibility of error: (1) The pupae from which the normal material was obtained were siblings of translocation-bearing individuals; of the 11 normal pupae whose testes were used for scoring, five were derived from T23 material, two from T1, two from T32, one from T29, and one from T70. (2) Prometaphases and polar views of MII were excluded, as were those cells in which the chromosomes had obviously undergone disarrangement during squash preparation. (3) The scoring was done on a Wild M20 research microscope using a 50X oil fluotar objective and 12.5× oculars; each area selected was scored three times prior to recording the final assessment. In spite of these precautions there were a

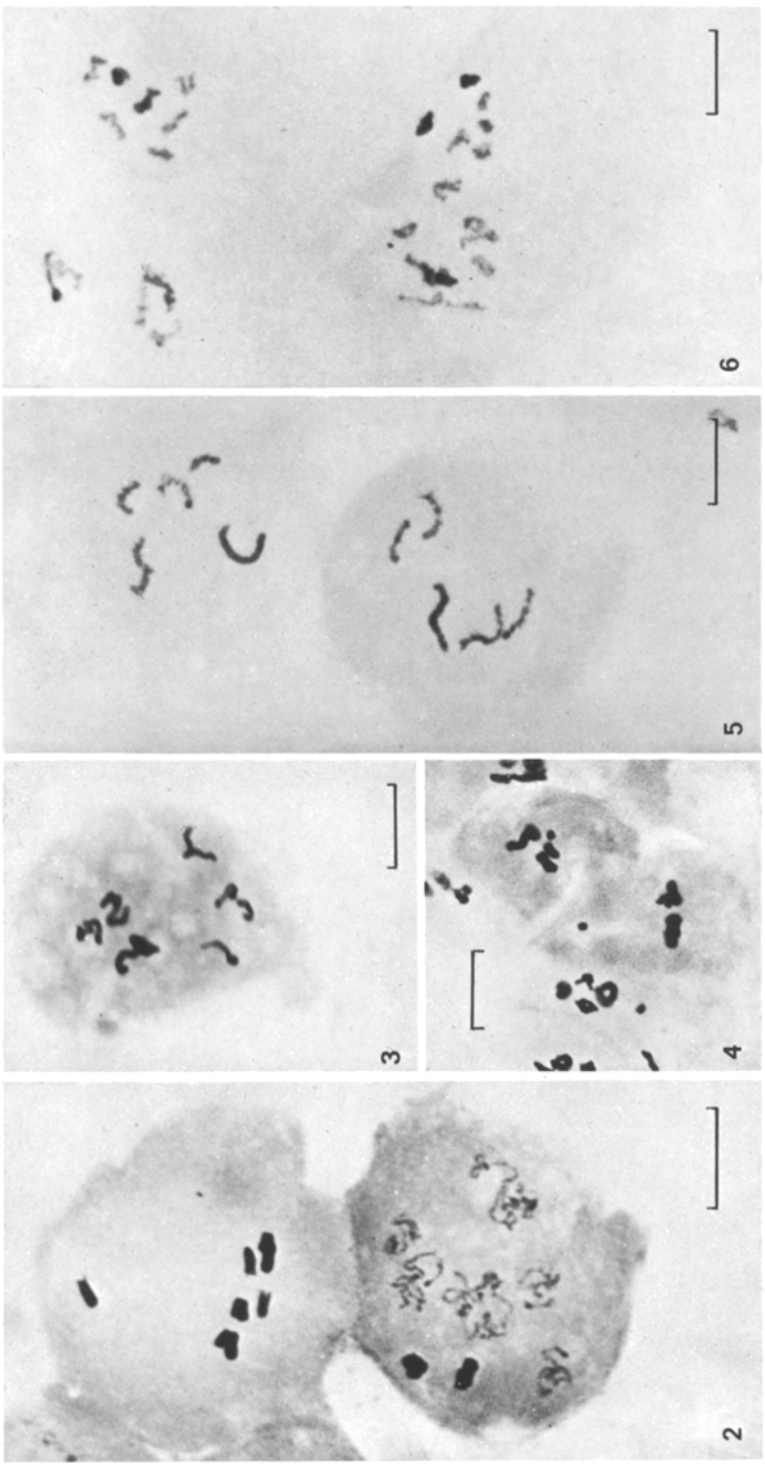
Table 1

Material	PC Identification	Translocation Chromosome On Plate	No. of Slides Counted	Cells With PC	PC On Plate	PC Position Uncertain	PC Dividing Equally	% Cells With PC
Normal	X Rod	–	11	1,037	17	0	0	98%
T1	II^X Very short rod	X^{II} v-shape	3	581	34	35	2	94–89%
T32	II^X Short rod	X^{II} v-shape	4	460	33	33	6	92–86%
T23	X^{III} "Broken" v	III^X Very short rod	3	184	7	0	0	96%
T29	IV^X Small j	X^{IV} v-shape	4	186	227	0	2	45%
T70	X^{IV} "Broken" j	IV^X j-shape	2	144	1	0	7	95%

number of cells in both T1 and T32 in which the position of the PC remained uncertain (column 7 of the Table). (4) No slide was used for scoring unless it was possible to establish with certainty that the chromosome complement was heterozygous for the translocation in question and did not include an extra autosome. For such complement analysis the smear had to provide various stages of spermatocyte development including excellent PIs and MIIs. This precaution was necessary because of the occurrence of a few males derived from non-disjunctional eggs (see Crouse, 1965 on non-disjunction in X-translocations) in which, as a result of erroneous chromosome elimination during development, the extra chromosome is retained in the germ cells but not in the soma. Males which have an extra chromosome (Ls excluded) in the germ cells regularly show reduced PC efficiency (unpublished studies).

