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Heterologous Synapsis and Crossover Suppression in Heterozygotes for a Pericentric Inversion in the Zebra Finch

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Chromosome inversions · Crossing over · Meiosis · Synapsis · Zebra finch

Abstract
In the zebra finch, 2 alternative morphs regarding centromere position were described for chromosome 6. This polymorphism was interpreted to be the result of a pericentric inversion, but other causes of the centromere repositioning were not ruled out. We used immunofluorescence localization to examine the distribution of MLH1 foci on synaptonemal complexes to test the prediction that pericentric inversions cause synaptic irregularities and/or crossover suppression in heterozygotes. We found complete suppression of crossing over in the region involved in the rearrangement in male and female heterozygotes. In contrast, the same region shows high levels of crossing over in homozygotes for the acrocentric form of this chromosome. No inversion loops or synaptic irregularities were detected along bivalent 6 in heterozygotes suggesting that heterologous pairing is achieved during zygotene or early pachytene. Altogether these findings strongly indicate that the polymorphic chromosome 6 originated by a pericentric inversion. Since inversions are common rearrangements in karyotypic evolution in birds, it seems likely that early heterologous pairing could help to fix these rearrangements, preventing crossing overs in heterozygotes and their deleterious effects on fertility.

The zebra finch *(Taeniopygia guttata)* is a model organism in behavior, endocrinology and neurobiology and is the second bird to have its genome sequenced [Warren et al., 2010]. In this species, the existence of a polymorphism for a putative pericentric inversion in autosome No. 6 was described in wild birds and also in a colony kept for research purposes [Christidis, 1986; Itoh and Arnold, 2005]. As a consequence of this rearrangement, this chromosome exists in 2 alternative morphs: one submetacentric and the other acrocentric. Current evidence, based on classic mitotic chromosome analyses, cannot exclude a different basis for these morphological variants, such as the formation of a neocentromere.

During the course of several meiotic studies in zebra finches, we observed misaligned centromeric signals along the 6th autosomal synaptonemal complex (SC) in immunostained pachytene cells from 3 individuals. This morphological feature is compatible with the presence of 1 acrocentric and 1 submetacentric homolog, similar

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to the morphological variant described in mitotic chromosome studies. A possible way to test if a chromosome segment was affected by an inversion is to analyze the pattern of synapsis and crossing over in SC spreads in heterozygotes. Extensive analyses of SC spreads in plants, mice and humans carrying inversions have shown that these rearrangements can lead to synaptic irregularities, loop formation and disruption of the normal crossover pattern [Ashley et al., 1981; Moses et al., 1982; Gabriel-Robez et al., 1986; Anderson et al., 1988; Koehler et al., 2004; Massip et al., 2010; Kirkpatrick et al., 2012].

In the present analysis, we compare the patterns of synapsis and crossing over in these 3 heterokaryotypic birds with those in homozygotes for the acrocentric chromosome 6. For this purpose, we used SC spreads immunostained for the meiotic axes and the mismatch-repair protein MLH1 which is a marker of the crossover sites along pachytene bivalents in birds and mammals [Barlow and Hultén, 1998; Anderson et al., 1999; Pigozzi, 2001]. Our results show that bivalent 6 always forms a straight SC in heterozygotes, without evidence of synaptic irregularities. In these individuals, no MLH1 foci were found in the chromosome segment between the misaligned centromeres supporting the idea that pairing at this region is non-homologous and, therefore, this condition is more likely a consequence of a pericentric inversion. Our results represent the first meiotic analysis of a chromosomal rearrangement in birds using molecular cytogenetic methods. We discuss the relevance of these data in connection with the growing evidence about the role of internal rearrangements during the course of avian karyotype evolution.

