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RESEARCH ARTICLE

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The germ-line-restricted chromosome in the zebra finch: recombination in females and elimination in males

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Abstract In the zebra finch (*Taeniopygia guttata*), there is a germ-line-restricted chromosome regularly present in males and females. A reexamination of male and female meiosis in the zebra finch showed that this element forms a euchromatic bivalent in oocytes, but it is always a single, heterochromatic element in spermatocytes. Immunostaining with anti-MLH1 showed that the bivalent in oocytes has two or three foci with a localized pattern, indicating the regular occurrence of recombination. In male meiosis, the single restricted chromosome forms an axis that contains the cohesin subunit SMC3, and the associated chromatin is densely packed until late pachytene. Electron microscopy of thin-sectioned seminiferous tubules shows that the restricted chromosome is eliminated in postmeiotic stages in the form of packed chromatin inside a micronucleus, visible in the cytoplasm of young spermatids. The selective condensation of the restricted chromosome during early meiotic prophase in males is interpreted as a strategy to avoid the triggering of asynaptic checkpoints, but this condensation is reversed prior to the final condensation that leads to its (ulterior) elimination. Recombination during female meiosis may prevent the genetic attrition of the restricted chromosome and, along with the elimination in male germ cells, ensures its regular transmission through females.

Introduction

Aberrations of typical chromosome behavior during cell division in the early embryo can result in different constitutions of the somatic and the germ lines, with some chromosomes being represented exclusively in the germ cells. In spite of their oddity, when these variations are thoroughly examined, they show elaborate control mechanisms that ensure the regular inheritance of the genetic material. Furthermore, they provide alternative experimental systems to study the basis of chromosome behavior in mitosis and meiosis (Pimpinelli and Goday 1989; Kloc and Zagrodzinska 2001).

A unique behavior has been described for a germ-line-restricted chromosome in the passeriform bird *Taeniopygia guttata* (Pigozzi and Solari 1998). This chromosome resembles B chromosomes (Jones and Rees 1982) because it is present in addition to the regular chromosome complement observed in somatic cells of the species, but it is not a typical B chromosome because it is restricted to the germ line and it seems functional, considering its regular presence in every meiocyte examined so far. In the light of our new findings about the meiotic behavior and inheritance of the chromosome, the term germ-line-restricted chromosome (GRC; Hennig 1986) is used throughout this paper to differentiate it from B chromosomes that are sometimes referred to as accessory chromosomes (Jones and Rees 1982).

The constant presence of a single GRC was described in oocytes and spermatocytes of zebra finches from domestic local populations (Pigozzi and Solari 1998). This finding agrees with previous unpublished observations in spermatocytes of Australian zebra finches (C. Gillies, personal communication). Remarkably, the GRC is the largest of the chromosomal complement, a surprising fact considering the trends toward reduced DNA contents among birds when compared to other vertebrates (Tiersch and Wachtel 1991). The large size of this chromosome allows its unambiguous identification in acid-fixed spermatogonial metaphases and in synaptonemal complex (SC) spreads from both male and female meiocytes. Also because of its large size, its absence is readily demonstrated in the karyotype of somatic cells, even though the zebra finch has a high number of chromosomes—as usually found among birds. Extensive cytological analysis of male meiosis using light and electron microscopy showed that a single GRC is pre-

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sent in spermatogonia that it assumes a condensed, presumably inactive, state during early meiosis and that it is eliminated from the nucleus in the form of a condensed round body of chromatin associated to nuclei of secondary spermatocytes (Pigozzi and Solari 1998). On the other hand, the GRC is absent in cells from the bone marrow (Pigozzi and Solari 1998) and skin and liver fibroblasts (Itoh and Arnold 2005).