1. The Normal Material

Because normal spermatogenesis in *S. coprophila,* as observed in squash preparations, has been illustrated previously (see Crouse, 1943, 1960, 1965 and 1966), we include only four photomicrographs of non-translocation material. One of them is presented to show the 4 paternal homologues as they move backward out of the primary spermatocyte at AI; this is the only stage at which one can view the haploid complement in the germ cells of this species in the absence of the supernumerary L chromosomes. The Ls are *not* subject to chromosome imprinting and consequently do *not* move backward out of the cell with the paternal set of homologues although they may likewise be of paternal origin (Crouse et al., 1971); instead, all of the Ls enter the nucleus of the secondary spermatocyte (Metz et al., 1926). In Figure 3 the metacentric chromosome retreating from the pole corresponds to salivary element IV. Whether viewed at AI or at PII (Fig. 5) the differences in length of the three rods are very slight; II is the shortest, while X and III are approximately of the same length (see Fig. 1 b); later, at MII, the X becomes identifiable by virtue of its precocious movement to one pole (Fig. 2). In the testis shown in Figure 2, two L chromosomes are present in each cell: in the MII nucleus they are the two large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metacentrics on the equator, and in the PI figure they are the two conspicuous dark-staining masses that contrast sharply with the 8 delicate, intricately-coiled chromosomes of the regular complement. Unlike organisms such as maize, PI in *Sciara* is not a very useful stage; the homologues fail to undergo meiotic pairing, and we have been unable to construct reliable chromomere maps. Nor do we find distinctive chromomere patterns in PII. In the PII nuclei seen in Figure 5 there is one L; it is more condensed and of greater diameter than the four regular chromosomes.

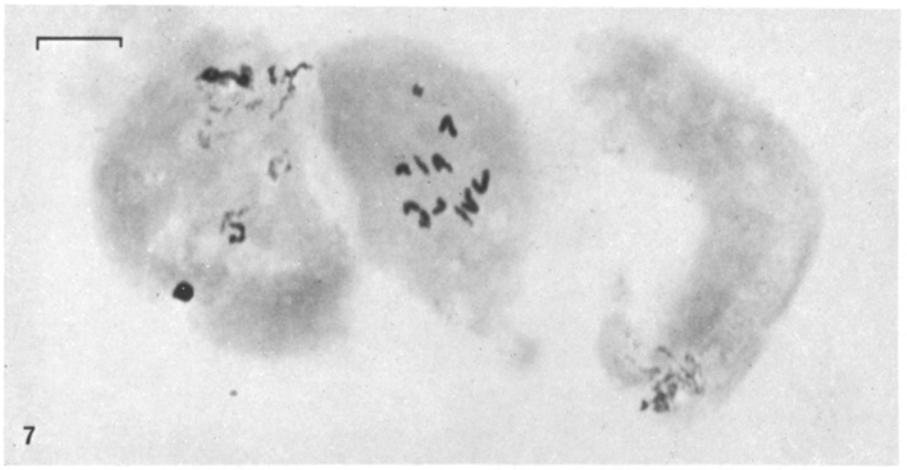
The behavior of the L chromosomes – their staining and replication cycles, their variation in number from one individual to another and from cell to cell within the same gonad, their mode of elimination from the embryonic germ cells – has been studied in *S. coprophila* (Rieffel and Crouse, 1966). In their condensation and staining they are out of step with the regular chromosomes except briefly during cell division. This asynchrony is striking not only during prophase of the primary spermatocyte (Fig. 2) but likewise at an early stage of spermatid development (Fig. 6). The cells shown in Figure 6 demonstrate that the Sciara spermatid nucleus contains *five* regular chromosomes (3 autosomes and 2 Xs) in addition to the Ls.

2. Translocations T1 and T32

T1 and T32 are very similar translocations. Both involve autosome II, the T32 exchange point being approximately 12 bands distal to that of T1. The breaks on the X are separated by one heterochromomere; the T1 break lies

Figs. 2–6[1]. **Figs. 2, 3, 5 and 6** from normal testis, **Fig. 4** from T32 testis. **Fig. 2.** The cell at the top is in MII and shows the PC at the upper pole; on the equatorial plate are two large v-shaped Ls, the metacentric autosome IV, and the rod-shaped autosomes II and III. The lower cell is a primary spermatocyte showing two heavily condensed Ls and eight intricately coiled regular chromosomes which still show the remnants of somatic pairing normally characteristic of Dipteran nuclei at prophase. **Fig. 3.** A primary spermatocyte at AI showing the selective elimination from the cell of the four paternal (genetic studies) homologues. Autosome IV is the V-shaped chromosome on the right; the three rods cannot be individually identified in normal material. Note that the chromosomes are moving backward out of the cell in spite of their spindle fiber attachments which are directed towards the single pole. **Fig. 4.** Three secondary spermatocytes at 2nd meiotic division. In the lower MII (side view) the small PC can be seen at the upper pole. In the early AII to the left the PC is at the lower pole; on the equatorial plate autosomes III (rod) and IV (small V) are in sharp focus; out of focus, and seemingly lying below the equator, there is a large L whose chromatids have repelled each other widely; also out of focus and somewhat displaced from the equator is translocation chromosome X^{II} in which the repulsion of chromatids is somewhat delayed as is often the case. In the third spermatocyte (upper right) the PC appears to be on the plate with the other chromosomes, but the stage is too early to judge; such cells have been excluded from the counts presented in Table 1. **Fig. 5.** Two secondary spermatocytes at PII showing one L (darker staining) and four regular chromosomes (maternal set). In the lower cell the V-shaped chromosome IV can be recognized at the lower right. The chromomere patterns at PII are not good enough for identifying individual chromosomes. **Fig. 6.** Spermatid formation. Three cells are passing through a very brief stage during which the *five* regular chromosomes (3 autosomes and 2 sister Xs) are clearly out of step with the two heavily condensed Ls

