Genome (2006) 49:269–274
DOI: 10.1139/g05-103

Chromosome elimination in germ line - soma differentiation of *Acricotopus lucidus* (Diptera, Chironomidae)

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Abstract

During germ line — soma differentiation in early syncytial embryonic development of the chironomid *Acricotopus lucidus*, a complement of supernumerary chromosomes, the so-called germ line limited chromosomes (Ks), is excluded from the future somatic nuclei in the course of elimination mitoses. The Ks lag behind in the equatorial plane, while the somatic chromosomes (Ss) segregate equally. After elimination mitoses, the Ks are only present in the pole cells, the primary germ cells. In the divisions before their elimination, the Ks frequently showed delayed separation of sister chromatids with high frequency formation of anaphasic bridges and lagging in pole movement as detected on 4′,6-diamidino-2-phenylindole (DAPI)-stained squash preparations of early embryos. To determine if all of the Ks are eliminated in one step during a single mitosis, a fluorescence in situ hybridization (FISH) analysis of early embryonic divisions was performed using probes of germ line specific repetitive DNA sequences, that specifically label the Ks in their centromeric regions. In most cases, all the Ks are lost in one mitosis; however occasionally one or several of the Ks can escape their elimination by segregating and moving poleward together with the Ss. The escaping Ks will then be eliminated in one of the following mitoses. This clearly indicates, that the specific conditions to eliminate the Ks are not restricted to only one division. Possible mechanisms of elimination of the Ks are discussed.

Key words*:* Germ line limited chromosomes, elimination mitosis, germ line soma differentiation, FISH

Introduction

A programmed elimination of a specific chromosome complement occurs with the exclusion of the germ line limited chromosomes (Ks) from the future somatic nuclei during germ line - soma differentiation in early embryonic development of the Orthocladiinae, a subfamily of the Chironomidae (Bauer and Beermann 1952; White 1973; Redi et al. 2001). In elimination mitoses, the Ks (K being derived from "Keimbahn"; Bauer 1970) remain behind in the equatorial plane, while the somatic chromosomes (Ss) segregate regularly. In the orthocladiid *Acricotopus lucidus*, this fundamental change in chromosome constitution of the future somatic nuclei begins in some nuclei at the time of pole cell formation in the 4th-6th nuclear division cycle (Staiber 2000). Therefore, only the pole cells, the primordial germ cells, carry the Ks together with two sets of Ss. The same way for eliminating germ line restricted chromosomes from the future somatic nuclei was also found in two other dipteran families, Cecidomyiidae and Sciaridae (White 1973; Gerbi 1986).

Recent painting studies on mitotic and meiotic Ks of *Acricotopus* using chromosome-specific probes of the three Ss clearly demonstrated that the Ks are derivatives of the Ss and are composed of large S-homologous sections and heterochromatic segments containing germ line specific repetitive sequences (Staiber and Schiffkowski 2000; Staiber and Wahl 2002). The knowledge of the cytogenetic behavior of the Ks during this early developmental period especially in elimination mitosis of *Acricotopus* and of other Orthocladiinae is very limited (Bauer and Beermann 1952). The present study reports on the behavior of the Ks in early syncytial nuclear divisions before and during their somatic elimination, first by 4′,6-diamidino-2-phenylindole (DAPI)-stained squash preparations of early embryos of *Acricotopus,* and then by fluorescence in situ hybridization (FISH) using K-specific repetitive DNA sequences.

Materials and methods

Embryos of *Acricotopus lucidus* (Diptera, Chironomidae) from 30 min to 4.5 h after egg deposition were dechorionated with 50% chlorox bleach (90 s), fixed in ethanol-glacial acetic acid (3:1; v/v), squashed in 45% acetic acid, and frozen on dry ice. Genomic DNA of germ cells were isolated from testicles of *Acricotopus* prepupae. Repeats of the germ line specific tandem repetitive AlKeRe1 family (Staiber et al. 1997; Staiber 2002) were amplified from germ cell DNA by PCR using the primer pair 5'-AAC ATA ATG TGA AAA ATA CA-3' and 5'-GAC AAT AAA TTT TAT CAC AA-3'. The PCR involved 40 cycles of 1 min at 94 °C, 1 min at 46.3 °C and 3 min at 72°C followed by a final extension at 72°C for 10 min. A second labeling amplification was made with the first PCR product as template in a reaction mixture containing 50 µmol/L fluorescein-12-dUTP. FISH was performed as described by Staiber and Schiffkowsky (2000). Chromosomes were counterstained with DAPI (0.1 µg/mL). Images were captured with an epifluorescence microscope (Zeiss, Jena, Germany) equipped with a Neofluar 100/1,3 objective and a CCD camera (Canon A 80, Tokyo, Japan), and processed and pseudocolored

with a Corel Draw version 8.0 software package (Corel, Ottawa, Ont.). The RGB images of DAPI stained early embryonic mitoses were first converted into 16 bit grey scale images and then inverted.