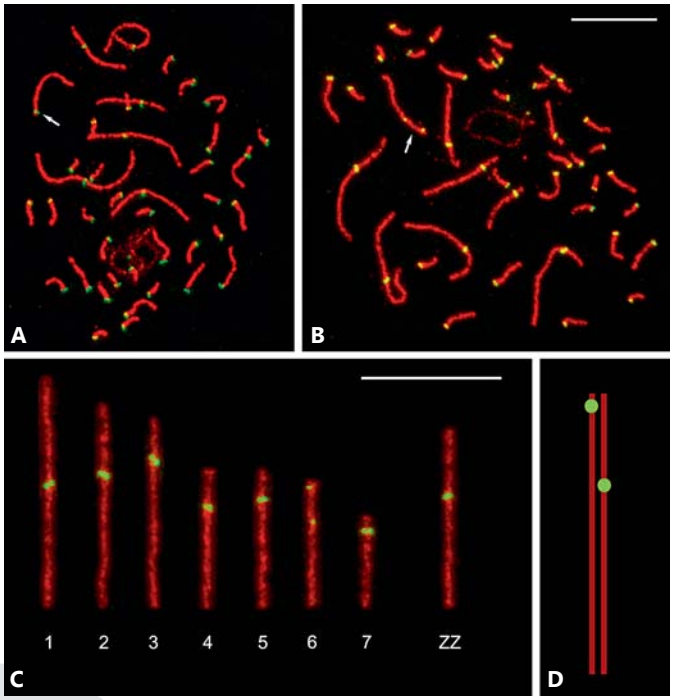

Fig. 1. Representative SC spreads from male zebra finches. SMC3 is stained in red, and centromeric proteins are in green. **A** Pachytene spread from an individual homozygous for the acrocentric form of chromosome 6. The arrow points to the autosomal SC 6 with a single centromeric signal close to one end. **B** Pachytene spread from an individual with misaligned centromeric signals along autosomal SC 6 (arrow). **C** The 7 largest autosomal SCs and the ZZ bivalent from the nucleus in **B** were digitally straightened to illustrate their differences in length. SC 6 has 2 clearly separated centromeric signals, each about half the size of those on other SCs. **D** Schematic representation of the rearranged bivalent with the centromeric signals depicted at their average positions according to averaged measurements. Bars = 10 μm.

Materials and Methods

Animals

All adult male zebra finches were purchased from the same local pet store; 4-day-old females were provided by a local breeder who keeps separated cages for our studies. Immunostaining of SCs and centromeres in 11 zebra finches revealed the presence of misaligned centromeres along the 6th autosomal SC in 3 individuals, while in the other birds the corresponding SC showed terminal centromeric signals. In 6 of these birds, the number of nuclei was sufficient to analyze SC lengths, centromeric indexes and MLH1 focus distributions.

Meiotic Spreads and Immunostaining

SC spreads were prepared for immunostaining employing previously described methods [Pigozzi and Solari, 2005; Goday and Pigozzi, 2010]. Slides were incubated with the following primary antibodies diluted in PBS containing 0.5% Tween 20: rabbit anti-SMC3 (Chemicon, Millipore) that recognizes a component of the cohesin axis during prophase I at a 1:3,000 dilution; mouse anti-MLH1 (BD Pharmingen) at a 1:30 dilution and a human anti-centromere serum at a 1:30 dilution (ImmunoConcepts). The incubations were carried out overnight at 37°C in a moist chamber. Then, the slides were rinsed for 3 × 5 min in PBS and incubated for 1 h with the appropriate secondary antibodies: FITC-conjugated goat anti-mouse IgG at a 1:100 dilution, TRITC-conjugated goat anti-rabbit IgG at a 1:100 dilution, Cy3-conjugated donkey anti-human at a 1:200 dilution or FITC-conjugated goat anti-human (all from Jackson ImmunoResearch). After rinsing the slides 3 × 5 min in PBS, they were stained with 2 μg/ml DAPI and mounted with a glycerol-based solution containing an antifade reagent. Separate images for each color were captured using a CCD digital camera (Olympus DP73) coupled to a Zeiss Axiophot microscope equipped with the appropriate filter sets. Images were captured separately for each color and merged with Adobe Photoshop.