[1] The bars represent 10 μm, as in all subsequent figures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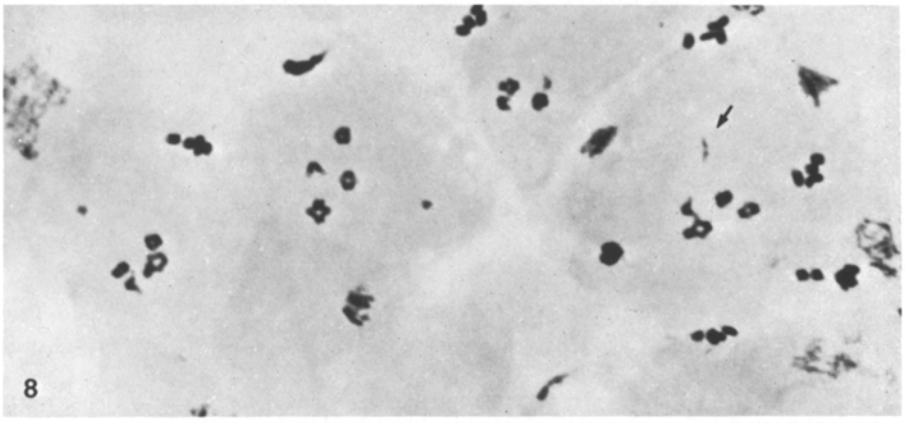
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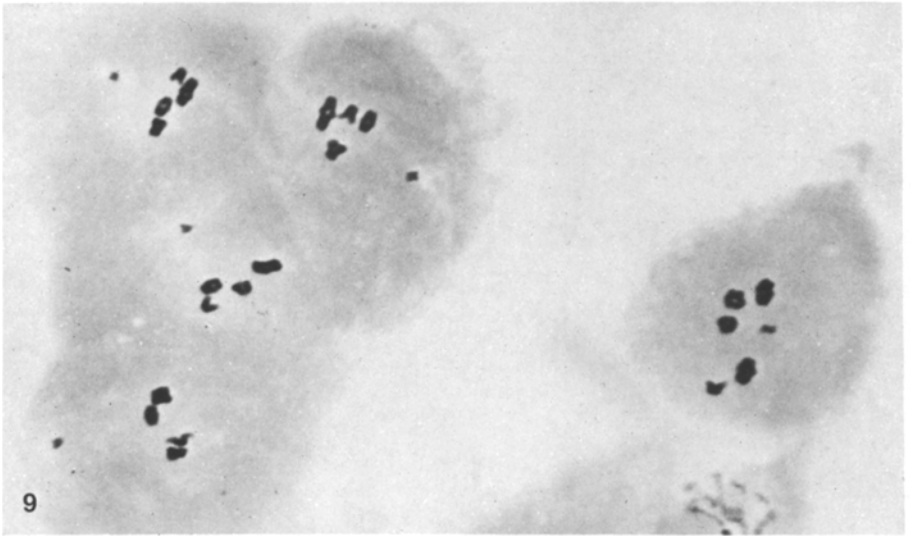
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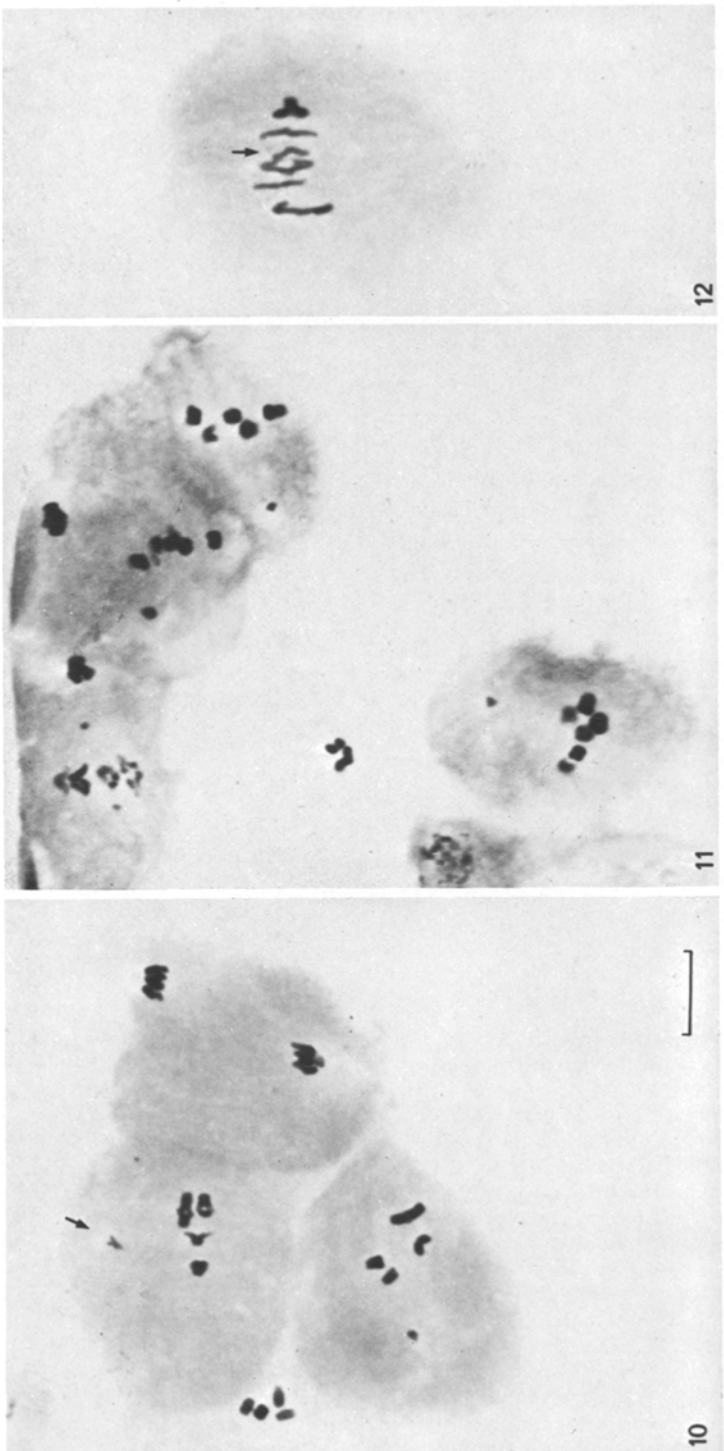
immediately to the right of the centromere and that of T32 between H1 and H2 (Fig. 1). In the case of T1, chromosome II^X is terminated by the three heterochromomeres and in the case of T32 by only two of them (H2+H3). In both translocations, chromosome II^X is the one which undergoes nondisjunction. The PC of T32 is slightly longer than the normal X. The other translocation chromosome, X^{II}, has an arm ratio of approximately 5:3. Thus, the complements of T1 and T32 form a sharp contrast at 2nd division to the normal: instead of two rods (autosomes II and III) and the metacentric IV on the equatorial plate with the Ls, there is only one rod (autosome III), all the other chromosomes being metacentric or nearly so; and the PC is so much smaller than any other chromosome of the complement, it is clearly identifiable whatever its position in the nucleus.