Results and discussion

Different stages of normal mitotic nuclear divisions before elimination of the Ks are shown in reverse DAPI fluorescence in Figs. 1a-1e (metaphases, front (Fig 1a.) and side view (Fig. 1b), mid (Fig. 1c) and late (Fig. 1d) anaphases, and a telophase (Fig. 1e)). The Ss (2n = 6; I-III) are smaller in size than most of the Ks and can be easily distinguished from each other in the metaphase in Fig. 1a by the different positions of their centromeres. The number of Ks can vary between different animals in *Acricotopus* as detected in a G-banding study of K complements of spermatogonial cells (Staiber 1988). In the side view of the metaphase in Fig. 1b, all Ss and Ks are accurately arranged along the equator. During anaphase (Fig. 1c) the S and K sister chromatids segregate equally and migrate to opposite spindle poles. The smaller Ss frequently move ahead of the Ks. Each of the pole-moving chromosome complements in Fig. 1d and Fig. 1e contain two S and two K chromosome sets. In contrast, during the anaphases of divisions just before the actual elimination mitosis, many K sister chromatids separate late, as they are still attached at the end of their arms, while their kinetochores are pulled in the opposite direction towards the spindle poles. Thus, those Ks form long, stretched, anaphasic bridges (Figs. 1f and 1g) extending from one chromosome group to the other. In late anaphases, as in Fig.1h, frequently lagging chromosomes occur showing delayed migration, probably as a result of late separation of the sister chromatids.

Figures 1i-1k show examples of telophases of elimination mitoses, in which the Ks are excluded from the future somatic nuclei. In this so-called somatic elimination of the Ks (Bauer and Beermann 1952, Redi et al. 2001), the Ks remained in the equatorial plane, as in the early telophase in Fig. 1i, whereas the S sister chromatids moved towards opposite poles. The insert in Fig. 1i (right above) views this elimination mitosis together with a neighbouring anaphase of a nucleus that has eliminated its Ks in a previous division. This anaphase with only segregating S chromatids is presented at the same magnification as the elimination mitosis in the insert in Fig. 1i (left below). In the telophases of elimination mitoses, in Figs. 1j and 1k, the Ks are still arranged in a condensed state at the equator, while the Ss in the daughter nuclei begin to decondense. In Fig. 1k, 2 large chromatin bridges range in opposite directions from the eliminated Ks to the daughter nuclei. It cannot be decided whether the bridges result from delayed pole-moving S or K chromatids. The result of an elimination is shown in Fig. 1l: 2 interphasic daughter nuclei containing the Ss and lumps of strongly condensed K chromatin showing bright DAPI fluorescence. In the enlarged view of the eliminated Ks of an anaphase in Fig. 1m, some K kinetochores are clearly under tension and are drawn in the direction of the poles (arrowheads in Fig. 1m). Two sister chromatids with oppositely oriented centromeres are