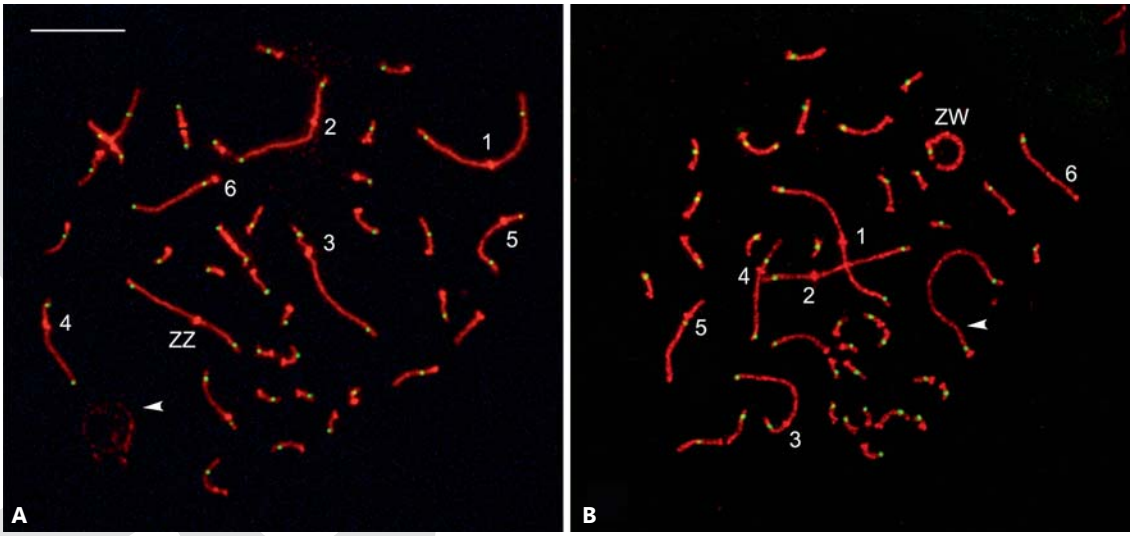

Fig. 2. Immunolocalization of meiotic recombination events in homozygotes and heterozygotes. SCs and centromeres are shown in red, MLH1 foci in green. The numbers close to the centromeric signals identify the largest autosomal SCs. The arrowheads in each image point to the germ-cell restricted chromosome. **A** A typical MLH1-focus distribution for this species is observed along the largest SCs: biarmed bivalents show 2 foci, located towards the ends of each arm; the acrocentric bivalent 6 shows 1 focus proximal to the centromere and another close to the opposite end. **B** Pachytene spread from a heterozygous female. The heterokaryotypic pair 6 forms a straight SC, with a single MLH1 focus close to the end of the long arm. Bar = ■■■.

Statistical Analysis

Only complete, well-spread SC sets with minimal overlapping were used for measurements. SC lengths, centromere positions and MLH1 foci were scored using the computer application MicroMeasure version 3.3 (http://www.colostate.edu/Depts/Biology/MicroMeasure). Statistical analysis was performed by nonparametric Mann-Whitney exact test using GraphPad Prism 6.01.

Results

Identification of Carrier Birds and Characterization of the Rearrangement

The zebra finch has a diploid chromosome number of 2n = 80 including 7 pairs of macrochromosomes, 32 pairs of microchromosomes and the sex chromosome pair. In addition to the regular chromosomal complement, a germ-cell restricted chromosome can be recognized as univalent in spermatocytes and as a bivalent in oocytes [Pigozzi and Solari, 2005]. After immunostaining of meiotic axes with anti-SMC3 and anti-centromere serum, it is possible to identify the SCs of the largest bivalents by their relative lengths and centromeric indexes (fig. 1A–C). In 8 birds, we observed an acrocentric bivalent 6 (fig. 1A), while in 3 individuals this bivalent had 2 centromeric signals at different distances from the end (fig. 1B, C). Figure 1D is a schematic illustration interpreting this variant pair 6 with the straight lines representing the lateral elements corresponding to 1 acrocentric and 1 submetacentric chromosome and their respective centromeric signals (green) at the average location according to SC measurements. Previous studies using mitotic spreads defined pair 6 as telocentric, because of the presence of a negligible short arm [Christidis, 1986; Itoh and Arnold, 2005]. We prefer to use the term acrocentric because a very short SC arm is observed with electron microscopy and SMC3 labeling [Pigozzi and Solari, 1998; Calderón and Pigozzi, 2006; this report]. We did not find birds homozygous for the submetacentric form of this chromosome; therefore, individuals with misaligned centromeres are hereafter called heterozygotes and those with an acrocentric bivalent 6 are called homozygotes. In 230 images of pachytene cells from heterozygotes, the autosomal SC 6 with non-aligned centromeres was always present as a straight bivalent, without evidence of loop formation or synaptic irregularities. Measurements of selected pachytene cells with complete sets and minimal SC overlapping showed that SC 6 represents about 5% of the autosomal set without significant differences between homozygotes (5.2 ± 0.04; n = 111) and heterozygotes (5.3 ± 0.04; n = 107) (p = 0.08). The average relative distance between