In the course of additional meiotic analyses in zebra finch oocytes, we uncovered the existence of females that bear two GRCs instead of one. In these females, the GRCs synapse and form a euchromatic bivalent that recombines regularly during the first meiotic prophase. Moreover, the presence of the GRC as a bivalent seems to be the most frequent situation among females, and its finding as a univalent seems to be the exception among females from the sources available in this laboratory. On the other hand, analysis of meiosis and spermatogenesis in a larger number of males established the presence of a single GRC in spermatocytes and its regular elimination from male germ cells. The behavior of the univalent during meiotic prophase is described in immunostained nuclei from males and females, and the elimination from the male germ line is shown with electron microscopy of the seminiferous tubules. The unorthodox, yet regular, mechanisms involved to maintain the regular inheritance of this chromosome suggest that it is necessary for oogenesis and that its transcription is dispensable during spermatogenesis.

Materials and methods

A total of 20 birds that came from three unrelated commercial breeders were examined during the course of different projects separated over time. Nine newly hatched females were used for light and electron microscopy preparations in both the previous (Pigozzi and Solari 1998) and the present report. The ovaries from two females were subject to acid fixation and Giemsa staining to assess the euchromatic nature of the GRC during female meiosis in our first study. In this type of preparations, individual bivalents cannot be distinguished in pachytene nuclei; therefore, it was not possible to establish if the GRC was present as univalent or bivalent. In the other seven females, SC spreads were made from each ovary and used for electron microscopy or immunostaining. Spermatocytes were analyzed from 11 adult males in addition to the three used in the precedent study.

Meiotic spreads for electron microscopy

In females, meiosis was studied in 4- to 5-day-old hatchlings when the pachytene peak occurs in this species. The left ovary was excised, placed in Hanks' solution, and used for SC spreads for light and electron microscopy, as previously described (Solari 1998; Pigozzi 2001). Spreads on plastic-covered slides were stained either with silver nitrate or ethanolic phosphotungstic acid (PTA) to observe SCs and recombination nodules. Well-spread nuclei were photographed in a Siemens Elmikosp I electron microscope at magnifications ranging from 1,000× to 5,000×.

Immunostaining

Meiotic spreads were immunostained with antibodies against the SC protein 3 (SCP3) and the cohesin subunit SMC3 that label the lateral elements, CREST serum that labels the centromeres, and an anti-MLH1 antibody (Pharmingen) that recognizes a protein present at the sites of crossovers in a variety of organisms (Baker et al. 1996). Secondary antibodies were goat antihuman Texas Red, goat anti-mouse fluorescein isothiocyanate (FITC), and goat anti-rabbit Texas Red (Jackson ImmunoResearch). The secondary antibodies were diluted 1:100 in PBT [phosphate-buffered saline (PBS), 3% bovine serum albumin (BSA), and 0.05% Triton X-100]. Nuclei were counterstained with 4-6-diamidino-2-phenylindole·2HCl (DAPI) and mounted in glycerol containing antifade. Spreads were examined with a Leica fluorescence microscope and photographed with Kodak color film using suitable filters for each fluorochrome. Two exposures were done successively on the same frame: one using the FITC filter set to capture MLH1 foci and the second with the Texas Red set to capture SCs and centromeres. Alternatively, pictures were taken separately, and their scanned images were overlapped using Adobe Photoshop. No differences were observed between both methods regarding the number of MLH1 foci.

Results

The restricted bivalent during female meiosis

In six out of seven females examined with SC spreads, two homologous GRCs were found forming a typical SC with terminal kinetochores (Fig. 1a,b). The SC of the GRC bivalent is the largest of the SC set, measuring on average 22.3 μm. The SC of the GRC was identified in a total of 48 electron micrographs of pachytene oocytes, and its presence was observed in over 100 oocytes examined with electron microscopy or with light microscopy in immunostained spreads. In most oocytes, the autosomal macro- and microbivalents and the sex chromosomes synapse regularly (Fig. 1a), but about one third of the oocytes showed partial desynapsis or delayed synapsis of some autosomal SCs in pachytene nuclei. The synaptic anomalies affected mainly macrobivalents and rarely involved the shortest microbivalents. In contrast with irregular synapsis of some autosomal SCs, the homologous GRCs synapse regularly along their entire length during pachytene.