The behavior of T1 and of T32 during spermatogenesis, as the data indicate (Table 1 and Figs. 4, 7–11), are essentially alike in spite of the different distribution of heterochromomeres between the rearranged chromosomes. Chromosome X^{II} is programmed to undergo normal mitotic division in both cases. And chromosome II^X operates as PC in T32 nearly as efficiently as it does in T1 (86–92% versus 89–94%) despite the fact that the T32 chromosome lacks heterochromomere H1. The essential point established by the study, therefore, is that H1 does not contain the CE.

Additional points raised by the study relate to the centric activity of the PC and to the question of whether in normal material the X undergoes congression on the equatorial plate prior to its advance to the pole. In his classic study on spermatogenesis in several different species of Sciara (Metz et al., 1926) Metz examined sectioned material fixed in a variety of ways so as to preserve both chromosomal and spindle components. He found that the pole to which the PC moves is *predetermined* by the arrangement of cells within each cyst and that in most cells the PC is already at the pole *before* division. He observed also that there are spindle fibers leading from the PC to *both*

Figs. 7–9. *T1 testes*. **Fig. 7.** Second division and spermatid formation. The very short rod-like PC is at the upper pole with its two chromatids lying parallel and showing no evidence of repulsion in the centromere region. The five chromosomes undergoing. disjunction on the equatorial plate are all identifiable: the two large Ls at the extreme right; next, the rod-shaped autosome III; then the separating halves of metacentric IV; and on the extreme left, lying slightly below the equator, translocation chromosome X^{II}, the two halves of which have still not disjoined completely. – The cell to the left is in telophase II; the condensed heteropycnotic spherical body in the cytoplasm is the genetically-deficient nucleus which will be extruded as a "bud" and subsequently degenerate. – The cell on the right is a spermatid. (Compare Fig. 6.) **Fig. 8.** Three cells at MII, each showing the tiny PC at one pole and four chromosomes on the equatorial plate, including one L. The cell to the right demonstrates clearly that one chromatid of the PC (see arrow) has a spindle fiber which leads to the opposite pole; the opposing kinetic forces have pulled the chromatids completely apart so that they lie end to end rather than side by side. The chromosomes in the various nuclei are identifiable as in Figure 7. **Fig. 9.** Five secondary spermatocytes at MII, four of them showing the PC at one pole, and four chromosomes including one L on the equator. The cell at the far right contains 2 Ls and the PC has failed to take up its characteristic position at one of the poles. All of the chromosomes are identifiable as in Figure 7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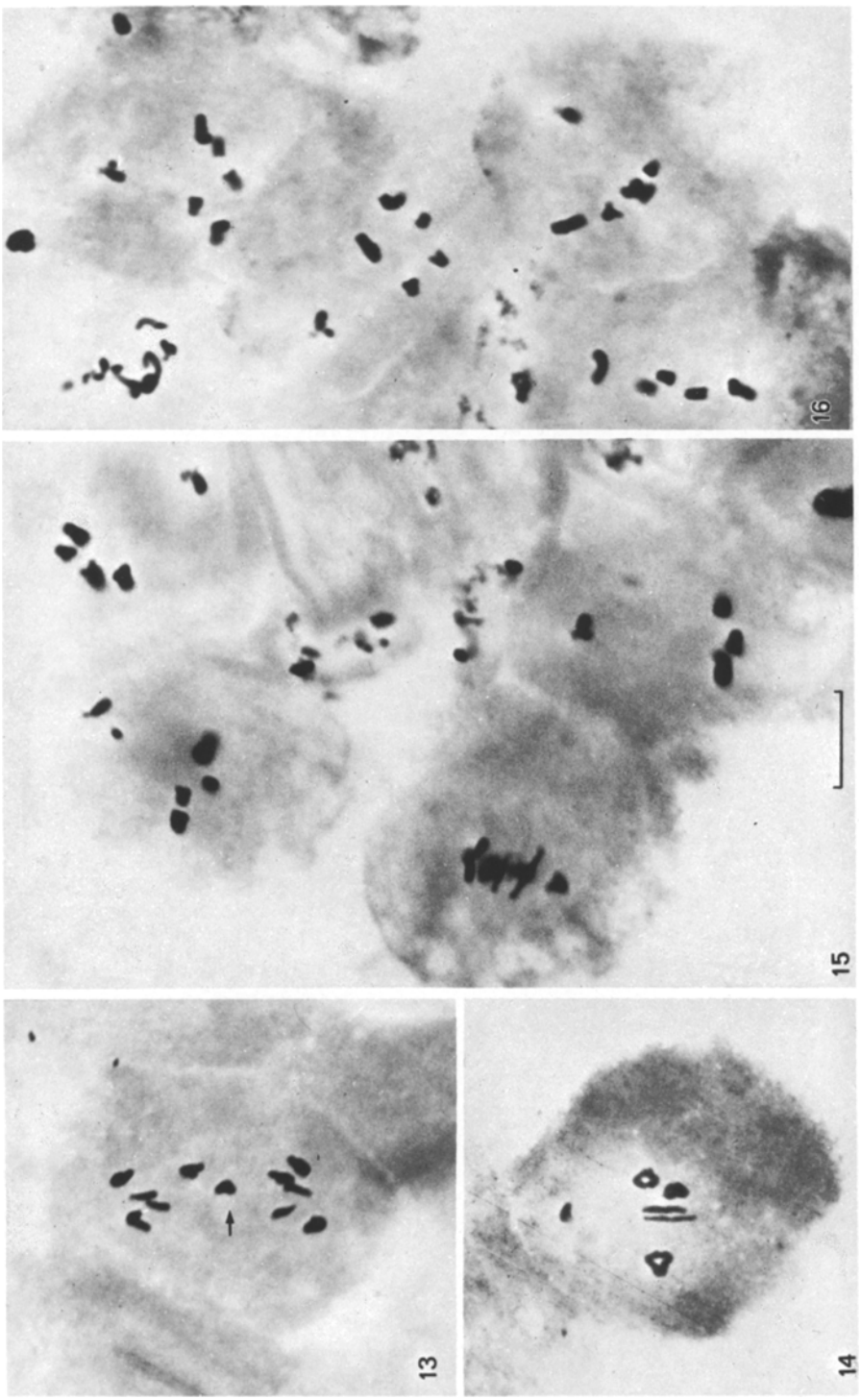
poles and that the opposing forces are great enough to distort the chromosome in species having a metacentric X (*S. pauciseta, S. similans, S. prolifica*). In acetic squash preparations, such as those used in the present study, one rarely finds any evidence that the centromere of the PC in normal *S. coprophila* is subjected to opposing forces. This is not true in *S. pauciseta* (Fig. 24) or in T1 and T32. Although of relatively rare occurrence, the chromatids of the T1 PC may be pulled completely apart so that they are aligned end to end (Fig. 8) instead of side by side. More often a separation of chromatids is observed only in the proximal region of the chromosome (Fig. 10; also Figures 8 and 9 in Crouse, 1960). In those cells where chromosome II^X fails to move to the predetermined pole and becomes aligned instead on (columns 6 and 8 of Table 1) or near (column 7) the equator, it is clearly subjected to bipolar forces that ultimately may result in its equational division (Fig. 11). Chromosome II^X appears to assume an equatorial position more frequently than does the X in normal material. We do not know why this is so, but we believe it is related to size; chromosome II^X is by far the shortest chromosome in the nucleus and completes its condensation relatively early; in this state it apparently responds more readily to the forces of attraction of the equatorial zone. Whenever chromosome II^X is observed undergoing division, it is invariably ahead of the other chromosomes (Fig. 11). The same phenomenon of early condensation and division is encountered in translocation T23; the exceedingly short chromosome, III^X, which behaves like an autosome, is always far in advance of the other chromosomes in its poleward movement (Fig. 23).