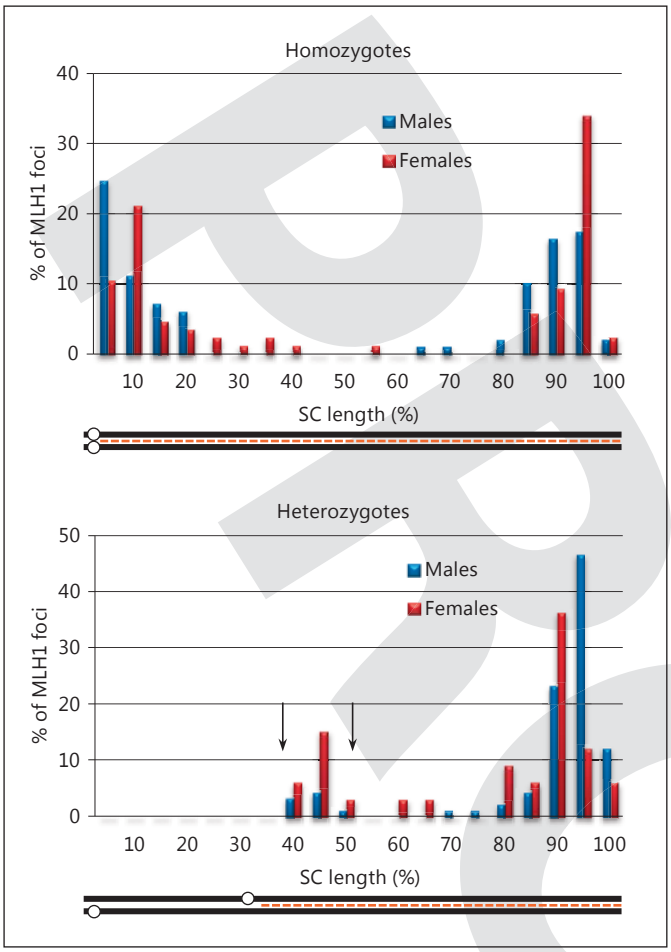

Fig. 3. Comparative distribution of recombination events along SC 6 in homozygotes and heterozygotes. In each graph, the X-axis represents the length of the SC, and the Y-axis shows the percentage of total MLH1 foci. The arrows mark a segment with higher focus frequency in heterozygotes compared to the same region in homozygotes. In this region, the first focus is at 38% from the centromere of the acrocentric element. The chromosome is divided into 10% intervals of SC length. Below each graph, there is a representation of the SC in homozygotes and heterozygotes showing the average position of the centromeres in each case. The dashed line indicates the probable extent of homologous synapsis.

centromeric signals is 31% of the total SC length with insignificant differences between male and female heterozygotes. If this centromere repositioning was caused by a pericentric inversion, then the distance between centromeric signals in heterozygotes can provide an estimate of the minimum size of the inverted segment. The average centromere positions along the acrocentric/submetacentric elements of the SC in heterozygotes (see fig. 1D) are consistent with one breakpoint located near the telomere of the short arm and the other ~31% from the former.