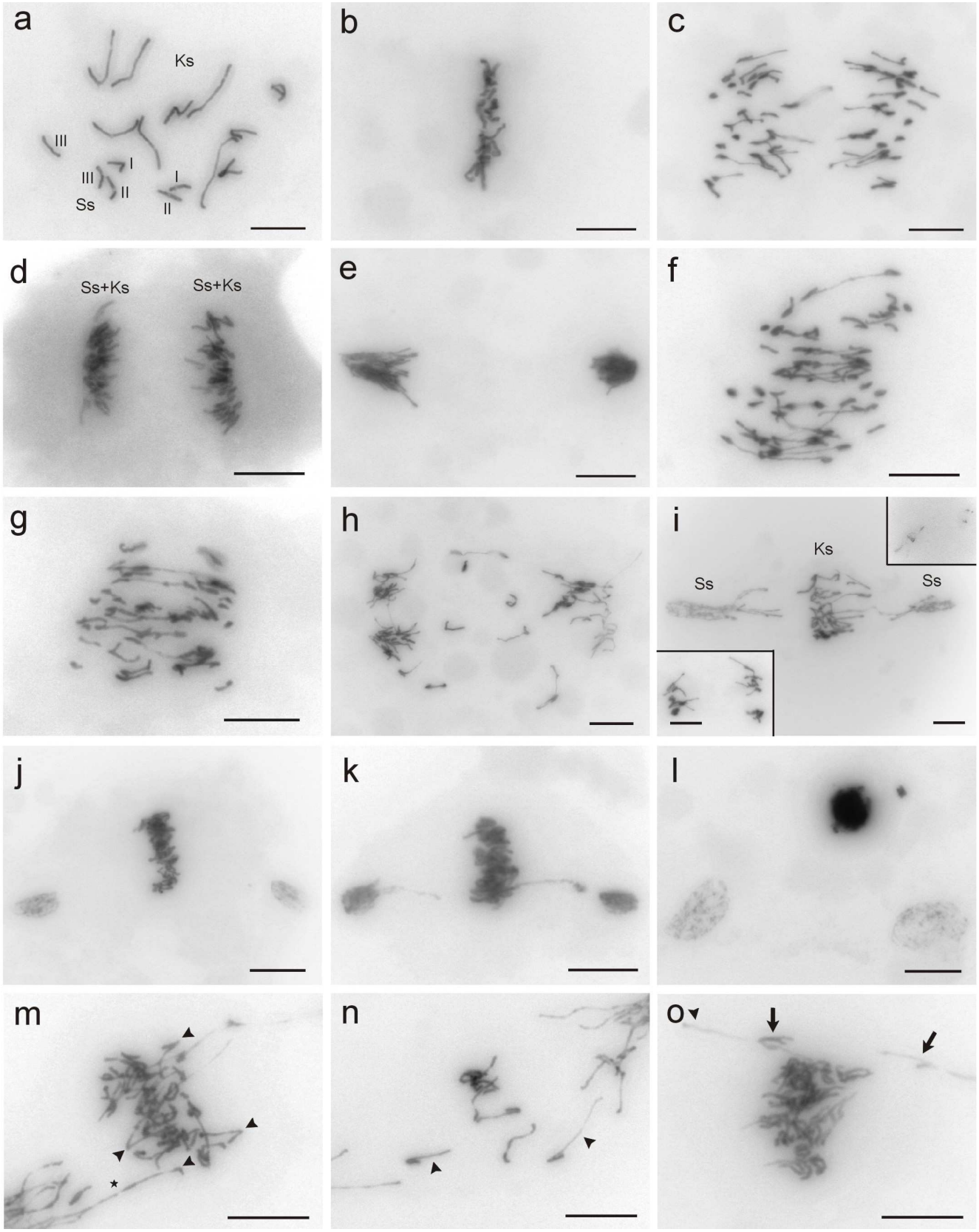

Fig. 1. Different stages of early embryonic mitoses of *Acricotopus lucidus* before (**a**-**h**), during (**i**-**k**), and after (**l**) elimination of the germ line limited K chromosomes from the future somatic nuclei, and enlarged views (**m**-**o**) on eliminated Ks all seen in inverted DAPI fluorescence. (**a**) Metaphase plate with six Ss (2n = 6: I-III) and 10 Ks. The Ss are smaller than the Ks. (**b**) Lateral view of a metaphase plate with Ss and Ks. (**c**) Anaphase of a regular mitosis. All S and K sister chromatids have separated and move poleward. (**d**) Early and (**e**) late telophase of regular mitotic divisions. (**f** and **g**) During the anaphases of divisions