3. Translocations T23 and T70

These two translocations, although involving different autosomes, will be considered together since the PC in both of them includes the X centromere and heterochromomeres H1 and H2 (Fig. 1). The third block of heterochromatin, H3, in both cases is part of the chromosome that undergoes division, demonstrating that H3 is non-essential to PC activity. Thus, T23 and T70, together with T1 and T32, establish heterochromomere H2 as the site of the CE.

Translocations T23 and T70 are also of interest with reference to a point raised by Metz in his study on the metacentric X of *S. similans* and *S. pauciseta*

Figs. 10–12. Figs. 10 and 11. T1 testes. **Fig. 10.** Cluster of three secondary spermatocytes. In the upper MII the PC (indicated by arrow) shows evidence of opposing kinetic forces acting on the centromere; the chromatids, however, have not been pulled completely apart as in Figure 8. **Fig. 11.** An interesting group of four secondary spermatocytes showing complete separation of the PC chromatids to opposite poles (early AII cell); such disjunction is very rarely observed. Most likely this particular PC was aligned on the equatorial plate with the other chromosomes (as is the case in the adjacent MII) and then, because of its relatively small size, it underwent disjunction well in advance of the other chromosomes. (Compare Fig. 23.) Variation in the number of Ls (2 or 3 per cell) is demonstrated here. **Fig. 12.** T29 testis. An early AII showing the PC (chromosome IV^X) on the equator with the other five chromosomes. The two translocation chromosomes are in the center, flanked on either side by a rod and an L. The arrow identifies the PC; note its relative arm lengths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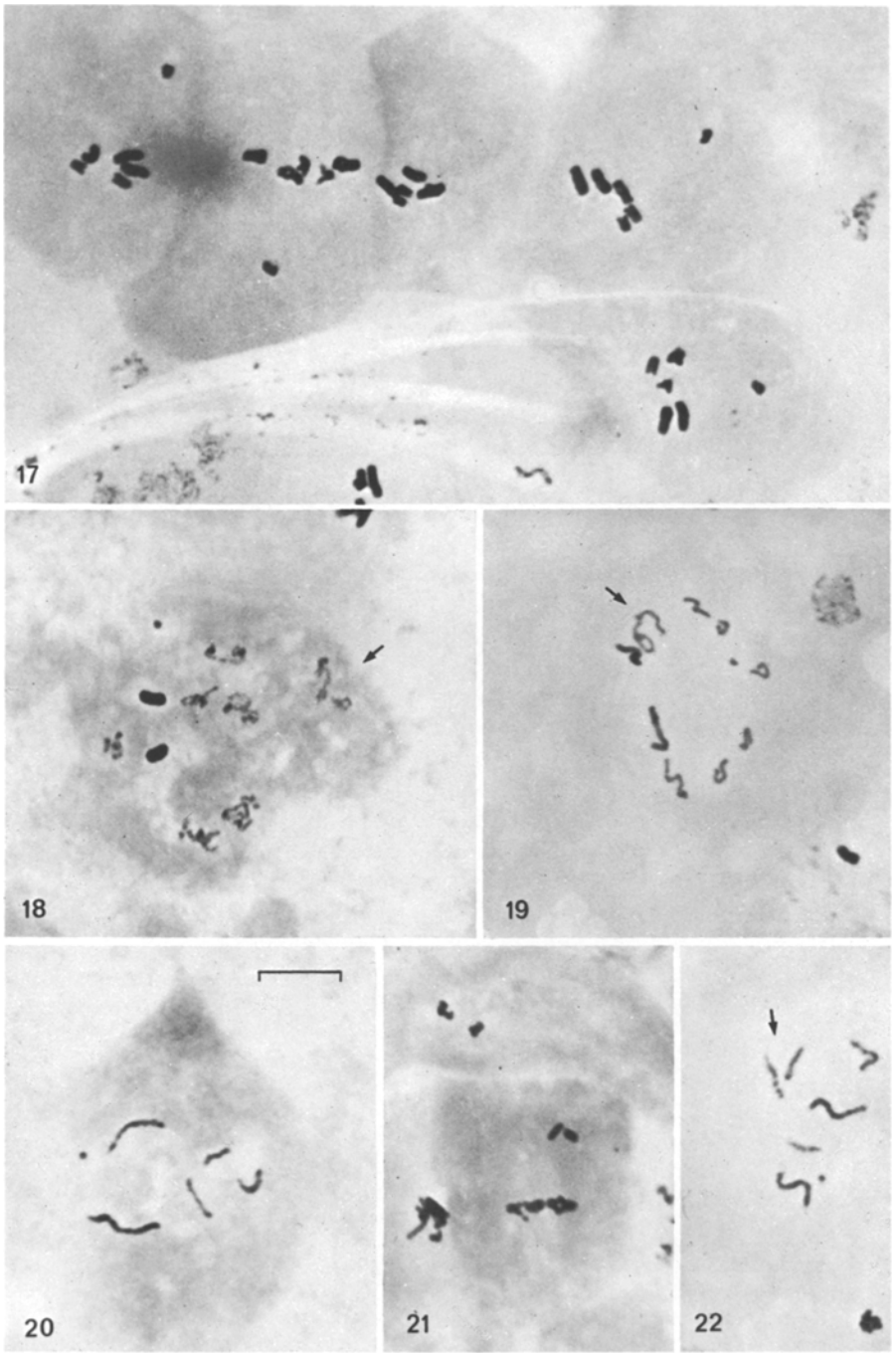
(Metz et al., 1926). Whereas in mitotic divisions in somatic and gonial tissue this chromosome was found to be v-shaped, at the 2nd meiotic division in the testis it resembled a "double hook" or was broken transversely into two rod-shaped elements (see Fig. 24). Essentially the same condition is found in the spermatocytes of T23 (Figs. 21 and 23) and T70 (Fig. 15–16), the similarity being more striking in the case of the T23 PC which has an arm ratio of 1:1 like the X of *pauciseta* and *similans*. In T70, where the ratio is 2:1, the PC looks like a broken J (see Table 1), very much resembling an SAT-chromosome (terminology by Heitz, 1931). The explanation of this interesting phenomenon is to be found in the chromomere make-up of the translocation chromosomes. Referring to the diagrams in Figure 1, we find that heterochromomeres H1 and H2 are joined in the case of T70 to a short segment of autosome IV and in the case of T23 to a very long segment of autosome III. More than half of the ribosomal RNA cistrons are located in H1 and H2 (Crouse et al., 1977), and these two heterochromomeres lie immediately adjacent to the centromere. Thus, the non-staining region which looks like a gap or break in the PC coincides with the location of the rDNA. This is apparently also the case in normal *S. pauciseta* (Gabrusewycz-Garcia, 1971).