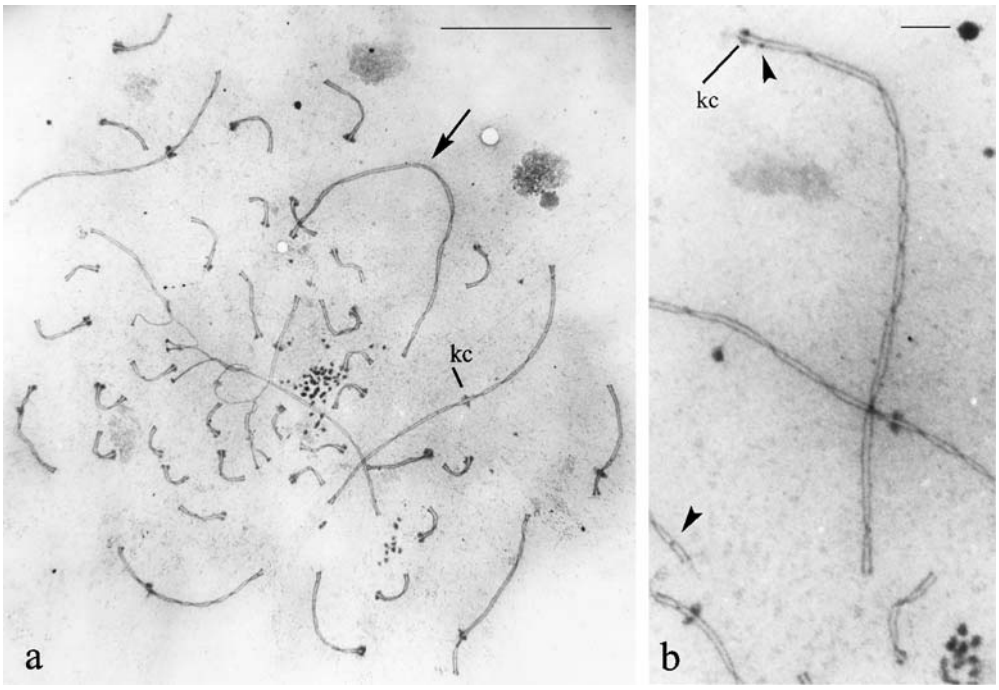

Fig. 1 Electron micrographs of pachytene oocytes stained with PTA. **a** Complete SC set showing the 39 autosomal SCs and the SC of the restricted bivalent (*arrow*). The largest autosomal SC has a median kinetochore (*kc*), and it is slightly shorter than the SC of the GRC. The Z and W axes are close to each other but do not form an SC. The Z axis is interlocked with an autosomal SC and hooked with the W axis. *Bar*, 10 μm. **b** Micrograph at larger magnification showing the SC of the GRC and its near terminal kinetochore (*kc*). *Arrowheads* point at recombination nodules on the GRC and an autosomal SC. *Bar*, 1 μm

Crossover pattern of the GRC

In the zebra finch, recombination nodules are visible as small electrondense dots in PTA-stained nuclei, lying at the central space of the SC (Fig. 1b). Since the analysis of MLH1 foci on immunostained spreads is considerably easier compared to that of recombination nodules in electron micrographs, the former method was preferred to determine the number and distribution of crossovers in the GRC.