Number and Distribution of MLH1 Foci along Bivalent 6

We explored the distribution of crossovers in homozygous and heterozygous individuals using immunostaining of the MLH1 protein (fig. 2). Almost invariably, bivalent 6 carried 2 MLH1 foci in homozygotes with no statistical differences between males (1.9 ± 0.06) and females (1.9 ± 0.03); in contrast, the number of foci was drastically reduced in male (1.1 ± 0.03) and female (1.3 ± 0.09) heterozygotes. In heterozygotes, most bivalents (85%) had 1 focus; when 2 foci were present, they were more commonly observed in the female. Focus frequency distributions indicate that crossovers occur more frequently towards opposite ends of the bivalent in homozygotes, with 1 focus at the proximal region and another close to the telomere of the long arm (fig. 3). In heterozygotes, the preferred location of the distal crossover is maintained, but there is an increment of foci in the middle of the SC compared to the same region in homozygotes (fig. 3). In the heterozygotes, no foci were observed in the region between 0 to 38% measured from the centromere of the acrocentric element; therefore, it is possible that one of the inversion breakpoints is located close to the distal point of this segment.

Discussion

Suppression of Crossing Over in Heterozygotes Is Consistent with the Presence of a Pericentric Inversion

We show that in zebra finches heterozygous for a centromeric repositioning on chromosome 6, crossover is completely suppressed in the region involved in the rearrangement. This evidence favors the idea that the nonrecombining region is synapsed heterologously as a consequence of a pericentric inversion in one of the homologs. SC studies of inversion heterozygotes in a variety of organisms showed that strict homologous pairing results in the formation of inversion loops [Chandley, 1982; Moses et al., 1982; Batanian and Hultén, 1987; Anderson et al., 1988; Koehler et al., 2004; Torgasheva and Borodin, 2010]. However, inversion loops are not always formed, and in several cases a straight bivalent is present because heterologous synapsis takes place at the inverted segment even at early stages of the meiotic prophase [Ashley et al., 1981; Greenbaum and Reed, 1984; Hale, 1986; Ashley, 1988; Gabriel-Robez et al., 1988]. In the present analysis, inversion loops or asynapsis were not observed suggesting that the linear bivalents in heterozygotes are the product of direct heterosynapsis at late zygotene/early pachy-

Table 1. Pericentric inversions in birds analyzed for synapsis and/or crossing over

Species	Chromosome/ linkage group	Inversion size, %[a]	SC analysis	Crossover analysis	Reference
Z. albicollis (ZAL)	ZAL2	~80	no	metaphase I and genotyping chiasma/crossover suppression	Throneycroft, 1975; Thomas et al., 2008; Huynh et al., 2011
J. hyemalis (JHY)	2/5	NA	no	chiasma suppression	Shields, 1976
G. gallus domesticus (GGA)	GGA1	~75	no	metaphase I: inversion loop (>90% of cells) 1 chiasma in the inverted segment	Bitgood et al., 1982
	GGA2	14	mainly non-homologous pairing (77%)	chiasma suppression (?)	Kaelbling and Fechheimer, 1985
T. guttata (TGU)	6/TGU5[b]	~31	non-homologous pairing	MLH1 foci: crossover suppression	this report

[a] Expressed as percentages of the chromosome length. In ZAL2 and GGA1, the inversion length was calculated from chromosomes and/or drawings available in the references.
[b] In *T. guttata*, the sixth autosome of the karyotype corresponds to TGU5 in the linkage map and the genome assembly.

tene. It might be argued that inversion loops may have formed and adjusted before pachytene. However, in the event of homologous synapsis followed by loop adjustment some level of crossing over could be expected in the inverted segment because this region of bivalent 6 shows high recombination levels in homozygotes (see Results; fig. 3). If a crossover occurs within an inversion, complete synaptic adjustment is mechanically impaired, leading to the observation of inversion loops with different degrees of adjustment depending on the location of the crossover event [Koehler et al., 2004; Torgasheva et al., 2013]. As shown here, bivalent 6 was present as a straight SC in all nuclei from heterozygotes, without MLH1 foci in a segment spanning more than one-third of the bivalent supporting the idea that this SC segment does not engage in homologous synapsis during early prophase stages. Although this evidence cannot completely rule out a neocentromere formation, it should be pointed out that neocentromeres do not change the sequence order, so there should be no hindrance to crossover within the homologous segment between the shifted centromeric signals. The use of FISH with locus-specific probes in homozygotes and heterozygotes will provide definite proof about this issue.