The question of why the rDNA is detectable at the 2nd spermatocyte division and not during ordinary mitosis in somatic and gonial tissue (Metz's observation, see above) is an interesting one. Is it simply a matter of differential transcriptional activity of the ribosomal RNA cistrons? Or is the phenomenon of precocity important; i.e., if the chromosome (X^{III} in T23; X^{IV} in T70) moved to the equator and underwent division, would it appear "broken"? Because the PC in both translocations operates with such a high efficiency (95–96%, Table 1), the anwer to this question had to be sought in the few cells of T70 listed in column 8 of the Table. Fortunately, such a spermatocyte was found at late anaphase in which all the chromosomes except X^{IV} had undergone separation: X^{IV} lagged on the equatorial plate, its long arm still showing no evidence of chromatid disjunction; the two halves of the short arm, however, had been completely pulled apart, revealing its SAT (trabant) character.

In her classic study on the nucleolus of *Zea mays*, McClintock demonstrated that the length of the non-staining region (the "stalk" in her terminology)

Figs. 13–16. Fig. 13. T29 testis. A spermatocyte at AII in which five chromosomes (3 Vs and 2 rods) are moving to each pole. The PC is not at either pole but lags on the equator (see arrow) as though its chromatids are unable to disjoin. **Fig. 14.** T29 testis. An excellent early AII showing the PC at the upper pole; note that the PC does not appear "broken" in the proximal region (compare Figs. 15 and 21). The five chromosomes on the equator include 2 Ls, the two rods, and translocation chromosome X^{IV} (far right position on spindle). **Fig. 15.** T70 testis. Four spermatocytes at MII including one in which the PC is lying on the equator with the other chromosomes. This cluster of cells is not sufficiently flattened for individual chromosome identification except in the case of the three PCs which have taken up their characteristic polar position. The morphology of the PC (translocation chromosome X^{IV}) is very well revealed in these cells and in Fig. 16; note that the PC gives the appearance of a "broken" J (see text). **Fig. 16.** T70 testis. Three spermatocytes at MII, each with a PC at the pole and five chromosomes, including 2 Ls, on the equator. The translocation chromosome IV^{X}, which is J-shaped, the two rods, and the 2 Ls can all be identified in the cell occupying the center of the cluster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on chromosome 6 is a measure of nucleolus size in the previous interphase (McClintock, 1934). The interphase between the 1st and 2nd meiotic divisions in Sciara is very brief. We have examined the condition of chromosome X^{III} in translocation T23 at PII and also at several stages of the primary spermatocyte. At PII the gap or secondary constriction is so large that the chromosome readily breaks into two segments (Figs. 20 and 22). During PI (Figs. 18 and 19), on the other hand, a stage at which the chromosomes are much more fragile and susceptible to rupture in smearing, we could not detect any chromosomal discontinuity. In other words, the increased transcriptional activity of the rRNA cistrons is clearly correlated with the transition from first to second spermatocyte.