Immunostaining of pachytene nuclei showed 39 autosomal bivalents, the ZW pair and the large SC of the GRC that has a terminal CREST signal corresponding to the kinetochore (Fig. 2a). In most oocytes, the GRC bivalent showed two MLH1 foci, but three foci were observed in 20% of the nuclei (Fig. 2b). When two foci are present, they lie invariably on opposite ends of the bivalent: one close to the centromere and the other next to the distal telomeres. In bivalents with three foci, two foci were always closer to each other and the third one lied at the opposite end. The pair of closer foci was commonly located near the centromere (Fig. 2b), but, in a few bivalents, the pair was near the distal end (not shown). In a total of 41 bivalents analyzed, no foci were observed in the midregion, spanning about 45% of the SC length. The histogram in Fig. 3 summarizes the frequency distribution of foci in GRC bivalents with two or three foci. The location of each individual focus was expressed as its relative distance to the kinetochore to compensate for variations in the absolute length of the bivalent in different nuclei.

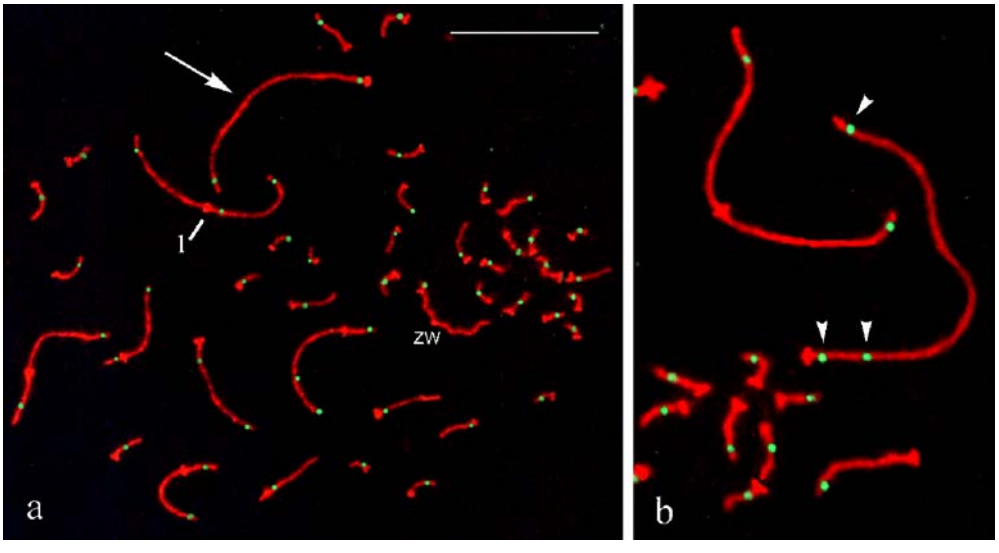

Fig. 2 Immunostaining in pachytene oocytes. **a** Complete SC set immunostained with anti-SCP3 antibody and CREST serum (*red*) and anti-MLH1 antibody (*green*). The *arrow* points at the GRC and the number (*1*) indicates the largest autosomal bivalent with its medially located kinetochore. The ZW pair has a single MLH1 focus at one end, and the lengths of their axes are equalized. *Bar*, 10 μm. **b** Partial view of another oocyte showing a GRC bivalent with three MLH1 foci (*arrowheads*)

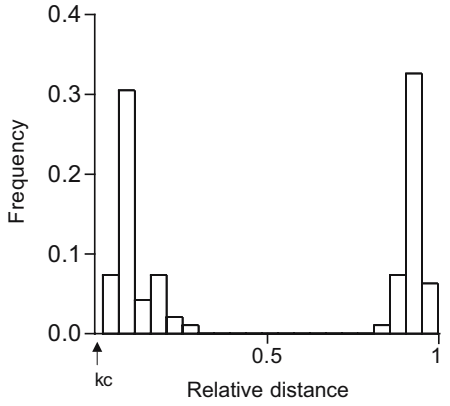

Fig. 3 Frequency distribution of MLH1 foci on the SC of the GRC. Foci show localization on the proximal and distal ends. The distance of each focus to the kinetochore (*kc*) was expressed as a percentage of the total SC length of GRC. Data from 41 GRC bivalents