before their elimination, the K sister chromatids frequently form long, stretched, anaphase bridges resulting from delayed separation. (**h**) As a consequence, these K chromatids show delayed pole migration in late anaphase. (**i**) Early telophase of an elimination mitosis. The separated Ss move poleward, while the Ks remain in the equatorial plane. Insert at top right shows an overview of this elimination mitosis and an anaphase with only segregating Ss. Here the Ks were eliminated in one of the divisions before. Insert at bottom left shows latter anaphase in the same magnification as the elimination mitosis. (**j** and **k**) Late telophases of elimination mitoses. The eliminated Ks are still arranged at the equator and remain condensed, while the Ss in the daughter nuclei begin to decondense. (**k**) Chromatin bridges ranging from both sides of the eliminated Ks to the daughter nuclei. (**l**) Two interphasic daughter nuclei of an elimination mitosis containing the Ss. The eliminated Ks have formed a very large block and a small clump of strongly condensed chromatin showing bright DAPI fluorescence. (**m**) Late anaphase of an elimination mitosis. The kinetochores of some K sister chromatides clearly appear to be under tension (arrowheads). In the lower part, 2 still-attached K sister chromatids (asterisk) have widely moved in the direction of the pole. (**n**) Late anaphase. Some Ks are eliminated by remaining in the equator, while others have separated (arrowheads). (**o**) Two just-separated K sister chromatids (arrows) with highly stretched centromeres (arrowhead) are drawn in opposite directions. Bars represent 10 µm.

still attached at the ends of their arms (asterisk in Fig. 1m) and have moved far towards the spindle pole. Unfortunately, it is not possible to analyse the arrangment of the individual chromatid arms in the entangled Ks that remained behind in the spindle equator.

In the anaphase (Fig. 1n) only some of the Ks seem to be eliminated while others have separated and show delayed migration to the poles (arrowheads in Fig. 1n). In Fig. 1o, the kinetochores of 2 recently separated K sister chromatids (arrows in Fig. 1o) are clearly under tension. The kinetochore and adjacent centromeric chromatin are pulled out from the chromosomes (arrowhead in Fig. 1o).

It is not possible to say with certainty on DAPI-stained elimination mitoses, whether all Ks are eliminated during a single mitosis, or whether some of them can escape the elimination. A prerequisite to decide this is the specific labeling of the Ks i.e. by FISH. In this study, fluorescein-labeled sequences of the germ line specific tandem repetitive AlKeRe1 family were used as probes to label the Ks specifically in their centromeric regions (Staiber et al. 1997). This is clearly documented in the metaphase in Fig. 2a, in which only the Ks exhibit fluorescent signals, while the Ss do not. In the anaphase of an early normal mitosis before elimination of the Ks (Fig. 2b) all K sister chromatids segregate regularily and move with their centromeres ahead (see signals) to the spindle poles. Two unlabeled Ss (asterisks in Fig. 2b) migrate in front of the left group of pole-moving chromosomes. In normal elimination mitoses (as shown in Figs. 2c-2e), all labeled chromosomes (Ks) remain in the equator and only the unlabeled S chromatids separate, move poleward, and are included in the daughter nuclei. This equatorial arrangement of eliminated Ks persists from anaphase to telophase. Following elimination mitoses, the condensed labeled chromatin blocks consisting of the eliminated Ks are clearly visible between the unlabeled interphasic S nuclei (Fig. 2f).

Besides elimination mitoses eliminating all Ks in the course of only one division, incomplete elimination mitoses can also occur; only a portion of the Ks are eliminated, while other Ks segregate and move to the poles like the Ss (Figs. 2g-2j). In the anaphases, one K (Fig. 2g) and some Ks (Fig. 2h) have separated and the sister chromatids migrate poleward behind the Ss.