It should be noted that the nucleolar condition in Sciara differs markedly from that in maize or Drosophila (see Gerbi and Crouse, 1976). Instead of a well-formed nucleolus attached to the proximal end of the X in the salivary gland nucleus of *S. coprophila,* one finds a variable number of "micronucleoli" which apparently become detached from the chromosome and are released into the nuclear sap (Gabrusewycz-Garcia and Kleinfeld, 1966). In the T23 and T70 material there appears to be built into the chromosome a device for monitoring rRNA cistron activity, namely, the size of the secondary constriction. A careful study should be made comparing the nucleolar condition in the secondary spermatocyte of T23 and T70 to that in the salivary gland nucleus.

4. Translocation T29

As in all X-translocations studied to date, only one of the T29 chromosomes displays precocious behavior, namely IV^{X} (Figs. 14 and 17); yet it does so

Figs. 17–22. Fig. 17. T29 testis. Five spermatocytes at MII in four of which the morphology of the PC is shown clearly. This J-shaped chromosome (IV^{X}) is not "broken." (Compare with T70 in Fig. 16, also in Fig. 1b). **Figs. 18–22.** T23 testes. **Fig. 18.** Primary spermatocyte showing 8 regular chromosomes and 2 Ls. The arrow indicates the long metacentric PC (translocation chromosome X^{III}). The other translocation chromosome (III^{X}), which is very short and stains nearly as heavily as the Ls (see text), can be seen in the upper left periphery of the cell. **Fig. 19.** Primary spermatocyte just before AI. The arrow designates the PC. The two Ls can still be distinguished by their staining although they are nearly completely uncoiled. **Fig. 20.** Secondary spermatocyte at PII. The nucleus includes 1 L, the thicker dark chromosome at the lower left. The short translocation chromosome III^{X} is seen at the far left. The two segments of the PC have been pulled completely apart and cannot be identified with certainty. (Compare Fig. 22 and refer to description of Fig. 21.) **Fig. 21.** Secondary spermatocyte at MII. The PC is at the upper pole and appears to be "broken" (see text) in the region of the centromere. The short translocation chromosome III^{X} lies to the left and below the equator in close proximity to the proximal end of autosome II. The only L in the cell lies on the plate at the far right. The metacentric IV lies between II and the L. The mass of chromatin at the left edge of the cell is extruded chromatin from adjacent spermatocytes which have completed the 2nd division (see Fig. 7); such so-called "buds" are lacking a chromatid of X^{III} because of PC behavior. **Fig. 22.** Secondary spermatocyte at PII. The nucleus includes two dark-staining Ls; the short translocation chromosome III^{X} lies close and to the right of the s-shaped L. The short chromosome between the Ls is believed to be autosome II and the one to the upper right, autosome IV. The PC (arrow) can be clearly identified, its two segments being only slightly pulled apart in the proximal region of the chromosome (compare Fig. 20)

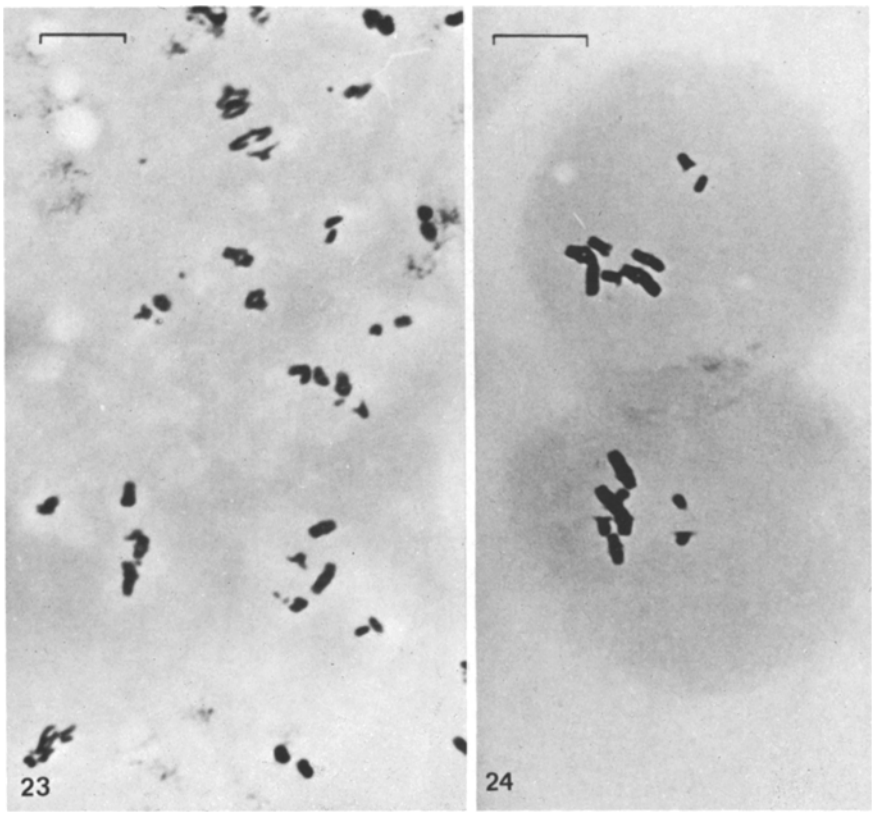

Fig. 23. T23 testis. Five secondary spermatocytes each showing the "broken" v-shaped PC at one pole of the spindle. In the early AII at the top of the figure the two halves of the short III^X chromosome have already completed their movement to opposite poles. The rod-shaped II, the metacentric IV, and the two Ls lined up on the equator, are just beginning their anaphase separation (compare Fig. 24)