The univalent GRC in male meiosis

In the present study, the single GRC was observed in pachytene spermatocytes from eight adult males, in agreement with observations from three males analyzed in our first report. As previously shown, the single GRC forms an axial core that stains with silver and has a densely-packed chromatin (Pigozzi and Solari 1998). This chromatin condensation of the GRC is noticeable since leptotene in acid-fixed spermatocytes is stained with Giemsa (not shown). In microspread spermatocytes, the long single axis of the GRC and the associated chromatin can be singled out in midlate zygotene using an antibody against SMC3 and DAPI as counterstain (Fig. 4a–c). During early prophase, the single axis of the restricted chromosome is folded over itself, and it is more faintly stained compared to the nonsynapsed autosomal lateral elements still visible during late zygotene. During pachytene, the GRC axis is visibly shorter and the chromatin maintains its differential packing, but this axis is no longer distinguishable in diplotene nuclei (Fig. 4d–i). We failed to detect the GRC axis in spermatocytes using two different anti-SCP3 antibodies raised against the mouse protein (Sycp3), possibly because of a lesser affinity of the antibodies against the avian homologous SCP3p (not shown).

Elimination of the GRC from the male germ cells

In thin sections of seminiferous tubules, the cytoplasm of some young spermatids has a round dense body that

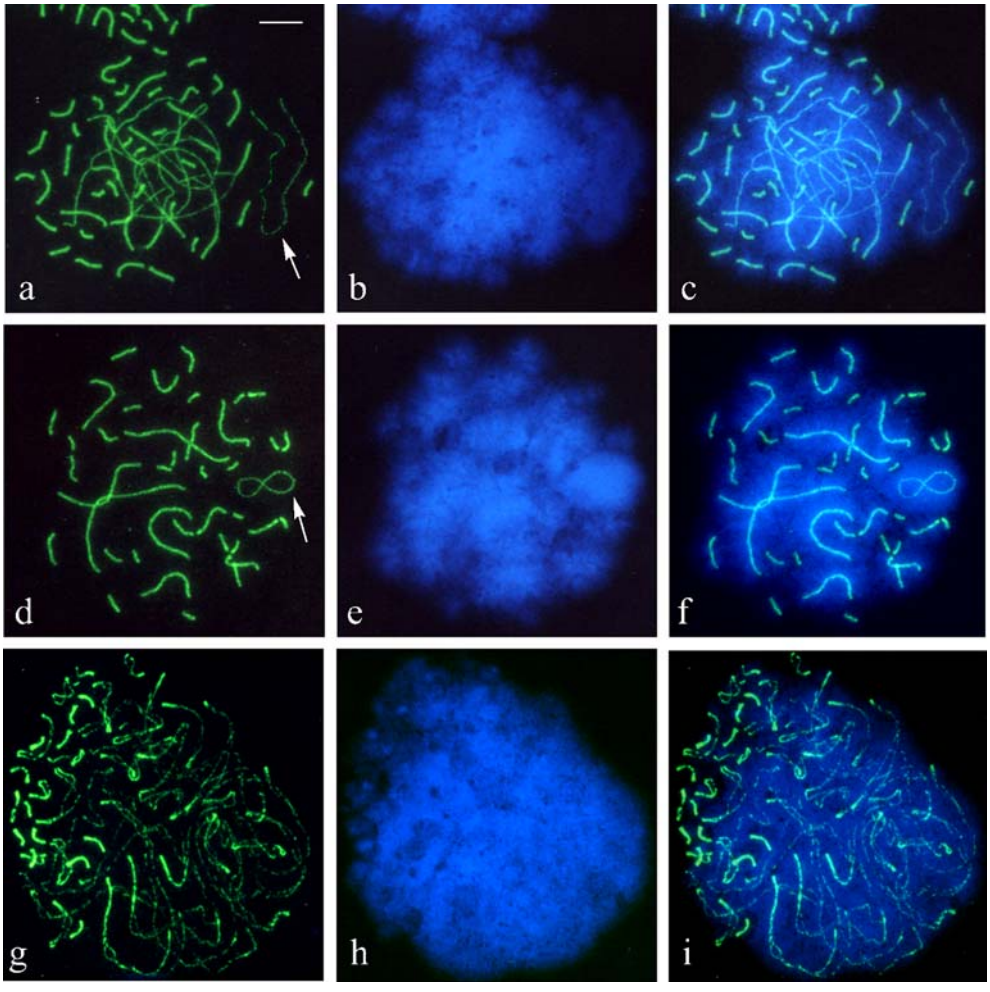

Fig. 4 Spermatocytes during early meiotic prophase immunolabeled with SMC3 (*green*). Chromatin is counterstained with DAPI. **a**–**c** During late zygotene, the axis of the GRC is more faintly stained than single autosomal axes and SCs (*arrow*). The restricted chromosome forms a peripheral body, with its chromatin differentially packed. **d**–**f** During pachytene, the GRC reaches a maximum condensation, and its axis is noticeably shorter. **g**–**i** In diplotene, the GRC is no longer distinguishable as a compact chromatin body. *Bar*, 10 μm