Inversion Heterozygosity and Meiosis in Birds

In contrast to extensive analyses in mammals, studies on the consequences of the heterozygote condition for an intrachromosomal rearrangement are scarce among birds. As far as we know, in addition to the present study, synapsis or crossing over were analyzed in heterozygotes for pericentric inversions in 3 other avian species (table 1). With 1 exception, the presence of an inversion disrupts crossing over, more likely due to non-homologous pairing, although cytological evidence of the synaptic behavior is not available in every case. In 2 species of passerines *(Junco hyemalis* and *Zonotrichia albicollis)*, pericentric inversions were described as stable polymorphisms, and chiasma analysis at metaphase I showed that crossing over is disrupted within the borders of the rearrangements [Throneycroft, 1975; Shields, 1976]. Cytogenetic mapping in *Z. albicollis* (ZAL) revealed that the chromosome polymorphism originated by a pair of nested inversions in chromosome 2 (ZAL2), and genetic analysis showed that gene flow is limited to one chromosome end in heterozygotes [Thomas et al., 2008; Huynh et al., 2011]. In *Gallus gallus domesticus* (GGA), SC analysis in heterozygotes for a pericentric inversion in GGA2 revealed a high incidence of non-homologous pairing, with consequent disruption of crossing over [Kaelbling and Fechheimer, 1985]. These observations agree with the occurrence of heterologous synapsis and crossover suppression in zebra finches as shown in the present report. An exception to this meiotic behavior was observed in heterozygotes for a pericentric inversion in GGA1 [Bitgood et al., 1982] where heterozygotes have a single crossover within the inverted segment. The inversion in GGA1 was large compared to the inversions in GGA2 and those presently analyzed in chromosome 6 of the zebra finch (see table 1). This circumstance might offer an explanation for the prevalence of homosynaptic behavior in this rearrangement. In ZAL2 the rearrangement also com-

prises most of the chromosome, but it is composed of 2 inversions that may cause additional mechanical constraint to homology search during pairing, explaining the lack of gene flow within the limits of the rearrangement.

A well-known consequence of crossing over in heterozygotes for pericentric inversions is the presence of deletions and duplications in the recombinant products. Consequently, it should be expected that inversions would be rapidly eliminated from natural populations, unless a mechanism evolves to reduce effective recombination rates, either through decreased pairing and crossing over between inverted regions or selection against recombinant gametes [Noor et al., 2001; Rieseberg, 2001; reviewed in Hoffmann and Rieseberg, 2008]. Here, we show that early non-homologous synapsis and crossover disruption seems to be prevalent in heterozygotes for intrachromosomal rearrangements among birds, with the possible result of reduced deleterious effects and minimal reproductive impairment in carriers. These observations are significant because comparative genomic and cytogenetic studies show that inversions are especially common during the evolution of avian karyotypes in distantly related taxa [Skinner and Griffin, 2012; Kawakami et al., 2014], as well as in closely related species [Kretschmer et al., 2014; Dos Santos et al., 2015]. Moreover, it has been suggested that the relatively high rate of inversions detected in interspecific comparisons agrees with the observation of segregating inversion polymorphisms among birds [Ellegren, 2013]. Further investigations of the meiotic behavior of intrachromosomal polymorphisms in birds will help to determine if the size of the affected segment is central to heterosynaptic behavior and crossover suppression in carriers. If so, small inversions could be preferentially fixed as stable polymorphisms because they might avoid the detrimental effects on fertility and zygote viability that arise when a crossover occurs within an inversion.

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Statement of Ethics

All procedures involving animals were approved by the Animal Care and Use Committee (Approval no. 2833/10), School of Medicine, University of Buenos Aires.

Disclosure Statement

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