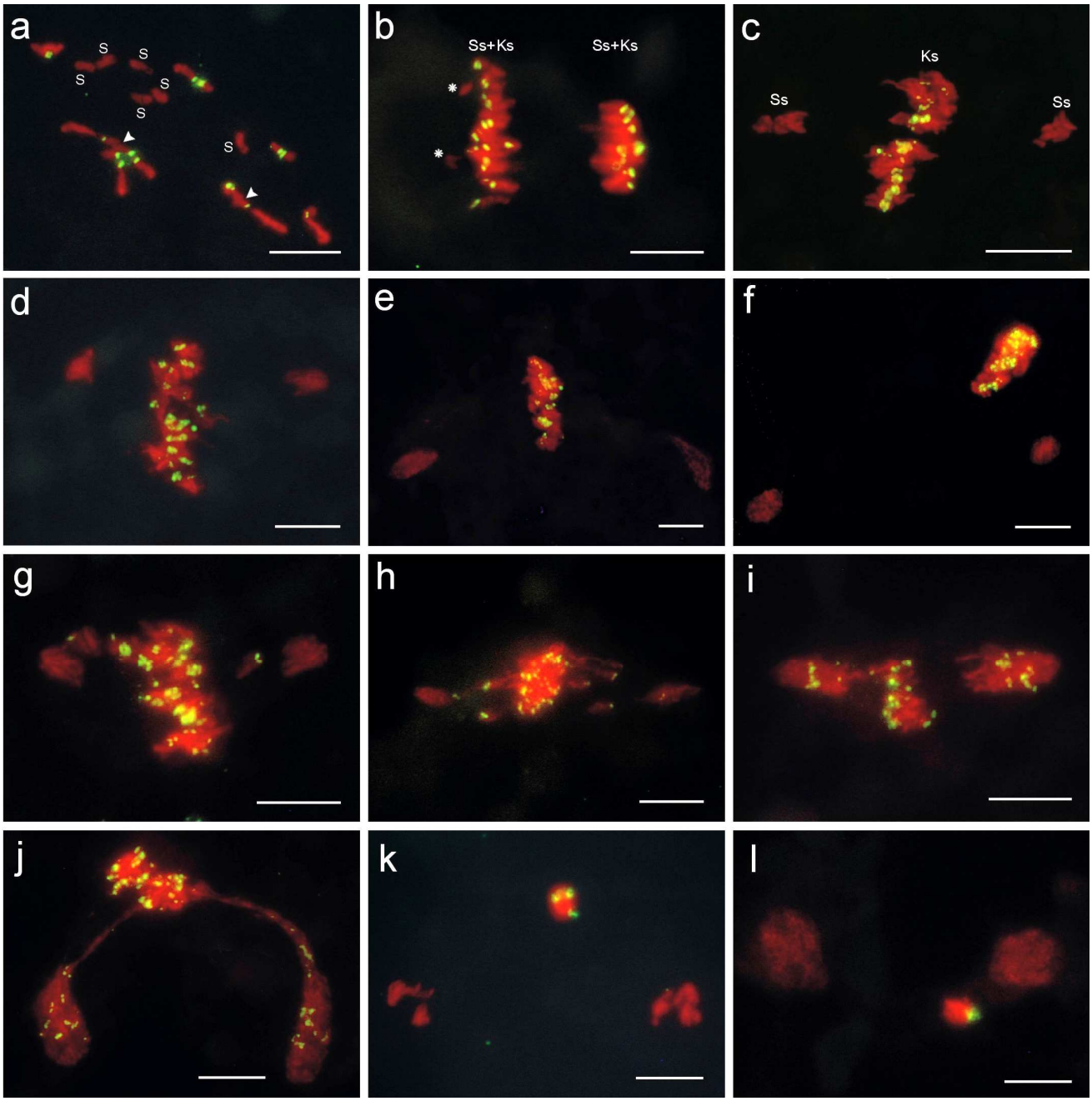

Fig. 2. (**a**-**l**) FISH with probes of germ line specific repetitive AlKeRe sequences on different stages of early embryonic mitoses in early *Acricotopus lucidus* embryos. The fluorescein-labeled probes hybridized specifically in the centromeric heterochromatin of the Ks. Chromosomes are counterstained with DAPI; its blue fluorescence was pseudocolored red. (**a**) Metaphase with the 6 unlabeled Ss and nine Ks showing clear AlKeRe signals on their centromeric regions. Arrowheads mark two pairs of Ks lying directly adjacent to each other. (**b**) Normal, pre-elimination anaphase with regularly segregating S and K chromatids. Two unlabeled Ss (asterisks) move ahead of the other chromosomes. (**c** and **d**) Telophases of elimination mitoses. All Ks (i.e. all labeled chromosomes) remain in the equatorial plane, while the separated Ss move to the opposite poles. (**e**) Late telophase of an elimination mitosis. The Ks are still arranged along the equator, while the Ss begin to decondense. (**f**) Result of an elimination mitosis: 2 somatic nuclei (in interphase) each containing only sets of Ss (2n = 6). Bright AlKeRe signals indicate the eliminated Ks that are condensed in a chromatin block. (**g**-**i**) Anaphases of incomplete elimination mitoses. The sister chromatids of one (**g**) or some (**h** and **i**) Ks have separated and move poleward behind the Ss chromatids. (**j**) Telophase with an equatorial mass of condensed chromatin formed by the eliminated Ks and two daughter nuclei each containing a considerable number of non-eliminated Ks (see signals). The escaped Ks will be eliminated in one of the subsequent divisions forming smaller-sized labeled chromatin droplets between the somatic nuclei (**k** and **l**). Bars represents 10 µm.