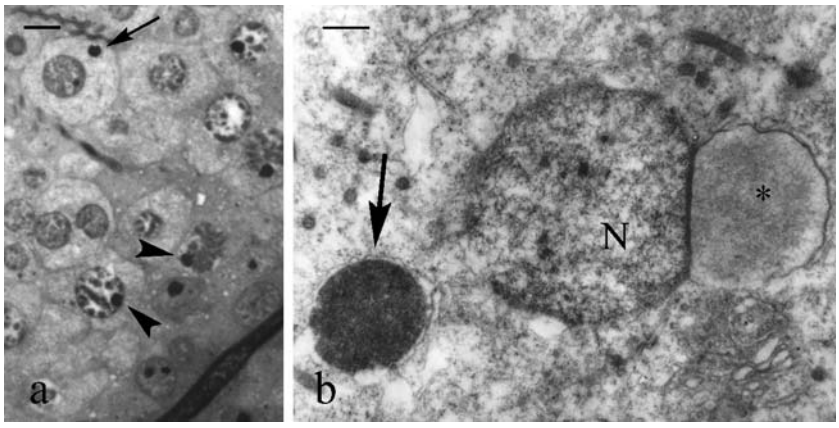

Fig. 5 The heterochromatic GRC in male meiosis. **a** Light micrograph of a seminiferous tubule in a thick section stained with toluidine blue. The *arrow* points to the round body of chromatin (GRC) in the cytoplasm of a young spermatid. Spermatocytes at early meiosis show the condensed chromatin of the GRC attached to the inner side of the nuclear envelope (*arrowheads*). *Bar*, 10 μm. **b** Electron micrograph of a developing spermatid showing the micronucleus (*arrow*) in the cytoplasm of this young spermatid. *N* Spermatid nucleus, * acrosome. *Bar*, 1 μm

has similar size and staining affinity than the condensed restricted chromosome associated to the nuclear membrane in spermatocytes during the first meiotic prophase (Fig. 5a). This round body of chromatin body is different from the chromatoid body observed in the cytoplasm of spermatocytes and spermatids in several species, because the round body observed in zebra finch spermatids is DAPI positive (Pigozzi and Solari 1998) and has a later timing of appearance. Observations of young spermatids at the electron microscope showed that the dense body is a micronucleus surrounded by a double membrane and revealed the fibrilar constitution of the packed material and its width similar to chromatin fibers from other nuclei (Fig. 5a). In thick sections (Fig. 5a), the presence of the packed chromatin body cannot be ascertained in most spermatids because of its random location as regards to the main nucleus. However, the size, shape, and staining affinity of the round dense body in sections agree with the previously described round, DAPI positive body that is present in about 40% of secondary spermatocytes and young spermatids in whole mounts (Pigozzi and Solari 1998).