Fig. 24. *S. pauciseta*. Normal material showing characteristic behavior of metacentric X chromosome at 2nd meiotic division. Note clarity of spindle fibers leading from centromere of PC to *opposite* poles. Compare "broken" condition to that of PC in T23 spermatocytes (Fig. 23)

in only 45% of spermatocytes (Table 1). The remaining 55% of the cells apparently do not form functional sperm. Their exact history is difficult to trace; in some of them (Fig. 13) chromosome IV^X lags on the equator while in others (Fig. 12) it undergoes division. In late pupae testes there are signs of cellular disintegration, and the adult males are highly sterile.

Why the T29 chromosome fails to behave precociously in more than half the cells is not understood. The exchange point on the X coincides with that of T32 (Fig. 1); consequently, the PC in the two translocations have the same heterochromomere make-up, i.e., H2+H3. Yet in operational efficiency as PC (see Table 1), the two differ by at least 40% (86% − 45% = 41%). Since the T29 chromosome contains the essential element, H2, barring mutation therein

or a position effect, the defect must lie in the autosomal centromere or in some other part of the system which normally allows these two components to interact effectively. On chromosome IV at the T29 exchange point there is a 5–6 band deficiency which might be accountable. There is some evidence to support this view; although one is not aware of any gross abnormality in the T29 spermatocytes, in some of them at MII the chromosomes are not aligned in a single equatorial plane but appear to be distributed at different levels between the poles. Such an alteration in chromosome arrangement would probably affect adversely the early movement of the PC to the pole.

In connection with the low operational efficiency observed in T29, mention should be made of Oak Ridge T5, a reciprocal translocation between X and IV, in which the operation of the PC was likewise defective. In T5 the exchange point on X was approximately 25 bands distal to the heterochromatin (between repeats R1 and R2) and that on IV was close to the right end (see Fig. 1 in Rieffel and Crouse, 1966). The PC, chromosome X^{IV}, was nearly as short as the PC of T1; although it contained the X centromere and all of the heterochromatin (H1+H2+H3), it operated with an efficiency of only 15% (Crouse, unpublished); consequently, T5 males were almost completely sterile. In salivary smears of T5 heterozygotes, figures well-flattened in the exchange region were difficult to obtain because of the characteristic lateral synapsis of the two repeats (R1+R2) located on either side of the exchange point; thus, it is not certain whether a small deficiency remained undetected. Conceivably, the defect may have been due to the location of the T5 exchange point between the repeats; rarely have rearrangements with a break point in proximity to R2 been recovered and those which were, proved to be highly inviable.

Finally, in relation to T29 it should be pointed out that neither of the translocation chromosomes in the secondary spermatocyte has the appearance of an SAT-chromosome (Figs. 14 and 17); on IV^{X}, heterochromomeres H2+H3 are terminal in position; and although chromosome X^{IV} is metacentric and has H1 adjacent to its centromere there are very few rRNA cistrons located in H1 (approximately 10%).

Discussion

In her monumental work on mutable loci in maize, McClintock (1951) developed a theory of far-reaching significance regarding the rôle of heterochromatin in gene activation and control and in nuclear differentiation. Now, nearly 30 years later, her "revolutionary" views and insights have been vindicated by a variety of molecular studies (Endow and Gall, 1975; Bukhari et al., 1977).

In the experiments described above we have localized on the polytene X of *S. coprophila* the heterochromatic element that controls the pattern of chromosome behavior leading to the differentiation of sex. Although the *Sciara* system of chromosome imprinting, recognition, and elimination was discovered nearly half a century ago, we still know nothing about it in molecular terms or precisely when it takes place. Nor do we know whether the Sciara system is essentially similar to the systems of chromosome identification and inactivation by hetero-

pycnosis which are found in the coccids (Hughes-Schrader, 1948; Brown and Nur, 1964) and in the mammals (Lyon, 1961; Cooper et al., 1971). The comparative aspects of these different chromosomal manifestations have been discussed fully in two recent publications (Brown and Chandra, 1973; Chandra and Brown, 1975).

There are two points related to the data presented here which merit attention: (1) the first concerns the fact that the centromere of autosome III has not been brought under CE control; and (2) the second concerns the location of both CE and rDNA in the same heterochromomere.

(1) On the basis of relative chromosome length, one would expect the X-III exchange rate to exceed that of X-II. Actually two X-III translocations were recovered which are not recorded in Figure 1; they were lost before cytological analysis could be completed. In one of them, designated T60, the X break coincided with that of T1, and in the other, designated T31, the break was located either between H1 and H2 or between H1 and the centromere. In neither case was it possible to define the break on III or to study spermatogenesis prior to the loss of the stocks. Thus, we are uncertain as to whether the centromere of chromosome III can be brought under CE control; nevertheless, we consider it significant that of a total of 90 X-translocations studied to date, T31 and T60 were the only two lost after the second generation in culture.

According to the molecular studies of Gerbi (Gerbi and Crouse, 1976) the X of *S. coprophila* contains only 45 rRNA cistrons, one of the lowest rDNA values known for any eukaryote. That this small number of cistrons should be distributed among all three of the heterochromatic chromomeres (Crouse et al., 1977) is indeed surprising. Equally surprising was the discovery that both the CE and 40% of the rRNA cistrons are located in the same heterochromomere (H2). It is true that H2 is very large in comparison to both H1 and H3 and probably exceeds their combined volumes; in some preparations there is the slight suggestion that H2 is really compound in nature (Crouse, 1977; Crouse et al., 1977). In any event it is likely that future molecular studies will resolve some of these problems.

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