Clear hybridization signals in the pole-moving groups of chromosomes and in the equatorial arrested chromosome groups in anaphase (Fig. 2i) and in telophase (Fig. 2j) clearly demonstate that one portion of the Ks is eliminated while the other escapes elimination. Small labeled chromatin droplets observed in later embryonic stages between the somatic nuclei, as shown in Figs. 2k and 2l, indicate that those Ks, which escaped at first, will be eliminated in one of the subsequent divisions. Interestingly, normal mitoses with equally segregating Ss and Ks, as well as complete and incomplete elimination mitoses can occur side by side in the same embryo.

The observed lagging of the Ks in the ultimate mitosis just ahead of the K-eliminating division appears to suggest that a chromosome-discriminating factor has to accumulate. K elements become actually excluded from daughter nuclei when the unknown factor exceeds a threshold. The finding of incomplete K elimination indicates that it is not a rigid all-or-none mechanism; this rather supports a threshold hypothesis. Bridging and lagging in mitoses before their elimination were also reported for the germ line limited (L) chromosomes in *Sciara* (De Saint Phalle and Sullivan 1996). The observations in *Acricotopus* that some nuclei enter into elimination mitosis, while others had already eliminated their Ks in a previous mitosis (Fig. 1i), strongly indicate, that prospective somatic nuclei initiate the elimination of their Ks autonomously in early syncytial embryonic divisions. Analysing the process of elimination of X chromosomes in *Sciara,* De Saint Phalle and Sullivan (1996) concluded, that the decision to eliminate X chromosomes falls at the level of the individual nucleus. They suggested that incomplete sister chromatid separation is the mechanism of X elimination, which is also effective in the elimination of the L chromosomes in *Sciara*.

Experiments to ascertain a functional role of the germ-line limited chromosomes were carried out on embryos of the cecidomyiids *Wachtliella persicariae* (Geyer-Duszynsca 1966) and *Mayetiola destructor* (Bantock 1970). Embryos with pole cells, the primordial germ cells, containing only Ss but no Ks were produced. Such embryos developed into normal, but sterile adults. These results strongly suggested that the Ks are indispensable for normal gametogenesis.

For a correct alignment of the Ks in the spindle equator during prometaphase of elimination mitosis it is necessary that the kinetochores of the K sister chromatids be attached to microtubules extending in opposite directions (Zhou et al. 2002). Immunostaining of spindle microtubules in elimination mitosis of *Acricotopus* demonstrated that Ks remaining behind in the equatorial plane are also connected to microtubules (Staiber 2000). This was confirmed in this study. During the early and mid anaphases of elimination mitosis at least some of the kinetochores of the remaining K sister chromatids were clearly under tension, as seen in Fig. 1m, and therefore attached to the spindle. Extensive formation of anaphase bridges between segregating Ks resulting from apparent stickiness or persisting cohesion at the ends of their arms in the division preceding their loss (Figs. 1f and 1g) and observations of linked K sister chromatids in elimination mitoses (Fig. 1m) suggests that elimination of Ks in *Acricotopus* may be caused by non-separation of sites in distal parts of the chromosome arms.

Different mechanisms could be responsible for an incomplete separation of the K sister chromatids. The failure of sister chromatid separation may result from the activity of chromatin-modifying enzymes. Chromatin modifications can affect local chromatin structure (Suja et al. 1999; Cimini et al. 2003). Such modifications selectively present on sites of the chromosome arms of the Ks may lead to unresolved catenations holding sister chromatids together. For the decatenation of sister chromatids, topoisomerase II activity is required (Shamu and Muray 1992; Coelho et al. 2003). The difficulties in separating chromosome arms of the Ks during anaphases in the last divisions just before elimination mitosis may be due to residual topological links between sister chromatids that have not been resolved properly by topoisomerase II. It is possible that a checkpoint mechanism may selectively prevent the release of the last catenations linking the K sister chromatids in early anaphase of elimination mitosis. Specific proteins may also mask specific chromosomal sites so that topoisomerase II cannot resolve these catenations. It would be of interest to search for molecular and biochemical differences between chromosomal regions of K sister chromatids that have separated versus those that remain connected in elimination mitosis to elucidate the molecular basis of this case of programmed non-segregation of sister chromatids.

Acknowledgements

The author gratefully acknowledges the support of Professor Anette Preiss. Also, thanks to the referees for their helpful suggestions.

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