Discussion

Regular meiotic behavior of the GRC in oocytes

Most of the previously reported instances of germ-line-restricted chromatin refers to C-banding positive, heterochromatic chromosomes, or highly repetitive sequences located in *C*+ regions, for instance, in seven species of hagfish of the Baltic Sea and the Pacific Ocean (Nakai et al. 1995; Kubota et al. 2001) and in the chironomid fly *Acricotopus lucidus* (Staiber et al. 1997). In *Acricotopus*, further studies showed that the eliminated chromosomes have distal segments homologous to regions of regular chromosomes, and this fact suggests that these segments determine the regular occurrence of crossover events and the correct segregation of the restricted chromosomes during meiosis (Staiber and Schiffkowski 2000).

In the zebra finch, the restricted chromosome is eliminated from the male germ line, and thus, the transmission of the chromosome depends on its regular behavior during female meiosis. This is achieved through synapsis and recombination as shown by the regular formation of an SC and the presence of MLH1 foci. MLH1 foci occur at the sites of crossover, as shown by their correspondence with the pattern of chiasmata or genetically mapped crossovers in several vertebrates, including birds (Barlow and Hulten 1998; Anderson et al. 1999; Pigozzi 2001). The recombination events on the GRC will result in the formation of chiasmata (generally on opposite ends of the bivalent) that will ensure proper chromosome orientation and disjunction at metaphase I (Nicklas 1997). The localized pattern of MLH1 foci suggests that an extended interstitial region of the GRC lacks the needed requirements for recombination initiation. Theoretically, the occurrence of regular meiotic recombination may have evolutionary implications. It is widely accepted that the lack of recombination has detrimental effects on the chromosomes or chromosome segments having recombination restriction (Felsenstein 1974; Haig 1978; Barton and Charlesworth 1998). Chromosome segments devoid of regular recombination undergo processes of gene attrition and enrichment of repeated sequences through several mechanisms, including Muller's ratchet (Haig 1978) and the accumulation of harmful mutations (Rice and Chippindale 2001). Thus, in principle, the euchromatic GRC element is protected in the female sex of zebra finches and regular recombination may help in the permanence of this element.

On the other hand, considering that in female meiosis, the GRC recombines with an assumed copy of itself, it might be concluded that the duplication of the restricted chromosome and its recombining activity are features selected to provide a stable behavior during female meiosis and maternal inheritance and not to gain genetic variation.

Synaptic irregularities in regular autosomes

Compared to the regular synapsis of the restricted bivalent, synaptic abnormalities were observed among macrobivalents in a significant number of oocytes. In other domestic and wild species of birds analyzed in our laboratory with similar methods, the autosomal set synapses in a regular fashion, with the microbivalents forming complete SCs earlier in zygotene and the largest autosomes completing SC formation later (Solari 1977; A.J. Solari, unpublished data). Thus, the irregular synaptic behavior observed in zebra finch oocytes can be ascribed solely to the presence of the euchromatic GRC. The precise factor, other than the presence of the GRC that alters the synapsis of autosomal bivalents in some oocytes, is not known. A direct interaction between the accessory axis and the desynapsed bivalents was not observed, although the spreading procedure may disrupt such an association. Alternatively, the synapsis of autosomal bivalents may be susceptible to a presumed transcriptional activity of the euchromatic GRC. In spermatocytes, the single GRC does not trigger any synaptic disturbance, probably because it is densely packed forming a heterochromatic body since early meiotic prophase (Pigozzi and Solari 1998). This heterochromatic state might prevent its recognition by checkpoint proteins that monitor the completion of synapsis during early meiotic prophase I (Roeder and Bailis 2000; Tarsounas and Moens 2001). The fate of the oocytes with synaptic irregularities cannot be determined with certainty, but they do not seem to compromise the fertility of the females.

Possible mechanisms of transmission and restriction to the germ line

There are a few well-known instances in which some chromosomes are differentially sorted between somatic and germ cells. Most of the cases of germ/soma differences are observed in invertebrates, with fewer cases among plants and vertebrates (Goday and Esteban 2001; reviewed in White 1973). Among mammals, the creeping vole *Microtus oregoni* has XY somatic cells and Y0 germ cells in male and X0 somatic cells and XX germ cells in females (Ohno 1967); and some marsupial species eliminate one X chromosome in females and the Y chromosome in males from selected somatic tissues (Hayman and Martin 1974). In the zebra finch, the absence of the GRC has been so far demonstrated in several somatic tissues, such as bone marrow (Takagi 1972; Pigozzi and Solari 1998; Christidis 1986), liver, and muscle (Itoh and Arnold 2005), both in male and female individuals. These evidences strongly suggest that the GRC is restricted to the germ cells at the moment of their differentiation in early embryogenesis. Furthermore, to accomplish the final result of one GRC in males vs two in females, the chromosome should have a sex-dependent behavior during the mitotic divisions immediately previous to germ line development during early embryogenesis.

In the absence of data on the chromosome behavior during early development, which are hard to obtain due to the large amount of yolk compared to the embryo proper, the soma/germ line differentiation is necessarily speculative. In human and marsupial cells in culture and released from mitotic arrest, lagging chromosomes during anaphase may lead to the formation of a monosomic daughter cell and a trisomic one as well as the loss of an extra chromosome in a micronucleus (Catalan et al. 1998; Cimini et al. 2002). In zebra finches, the lagging of the GRC during second meiotic division (Pigozzi and Solari 1998) suggests that this element is also able to undergo anaphase lagging in mitotic divisions of the early embryo. If this hypothesis is true, the germ line/soma differentiation in females is associated with nondisjunction of the whole GRC, giving germ cells with two GRCs, while the soma/germ line differentiation in males is associated to the lagging of single chromatids, and the result of a germ line with a single GRC. Hypothetically, a differential, sex-linked transcription of cohesin-related genes may be involved in this behavior.

Another sex-specific feature of the restricted chromosome is its postmeiotic elimination exclusive of the male germ line. The fact that the chromosome is heterochromatic in spermatocytes since early meiotic prophase indicates that it is dispensable for male spermatogenesis and its early inactivation might be a way to prevent the meiotic abnormalities caused by a single unpaired element. Its apparently degenerate, pycnotic state in secondary spermatocytes (Pigozzi and Solari 1998) and spermatids (see "Results") seems to be independent from the chromatin compaction during the first meiotic prophase. This idea is supported by the absence of a compacted GRC chromatin since diplotene and also by the isopycnotic staining of the restricted univalent during metaphase I compared to autosomal bivalents (Pigozzi and Solari 1998). The euchromatic state of the male GRC after early prophase also agrees with its regular segregation to one pole during the first meiotic anaphase, keeping together its sister chromatids due to the presence of cohesins (see "Results"). Sister-chromatid cohesion is essential for proper chromosome disjunction during mitosis and meiosis (Nasmyth 2001), and, in meiotic chromosomes, the heterodimer SMC1/SMC3 is part of a structural backbone for each chromosome, along with the meiosis-specific cohesin REC8 and the axial element components (Jessberger 2002; Eijpe et al. 2003). Part of this cohesin complex persists at centromeric regions during anaphase I with the net result of the reductional division of homologous chromosomes. In male zebra finches, the round bodies of chromatin representing the pycnotic GRC are associated to near half of the spermatocytes in metaphase II (Pigozzi and Solari 1998), as predicted from a reductional division of the GRC during first meiosis. Altogether, these evidences support the idea that two types of spermatids are formed—with and without the restricted chromosome—and that in the end the whole GRC is eliminated from spermatids in the form of the round dense body, and therefore, spermatozoa do not carry the restricted element.

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